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Tesi di Dottorato

Sonic Hedgehog pathway impairment in Neural Precursor Cells of the Ts65Dn mouse, an animal model of Down syndrome

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1. AIM OF THE STUDY

Down syndrome (DS), caused by trisomy of human chromosome 21 (HSA21), is one of the most common genetic causes of mental retardation, occurring in one out 800-1000 live births (Hayes and Batshaw 1993; Roizen and Patterson 2003; Shin, Besser et al. 2009). Although a wide range of variable traits characterizes Dow syndrome, intellectual disability is the unavoidable hallmark of the pathology, with a heavy impact on public health. Mental retardation in DS is likely due to widespread brain hypoplasia and hypocellularity that start to be present from early developmental stages and are retained in adulthood. Recent evidence from our laboratory shows that the fetal DS brain is characterized by severe proliferation impairment that involves numerous brain regions (Contestabile, Fila et al. 2007; Guidi, Bonasoni et al. 2008; Guidi, Ciani et al. 2011). These findings clearly indicate that **neurogenesis impairment is a key determinant underlying brain malfunctioning in DS**.

Mouse models that mimic human pathologies as closely as possible are becoming invaluable tools because they can be exploited to identify the mechanisms underpinning a given pathology and to examine the outcome of targeted therapies. Various mouse models for DS are now available but the Ts65Dn mouse is one of the most widely used because it displays several neuroanatomical and behavioral defects that closely recapitulate the human condition (Reeves, Gearhart et al. 1986). In the Ts65Dn mouse neurogenesis is severely impaired in the embryonic ventricular zone and hippocampus (Chakrabarti, Galdzicki et al. 2007) and in the hippocampus, cerebellum and subventricolar zone (SVZ) of neonate and adult mice (Baxter, Moran et al. 2000; Contestabile, Fila et al. 2007; Contestabile, Fila et al. 2009). This evidence validates the use of the Ts65Dn mouse model to get insight into the mechanisms underlying proliferation impairment in DS.

The mechanisms by which trisomy 21 interferes with brain development are still largely unknown. It has been recently shown that the reduced proliferation

of cerebellar granule cell precursors from Ts65Dn mice is related to an attenuated response to sonic hedgehog (Shh) (Roper, Baxter et al. 2006), a potent mitogen that controls cell division during brain development (Ishibashi, Saitsu et al. 2005). The wide brain expression of the Shh pathway (Ishibashi, Saitsu et al. 2005; Arsic, Beasley et al. 2007), suggests that it may exert a widespread role in the regulation of neural precursor proliferation in different brain neurogenic zones. If so, a generalized attenuation of the response to Shh might account for the widespread neurogenesis impairment that characterizes the trisomic brain.

Although in vivo models reflect in a more realistic fashion the environment of the diseased brain, in view of the complexity of the mechanisms underlying neurological diseases, simplified in vitro approaches are often essential tools to better dissect the molecular mechanisms that take part in the pathology.

The overall goal of the present study was to create an in vitro model suitable to understand the mechanism/s underlying neurogenesis impairment in DS. The specific goals were to:

i) establish whether cultures of neural precursor cells (NPCs) from Ts65Dn mice exhibit the same proliferation defects as the *in vivo* condition;

ii) establish whether NPCs exhibit a defective response to Shh, similarly to cerebellar granule cell precursors;

iii) dissect the molecular mechanisms that underlie deregulation of the Shh pathway and, consequently, the proliferation impairment of trisomic NPCs.

2. INTRODUCTION

2.1 DOWN SYNDROME (DS)

2.1.1 History of DS

The history of the discovery and characterization of the DS has been summerized in very fine details by Pierre L. Roubertoux and Bernard Kerdelhué in a review of 2006 (Roubertoux, Bichler et al. 2006). They describe that in 1838, Jean-Etienne-Dominique Esquirol, specialized in ‘‘mental insanity’’, published the first handbook of psychiatry, entitled “Des maladies mentales considérées Les sous les rapports médical, hygiénique et médico-légal” [Mental Disease: medical, health/hygiene and medical-legal considerations] (Esquirol 1838). Esquirol, who had previously published “Des Passions considerées comme causes, symptomes et moyens curatifs de l’aliénation mentale” [The passions considered as causes, symptoms and means of curing cases of insanity] (Esquirol 1805), had also set up a course on ‘‘mental disorders’’ in 1811, at Salpétrière Hospital in Paris, where he was an ‘‘ordinary physician’’ working under Pinel. A large section of the 1838 book was devoted to ‘‘Idiocy’’, covering what is now referred to as ‘‘mental deficiency’’, ‘‘mental retardation’’ or ‘‘feeble mindedness’’. Esquirol described a particular category of patient characterized by oblique eye fissures, epicanthic eye-folds, a flat nasal bridge and protruding tongue. Esquirol noted their short, stocky stature with virtually no neck, with malformed limbs and mental retardation. The description clearly tallies with the modern symptomatology of trisomy 21. Eight years later, Edouard Séguin (1846) took up this description of the symptomatological group, adding a detailed description of the small nose and open mouth; he described the morphology of the tongue, that was thick and cracked, and the susceptibility of the lungs and integuments to infection. In two later papers, first published in English (Séguin 1856; Seguin 1866), Séguin wrote that in spite of ‘‘profound idiocy’’, these ‘‘good kids’’ had language and were able to gain some basic knowledge. He described the mental pathology reported by Esquirol in greater detail and named it ‘‘furfuraceous cretinism’’ because of the bran-like appearance of the skin of trisomic 21 persons.

A classification based on ‘‘races’’ devised by the German Blumenbach was popular in England in the mid-nineteenth century and featured five main groups: Mongolians, Aztecs, Caucasians, Malayans and Ethiopians. The British alienist, **John Langdon Haydon Down**, classified patients in the mental hospital where he was working and assigned them to these ethnic groups. ‘‘A very large number of congenital idiots are typical Mongols. So marked is this that, when placed side by side, it is difficult to believe the specimens compared are not children of the same parents.’’ (Down 1866). The description of what was later referred to as trisomy 21 was made using Down’s obsolete racial framework. ‘‘The Mongolian type of idiocy occurs in more than ten per cent of the cases which are presented to me. They are always congenital idiots and never result from accidents after uterine life. They are, for the most part, instances of degeneracy arising from tuberculosis in the parents. They have considerable power of imitation, even bordering on being mimics. They are humorous and a lively sense of the ridiculous often colors their mimicry. This faculty of imitation can be cultivated to a very great extent and a practical direction given to the results obtained. They are usually able to speak; the speech is thick and indistinct, but may be improved very greatly by a well-directed scheme of tongue gymnastics. The coordinating faculty is abnormal, but not so defective that it cannot be strengthened. By systematic training, considerable manipulative power may be obtained. The circulation is feeble and however much advance is made intellectually in the summer, some amount of retrogression may be expected in the winter. Mental and physical capabilities are, in fact, directly as the temperature. The improvement which training effects in them is greatly in excess of what would be predicted if one did not know the characteristics of the type. The life expectancy, however, is far below the average, and the tendency is to tuberculosis which I believe to be the hereditary origin of the degeneracy.’’ The term ‘‘mongolism’’ was adopted from Down’s classification and was in common usage by the late nineteenth century, but as it refers to clearly non-scientific etiology, it has been discarded. The term ‘‘mongolism’’ was gradually seen as an embarrassment and in 1961 the term ‘‘Down’s syndrome’’ was suggested by Allen (as reported by Ward, 1998).

It was not until 1959 that **Dr. Jerome Lejeune**, a French physician, made the discovery that Down's Syndrome was the result of a chromosomal abnormality. His research led him to the fact that the cells of people with Down's Syndrome had 47 chromosomes instead of 46. Dr. Jerome Lejeune identified in subjects with Down's syndrome a triplication of the chromosome 21. In this way, the term ''trisomy 21'' (TRS21) was introduced.

2.1.2 Epidemiology

In the world, 1 of every 800-1000 live births has trisomy 21 (Winter, Ostrovsky et al. 2000). Worldwide about 220,000 infants with trisomy 21 are born each year with phenotypes collectively referred to as DS (Christianson, Howson et al. 2006). The prevalence of DS does not correlate to geographic location, socio-economic level or ethnicity (Lejeune 1959; Czarnetzki, Blin et al. 2003). The increasing probability of having a DS child correlates to the increasing age of the mother, as was first observed by Penrose (Penrose 1951).

MATERNAL AGE	INCIDENCE
< 30 years old	1 of 1500
30-34 years old	1 of 580
35-39 years old	1 of 280
40-44 years old	1 of 70
> 45 years old	1 of 38

2.1.3 Etiology

Genomic aneuploidy, defined as an abnormal number of copies of a genomic region, is a common cause of human genetic disorders. Classically, the term aneuploidy was restricted to the presence of supernumerary copies of whole chromosomes (trisomy), or absence of chromosomes (monosomy), but now we can extend this definition to include deletions or duplications of subchromosomal regions (Antonarakis, Lyle et al. 2004). Trisomy 21 is a model of all human disorders that are the result of supernumerary copies of a genomic region (Antonarakis, Lyle et al. 2004).

Non-disjunction

Several hypotheses were formulated before Lejeune discovered the path to the genetic/chromosomal origin of what was then called ‘‘Mongolism’’, with an extra copy of one chromosome but two other advances were needed before the discovery could be made. Cytogenetic studies were needed: 1) to determine the exact number of human chromosomes; 2) for the individual cytological characterization of each chromosome. Tijo and Levan (Tijo 1956) established that the human genome has 46 chromosomes. Two years later, in 1958, Lejeune reported that a patient had 47 instead of 46 chromosomes, the hypothesis being either a translocation on chromosome 4 or an extra chromosome present. Lejeune confirmed the finding of an extra chromosome 21 in two patients and went on to report an extra chromosome 21 in 9 ‘‘mongoloid’’ patients (Lejeune 1959). The year after this discovery, the observation of a triple chromosome 21 in patients with the same clinical diagnosis was reproduced by other independent groups (Fig.1).

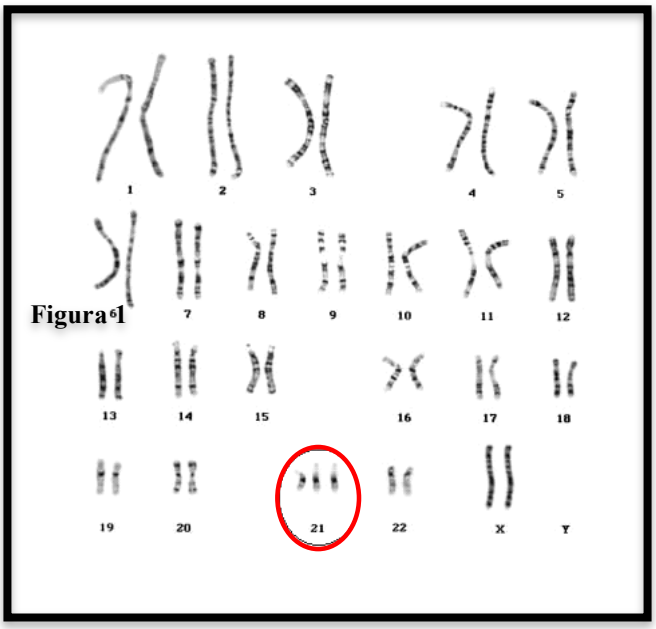


Figure 1 Karyotype for trisomy Down syndrome. *G-banded karyotype of a trisomy 21 female, showing three copies of human chromosome 21 (HSA21).*

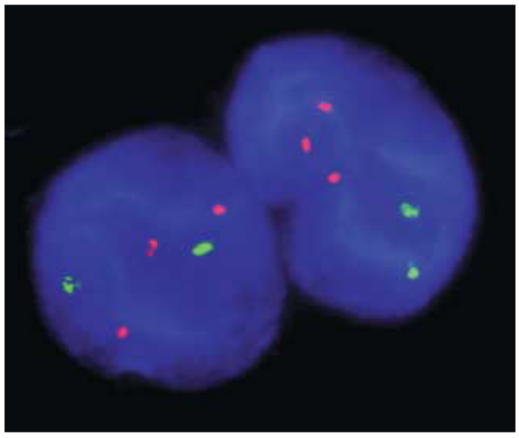


Figure 2: Fluorescent in situ hybridization (FISH) of interphase nuclei of a trisomy 21 fetus. *In red the trisomic chromosome 21.*

More recent analyses using molecular tools (Fig. 2) have confirmed that in DS, 95% of all cases are caused by full trisomy of chromosome 21, whereby all HSA21 genes are present in 3 copies, resulting from a chromosomal non-disjunction. One cell has two HSA21 instead of one, so the resulting fertilized egg has three HSA21 chromosomes. If a sperm or egg with an abnormal number of chromosomes merges with a normal mate, the resulting fertilized egg will have an abnormal number of chromosomes. Hence the scientific name, trisomy 21. Recent research has shown that in these cases, approximately 90% of the abnormal cells are eggs (Antonarakis 1996). The cause of the non-disjunction error remains still unknown. Research is currently aimed at trying to determine the cause and timing of the non-disjunction events.

Robertsonian Translocation

Three to four percent of all cases of trisomy 21 are due to Robertsonian Translocation. In this case, two breaks occur in separate chromosomes, usually the 14th and 21st chromosomes. There is a rearrangement of the genetic material so that some of the 14th chromosome is replaced by extra 21st chromosome. So while the number of chromosomes remains normal, there is a triplication of the 21st chromosome material. Translocations resulting in trisomy 21 may be inherited.

Mosaicism

The remainder of cases of trisomy 21 is due to mosaicism**.** In genetics, a mosaic or mosaicism denotes the presence of two or more populations of cells with different genotypes in one individual who has developed from a single fertilized egg. Mosaicism may result from a mutation during development which is propagated to only a subset of the adult cells.

2.1.4 Somatic traits and clinical features

Children with DS exhibit chatacteristic and peculiar physical traits:

- Short stature : children with DS usually have slow growth rate, and in adulthood their height is lower than average;

- Low muscle tone : children may have less muscle strength than other children of the same age;
- Short neck, thick with fat and excess skin : usually this feature becomes less obvious as the children grow;
- Short and stocky limbs, some children may have a wider space between the thumb and second finger of the foot;
- One fold in the central part of the palm : it is called the simian line.

Facial features. They show typical facial features which include, for example, ears with modified form, usually small and with a low placement (Fig. 3 E). They present, also, abnormal mouth and tongue, with mouth often open (Fig. 3 F). It is typical to find children with exfoliative glossitis and tongue with scrotal appearance (in adolescents and adults). Furthermore, it is frequrent pseudomacroglossia (Fig. 3 F). It is very characteristic a flattened nasal bridge and this flat nose portion, located between the two eyes (nasal bridge), is frequently clogged (Fig. 3A). There are, often on their faces Brushfield's spots. They are colored spots on the iris, without affecting the sight. Moreover, malformation of the teeth are very frequent: baby teeth may grow later and in an unusual way with, often, agenesis of lateral incisors.

Skeleton and skin features. Besides the short arms, they exhibit a finger clinodactyly of the fifth finger with a single flexion crease, flat foot and increased space between first toe and second toe.Tipically their skin is dried with xerosis. There are recurent cases, also, of hyperkeratotic lesions, alopecia, vitiligo, foliculitis and skin infections.

Psychomotor retardation. Hypotonia occurs soon at birth.

Congenital abnormalities associated with DS. Although DS is usually linked to intellectual disability, the morbidity from other associated congenital abnormalities is considerable. Many infants with DS have co-occurring congenital malformations (Fig. 4) requiring intensive surgical and medical management. To anticipate the care needed by these infants, providers and parents require accurate

information about birth defects that may be present. With this purpose in mind, many articles use different datasets to identify the rate at which structural birth defects are identified among children with DS. Overall, about one in five live born children with DS dies before the age of 5 years, and about two of five survivors have major health problems in addition to mental retardation in early childhood (Noble 1998).

Congenital heart diseases were the most frequently documented birth defects in live born infants with DS and approximately 46% of infants with DS were born with one or more heart defects. The three most common types of congenital heart defects were atrioventricular canal defects (45%), ventricular septal defects (35%) and patent ductus ateriousus (Roizen and Patterson 2003). The atrioventricular canal defect is the most serious of these defects. In the normal formation of the heart the endocardial cushions grow toward each other and leave openings between the atria and the ventricles, where the mitral and tricuspid valves form. The atrioventricular canal defect is caused by a failure of this process and results in the formation of a single valve structure with a septal defect above and below it. Survival can be improved with surgery, but the prognosis in children with these abnormalities is still poor because of the increased risk of heart failure. The ventricular septal defect, a defect in the ventricular septum, which divides the left and the right ventricles, may require surgery, though about 15% close spontaneously. The patent ductus is a congenital defect wherein a neonate's ductus arteriousus, the shunt connecting the pulmonary artery to the aortic arch, fail to close after birth. It is not a serious abnormality and can be corrected by a relatively simple surgical procedure. Assessment of all newborns with DS with an echocardiogram is the standard recommendation. Symptoms of serious heart disease may be absent or hidden due to the tendency of children with DS to develop pulmonary vascular resistance. Adolescents and young adults with no known intracardiac disease can develop mitral valve prolapse (46%) and aortic regurgitation (17%), and most experts recommend assessment also of adult patients.

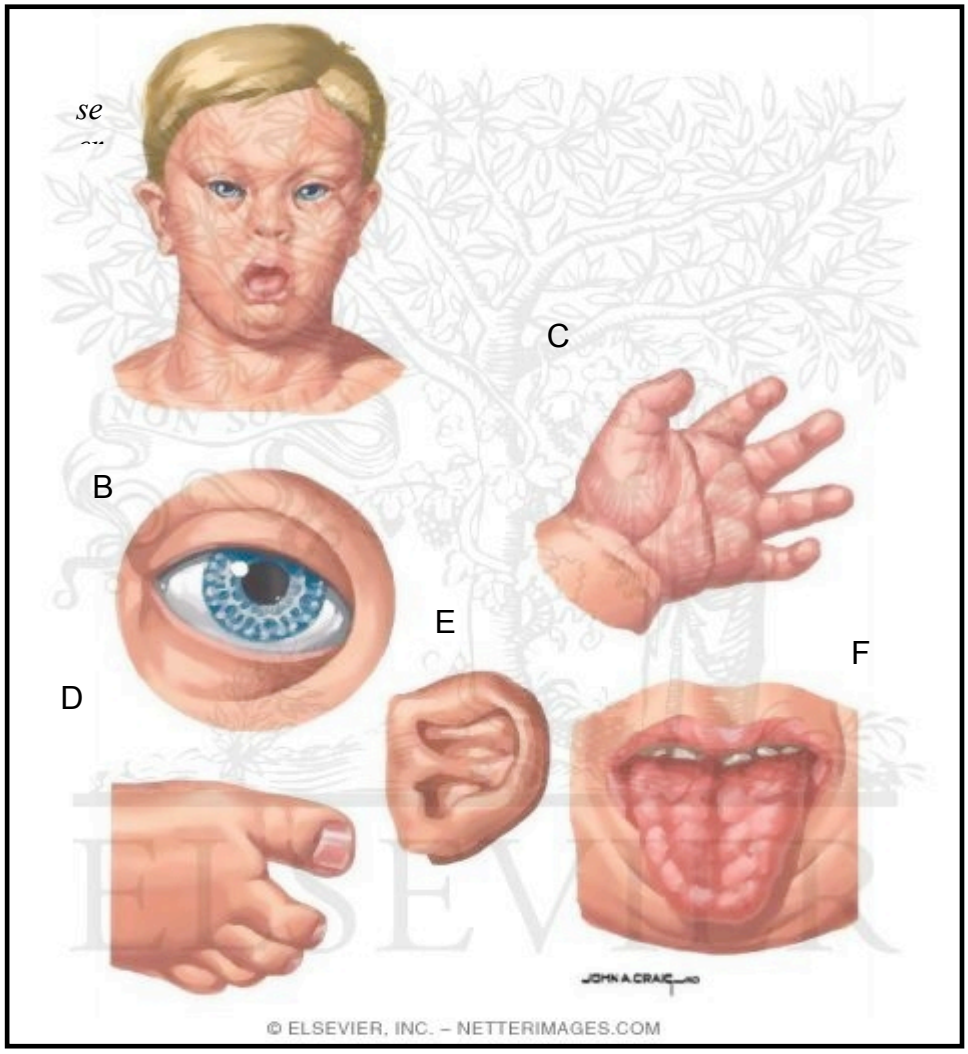


Figure 3: Down syndrome main features. *A) Typical face, B) eye of a DS subject. Note the oblique eye fissures and the epicanthic eye-folds , C)-D) hand (C) and feet (D). Note the shortened, incurved fifth fingers. E) small ear, F) protruding tongue.*

After the co-occurring heart abnormalities, the second most common malformations in children with DS are those of the gastrointestinal tract (11%) (Fig. 4) like duodenal atresia, Hirschsprung's disease and tracheo-oesphaegal fistula (Noble 1998). Duodenal atresia is the congenital absence or complete closure of a portion of the lumen of the duodenum. Approximately 8% of all infants with DS show this disease and it's 300 times more common in individuals

affected than in normal newborns. The Hirschsprung's disease or aganglionic megacolon, involves an enlargement of the colon by bowel obstruction resulting from absence of the enteric innervation in this section of bowel.

Other congenital abnormalities have been reported in association with DS: malformations in the genitourinary system (6%), including stenosis or atresia of the urinary tract at any location, malformations of the abdominal wall, limb defects (9%) (reduction of both the lower and upper extremities) and congenital cataract (1%) (Noble 1998).

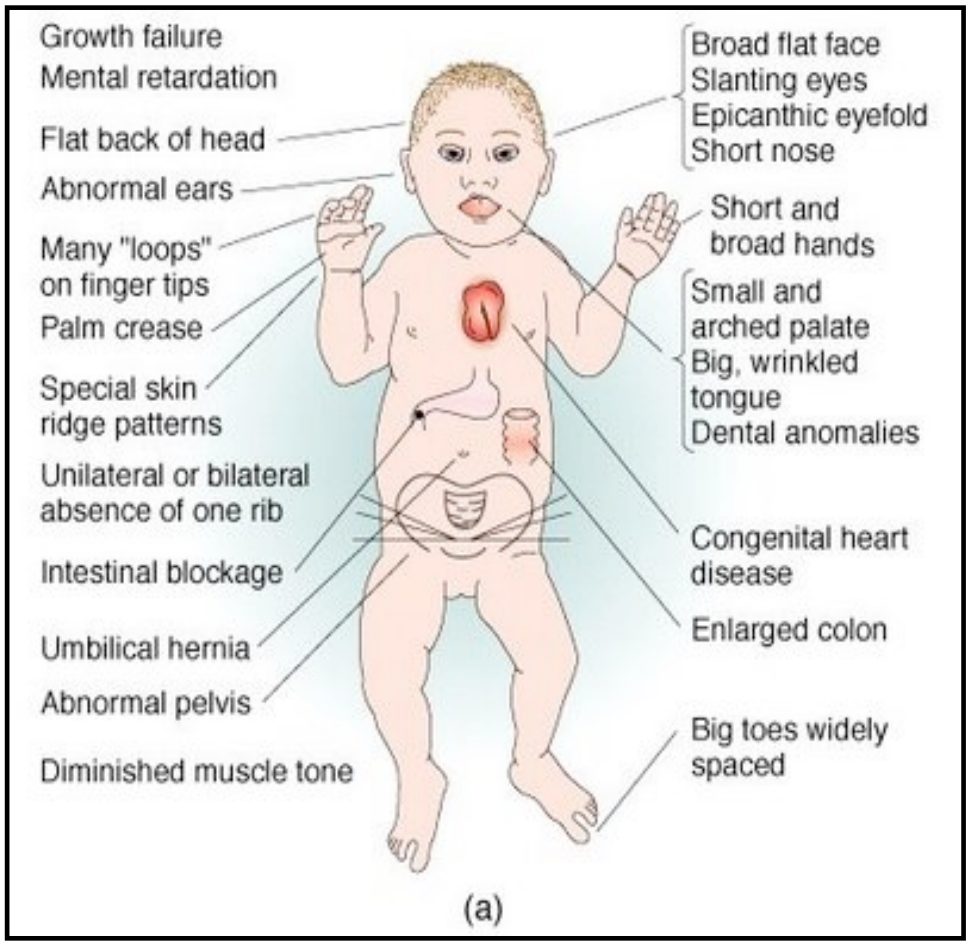


Figure 4: Variable traits and clinical features in Down syndrome.

2.1.5 Behavioural defects in DS

Mental retardation remains the invariable hallmark of DS and its more

invalidating pathological aspect with a hard impact in the public health. In DS patients, the average IQ score is around 50, with individual values ranging from 30 to 70. (Chapman and Hesketh 2000; Vicari 2006). The early infancy is characterized by a delayed cognitive development, leading to mild-to-moderate mental retardation. The IQ declines from early in the first year to late childhood (Brown, Greer et al. 1990; Hodapp, Dykens et al. 1990) and the decline in cognitive performance that occurs in the adult DS patients has been considered as the consequence of accelerated aging *(Devenny, Silverman et al. 1996; Lott and Head 2005)*. Mental retardation in DS is characterized by major neurological dysfunctions in the short- and long-term memory (Carlesimo, Marotta et al. 1997; Nadel 2003). It has been also demonstrated a decline with age on measures of long-term memory, providing behavioral evidence of hippocampal dysfunction by adolescence *(Carlesimo, Marotta et al. 1997),* consistent with the structural magnetic resonance imaging (MRI) finding of reduced hippocampal volumes in adolescents with DS *(Jernigan, Bellugi et al. 1993; Pennington, Moon et al. 2003)*. There is also evidence for impairment of prefrontal cortex and cerebellar function (Nadel 2003) and speech and articulation are also particularly affected. The lower performances of DS in linguistic tasks may be partially explained in terms of impairment of the frontocerebellar structures involved in articulation and verbal working memory *(Fabbro, Libera et al. 2002)*. Moreover, learning can be complicated by avoidance strategies when faced with cognitive challenges (Epstein 1995). Psychometric examinations of DS patients have shown that not all skills are affected in all persons or to the same extent. (Crnic 2002; Krinsky-McHale, Devenny et al. 2002; Brown 2003; Clark and Wilson 2003; Pennington, Moon et al. 2003; Vicari 2006). Although all domains of development follow the usual sequence, a deficiency in language production relative to other areas of development often causes substantial impairment (Chapman, Seung et al. 1998). Children with DS have more behavioral and psychiatric problems than in other children, but fewer than in other individuals with mental retardation. In adult age, DS patients can have a similar prevalence of psychiatric problems to other people with intellectual disability. A raised frequency of psychiatric problems is also

related to the increased prevalence of depression in people with DS. However, they seem protected from some psychiatric disorders such as personality disorder, schizophrenia and anxiety. *(Collacott, Cooper et al. 1998).* Elsewhere, DS children show continuous but gradual improvement in mental age throughout childhood; intelligence quotients generally decline from early in the first year to late childhood (Hodapp, Dykens et al. 1990). Consequently, early implementation of special education programs results in improved cognitiveabilities in DS individuals. *(Chapman, Seung et al. 1998).*

By age 40 years, there is a ubiquitous occurrence of plaques and tangles suggestive of Alzheimer disease (AD), with an increase in dementia. The earliest manifestations of dementia in DS appear to reflect frontal lobe dysfunction with changes in sociability, emotional-based language, and depressive symptoms. However, physical–chemical dating of amyloid has suggested that it is first deposited in the frontal and entorhinal cortices. The amyloid burden in DS is, in part, related to increases in the expression of the amyloid precursor protein gene, but other factors are likely involved. Oxidative stress secondary to critical-regionmutations in mitochondrial DNA is associated with an increased brain concentration of oxidized β-amyloid.

2.1.6 Neuroanatomy of DS

The DS brain is characterized by numerous structural alterations that start to be present from early developmental stages and are retained in adulthood. Reduced thickness of cortical layers, diffuse hypocellularity, and astrocytic hypertrophy are a characteristic feature of the DS brain (Bartesaghi, Guidi et al. 2011). Autopsy data on the neuroanatomy of DS have been complemented by increasing studies from in vivo structural imaging using computer assisted tomography, voxel-based morphometry and to a larger extent MRI. The brain of DS subjects is characterized by several postmortem macroscopic features that are related to pre- and post-natal abnormalities in synaptogenesis leading to retardation of brain growth *(Schmidt-Sidor, Wisniewski et al. 1990),* such as lower brain weight and

brachycephaly, with a small cerebellum, frontal and temporal lobes reduced number and depth of the cerebral sulci, and a narrow superior temporal gyrus. (Coyle, Oster-Granite et al. 1986; Schmidt-Sidor, Wisniewski et al. 1990; Becker, Mito et al. 1991). MRI studies show regional brain abnormalities in young and non-demented adult DS subjects, consistent with postmortem data. Overall, brain volume is reduced in DS subjects, including cerebellar and cerebral gray and white matter. *(Schapiro, Haxby et al. 1992; Pinter, Eliez et al. 2001)*. In particular, the reduced cerebellum shows a decreased volume of lobules VI–VIII (Avraham, Sugarman et al. 1991; Jernigan, Bellugi et al. 1993; Raz, Torres et al. 1995). In the temporal neocortex of fetuses with DS the emergence of lamination is both delayed and disorganized (Golden and Hyman 1994), total neuron number is reduced in the hippocampal dentate gyrus (DG), hippocampus and parahippocampal gyrus (Guidi, Bonasoni et al. 2008) and the cellular layers of the cerebellum are characterized by prominent hypocellularity and reduced thickness (Guidi, Ciani et al.).

In contrast, an increased volume is found in other brain areas, such as ventricles (Kesslak, Nagata et al. 1994; Pearlson, Breiter et al. 1998¸ Ikeda, 2002 #630; Schimmel, Hammerman et al. 2006) parahippocampal gyrus after adjustment for overall brain volume *(Kesslak, Nagata et al. 1994; Raz, Torres et al. 1995; Teipel, Schapiro et al. 2003; Teipel and Hampel 2006)* temporal, parietal and posterior cortex, lenticular nucleus, thalamus and hypothalamus *(Jernigan, Bellugi et al. 1993; Pinter, Eliez et al. 2001),* while the occipital lobe and superior temporal gyrus do not show volume changes after adjustment for overall brain volume (Frangou, Aylward et al. 1997; Pinter, Eliez et al. 2001). The second phase of cortical development and the emergence of lamination are delayed and disorganized in fetal DS brain. In addition to the known effects of the hippocampal formation in spatial memory, the altered cortical layer and cerebellum also may participate to cognitive and behavioral phenotypes in DS (Funahashi, Takeda et al. 2004).

2.1.7 Causes of brain hypotrophy in DS

The widespread hypoplasia of the DS brain is considered to be the main cause of mental retardation. **Several lines of evidence show that two major determinants underlie brain hypotrophy in DS are neurogenesis reduction and dendritic hypotrophy** *(Coyle, Oster-Granite et al. 1986; Becker, Mito et al. 1991)***.** In addition, an increase in cell death at more advanced life stages contributes to further reduce neuron number.

Neurogenesis alterations

The fact that brain and cerebellar volume reduction and cortical hypocellularity are already present in children and fetuses with DS (Sylvester 1983; Schmidt-Sidor, Wisniewski et al. 1990; Golden and Hyman 1994; Winter, Ostrovsky et al. 2000; Pinter, Eliez et al. 2001) strongly suggests that defective *neuronogenesis* during critical phases of brain development may be a major determinant of microencephaly.

Due to the obvious difficulties in obtained fetal material, very little information is available concerning neurogenesis in the fetal DS brain. Our group recently obtained evidence that in fetuses with DS cell proliferation is severely impaired in the DG (most likely due to alteration of the cell cycle), in the germinal matrix of the inferior horn of the lateral ventricle and in the germinal zones of the hippocampus proper and parahippocampal gyrus (Contestabile, Fila et al. 2007; Guidi, Bonasoni et al. 2008). Quantification of the number of mature neurons and astrocytes in the hippocampus and parahippocampal gyrus showed that in all these regions fetuses with DS had proportionally fewer neurons and a larger number of astrocytes compared with normal fetuses (Guidi, Bonasoni et al. 2008). In trisomic fetuses, our group found a defective neurogenesis in the EGL of the cerebellum, and in a region of the fifth lobe that is the remnant of the cerebellar VZ (Guidi, Ciani et al.). This evidence clearly shows proliferation impairment in numerous regions of the fetal DS brain. Importantly, these defects are shared by trisomic mice (see below) which validates the use of mouse models for DS and renders evidence obtained in mouse model transferable to the human condition.

Dendritic hypotrophy

Dendritic pathology is a typical feature of the DS brain and DS neurons exhibit patent alterations in dendriitic pattern and spine density and shape. In the visual cortex of fetuses with DS neuronal morphology is similar to that of control fetuses (Takashima, Becker et al. 1981). Likewise, the dendrites of layer IIIc pyramidal neurons of the prefrontal cortex in 2.5-month-old infants with DS is similar to that of normal cases (Vuksic, Petanjek et al. 2002). Dendritic abnormalities, however, begin to appear at later ages. A study in a 3 month-old infant with DS shows that interneurons in the motor cortex have a lower dendritic area (Prinz, Prinz et al. 1997). The pyramidal neurons of the visual cortex of newborns older than 4 months and older infants with DS have shorter basilar dendrites (Takashima, Becker et al. 1981). Dendritic hypotrophy is also present in pyramidal neurons of the parietal cortex of children with DS (Schulz and Scholz 1992). The dendritic hypotrophy seen in childhood continues into adulthood, with a marked decrease in dendritic branching and dendritic length in elderly adults with DS (Takashima, Ieshima et al. 1989). In the visual cortex of children with DS, the total dendritic length is above normal in the infantile period (6 months old or less) but drops steadily to below normal in juvenile subjects (older than 2 years) (Becker, Armstrong et al. 1986). This reduction contrasts with expanding dendritic arborization in normal children. These data indicate that in individuals with DS the dendritic tree atrophies in early childhood without a recovery at subsequent life stages.

While in the visual cortex of fetuses with DS spine counts (basilar dendrites) are similar to those of control fetuses, newborns and older infants with DS have a decreased number of spines and spines exhibit an altered morphology (Takashima, Becker et al. 1981). In normal subjects, spine density on the basal dendrites of cortical pyramidal neurons increases from neonate to 15 years of age and gradually decrease after 20 years. In contrast, spine density poorly increases in children and rapidly decreases in adults with DS (Suetsugu and Mehraein 1980; Takashima, Ieshima et al. 1989; Takashima, Iida et al. 1994). A reduced spine density has been found in the apical dendrites of pyramidal neurons of the

hippocampus and cingulate cortex and in both the apical and basilar dendritic arbors of CA1 and CA2-3 pyramidal neurons in patients with DS when compared to age-matched control (Suetsugu and Mehraein 1980; Takashima, Ieshima et al. 1989; Takashima, Iida et al. 1994). An additional decrease in spine density occurs in DS patients with associated AD, when compared to age-matched controls, and DS with no AD (Takashima, Ieshima et al. 1989; Ferrer and Gullotta 1990). The dendritic spines of the DS brain exhibit, in addition to reduced density, also aberrant morphology. Studies of infants with DS demonstrated that spines were small, had short stalks and were intermingled with unusually long spines (Marin-Padilla 1976; Purpura 1979). Considering that drebrin expression is reduced in DS individuals (Shim and Lubec 2002), a reduced excitatory-inhibitory ratio is expected in DS individuals, which may lead to neuron hypoexcitation.

Biochemical alterations

Biochemical alterations also occur in fetal DS brain, which could serve as substrates for the morphological changes *(Engidawork and Lubec 2003).* In addition to the cholinergic system, that undergoes alterations at advanced life stages, the glutamatergic, serotonergic, noradrenergic and GABAergic systems are profoundly altered in DS individuals and in trisomic mice (Wisniewski and Bobinski 1991; Risser, Lubec et al. 1997). Alterations involve neurotransmitter levels as well as defects of receptor expression/function.

Although young children with DS appear to be born with a normal septohippocampal cholinergic system (Kish, Karlinsky et al. 1989), an aging-dependent neurodegeneration of the basal forebrain cholinergic neurons (BFCNs) was observed (Yates, Simpson et al. 1983; Casanova, Walker et al. 1985). Because BFCNs provide the major cholinergic input to the hippocampus and neocortex, the degeneration of these neurons may have functional consequences at level of cholinergic receptors. These dysfunctions could produce additional learning and memory deficits in older individuals with DS (Yates, Simpson et al. 1983) and could be an outgrowth of the Alzheimer disease in these patients. In addition, an early onset of an Alzheimer disease-like neurohistopathology is

systematically observed by the fourth decade (Dalton and Crapper-McLachlan 1986).

2.1.8 The Human Chromosome 21, genotype–phenotype correlations

Chromosome 21 (HSA21) is the smallest human autosome that represents around 1±1.5% of the human genome. Different hypotheses have been presented to account for the relationship between trisomy 21 and the occurrence of DS phenotypes. The effects of trisomy on brain phenotypes have been explained by two hypotheses: 1) the "gene-dosage effect" hypothesis claims that DS phenotypes are determined by increased dosage of a subset of dosage-sensitive genes, and of their encoded proteins, especially during development (Delabar, Theophile et al. 1993; Korenberg, Chen et al. 1994; Ait Yahya-Graison, Aubert et al. 2007; Lyle, Bena et al. 2009), and *2*) the "amplified developmental instability" hypothesis holds that Hsa21 trisomy determines a general alteration in developmental homeostasis (Roizen and Patterson 2003; Antonarakis, Lyle et al. 2004). The gene-dosage hypothesis proposes that the 50% increase in expression at the RNA level of trisomic genes is the initiating cause of the DS phenotype. Recent experiments using microarrays and quantitative RT-PCR of human DS and mouse model samples indicate that the majority of trisomic genes in the majority of tissues indeed show increased expression, although not always by precisely 50% (Mao, Zielke et al. 2003; Amano, Sago et al. 2004; Kahlem, Sultan et al. 2004; Lyle, Gehrig et al. 2004; Dauphinot, Lyle et al. 2005). The triplicated genes could modulate directly or indirectly the expression of target genes on both the Hsa21 and the other chromosomes, determining a secondary gene dosage effect. Thus, the gene expression alterations in the brain, resulting from the different genetic mechanisms in response to the gene over-dosage in DS, may induce primary phenotypes at cellular level consisting in alterations in cellular processes, such as proliferation, differentiation, synaptogenesis, dendritogenesis and apoptosis in neuronal and glial cells. Even though several genes have been identified that are deregulated in DS brains, the challenge in DS research is to

establish a correlation between the functions of triplicated genes and features of the cognitive and behavioral phenotypes seen in DS.

2.2. MOUSE MODELS FOR DS

Although studies in DS cases with partial trisomy have helped to suggest chromosomal regions and HSA21 genes that may contribute to various phenotypes, the ability to resolve the basis of neurological and other phenotypes is limited. The use of animal models, particularly the mouse, is an invaluable tool for understanding underlying genetic and molecular mechanisms of DS. The advantages are: (1) the ability to study a large number of animals in a short time; (2) the availability of mice engineered to contain a specific gene(s); (3) the ability to control for genetic background; (4) the low cost of screening; (5) the ability to carry out studies on both developing and mature subjects; (6) the ability to design and execute a variety of therapeutic interventions; and (7) the lack of postmortem delay. The number and variety of these models is growing (Fig 5). At the genomic level, the long arm of the human chromosome 21 (Hsa21) is approximately 33.7 Mb in length and contains about 430 protein-coding genes of which 293 have a homolog in the mouse genome. These genes are found in syntenic regions localized on three different mouse chromosomes. From the centromeric to the telomeric end of the Hsa21, the first and largest region is found on Mmu16 (about 37 Mb in length with 224 orthologous genes), followed by a smaller region on Mmu17 (1.1 Mb in length with 22 orthologous genes) and finally a region on Mmu10 (2.3 Mb in length with 47 orthologous genes). In these three syntenic regions, relative order and orientation of the genes are preserved between the two species. With the introduction of new genetic engineering methods, it will be possible to produce additional models in which triplication of smaller genetic segment is readily accomplished.

2.2.1 Mice Trisomic for the entire MMU16

Ts16 Mice trisomic for the entire 16th chromosome (Ts16) were generated by spontaneous Robertsonian translocation. Unfortunately, these mice die in utero, thus limiting research during the postnatal period. Death probably occurs as the result of cardiovascular abnormalities and insufficient placenta function (Miyabara, Gropp et al. 1982). A number of studies focused on nervous system abnormalities in Ts16 fetuses showed similarities with DS. These changes ranged from delayed development to degenerative processes. There is delayed cortical development in Ts16 mice. The cross-sectional area of cortical layers appears normal at E13. However, at E16, the cortical thickness is significantly (12–48%) smaller when compared with that of disomic mice (Haydar, Blue et al. 1996). Interestingly, 2 days later (E18) no such a difference could be detected. In addition to delayed development, a degenerative process has been found in these mice. Sweeney et collaborators (Sweeney, Hohmann et al. 1989) quantified the number of acetylcholinesterase (AChE)-positive neurons in the basal forebrain of Ts16 mice and found a significant (40%) loss when compared with controls. In a recent study, Dorsey et al.(Dorsey, Bambrick et al. 2002) studied brain derived neurotrophic factor (BDNF) signaling in Ts16 cultured hippocampal neurons and found evidence supporting failed tyrosine receptor kinase (TrkB)-mediated BDNF signaling in these cells. Accordingly, TrkB phosphorylation was significantly (33%) reduced in response to BDNF in Ts16 mouse hippocampal neurons. Moreover, trisomic cells were characterized with increased levels of a truncated, kinase-deficient isoform (T1) of the TrkB (TrkB T1). Interestingly, disruption of a gene encoding this protein restored the survival of cultured cortical and hippocampal Ts16 neurons. Although Ts16 mice have been extremely helpful in directing our attention to the probability that mouse model scan be used to study DS, this model suffers from severe practical and theoretical limitations. Because they do not survive birth, we are largely prevented from studying age-dependent degenerative effects. Furthermore, in addition to carrying orthologues of HSA21, MMU16 harbors orthologues of genes on humanchromosomes 3, 16, and 22.

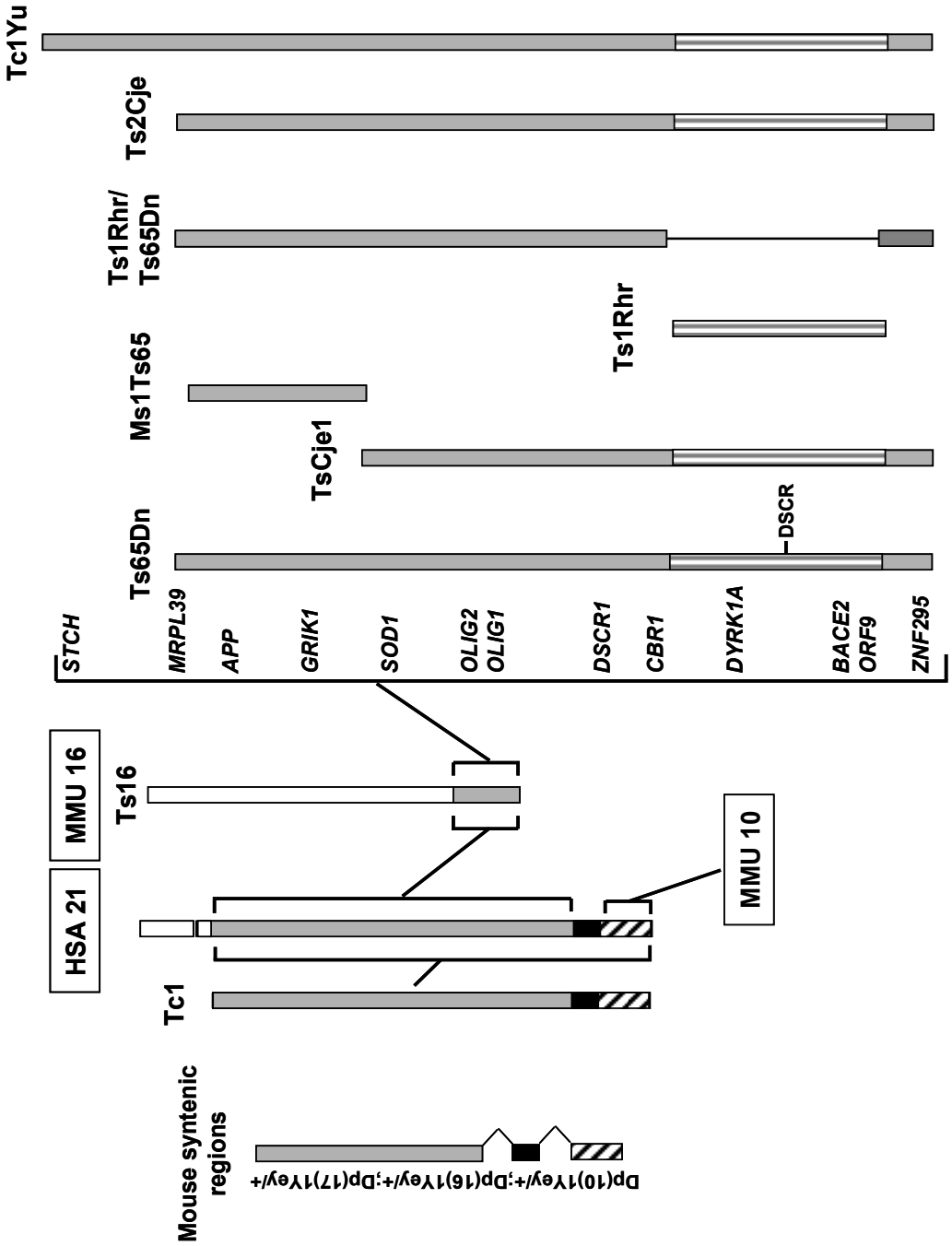


Figure 5: Mouse models of Down syndrome. *Schematic representation of Hsa21 and corresponding regions of Mmu10 (hatched region), Mmu16 (gray region) and Mmu17 (black regions). Different mouse models that carry different triplication of different sets of genes orthologous to those of Hsa21 are indicated.*

2.2.2 Segmental MMU16 trisomic mice

Ts65Dn Davisson et collaborators (Davisson, Schmidt et al. 1990), using irradiation, induced reciprocal translocation of T(16C3-4;17A2) and generated a mouse segmentally trisomic for MMU16 entitled Ts65Dn (Fig 6). The triplicated region of MMU16 in Ts65Dn extends from Znf295 to Mrp139 and contains at least 132 genes (Fig. 6). Ts65Dn mice recapitulate a variety of DS structural and functional changes. Similar to DS, basal forebrain cholinergic neurons (BFCNs) undergo age dependent degeneration in these mice (Cooper, Messer et al. 1999; Hunter, Bimonte et al. 2003; Salehi, Delcroix et al. 2004; Seo and Isacson 2005). The degeneration was linked to a marked decrease in the retrograde transport of nerve growth factor (NGF), a keytrophic factor for these neurons. By delivering NGF via intracerebro ventricular injection of NGF, and thus bypassing the retrograde transport, degenerative changes were rapidly reversed, even in very old Ts65Dn mice (Cooper, Messer et al. 1999). BFCNs project extensively to the hippocampus and cortex (Salehi, Delcroix et al. 2003). In a series of careful studies, marked synaptic structural abnormities were detected in these and other brain regions in Ts65Dn mice (Belichenko, Masliah et al. 2004; Kleschevnikov, Belichenko et al. 2004). Kurt and collaborators (Kurt, Kafa et al. 2004) studied neuronal density in the CA1 area of the hippocampus in old Ts65Dn mice and found a significant reduction in cell density. No such a difference was found at 3 months of age (Lorenzi and Reeves 2006). Furthermore, a significant reduction in the area and number of dendritic branches, spine density and in the layer III pyramidal cells in Ts65Dn mice has been reported (Kurt, Kafa et al. 2004). Alterations in spine size and density in Ts65Dn hippocampus were associated with failure to induce long-term potentiation (LTP) in the dentate gyrus and CA1 area (Kleschevnikov, Belichenko et al. 2004; Costa and Grybko 2005). In behavioral analyses, Ts65Dn mice showed significant spatial and non spatial learning disabilities, as shown by the hidden platform and probe tests in the Morris water maze (MWM) (Sago, Carlson et al. 2000; Hyde, Crnic et al. 2001; Hyde, Frisone et al. 2001). Ts65Dn mice are the most used model for DS.

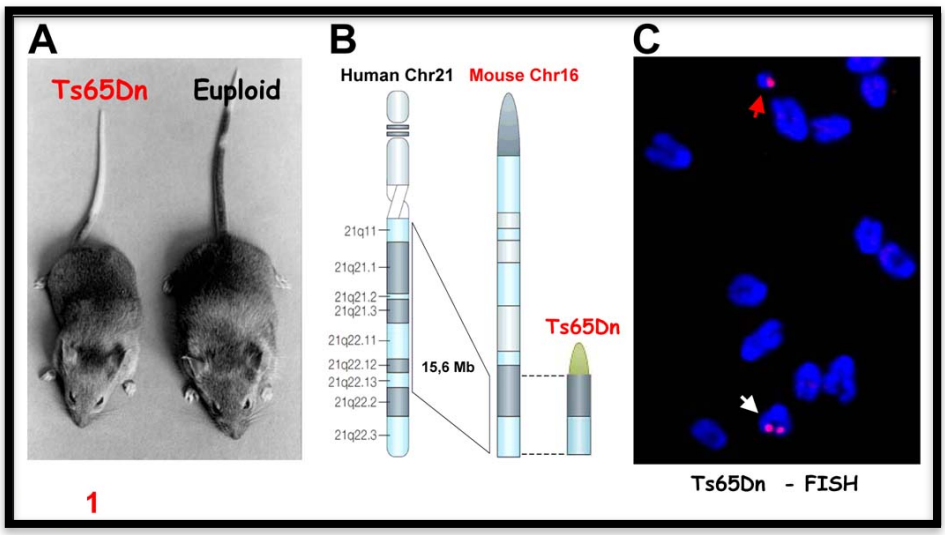


Figure 6: Ts65Dn mouse model. *A) Ts65Dn and control mouse. B) Ts65Dn mouse model shows segmental trisomy for a distal region of chromosome 16, a region that shows perfectly conserved linkage with human chromosome 21. C) Fluorescent in situ hybridization (FISH) in Ts65Dn mouse.*

Ts2Cje Since male Ts65Dn are infertile, only females can be used for breeding. Furthermore, the occurrence in progenyis ~20–25%. The Epstein Laboratory discovered a new mouse model called Ts2Cje that was generated by spontaneous Robertsonian fusion in which the triplicated segment in Ts65Dn was fused to mouse chromosome12 (Villar, Belichenko et al. 2005). Although the litter size in Ts2Cje mice is similar to those of Ts65Dn, the frequency of progeny bearing Rb (12.1716) has been reported to be higher than Ts65Dn mice (~43%). Furthermore, both male and female Ts2Cje mice are fertile. In terms of central nervous system changes, there was a significant decrease in spine density on the dendrites of dentate granule cells together with enlarged dendritic spines (~38%) in Ts2Cje mice (Villar, Belichenko et al. 2005), very similar to the changes described in Ts65Dn mice (Belichenko, Masliah et al. 2004). The functional consequences of these changes in Ts2Cje mice are yet to be explored.

Ts1Cje Crossing balanced T(12:16)1Cje to wild type mice produced trisomic mice for a fragment of MMU16 extending from Sod1 to Znf295, but not including

a functional copy of Sod1 (Sago, Carlson et al. 1998; Sago, Carlson et al. 2000). Ts1Cje mice harbor three copies of ~78% of genes triplicated in Ts65Dn mice (Olson, Roper et al. 2004). There are important similarities between Ts1Cje and Ts65Dn mice in regards to CNS structural changes as enlargement of dendritic spines, decreased density of spines, and selective reorganization of inhibitory afferents (Belichenko, Kleschevnikov et al. 2007). Unlike hippocampal changes, no evidence of shrinkage and loss of markers in BFCNs in old Ts1Cje mice have been shown (Salehi, Delcroix et al. 2006). In accordance with this finding, NGF transport in the septo-hippocampal pathway in Ts1Cje mice was significantly increased relative to that in the Ts65Dn mouse suggesting that one or more genes in the segment that distinguishes Ts65Dn and Ts1Cje mice contribute significantly for the dramatic reduction of NGF transport.

Failure in synaptic plasticity as measured by LTP have been reported in the both CA1 area (less severe than Ts65Dn) (Siarey, Villar et al. 2005) and dentate gyrus (Belichenko, Kleschevnikov et al. 2007) of hippocampus in Ts1Cje mice. The T maze spontaneous alternation task is abehavioral paradigm known to reveal dysfunction of the hippocampal system (Gerlai 2001; Lalonde 2002). Comparing Ts65Dn and Ts1Cje mice, both mice differed significantly from that of the corresponding 2N mice (Belichenko, Kleschevnikov et al. 2007). In the context of Morrison water maze, while Ts1Cje mice do show abnormalities in spatial learning and memory, they are less severe than in Ts65Dnmice (Sago, Carlson et al. 1998; Sago, Carlson et al. 2000).

Ms1Ts65Dn Ms1Ts65Dn mice are produced by crossing Ts65Dn mice with T(12:16)1Cje mice (Sago, Carlson et al. 1998; Sago, Carlson et al. 2000). They are segmentally trisomic for the genetic segment from Mrp139 to Sod1, but not including a functional copy of Sod1. Unlike Ts65Dn, which are found to be hyperactive, and Ts1Cje, which are hypoactive, Ms1Ts65Dn mice were found to be normally active. Sago et al. (Sago, Carlson et al. 2000) compared the performance of these mice with Ts65Dn and Ts1Cje mice in the Morrison water maze and found that Ms1Ts65Dn mice did significantly better. In the reverse

probe test (preference score), and unlike Ts65Dn mice, neither Ts1Cje nor MS1Ts65Dn mice showed a significant difference with 2N mice (Sago, Carlson et al. 2000). Ms1Ts65Dn mice have not yet been studied extensively for changes in the structure and function of circuits involved in cognition.

Ts1Rhr Comparing DS patients with segmental trisomy 21 has suggested that triplication of a 5.4 Mb region ofHSA21 called DS critical chromosomal region (DSCR) is associated with various DS phenotypes (Korenberg, Chen et al. 1994). In 2004, Olson et al. (Olson, Roper et al. 2004) reported generation of a new mouse called Ts1Rhr. These mice have a 3.9-Mb duplication of DSCR. This region contains mouse orthologues of the 33 genes in the human DSCR (i.e., around 32% of triplicated genes in Ts65Dn mice), with boundaries at Cbr1 andMx2 genes. Surprisingly, it was found that, unlike Ts65Dn, Ts1Rhr mice do not show a smaller skull or mandible. This indicates that the genes causing facial features of DS are not located inthe DSCR. Relatively a very few studies have focused on brain abnormalities in Ts1Rhr mice. Recently, Aldridge et al. (Aldridge, Reeves et al. 2007) studied brain volume and found a significant (~20%) reduction in the entire brain as well as the cerebellum.

Ts1Rhr/Ts65Dn Using a similar strategy as for Ts1Rhr, Olson et al. (Olson, Richtsmeier et al. 2004) deleted DSCR to produce a mouse trisomic for most of the genes trisomic in Ts65Dn (70%) but with only the two copies of DSCR (Ts1Rhr/Ts65Dn). No results of nervous system function have yet been reported. Macroscopic analysis of Ts1Rhr/Ts65Dn mice showed ~18% reduction in the brain volume and abnormalities in around 80% of linear brain distances (Aldridge, Reeves et al. 2007) indicating a significant changes in the brain shape. Studies on craniofacial features showed that with the exception of brachycephaly, Ts65Dn and Ts1Rhr/Ts65Dn mice have similar craniofacial measurements. This suggests that DSCR is not sufficient to produce the phenotypes present in Ts65Dn. These studies are important because they chart the direction that future

studies will take in examining the contribution of gene segments and individual genes.

Tc1Yu Since Ts65Dn mice have three copies of a segment of MMU16 as well as a small portion of MMU17, Li and collegues (Li, Yu et al. 2007) recently reported generation of another DS mouse model with triplication of a larger segment of MMU16 without any contribution from MMU17. Using Cre/loxP-mediated chromosomal engineering, the Yu laboratory generated stem cells with duplication of MMU16 between D930038D03Rik and Zfp295genes (~22.9 Mb). Only 38% of Tc1Yu mice survive birth and no gross abnormality was detected through at least the age of 10 months. Around 37% of Tc1Yu mice suffer from cardiovascular abnormalities as early as E18.5. Furthermore, gastrointestinal abnormalities e.g., annular pancreas have also been reported. No studies on CNS related phenotypes have yet been reported.

Dp(10)1Yey/+;Dp(16)1Yey/+;Dp(17)1Yey/+ Recently a new mouse DS model that carries duplications spanning the entire Hsa21 syntenic regions on all Mmu10, Mmu16 and Mmu17 mouse chromosomes has been created (Dp(10)1Yey/+;Dp(16)1Yey/+;Dp(17)1Yey/+). This mouse mutant exhibits DS-related neurological defects, including impaired learning/memory and decreased hippocampal LTP (Yu, Li et al. 2010), very similarly to the Ts65Dn mouse. These results suggest that the critical genes associated with the DS brain phenotypes may reside within the Mrpl39–Zfp295 genomic segment of the Mmu16 and **support the use of the Ts65Dn mouse as the best genetic murine DS model**.

2.2.3 Mice trisomic for HSA21

Tc1 An additional category of mouse model for trisomy 21 resulted from the insertion of all or part of Hsa21. The Tc1 trans-species mouse strain, carrying an almost entire Hsa21, was developed using irradiation microcell-mediated chromosome transfer (O'Doherty, Ruf et al. 2005). This model exhibits several key phenotypes of DS (O'Doherty, Ruf et al. 2005; Morice, Andreae et al. 2008;

Galante, Jani et al. 2009). The most obvious CNS morphological changes found in Tc1 mice was a reduction in the density of cerebellar granule neurons in old Tc1 mice. Abnormalities in hippocampal dependent learning and memory as well as decreased LTP have been detected in physiological studies in the dentate gyrus of the hippocampus. Using a novel-object recognition task, Tc1 mice spent significantly shorter times exploring novel objects. Interestingly, no difference was found between Tc1 mice and their control littermates in the spontaneous alternation T-maze; another test of hippocampal-related cognition (O'Doherty, Ruf et al. 2005). One short coming of this model is a high degree of mosaicism within tissues. For instance, only approximatelyone-third of brain cells was found tocarry HSA21. Examining brain phenotypes will require careful attention to which cells maintain HSA21 and which do not. Furthermore, effective transmission of HSA21 is possible only by out crossing to achieve a mixed strain background (O'Doherty, Ruf et al. 2005). Thesefindings are evidence for the propensity of mouse cells to eliminate HSA21. Finally, the consequences of expression of a large number of human proteins in mouse micro-environment must be taken into account in interpreting the phenotypes detected.

2.2.4 Transgenic mice with triplication of individual genes

Several transgenic mice that express from one or a few genes to entire segments of HSA21 (YAC transgenic mice) have been produced. As expected, none of these recapitulates the entire phenotype of DS, but many have features that are reminiscent of aspects of the phenotype. Transgenic mice containing the human Cu-Zn Superoxide dismutase 1 (*SOD1)* gene were the first mice produced to contain a gene encoded on HSA21 (Epstein, Avraham et al. 1987). These mice have some features that are similar to those seen in people with DS (Avraham, Sugarman et al. 1991; Gahtan, Auerbach et al. 1998). Many investigators have produced transgenic mice that express normal and mutant forms of beta-amyloid peptide (*APP)*, and have demonstrated learning and memory deficits in these mice, including performance decline with age. In a recent experiment, compound transgenic mice that express human *SOD1* and *APP* were created (Harris-Cerruti,

Kamsler et al. 2004). Working memory and long-term memory are severely impaired in these double transgenic mice; they have defects in *APP* processing, lipofuscin accumulation and mitochondrial anomalies. These findings are intriguing in light of the increasing evidence for a link between mitochondrial dysfunction, oxidative stress, *APP* processing and DS (Busciglio, Pelsman et al. 2002). Other genes that cause phenotypes that are relevant to DS when overexpressed in transgenic mice include *PFKL* (Peled-Kamar 1998), the Drosophila minibrain homolog gene (*DYRK1A*) (Dierssen, Ortiz-Abalia et al. 2006), the neurotrophic factor *S100* β (Krapfenbauer, Engidawork et al. 2003), the transcription factor *ETS2* (Wolvetang, Wilson et al. 2003) and the transcription factor single-minded *(SIM2)* (Chrast, Scott et al. 2000). Sim2 is overexpressed in Ts1Cje mouse fetuses (Vialard, Toyama et al. 2000) and in trisomic tissues (Lyle, Gehrig et al. 2004).Variations of SIM2 expression level were found in the cerebellum, cortical layers, and hippocampus (Rachidi, Lopes et al. 2005), key human brain regions involved in learning and memory that also are altered in DS patients (Raz, Torres et al. 1995; Pinter, Eliez et al. 2001; Rachidi, Lopes et al. 2005). The transgenic mice overexpressing Sim2 (Tg Sim2) display reduced sensitivity to pain and mild impairment of learning (Ema, Ikegami et al. 1999; Chrast, Scott et al. 2000). These behavioral anomalies found in the Sim2 transgenic mice remembered some phenotypes observed in trisomic mouse models for DS, Ts65Dn and Ts1Cje (Coussons-Read and Crnic 1996; Sago, Carlson et al. 1998; Martinez-Cue, Baamonde et al. 1999) and in DS patients (Hennequin, Allison et al. 2005).

Two transgenic mouse models overexpressing DYRK1A have been generated. The first one, carried a human YAC 152F7, containing DYRK1A (Smith, Stevens et al. 1997); the second, carried the full-length DYRK1A cDNA (Altafaj, Dierssen et al. 2001). In the Morris water test, the transgenic lines carrying the YAC 152F7 showed lower performance in the probe test, in which the platform is removed. In the reverse learning paradigm, the transgenic mice showed the most severe deficits with no significant learning of the new platform position, indicating deficits in learning flexibility. Mouse line carrying a 152F7 YAC

fragment (152F7tel) containing only the DYRK1A gene, showed the same phenotype than the original YAC lines, indicating that the correct dosage of DYRK1A gene is crucial for brain function, learning and memory (Smith, Stevens et al. 1997). The two models show a significant impairment in spatial learning and memory, indicating hippocampal and prefrontal cortex function alteration. These transgenic mice showed increased brain weight and neuronal size (Branchi, Bichler et al. 2004) and dysfunction of reference memory (Smith, Stevens et al. 1997; Altafaj, Dierssen et al. 2001). They also exhibit neurodevelopmental defects, delayed craniocaudal maturation and motor dysfunction (Altafaj, Dierssen et al. 2001). All these alterations are comparable with those found in murine models of DS with trisomy of chromosome 16, and suggest a causative role of DYRK1A in mental retardation and motor anomalies in DS patients. Moreover, DYRK1A-deficient mice have defects in central nervous system development, and DYRK1A appears sensitive to gene dosage as heterozygous mutant mice show limited changes in central nervous system development (Fotaki, Dierssen et al. 2002). The YAC 230E8 mouse lines showed altered density of neurons in cerebral cortex (Rachidi, Lopes et al. 2005). This abnormal density of neurons may explain the learning and memory deficits of these animals in the Morris water test (Smith, Stevens et al. 1997).The YAC 230E8 contains the DOPEY2 gene, a new member of the Dopey family involved in morphogenesis that has been suggested as a candidate gene for the neurological alterations and mental retardation observed in these mice (Rachidi, Lopes et al. 2005). Ideally, transgenic mouse models of DS would contain a single extra copy of the relevant gene and regulatory elements that are similar enough to wild-type genes to assure typical spatio-temporal patterns of expression. These conditions are rarely achieved (Patterson and Costa 2005). Also, most transgenic mice contain human transgenes, and these might not be appropriately expressed in mice. Nonetheless, it is remarkable that these individual transgenes, and sometimes combinations of transgenes, have phenotypes that are reminiscent of DS. Construction of more transgenic mice seems well justified (Patterson and Costa 2005).

2.2.5 Segmental trisomic mice disomic for individual genes

Ts65Dn: App+/+/-. One critical question in understandingthe biology of DS is whether individual genes play a significant role in creating important phenotypes. To address this, Epstein et al. (Epstein, Berger et al. 1990) generated Ts65Dn mice with the normal two copies of specific genes (e.g., amyloidprecursor protein, App). This was achieved by breeding Ts65Dn female swith males in which one copy of the App gene was deleted. Ts65Dn: App+/+/- are viable and have no gross nervous system abnormalities with normal life span (Cataldo, Petanceska et al. 2003). Cataldo et al. (Cataldo, Petanceska et al. 2003) studied the endosomal system in Ts65Dn mice (with three copies of App) and found enlarged early endosomes in the basal forebrain as early as 2 months. Interestingly, these changes were not found in Ts65Dn mice withonly two copies of App. Significantly, they did not detect changes in endosomes in mice that are transgenic for mutant App. However, a recent report has documented an increase in early endosomes size and number (Laifenfeld, Patzek et al. 2007). Deleting one copy of App markedly improved NGF retrograde transport in Ts65Dn mice, reaching a level similar to that seen in Ts1Cje mice. Morphological analysis of BFCNs revealed that the increase in NGF retrograde transport was accompanied by a significant improvement in BFCN morphology in the medial septal nucleus (MSN). Indeed, this parameter did not distinguish the Ts65Dn: App+/+/- mice from their 2N controls (Salehi et al., 2006).

2.3 NEUROGENESIS ALTERATIONS IN DS ANIMAL MODELS

During the last few years various groups have tackled the issue of neurogenesis impairment in the DS brain by taking advantage of various mouse models.

In Ts16 mice the number of precursors of the future somatosensory cortex just before neuronogenesis begins, is notably reduced. At each cell cycle during neuronogenesis, a smaller proportion of Ts16 progenitors exit the cell cycle and the cell cycle duration is longer in Ts16 than in euploid progenitors (Haydar,

Nowakowski et al. 2000). In the Ts65Dn mouse a reduced proliferation has been detected at all examined life stages. Reduced neural precursor proliferation is already present during embryonic development in the hippocampal region and VZ, with a reduction in the number of neocortical and hippocampal neurons (Chakrabarti, Galdzicki et al. 2007). The reduced neurogenesis appears to be due to elongation of the cell cycle. Interestingly, the neurogenesis defects of Ts65Dn mice lead to an imbalance between production of excitatory and inhibitory neurons In particular, Chakrabarty et al. found that the medial ganglionic eminence (MGE) (which gives origin to inhibitory neurons) of Ts65Dn mice at E14.5 undergoes divisions at a normal rate but gives a higher neuronal output due to a large progenitor population. The large output from the MGE explains the observation that in the neocortex and hippocampus of neonate and young Ts65Dn mice there are fewer excitatory but more inhibitory neurons (Chakrabarti, Best et al. 2010). The excessive production of inhibitory interneurons is due to over-expression of the triplicated genes Olig1 and Olig2, as normalization of their expression rescues the Ts65Dn phenotype. In Ts65Dn mice, the postnatal SVZ of the lateral ventricle, which is the largest neurogenic area of the adult brain, exhibits a remarkably reduced proliferation rate that starts in the perinatal period and continues up to senescence (Bianchi, Ciani et al. 2010; Bianchi, Ciani et al. 2010; Trazzi, Mitrugno et al. 2011). A severe neurogenesis reduction has been also documented in the embryonic neocortex and VZ and in the SVZ of adult Ts1Cje and Ts2Cje mice (Ishihara, Amano et al. 2010). In the adult Ts1Cje SVZ trisomy does not appear to affect the number of neural stem cells but results in reduced numbers of neural progenitors and neuroblasts (Hewitt, Ling et al. 2010). Analysis of differentiating Ts1Cje neural progenitors shows a severe reduction in number of produced neurons, whilst the number of astrocytes is increased (Hewitt, Ling et al. 2010).

A severe neurogenesis impairment characterizes the DG of the Ts65Dn mouse at all examined life stages (Insausti, Megias et al. 1998; Lorenzi and Reeves 2006; Contestabile, Fila et al. 2007; Bianchi, Ciani et al. 2010). The study of the phenotype acquired by differentiating neural progenitor shows a reduction in the

number of new neurons with an increase in the number of astrocytes (Contestabile, Fila et al. 2007; Bianchi, Ciani et al. 2010). A severe neurogenesis reduction has been documented also in the DG of adult (3 months) Ts1Cje and Ts2Cje mice (Ishihara, Amano et al. 2010).

Neurogenesis in the EGL of the cerebellum, A reduced proliferation of cerebellar granule cell precursors in the EGL of Ts65Dn mice has been documented at P0 and P6 (Roper, Baxter et al. 2006). A reduced proliferation has been confirmed in P2 Ts65Dn mice, linked to elongation of the cell cycle (Contestabile, Fila et al. 2008). Moreover, in Ts65Dn mice the percentage of newborn cells that acquire a neuronal phenotype is smaller vs. euploid mice, while the percentage of cells that acquire an astrocytic phenotyoe is larger (Contestabile, Fila et al. 2008).

The widespread neurogenesis impairment observed in animal models and fetuses with DS during critical developmental stages strongly suggests that this defects may be a major determinant of the reduced brain size and, hence, of mental retardation. The proliferation impairment is worsened by reduction in neuronogenesis, (with a reduction in the production of excitatory neurons and an increase in the production of inhibitory neurons) and a relative increase in gliogenesis. Thus, both regulation of cell proliferation and differentiation are disrupted in the DS brain.

2.4 MOLECULAR MECHANISMS UNDERLYING NEUROGENESIS IMPAIMENT IN DS

A recent observation supports the possibility that Dyrk1a over-expression may be responsible for the generation of an inadequate number of neurons in cortical structures of the brain (Yabut, Domogauer et al.). *DYRK1A* is located within the DS critical region (Song, Sternberg et al. 1996), and is overexpressed in the DS brain (Guimera, Casas et al. 1999). Several studies using transgenicmice have demonstrated that Dyrk1a over-expression leads to cognitive impairment (Altafaj, Dierssen et al. 2001; Branchi, Bichler et al. 2004). Recently, it was

demonstratedthat Dyrk1a over-expression inhibits proliferation and inducespremature neuronal differentiation of neural progenitor cellsin the developing mouse cerebral cortex (Yabut, Domogauer et al.). Specifically, Dyrk1a over-expression probably inhibits cell cycle progression through nuclear export and degradation of cyclin D1 in neurogenic neuroepitheli. Another very recent report has shown that over-expression of Dyrk1a induces impaired G_1/G_0–S phase transition in immortalized rat hippocampal progenitor cells (de la Monte, Ng et al.). The proposed mechanism is mediated by the phosphorylation of p53, which leads to the induction of p21CIP1. In terms of the possible role of Dyrk1a in neuronal differentiation, a recent report shows that the interaction and phosphorylation of the intracellular domain of Notch by Dyrk1a attenuates Notch signaling in transfected neural cell lines (Fernandez-Martinez, Vela et al. 2009). Since during neurogenesis, the cells in which Notch signaling is activated remain as progenitors, whereas those in which Notch activity diminishes differentiate into neurons (Louvi and Artavanis-Tsakonas 2006), it can be hypothesized that Dyrk1a may regulate the onset of neuronal differentiation by inhibiting Notch signaling. In addition, the capacity of Dyrk1a to interact with and⁄or modulate different signaling pathways (EGF, FGF, NGF, Shh, NFAT, etc.) (Tejedor, Zhu et al.), supports a key role played by Dyrk1a in coordinating neural proliferation/differentiation.

Very recently, Chakrabarti et al. (Chakrabarti, Best et al. 2010) have shown that proper expression of two genes, *Olig1* and *Olig2*, is essential during embryonic development for regulating the numbers of inhibitory neurons, a type of neuron that controls signaling in the brain. The authors found in the forebrain of Ts65Dn mice a complex set of neurogenesis defects that led to an imbalance between excitatory and inhibitory neurons and to increased inhibitory drive. Alterations in the number of neuronsas well as in the timing of differentiation is likely to lead to theformation of abnormal cortical circuits and, consequently, to thecognitive deficits observed in transgenic mice and DS patients. The combined restoration of *Olig1* and *Olig2* to disomy in Ts65Ds embryos rescued the abnormal neurogenesis responsible for the increased interneuron phenotype and restored

spontaneous inhibitory postsynaptic currents in CA1 pyramidal neurons (Chakrabarti, Best et al. 2010), supporting a re-establishment of normal neuronal function. This is in accordance with a recent study where the pharmacological block of the hyperactivity of inhibitory interneurons rescues some of the learning and electrophysiological deficits present in this mouse model (Fernandez and Garner 2007).

It has been suggested that amyloid precursor protein (APP) over-expression may result in enhancement of the Notch signaling pathway (Fischer, van Dijk et al. 2005), which is crucial for the acquisition of a glial phenotype (Morrison, Perez et al. 2000). Indeed, the expression of Notch1 is up-regulated in the cortex of DS and AD patients and in DS fibroblasts (Fischer, van Dijk et al. 2005), indicating that enhanced APP processing interferes with Notch signaling and probably affects phenotype acquisition during brain development.

2.5 APP GENE AND NEUROGENESIS

2.5.1 The human gene APP

Human APP belongs to a highly conserved family of type 1 transmembrane glycoproteins which constitutes APP and the mammalian homologs APLP-1 and APLP-2, both homologs lacking the Aβ sequence (Goldgaber, Lerman et al. 1987; Wasco, Gurubhagavatula et al. 1993). The evolutionary conservation of APP gene family also extends to invertebrate species with its orthologs APPL in Drosophila and APL-1 in Caenorhabditis elegans respectively (Daigle and Li 1993). These proteins all share several conserved motifs within the large extracellular domain and a short cytoplasmic region which exhibits the highest sequence homology (Gralle and Ferreira 2007). The human APP gene contains 18 exons spanning more than 170 kbp. The region encoding the Aβ sequence comprises part of exons 16 and 17 and is composed of 40 to 43 amino acid residues that extend from the ectodomain into the transmembrane domain of this protein (Fig. 7). The presence of multiple distinct domains located within the extracellular portion includes a signal peptide (SP), a heparin-binding/growth-factor-like domain 1 (HPBD1), a copper binding domain (CuBD), a zinc binding domain (ZnBD), a Kunitz-type

protease inhibitor domain (KPI), a second heparin-binding domain 2 (HPBD2), a random coil region (RC) and the Aβ sequence (Fig. 7). The remaining region consists of the cytoplasmic tail of APP, including its intracellular domain, AICD. Several isoforms of APP that arises from alternative splicing have been identified and the most common forms differ mainly by the absence (APP-695) or presence (alternatively spliced APP-751 and APP-770) of a KPI domain (Kitaguchi, Takahashi et al. 1988; Konig, Monning et al. 1992).

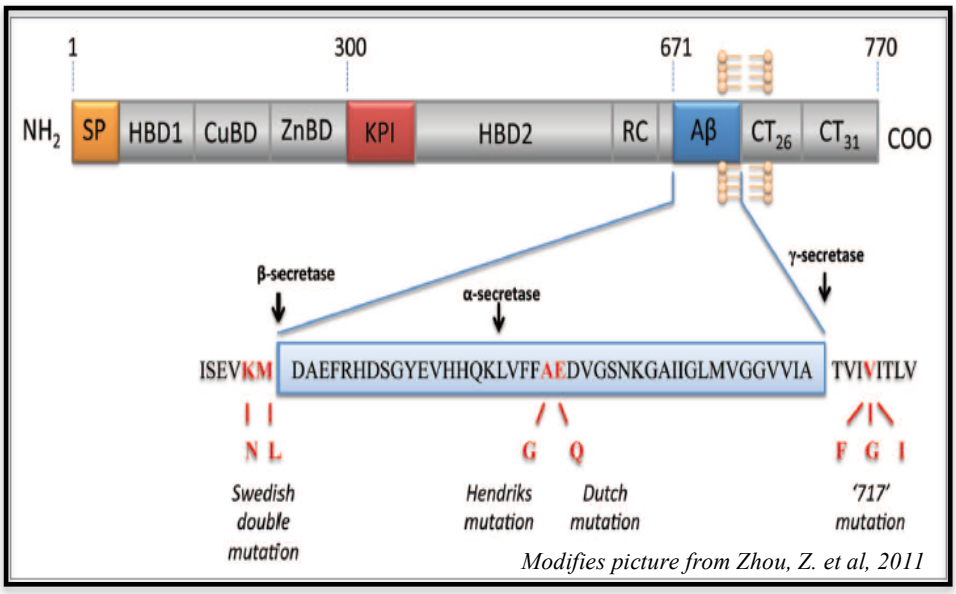


Figure 7: *Schematic diagram of APP consisting of a large extracellular domain, a hydrophobic transmembrane domain and a short cytoplasmic carboxyl terminus. The protein is proteolytically processed by different secretases via amyloidgenic and non-amyloidgenic proceeding pathways which either releases the Aβ peptide (cleaved by β- and γ-*

2.5.2 Proteolytic processing of APP

APP can undergo amyloidogenic or non-amyloidogenic processing via cleavage by different secretases (Ling, Morgan et al. 2003). The amyloidogenic processing of APP, cleaved initially by β-secretase, produces a long soluble secreted form of APP (sAPPβ) and a carboxy-terminal fragment (CTF99) containing the Aβ sequence and AICD (Fig. 8). In the brain, β-site APP cleaving enzyme (BACE1) has been found to be the major β-secretase (Vassar, Bennett et al. 1999). In the

alternative non-amyloidogenic pathway, APP could also be proteolytically processed by a presenilin-containing α-secretase complex, which cleaves at a site within Aβ sequence and consequently abrogates Aβ formation (Ling, Morgan et al. 2003). The non-amyloidogenic cleavage releases a carboxy-terminal fragment (CTF83) and another soluble fragment (sAPPα) which, in contrast to Aβ, may be neuroprotective (Furukawa, Sopher et al. 1996; Mattson 1997; Han, Dou et al. 2005). Both CTF99 and CTF83 fragments are then sequentially cleaved within the transmembrane domain by γ -secretase to generate AICD and Aβ or p3 respectively (Chow, Mattson et al. 2010). Elevated β -secretase levels were found to induce the increase of CTF99 and Aβ generation as well as the decrease of CTF83 and AICD generation in vitro. On the contrary, elevated levels of α -secretase was found to induce an increase in AICD levels (Kume, Maruyama et al. 2004). Therefore α -secretase and β –secretase cleavage of APP might influence subsequent AICD release differently. The details of amyloidogenic and non-amyloidogenic processing of APP are illustrated in Fig. 8.

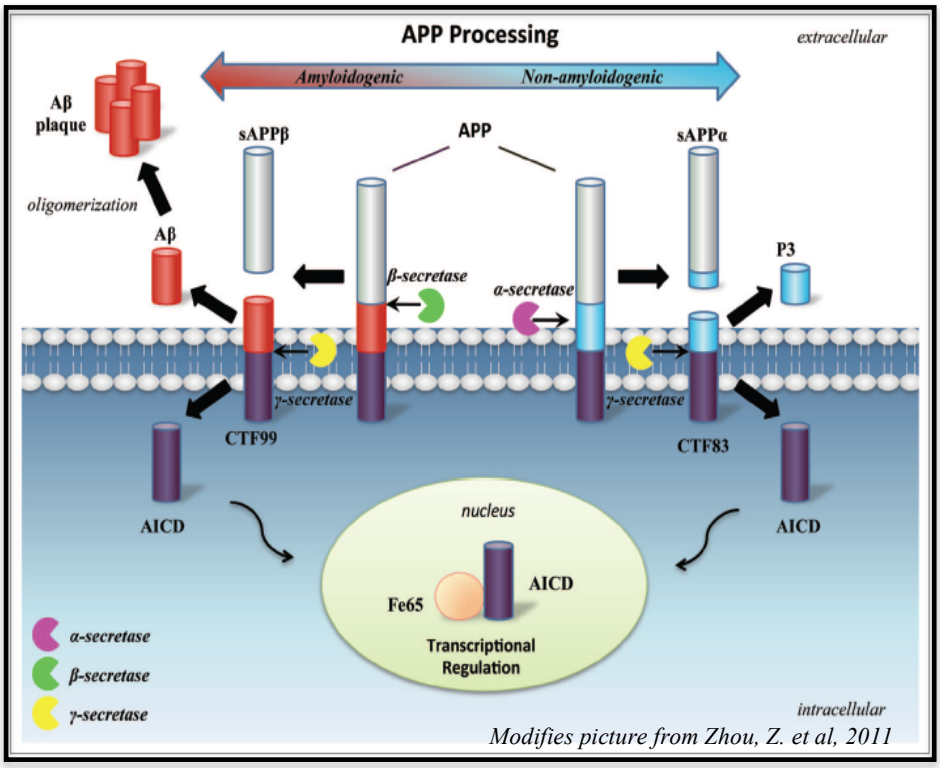


Figure 8: APP processing procedure and cleavage products. *The non-amyloidogenic APP processing pathway (right) involves proteolytic cleavages by α- and γ-secretases resulting in the generation of sAPPα and carboxyl terminal fragments including P3, CTF83 and AICD. The alternative amyloidogenic APP processing pathway (left) involves proteolytic cleavages by β- and γ-secretases resulting in the generation of sAPPβ and carboxyl terminal fragments including Aβ, CTF99 and and AICD. Aβ peptides could oligomerize and fibrillize leading to AD pathology. sAPPα could function to promote neurogenesis and survival, while AICD could have effects to inhibit neurogenesis possibly via forming complex with Fe65 and leading to transcriptional regulation.*

2.5.3 Role of APP in neurogenesis

Recent accumulative evidence demonstrated that APP is important for neuron generation, neuron differentiation and neural migration. In nematode *Caenorhabditis elegans*, loss of APL-1 by genetic inactivation resulted in postnatal lethality due to abnormalities in multiple developmental processes such as molting defects. This phenotype could be successfully rescued by expressing the extracellular domain of APL-1 in neurons (Hornsten, Lieberthal et al. 2007). Furthermore APP was found to be important in *Drosophila melanogaster*, as deletion of the APPL gene leads to behavioral defects in phototaxis that could be

partially rescued by human APP (Luo, Tully et al. 1992). Interestingly, highly elevated APPL levels were observed in regenerating neurons of a Drosophila brain injury model (Leyssen, Ayaz et al. 2005). In contrast, lack of this stress response in APPL mutant flies increased mortality (Vassar, Bennett et al. 1999). As an upregulation of APPL correlated with an increase in neurite arborization, a potential role in axonal outgrowth after traumatic brain damage was attributed to APP (Leyssen, Ayaz et al. 2005). Another study showed that APPL overexpression promoted synapse differentiation, while APPL mutants resulted in decreased synaptic bouton numbers at the neuromuscular junction in Drosophila (Torroja, Packard et al. 1999). APP is also ubiquitously expressed in mammalian cells and was found to have complicated physiological roles in cell adhesion, neuronal differentiation, neuronal migration, neurite outgrowth and synapse formation (Breen, Bruce et al. 1991; Young-Pearse, Bai et al. 2007). The immunoreactivity of APP was found to increase after brain injury of mice, which correlated well with traumatic brain injury (Johnson, Stewart et al. 2010). APP knockout mice showed reductions in weight, deficits in balance and strength, impairments in behavior and long-term potentiation (Zheng, Jiang et al. 1995; Dawson, Seabrook et al. 1999). The evidence from other APP knockout in vivo animal model systems demonstrated potential roles of APP in neuron generation, differentiation as well as neural migration (Bergmans, Shariati et al. 2010). **Taken together, these findings corroborate a potential crucial role for APP as part of a complex mechanism involved in a wide variety of neuronal functions, including normal neural development or response to traumatic brain injuries.** Cumulative evidence suggests that the soluble sAPPα is neuroprotective and is associated with growth factor-like functions, while the interaction of AICD with a myriad of proteins links it with diverse processes such as axonal transport and transcriptional regulation.

2.5.4 Role of sAPPα in the positive regulation of neurogenesis

The physiological functions of sAPPα have been implicated in the enhancement of synaptogenesis, neurite outgrowth, cell survival and cell adhesion (Mattson

1997; Gakhar-Koppole, Hundeshagen et al. 2008). In separate reports, sAPPα has been observed to exert proliferative effects on NPC isolated from the embryonic brains (Ohsawa, Takamura et al. 1999; Caille, Allinquant et al. 2004). In 2005, Caille et al. first acquired evidence suggesting the in vivo role of sAPPα in adult neurogenesis (Caille, Allinquant et al. 2004). The authors found that sAPPα binds prominently to cells of the subventricular zone (SVZ), one of the two adult central nervous system sites harboring NPC that are capable of regeneration in the adult brain (Caille, Allinquant et al. 2004). Their findings suggested that sAPPα were likely to participate in the EGF-induced proliferation of type A cells, although sAPPα alone fails to induce proliferation of these cells (Caille, Allinquant et al. 2004). The authors also observed that infusion of sAPPα into the lateral ventricle of mice led to an increase in number of progenitor cells (Caille, Allinquant et al. 2004). Conversely, blocking sAPPα secretion by α-secretase inhibitor or downregulating APP synthesis by antisense oligonucleotide against APP decreases the proliferation of EGF responsive cells, which leads to a reduction of the pool of progenitors (Caille, Allinquant et al. 2004). Their results also showed that sAPPα activity may be delivered in an autocrine/paracrine manner (Caille, Allinquant et al. 2004). The crystal structure analysis at 1.8 Å resolution of APP further demonstrated that its cysteine-rich N-terminal heparin-binding domain is similar to other cysteine-rich growth factors, which is conceived to be responsible for its function to stimulate neurite outgrowth (Rossjohn, Cappai et al. 1999). These growth-promoting properties of sAPPs and its structural similarities with cysteine-rich growth factors suggest that sAPPs may function as a growth factor in vivo (Rossjohn, Cappai et al. 1999). Early in vitro studies have also demonstrated that sAPPα protects cultured neurons against hypoglycemia damage and glutamate neurotoxicity, through the activation of potassium channels which in turn mediates the ability of sAPPα to inhibit calcium influx and thus modulates neuronal excitability (Furukawa, Sopher et al. 1996), (Mattson, Cheng et al. 1993). Taken together, these results suggest that sAPPα might function as specific growth factors or as a mediator for adult NPC proliferation. However, to date, no sAPPα receptors have been identified yet and the signaling pathways triggered

have not been thoroughly investigated. To this extent, it is of interest to note that two in vitro studies have reported a stimulation of MAP kinase activity by sAPPα and it would thus be of importance to dissect this signaling pathway triggered by sAPPα in detail (Yogev-Falach, Amit et al. 2002; Youdim, Amit et al. 2003). Intriguingly, sAPPα levels were shown to decrease in the cerebrospinal fluid (CSF) of AD individuals, while infusion of sAPPα into the brain increased synaptic density and improved memory retention (Meziane, Dodart et al. 1998). Therefore, these findings raised the possibility that sAPPα may contribute to neurogenesis in adult brain and sAPPα might be used for AD patients clinically, while decrease of sAPPα levels in brain may be an indispensable precondition for AD pathogenesis.

2.5.5 Role of AICD in the negative modulation of neurogenesis

AICD was termed by analogy to NICD (Notch intracellular domain) formed by the regulated intramembrane proteolysis (RIP) of another type I transmembrane glycoprotein Notch. Both AICD and NICD were produced via cleavage of APP or Notch by the same γ-secretase complex respectively. Extracellular binding of Notch to its ligand is one of the mechanisms responsible for this regulation of cleavage, stimulating release of NICD in cells (Selkoe and Kopan 2003).The NICD translocates into the nucleus and leads to a series of downstream signaling cascades.

Although multiple proteins have been reported to interact with AICD including Fe65 that may be necessary for AICD-dependent signaling, no functional ligands for APP have been characterized so far (Ma, Futagawa et al. 2008). Recently Ma et al. discovered that transient axonal glycoprotein 1 (TAG1), a neural cell adhesion molecule of the F3 family, acts as an extracellular binding partner for APP through the immunoglobulin (Ig) and fibronectin repeat (FNIII) domains of TAG1 (Ma, Futagawa et al. 2008). The author found that TAG1 and APP co-expressed in NPC in the neurogenic niche of the ventricular zone in developing mouse brains (Ma, Futagawa et al. 2008). It was also found that the extracellular interaction between APP and TAG1 was essential for initiating the release of

AICD which could be abrogated by the presence of specific γ-secretase inhibitors (Ma, Futagawa et al. 2008). It was further confirmed in knockout in vivo studies that the interaction between TAG1 and APP negatively modulates neurogenesis through release of AICD and triggers a Fe65-dependent molecular event (Ma, Futagawa et al. 2008). These findings provided valuable insights that APP could function as a transmembrane receptor protein which negatively mediates neurogenesis through recognition of its specific cell surface-associated ligands (Ma, Futagawa et al. 2008).

However, the detailed mechanism by which AICD suppresses neurogenesis still remains to be elucidated. Several questions regarding TAG1-APP signaling pathway including its potential contributory roles in adult brain development and AD pathogenesis also remain unanswered. What physiological functions does this signaling pathway eventually mediate? As yet, few immediate downstream target genes have been identified for AICD and Fe65. Based on the resemblance of molecular structure and processing procedure between Notch and APP as well as known knowledge of the Notch cascade, it would be tempting to speculate that the AICD generated by γ-secretase cleavage may be capable of inducing an intracellular signaling pathway via modulationof gene expression after interaction with its adaptor protein Fe65. The interaction between AICD and Fe65 may promote the translocation of AICD directly to the nucleus or may initiate a Fe65-mediated nuclear signal independently of AICD translocation. However, the hypothesis that AICD could function to modulate transcriptional activity in cells appears highly controversial so far. Although numerous studies have suggested that AICD can regulate expression of various endogenous genes including KAI1, GSK-3b, APP and neprilysin, other groups were unable to replicate these findings (Ryan and Pimplikar 2005; Hebert, Serneels et al. 2006). To this extend, it is interesting to note that several recent studieshave reported AICD-expressing transgenic mice recapitulate AD-like pathological features as activation of GSK-3β, phosphorylationand aggregation of tau, memory deficits and aberrant neural activity and seizure susceptibility (Ryan and Pimplikar 2005; Vogt, Thomas et al. 2011). **The authors showed that overexpressed AICD impairs adult**

neurogenesis intransgenic mice through induction of neuroinflammation, which could be prevented by treatment with anti-inflammatory drugs (Vogt, Thomas et al. 2011). However although inflammation may play a pivotal role in impaired neurogenesis in AICD transgene mice, abnormally expressed AICD may also confer its deleterious effects via stimulation of GSK-3β activity and alteration of the activity of Wnt signaling pathway, which has been shown to perturb neurogenesis in mice and in AD patients (Goodger, Rajendran et al. 2009). This hypothesisties in with previous studies suggesting that AICD is capable of regulating the expression of various endogenous genes including GSK-3β (Ryan and Pimplikar 2005). In contrast, other groups were unable to replicate these findings (Aydin, Filippov et al.; Hebert, Serneels et al. 2006). **Therefore a conclusive understanding of the APP signaling pathway related to regulation of neurogenesis by AICD would be necessary** as it may offer unique opportunitiesfor pharmacological intervention of AD in the future.
The influence of neurogenesis by APP fragments was illustrated in Figure 9.

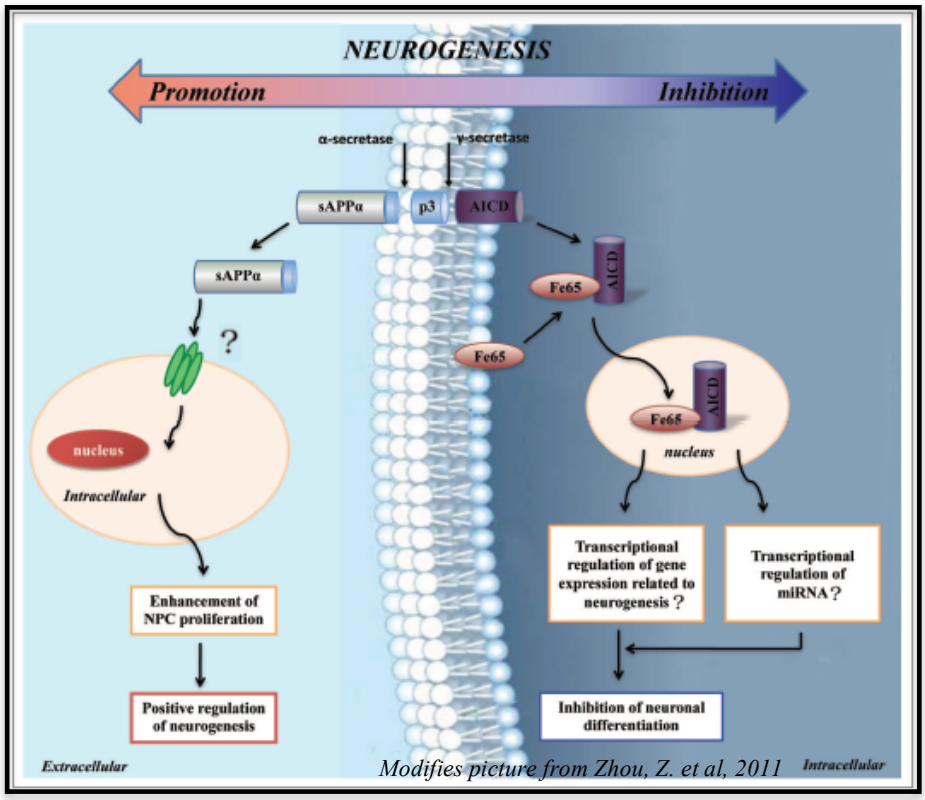


Figure 9: Schematic diagram for influence of neurogenesis by sAPPa and AICD. *The α secretases cleavage of APP could release sAPPa into extracellular space, which could function on cells possibly via binding with its specific receptors. The sAPPa could function to promote NPC proliferation and positively regulate neurogenesis. On the contrary, g-secretases cleavage of APP could release AICD into cytoplasm, which could complex with Fe65 and enter nucleus. The AICD and Fe65 complex might function to transcriptionally regulate gene expressions related to neurogenesis, including miRNAs genes. Finally AICD will function to inhibit neuronal differentiation via so far unknown mechanisms, hereby negatively regulate neurogenesis.*

2.5.6 APP and DS

Over-expression of several genes localized in chromosome 21 has been linked to neuronal death, including APP (Busciglio, Pelsman et al. 2002). Fibrillar amyloid beta-protein (Aβ) is a major component of amyloid plaques in the brains of individuals with AD and of adults with DS. Some effects of Aβ are linked to oxidative stress-dependent apoptosis. Tamagno et al. (Tamagno, Robino et al.

2003) proposed the existence of a sequence of events in Aβ-induced apoptosis, involving simultaneous generation of a lipid-peroxidation products such as 4-hydroxynonenal, H_2O_2, and oxidative stress-dependent activation of c-Jun aminoterminal kinase (JNK) and the mitogen-activated protein kinase p38 (MAPK) (Fig. 10). On its way to deposition in the brain, Aβ induces oxidative changes causing nerve cell insults. Increasing evidence indicate that Aβ overproduction, besides inducing extensive amyloidosis, possibly triggers early cognitive impairment in dementia in DS by altering synaptic activity (Conti and Cattaneo 2005; Gasparini and Dityatev 2008). In fact, Aβ aggregated oligomeric species profoundly affect synaptic plasticity of excitatory synapses by blocking LTP in vitro and in vivo (Lambert, Barlow et al. 1998; Walsh, Klyubin et al. 2002; Wang, Pasternak et al. 2002; Townsend, Shankar et al. 2006; Shankar, Li et al. 2008) and enhancing LTD (Li, Hong et al. 2009) via down-regulation of NMDARs and inhibition of glutamate reuptake (Li, Hong et al. 2009). Fibrillar oligomeric Aβ species have been detected within thioflavine S-negative diffuse Aβ plaques in AD and DS brain (Sarsoza, Saing et al. 2009). Importantly, it has been shown that amyloid dimers isolated from AD brain impair synaptic plasticity and reduce dendritic spine density in rodent hippocampus (Shankar, Li et al. 2008). However, further investigation is needed to ascertain whether similar Aβ soluble aggregates contribute to alteration of synaptic activity in DS. βAPP upregulation is also causally linked to BFCN (basal forebrain cholinergic neurons) degeneration in trisomic mice. It has been demonstrated that βAPP overexpressionin Ts65Dn mice impairs the retrograde transport of nerve growth factor (NGF) from the hippocampus to the basal forebrain, causing degeneration of BFCN (Salehi, Delcroix et al. 2006) through axonal mechanisms resulting in enlargement of early endosomes and βAPP overload (Cataldo, Petanceska et al. 2003; Chang and Gold 2008) (Salehi, Delcroix et al. 2006). Indeed, the degenerative phenotype is reversed by NGF infusion (Cooper, Salehi et al. 2001). Ts1Cje mice, which are trisomic for a smaller MMU16 segment missing βAPP, lack any evident BFCN degeneration (Cataldo, Petanceska et al. 2003; Salehi, Delcroix et al. 2003, Salehi, 2006 #2403). BFCN degeneration and endosomes

abnormalities are also prevented by restoring βAPP gene dosage to two copies in Ts65Dn mice (Cataldo, Petanceska et al. 2003; Salehi, Delcroix et al. 2006), suggesting that βAPP is one of the dosage sensitive gene the orized by the ‘‘dosage imbalance hypothesis’’ and is the main responsiblefor AD pathology in DS. Aβ overproduction may also contribute to the development of tau inclusions. In fact, it has been shown that the formation of cerebral tau aggregates is accelerated by injecting Aβ in the brainof tau transgenic mice or by crossing mice bearing human mutated tau with mutant βAPP overexpressing mice (Gotz, Chen et al. 2001; Lewis, Dickson et al. 2001). Finally, βAPP-mediated pathological mechanisms may also occur during development. It has been suggested that βAPP overexpression may result in increased Notch signalling pathway, which is crucial for neuronal and glial differentiation (Fischer, van Dijk et al. 2005). Indeed, the expression of Notch1, Dll1 (delta-like 1) and Hes1 (hairy and enhanced of split 1) is up-regulated in the cortex of DS and AD patients and in DS fibroblasts (Fischer, van Dijk et al. 2005), indicating that enhanced βAPP processing interferes with Notch signalling. **Importantly, emerging evidence indicates that βAPP and its secreted ectodomain βAPPs play a crucial role in regulating the proliferation of neural progenitors during development (Chen and Tang 2006) and adulthood (Conti and Cattaneo 2005), strogly suggest a potentiallink between βAPP overexpression and altered brain developmentin DS.**

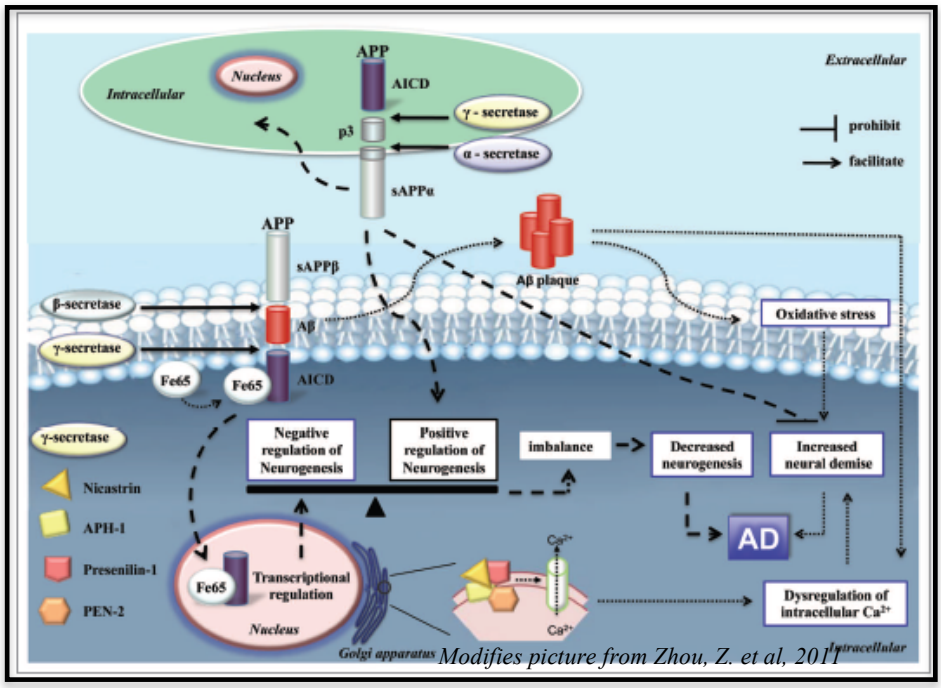


Figure 10: *The pathogenesis of AD involving Aβ induced toxicity via increased ROS and dysregulation of intracellular calcium as well as altered neurogenesis due to imbalance between positive regulation of neurogenesis by sAPPα and negative regulation of neurogensis by AICD.*

2.6 HEDGEHOG SIGNALING

The Hedgehog (Hh) signaling, through its family of secreted proteins, governs a wide variety of processes during embryonic development and adult tissue homeostasis. Hedgehog (hh) was initially discovered by Christiane Nusslein-Volhard and Eric Wieschaus nearly 30 years ago as a ‘‘segmentpolarity’’ gene that controls Drosophila embryonic cuticle pattern (Nusslein-Volhard and Wieschaus 1980). Since the molecular cloning of hh and the discovery of its vertebrate counterparts in the early 1990s, enormous progress has been made in revealing the role of Hh signaling in development and disease as well as the molecular underpinning of the Hh signaling cascade. It is well known that Hh signaling is not only important in fruit flies to pattern their embryonic cuticles and adult appendages, but vital for diverse aspects of animal development and essential in humans to regulate cell fate and number in their brains and spinal

cords, to pattern their limbs and internal organs, and even to control their body heights (Ingham and McMahon 2001; Weedon and Frayling 2008). Recent studies have also implicated Hh signaling as essential for stem cell maintenance (Beachy, Karhadkar et al. 2004). Not surprisingly, malfunction of Hh signaling contributes to numerous human disorders including birth defects, such as Gorlin syndrome and Greig cephalopolysyndactyly syndrome, and cancer including basal cell carcinoma and medulloblastoma (McMahon, Ingham et al. 2003; Nieuwenhuis and Hui 2005).

2.6.1 Sonic Hedhehog (Shh) signaling

Sonic Hedhehog (Shh), along with Indian hedgehog (Ihh), and Desert hedgehog (Dhh) is a member of the Hedgehog family of secreted glycoproteins, which affects the development of many organs and cell groups (Riddle, Johnson et al. 1993; Porter, Young et al. 1996). Synthesized as a large precursor protein, Shh undergoes autoproteolysis and lipid modifications of its biologically active aminoterminal domain (ShhN). Shh mediates its action via a receptor complex associating two transmembrane proteins: Patched (Ptch) displaying a transporter-like structure and Smoothened (Smo), a putative member of the G protein-coupled receptor superfamily. The repression exerted by Ptch on Smo is relieved when Shh binds Ptch, which leads to a complex signaling cascade involving the transcription factors of the Gli family and finally, to the activation of target genes including Ptch and Gli themselves (Huangfu and Anderson 2006; Ruiz i Altaba, Mas et al. 2007). At the cell membrane, the glycoprotein Hedgehog-interacting protein (Hhip) binds Shh with high affinity and acts as a negative regulator of Hh signaling in the embryo (Fig. 11). Like Ptch, Hhip is a target gene positively regulated by the Hh pathway. Both participate in an inhibitory mechanism of Hh signal by sequestering, modifying, or degrading the ligand at the cell surface (Jeong and McMahon 2005). Ptch induces rapid endocytosis and subsequent lysosomal degradation of Shh (Incardona, Lee et al. 2000), whereas Hhip appears to only physically sequester Shh at the cell surface (Jeong and McMahon 2005).

After relief of the inhibition exerted by Ptch on Smo, the canonical Hh pathway

leads to the transcription of target genes. This activity is mediated through the inhibition of Gli transcription factor processing into their transcriptional repressor forms and the concomitant accumulation of transcriptional activators. Su(Fu), a negative regulator of Shh signaling, interacts with the three vertebrate Gli proteins (Gli1, Gli2, Gli3) to retain them in the cytosol (Merchant, Evangelista et al. 2005; Jia, Kolterud et al. 2009) and also to control Gli function at nuclear level (Cheng and Bishop 2002; Barnfield, Zhang et al. 2005). These proteins contain five zinc-finger DNA-binding domains and differ by their N-terminal domains (Varjosalo, Li et al. 2006; Riobo and Manning 2007; Ruiz i Altaba, Mas et al. 2007). Gli1 is considered as a mean to amplify the Hh response rather than a direct effector of the Hh transduction machinery. However, Gli1 is a target gene of the pathway and is classically used as a convenient readout for the pathway activation. Gli2 would function mainly as a transcriptional activator, but could also display a repressor activity in specific contexts. Conversely, Gli3 mainly functions as a transcriptional repressor even though recent studies have also shown that Gli3 can act as an activator in vivo (Varjosalo, Li et al. 2006; Riobo and Manning 2007; Ruiz i Altaba, Mas et al. 2007).

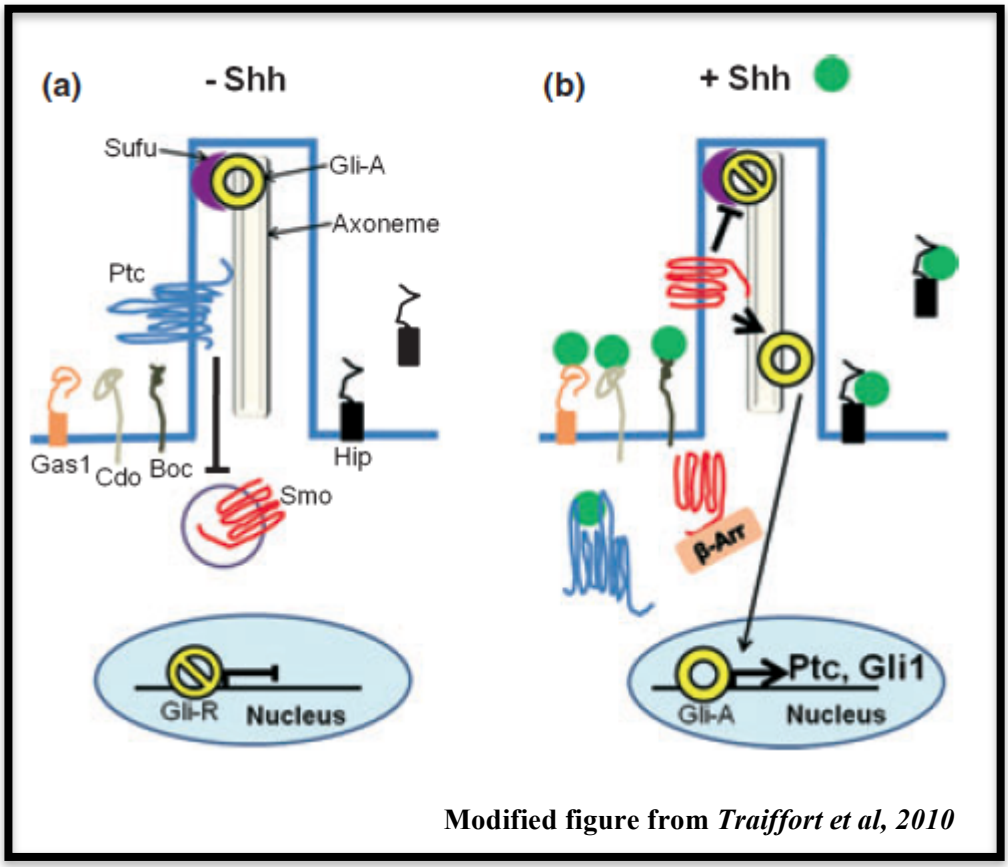


Figure 11: Proposed model for Sonic Hedgehog signaling pathway. *Shh uses the primary cilia to induce its signal. (**A**) In the absence of Shh ligand, the receptor Ptc is located in the cilium and represses Smo found mostly outside the cilium, by a yet unknown mechanism. The repressor forms of the transcription factors Gli (Gli-R) enter the nucleus and inhibit Shh target gene transcription. The negative regulator of the pathway Su(Fu) interacts with the activator (Gli-A) and repressor (Gli-R) forms of Gli maintaining them out of the nucleus and also controlling Gli function at nuclear level. Hip is a negative regulator able to directly bind Shh and is found as both membrane-associated and soluble forms. Gas1, Cdo and Boc are other membrane proteins able to bind Shh. (B) In the presence of Shh, Ptc is internalized, Smo inhibition is relieved allowing its translocation and accumulation to the cilium, which likely involves its interaction with β-arrestin (β-Arr). In the cilium, Smo may inhibit the formation of Gli-R, and activate Gli-A to promote the transcription of Hh target genes, such as Ptc and Gli1.*

2.6.2 Non-canonical Shh signaling

Several non-canonical mechanisms of Shh signaling have also been reported (Jenkins 2009). Hh responses, involving Hh components without any Gli-mediated transcription, have been proposed for Shh-induced cell migration (Bijlsma, Borensztajn et al. 2007), axonal guidance (Yam, Langlois et al. 2009), or modification of the electrophysiological properties of mature neurons (Bezard, Baufreton et al. 2003; Pascual, Traiffort et al. 2005). In the absence of Shh, Ptch may directly interact with Cyclin B1 (Barnes, Kong et al. 2001) or caspases (Thibert, Teillet et al. 2003), which inhibit cell proliferation or promote apoptosis, respectively.

Along the same lines, the repressor form Gli3 generated in the absence of Shh signaling is also able to inhibit the canonical Wnt/β-catenin signaling (Ulloa, Itasaki et al. 2007). Lastly, Shh effects occuring in adhesion or migration of neuroepithelial (Jarov, Williams et al. 2003) or neural crest cells (Testaz, Jarov et al. 2001) are independent of the Ptch–Smo–Gli cascade, while the repulsive activity of Shh on postcommissural axons during the neural tube development is mediated by Hhip (Bourikas, Pekarik et al. 2005).

2.6.3 Shh signaling in the developing Central Nervous System

Of the three vertebrate Hh homologs, only Shh is found in the mammalian CNS. It is expressed both during embryogenesis and, as is becoming more well appreciated, also in adults, where its function may be related to one that is among the most commonly conserved across species and tissues: maintaining reservoirs of stem cells that are located in specific niches (Traiffort, Angot et al.; Ahn and Joyner 2005; Palma, Lim et al. 2005; Balordi and Fishell 2007). Shh begins to exert an influence over CNS development at the very earliest stages of embryogenesis when neural tissue is first allocated during gastrulation. At that time, expression is seen in the embryonic node, the undifferentiated group of cells that are responsible for producing the signals that induce neural ectoderm and then for ventralizing this tissue. At presumptive spinal cord levels, Shh expression is maintained in the mesodermal notochord underlying the ventral neural plate and

then, shortly thereafter, in the floor plate (FP), a small group of cells located at the ventral neural midline that disappear later in development. In the forebrain, midline mesodermal cells expressing Shh form the prechordal plate, which does not coalesce into a rod-shaped structure like the notochord. Here, too Shh expression is initially induced secondarily in the midline, but is soon downregulated as the hypothalamus and pituitary begin to arise, after which time, it is only expressed in two lateral domains in the approximate boundary between the future hypothalamic/thalamic boundary. After brain vesicle formation, Shh expression is also upregulated in the zona limitans intrathalamica (ZLI), a morphological dividing line between emerging thalamic nuclei in the caudal diencephalon that extends dorsally from the FP in the midbrain, and later still in distinct foci in the preoptic area, presumptive amygdala, and the medial ganglionic eminence (MGE), the anlage to the basal ganglia (Sousa and Fishell 2010). Another region of the CNS where Shh expression has been characterized is in the developing cerebellum. Here, Shh is seen in postmitotic Purkinje cells, and from this source signals to granule cell precursors to support their extensive expansion (Wechsler-Reya and Scott 1999). This relationship has provided a unique opportunity to study the regulation of cell proliferation by Shh, a pursuit that has important clinical implications for understanding childhood brain tumors arising in the cerebellum (Hatten and Roussel; Vaillant and Monard 2009). Shh serves as an important early signal from all of these regions but its specific requirement varies. Early loss of Shh is associated with the most severe consequences, a failure to induce ventral midline neural tissue, resulting in holoprosencephaly and cyclopia, a condition where only a single forebrain vesicle and eye form (Chiang, Litingtung et al. 1996). Shh in the thalamic midline and ZLI is required to generate specific thalamic neuronal subtype (Jeong, Dolson et al. 2011). Loss of downstream Shh transduction components also results in the misspecification of ventral forebrain neuronal structures and subtypes (Carney, Mangin et al.; Cocas, Miyoshi et al. 2009; Xu, Guo et al. 2010).

2.6.4 Shh signaling and Neural Stem Cells

The Shh pathway has been extensively analyzed in neural stem and progenitor cells (Wechsler-Reya and Scott 1999; Dahmane, Sanchez et al. 2001; Lai, Kaspar et al. 2003; Machold, Hayashi et al. 2003; Ahn and Joyner 2005; Palma, Lim et al. 2005; Balordi and Fishell 2007). **Besides its multifaceted roles in the specification, proliferation, and differentiation of neural precursors** during embryogenesis, Shh signaling is required for the maintenance of Shh-responsive, Gli1-positive quiescent neural stem cells in the adult brain (Ahn and Joyner 2005; Palma, Lim et al. 2005; Balordi and Fishell 2007). During cerebellar development, Shh secreted by the Purkinje cells promotes rapid proliferation of granule cell precursors in the external granular layer (Fig. 12) (Wechsler-Reya and Scott 1999), and Ptch mutations are commonly found in both familial and sporadic medulloblastomas. Studies of Ptch heterozygous Gorlin syndrome patients, as well as analogous mutant mice, have strongly suggested that Shh pathway activation is critical for the transformation of granule cell precursors (Goodrich, Milenkovic et al. 1997). However, it seems likely that the ability of the pathway to act in this fashion is nonetheless dependent on cell-type-specific determinants. For example, although the Shh pathway is activated in lowand high-grade human gliomas, and Shh signaling positively regulates the self-renewal of glioma cancer stem cells (Clement, Sanchez et al. 2007; Ehtesham, Sarangi et al. 2007), Gorlin syndrome patients do not develop gliomas. Consistent with this, two recent studies have demonstrated that Shh pathway activation in mouse neural stem cells or restricted neural progenitors induces only medulloblastoma, not glioma (Schuller, Heine et al. 2008). The cell-autonomous determinants that control Gli action in the nucleus only represent a small fraction of the range of mechanisms that control the Hh response. For example, in many developmental contexts, there is considerable regulation at the level of production, dissemination, and presentation of Hh ligands.

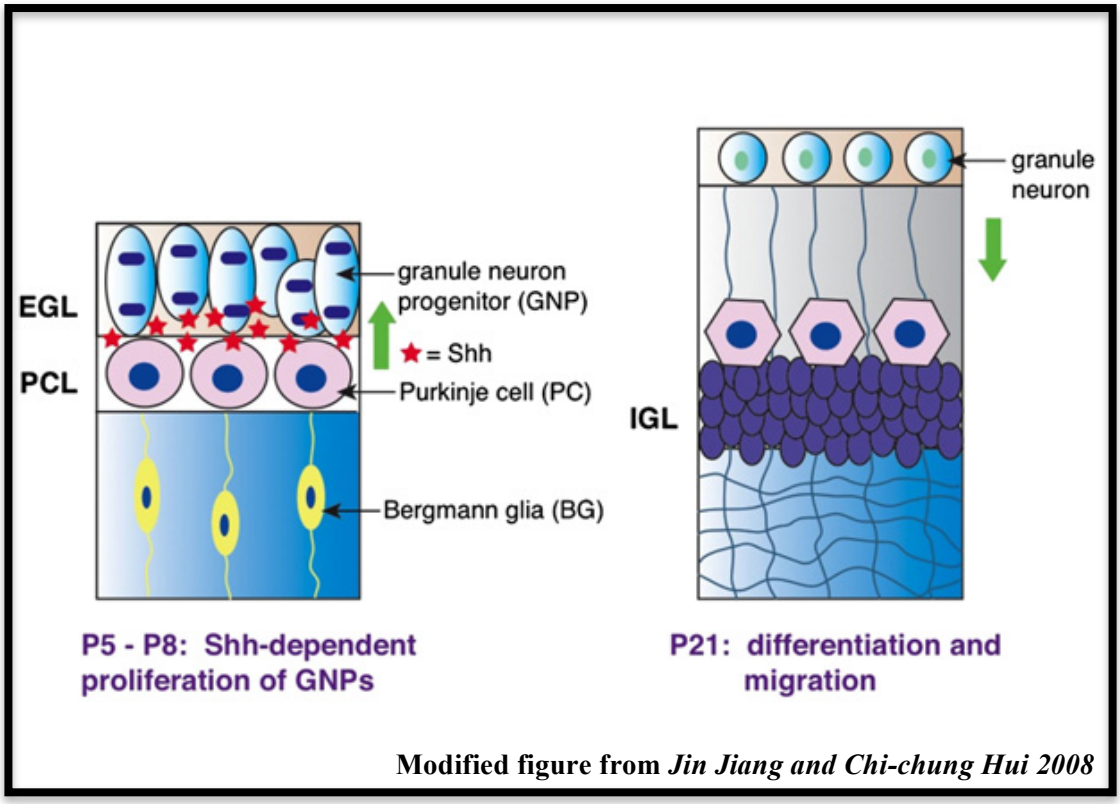


Figure 12: *In cerebellar development, Shh secreted by Purkinje cells controls the proliferation of granule neuron progenitors in the external germinal cell layer (EGL).*

2.6.5 Shh signaling and Neural Stem Cells in the SVZ and in the Hippocampus

The fact that neurogenesis and gliogenesis continue in the adult mammalian brain (Alvarez-Buylla, Garcia-Verdugo et al. 2001) might explain why Shh is expressed in some sites of the mature brain. The intriguing role for hedgehog signaling in regulating the proliferation of adult neural progenitors has been widely studied in the mammalian forebrain. It is an excellent system for the analysis of adult neural stem cells because it has two localized areas of postnatal neurogenesis, the dentate gyrus of the hippocampus and the subventricular zone (SVZ) of the lateral ventricles. Whereas the hippocampal progenitors locally populate the hippocampus, the SVZ progenitors migrate along the rostral migratory stream to populate the olfactory bulb. Work in mice has shown that this SVZ niche consists

of quiescent (slow-cycling) progenitors, transit-amplifying cells and migrating neuroblasts. Using AraC anti-mitotic treatment to ablate rapidly dividing cells in the precursor and neuroblast lineages, it was shown that astrocyte-like cells could divide to replenish these two populations, indicating that a slowly dividing glial population serves as the SVZ stem cell (Doetsch, Caille et al. 1999).

In vivo evidence highlighting the importance of hedgehog signaling in these two postnatal proliferative niches came from conditional genetic loss-of-function studies, viral gain-of-function studies and genetic fate mapping. Conditional removal of Smo in the brain at mid-gestation circumvents the early embryonic patterning defects seen in the Shh nulls and allows postnatal examination of the effects of Shh signaling in the dentate gyrus subgranular zone (SGZ) and SVZ proliferative regions (Machold, Hayashi et al. 2003). Both regions showed a marked reduction in the number of proliferating progenitors at 2–3 weeks after birth in these mutants, and the SVZ also showed increased cell death. As a result of these perturbations of the postnatal proliferative niches, the granule cell populations of both the hippocampus and the olfactory bulb were severely depleted. A similar decrease in proliferation was observed in the SVZ of juvenile mice that were administered a one week course of the Shh signaling inhibitor cyclopamine, and in the hippocampus of mice that had this inhibitor injected directly into the same region (Lai, Kaspar et al. 2003; Palma, Lim et al. 2005)In a series of experiments with interesting clinical implications, artificial stimulation of the hedgehog signaling pathway in the rodent forebrain led to increases in neural progenitor proliferation in both the SVZ and the dentate gyrus of the hippocampus. Activation of hedgehog signaling was achieved either through adenoviral delivery of the N-terminal active fragment of Shh (Lai, Kaspar et al. 2003), or through the use of a small-molecule hedgehog agonist that upregulates signaling in hedgehog responsive tissues through interactions with Smo (Machold, Hayashi et al. 2003). This suggests that enhancing hedgehog signaling might be effective as a means of promoting neural repair. Although it seems clear that hedgehog signaling has an essential role in maintaining adult neural progenitor proliferation, the downstream mechanistic details are still largely

unknown. Genetic inducible fate mapping (GIFM) studies in mice (Joyner and Zervas 2006) have begun to illuminate the developmental history and cell-type specificity of Shh signaling in these proliferative niches (Ahn and Joyner 2005). Using Gli1 as a marker of hedgehog responding cells, it was definitively shown that quiescent and transit-amplifying stem cells in the adult SVZ and SGZ normally receive GLI activator signaling. Furthermore, the quiescent cells expressing GLI1 continue to give rise to neurons that populate the olfactory bulb and hippocampus for over a year and are multipotent. In addition, it seems that the SVZ and SGZ niches are formed sequentially towards the end of embryonic forebrain development. With regard to the cellular mediators of this process, analysis has shown that periventricular astrocytes, the characterized SVZ stem cell population, express not only Gli1 but also other components of the hedgehog signaling cascade (Palma, Lim et al. 2005). In vitro analysis of dissociated cortices of Gli2 and Gli3 mutants indicates that these genes could in part mediate the role of hedgehog in adult progenitor proliferation (Palma and Ruiz i Altaba 2004). In addition to these issues, it will be interesting to better understand the source of the hedgehog signals that act in the two proliferative niches, as well as the instructive events in receiving cells that are directed by these signals. Finally, given the dichotomy between the proliferative response to Shh and its role in maintaining the adult stem cell niche, it will be interesting to determine whether this differential response reflects different mechanisms of Shh action or different functional effects in discrete lineages within the stem cell niche.

2.2.6 Shh and DS

Patients with Down Syndrom are characterized by a reduction of the granular cell layer volume in the cerebellum accompanied by hypotonia and fine motor control impairment (Latash and Corcos 1991). Similarly, Ts65Dn mouse model of DS has a reduction of the number of cerebellar granule cell precursors (CGPs), resulting from an insufficient mitotic rate. From exploring the mitotic deficit in Ts65Dn mice, Roper et collaborators (Roper, Baxter et al. 2006) found that trisomic GCP were less sensitive to the dually lipidated protein Shh. By injecting an analogue of

Shh into Ts65Dn pups at P0, the mitotic and GCP proficiency could be restored to that of euploid controls. **Yet, the cause of this insensitivity which leads to a reduction in mitosis and to a reduction in CGPs density remains undetermined.** Few clues can be found in transcriptome studies as to which aspect(s) of the Shh signalling pathway is affected. In the cerebellum, it is only in the postnatal external granule cell layer that genes involved in the Shh signalling pathway become expressed-genes such as Patched, Smoothened, Gli1 and Gli2 (Sotelo 2004). Therefore, it is likely that only at this time can a deficit in the pathway manifest and be observed in DS.

Roper et al, 2009 (Roper, VanHorn et al. 2009), found an attenuate response of NC (Neural crest) precursors to Shh in Ts65Dn mouse model of DS. NC contribute to the majority of the bone, cartilage, connective tissue and peripheral nervous tissue in the head (Santagati and Rijli 2003). The correct formation of the craniofacial skeleton is necessary for the proper development of the brain, sensory organs, and the normal functioning of the digestive and respiratory tracts (Santagati and Rijli 2003; Le Douarin, Brito et al. 2007). Because NC is a common precursor of many structures affected in DS, it **has been hypothesized that Shh response deficits may represent cellular and molecular "common denominators" of pathogenesis contributing to multiple aspects of the trisomic phenotype in DS**.

3. MATERIALS AND METHODS

3.1 Ts65Dn MICE COLONY AND TREATMENT

Female Ts65Dn mice carrying a partial trisomy of chromosome 16 **(Reeves, Irving et al. 1995, Davisson, 1993 #211)** were obtained from Jackson Laboratories (Bar Harbour, ME, USA) and maintained on the original genetic background by mating them to C57BL/6JEi x C3SnHeSnJ (B6EiC3) F1 males. Animals were karyotyped by real-time PCR (qPCR) as previously described (Liu, Schmidt et al. 2003). The animals had access to water and food ad libitum and lived in a room with a 12:12 h dark/light cycle. Experiments were performed in accordance with the Italian and European Community law for the use of experimental animals and were approved by Bologna University Bioethical Committee. In this study, all efforts were made to minimize animal suffering and to keep to a minimum the number of animals used.

The day of birth was designed as postnatal day (P) zero.On P2, three euploid and three Ts65Dn mice received a subcutaneous injection (150 μg/g body weight) of BrdU (5-bromo-2-deoxyuridine; Sigma), a marker of proliferating cells in 0.9% NaCl solution and were killed after 2 h to examine cell proliferation.

3.2 HUMAN FETUSES

Human fetal brains (17-21 weeks of gestation) were obtained after prior informed consent from the parents and according to procedures approved by the Ethical Committee of the St. Orsola-Malpighi Hospital, Bologna, Italy. Regulations of the Italian Ministry of Health and the policy of Declaration of Helsinki were followed. All fetuses derived from legal abortions and were collected with an average post-mortem delay of approximately 2 hours. The brains of control fetuses (n = 3) and fetuses with DS (n = 3) used in the current study are the same used in previous studies (Contestabile, Fila et al. 2007; Guidi, Bonasoni et al. 2008). Trisomy was caryotypically proved from the results of genetic amniocentesis procedures. Autopsies were performed at the Institute of Pathology

of the St. Orsola-Malpighi Hospital. The gestational age of each fetus was estimated by menstrual history and crown-rump length. Fetuses used in this study are summarized in the table below:

CASE	AGE (weeks)	SEX	CRL (cm)	BW (gr)
Control fetuses				
C164	19	F	14	285
C27	20	F	17	230
C178	21	M	n.a.	n.a.
Down syndrome fetuses				
C166	19	F	14	240
C133	20	M	16	300
C46	21	M	22	355

Table 1: *List of the cases used in the present study. AGE refers to gestional age in weeks, CRL: crown-rump length; BW: body weight; n.a.: not available.*

3.3 HISTOLOGICAL PROCEDURES

P2 mice were decapitated, brains removed, cut along the midline and fixed by immersion in Glyo-Fix (Thermo Electron Corp., Waltham, MA, USA) for 48 h and then dehydrated through a series of ascending ethanol concentrations. Each hemisphere was embedded in paraffin.Forebrainswere coronally sectioned in 8 μm thick sections that were attached to poly-lysine-coated slides. Fetal brains were fixed by subdural perfusion with Metacarnoy fixative (methyl alcohol: chloroform: acetic acid 6:1:1). After 24 h, the hippocampal region was coronally sectioned into two to three blocks, with a 2–3 mm thickness. The first block roughly corresponded to the rostral third of the hippocampal formation. The blocks were post-fixed in formalin (4% buffered formaldehyde) for at least 5 days, embedded in paraffin, according to standard procedures, and sectioned in 5 μm thick coronal sections.

3.4 CELL CULTURES AND TREATMENTS

Cells were isolated from the SVZ and hippocampus of newborn (P1–P2) euploid and Ts65Dn mice (Fig. 13) and neurosphere cultures were obtained as previously reported (Weiss et al, 1996; Pacey et al., 2006).

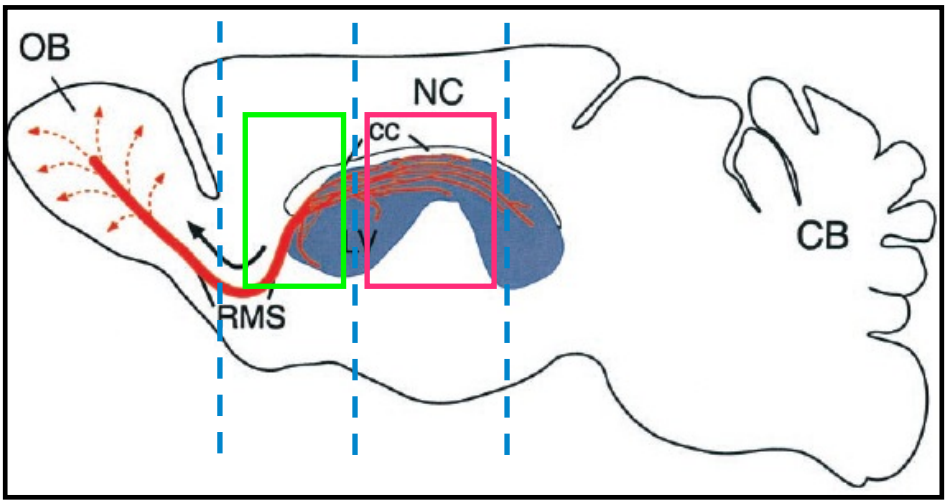


Figure 13: *Sagittal section of adult mouse brain. The subventricular zone is bounded by a green rectangle and the hippocampus by a red one. Scheme of the dissection protocol. The blue broken lines represent the coronal cuts at which the brain of P2 mice was cut for removing the rostral and caudal forebrain.*

Briefly, Ts65Dn and Euploid mice were decapited with sterile surgery scissors in the cell culture hood. The skulls were cut with a scalpel blade over the entire length. Gently skulls were broken and the brains removed and transferred to the dissection solution (Table 2)

Tissue dissection solution	
2.0 M NaCl	**15.5 ml**
1.0 M KCl	**1.25 ml**
1.0 M $MgCl_2$	**0.80 ml**
155 mM $NaHCO_3$	**41.90 ml**
1.0 M Glucose	**2.50 ml**
108 mM $CaCl_2$	**0.23 ml**
DDW (Double Distilled Water)	**188.0 ml**
Filter and store at 4°C and bubble with 95% O_2/ 5% CO_2 for 10 min before use	

Table 2: *Tissue dissection solution composition*

Any remaining meninges were stripped from the surface of the brain taking advantage of a dissecting microscope. Then, forebrains were coronally cut in order to isolate the subventricular zone and the hippocampus from both the emispheres (Fig. 13). Tissues were fine cut into small pieces using curved scalpel blades and forceps and centrifuged for 1 minute at 250g. After removing the supernatant, enzyme mix (Table 3) was added for 10 min. at 37°C.

Enzyme mix	
Trypsin	**2BAEE/ml**
Type 1-S Hyaluronidase	**12.12 μM**
Kynurenic Acid	**704.72 μM**
Enzyme mix components are dissolved in 30 ml of tissue dissection solution and filter with 0.22 μm filter Activities for trypsin are expressed in international units (IU) with BAEE as substrate. Unit definition: One BAEE unit will produce a ΔA_{253} of 0.001 per min at pH 7.6 at 25° C using BAEE as substrate	

Table 3: *Enzyme mix composition*

In order to neutralize the effect of the enzyme mix, after centrifugatin for 5 minutes at 250xg, tissues were resuspended in a solution containing trypsin

inhibitor (1.0 mg/ml in Serum Free Medium) and mechanically triturated (20 times) with a Pasteur pipette to produce a single cell suspension. Cells were cultured in suspension in Dulbecco's modified Eagle's medium (DMEM)/F12 (1:1) containing B27 supplements (2%), basic fibroblast growth factor (FGF-2, 20 ng/ml), epidermal growth factor (EGF, 20 ng/ml), heparin (5 μg/ml) and antibiotics (penicillin: 100 units/ml; streptomycin: 100 μg/ml) and counted by Trypan Blue dye exclusion (density of ~ 10-15 cells/μl). Primary neurospheres were dissociated at days 8–10 using Accutase (PAA, Pasching, Austria) to derive secondary neurospheres. The sub-culturing protocol consisted of neurosphere passaging every 7 days with whole culture media change (with freshly added FGF-2 and EGF). All experiments were done using neurospheres obtained after three to five passages from the initially prepared cultures. Most (98%) of the cells in neurospheres from the SVZ and hippocampus were positive for nestin, an established marker for neural and glial precursors. Cell cultures were kept in a 5% CO2 humidified atmosphere at 37°C. Treatments were performed for 3 days after cell plating adding fresh reagents every 24 h.

The following drugs were used: 3 μg/ml and 6 μg/ml recombinant mouse Sonic Hedgehog Peptide (Shh; Sigma), 250 nM benzo[b]thiophene-2-carboxamide,3-chloro-N-[4-(methylamino)cyclohexyl]-N-{[3(4 pyridinyl)phenyl] methyl}-(9CI) (SAG; Enzo life science), 10 μg/ml cyclopamine hydrate (Sigma), 5 μM 5-Aza-2′-deoxycytidine (AZA; Sigma), 150 nM TSA (Sigma), 10 μM N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT; Sigma) and 1 nM Compound E (Enzo life science).

3.5 NEUROSPHERES DIAMETER MEASURAMENTS

Dissociated neurospheres were plated at a low density (2 x 10^4 cells/ml) and phase contrast images of cells were taken almost every day for 8 days using an Eclipse TE 2000-S inverted microscope (Nikon, Tokyo, Japan). Neurosphere diameters were measured with the NIS-Elements AR software (Nikon).

3.6 BRDU IMMUNOCYTOCHEMESTRY IN NEUROSPHERES

For proliferation analysis, dissociated neurospheres were cultured for 3 days, treated with 10 μM BrdU for additional 16 h and harvested on microscope slides by cytospin centrifugation (215g, 5 min, Shandon, Thermo, Dreieich, Germany). Specimens, processed as previously described (Hendzel, Wei et al. 1997), were incubated with a mouse anti-5-bromo-2-deoxyuridine (BrdU) monoclonal antibody (1:100; Roche Applied Science) and a Cy3-conjugated anti-mouse secondary antibody (1:200; Sigma). Samples were counterstained with Hoechst-33258. Digital images were captured using an Eclipse TE 2000-S microscope and the NIS-Elements AR software (Nikon).

3.7 CELL CYCLE ANALYSIS IN NEUROSPHERES

For determination of total cell cycle length (TC) and S-phase length (TS), the BrdU cumulative method was applied (Takahashi, Nowakowski et al. 1993, Li, 2001). Neurospheres were treated with 10 μm BrdU for 1.5, 3.0, 4.5, 6.0, 24, 48 and 72 h, and fixed and processed for BrdU immunocytochemistry. The labeling index (LI; number of BrdU-positive cells over total cell number) was evaluated in 12 random fields for each sample. LI values obtained at each time point for control and trisomic neurospheres were then plotted as a function of time after BrdU administration as previously described (Contestabile, Fila et al. 2009; Contestabile, Fila et al. 2009). Using a linear regression analysis, a least-squares line was best-fitted to data points for each group. The *y*-intercept and the *x*-values for $y = 1$ extrapolated from the equation

$y = a*x + b$

were used to calculate the total length of the cell cycle (T_C) and the length of the S phase (T_S) as previously described (Fujita et al., 1967). The *y*-intercept (the b parameter of the equation above, i.e. the LI at $x = 0$) represents the length of the S phase over total cell cycle length:

$T_S / T_C = b$

and the time at which all cells are labeled ($y = 1$) represents the interval $T_C - T_S$:

$T_C - T_S = (1-b)/a$

By solving the system of the two equations above, T_C and T_S can be calculated:

$T_C = 1/a$

$T_S = b/a$

The number of cells in G_2 or M phase of cell cycle over total cell number is proportional to the length of G_2 (T_{G2}) or M (T_M) phase over total cycle length. Therefore, T_{G2} and T_M were estimated from the G_2I and MI obtained with pHH3 immunohistochemistry and total length of cell cycle ($T_{G2}/T_C = G_2I$; $T_M/T_C = MI$):

$T_{G2} = G_2I * T_C$

$T_M = MI * T_C$

Finally, the length of the G_1 phase was obtained by subtracting from the total length of the cell cycle the length of the other phases:

$T_{G1} = T_C - (T_S+T_{G2}+T_M)$

3.8 *IN VITRO* DIFFERENTIATION ANALYSIS OF NPCs

Neurospheres obtained after three passages in vitro were dissociated and plated on cover slips coated with 15 μg/ml poly-L-ornithyine (Sigma). Cells were grown for 2 days and then transferred to a differentiating medium (EGF and FGF free plus 1% foetal bovine serum) for 7 days. Every 3 days, half of the medium was replenished with fresh differentiating medium. Differentiated cells were fixed on slides by cytospin centrifugation and incubated with anti-glial fibrillary acidic protein (1:400; GFAP mouse monoclonal, Sigma) and anti-β-Tubulin III (1:100; rabbit polyclonal, Sigma), as primary antibodies, and with anti-mouse FITC-conjugated (1:100; Sigma) and anti-rabbit Cy3-conjugated (1:100; Jackson Laboratories), as secondary antibodies. Samples were counterstained with Hoechst-33258. Cells were counted in five different fields of each cover slip. Number of positive cells for each antibody was referred to the total number of Hoechst-stained nuclei.

3.9 PTCH1 QUANTIFICATION IN NEUROSPHERES

For Ptch1 immunocytochemistry, we used an anti-Ptch1 rat monoclonal antibody

(1:50; R&D systems) and, as a secondary antibody, a Cy3-conjugated anti-rat antibody (1:200; Jackson Laboratories). Samples were counterstained with Hoechst 33258. To quantify Ptch1 expression, we used the NIS-Elements AR software (Nikon). For each neurosphere, the perimeter was traced and the mean intensity of the Ptch1 signal was derived by the ratio between the sum of the intensity of positive (bright) pixels and the area of single neurospheres. Approximately 3 neurospheres were analyzed from each slide (three euploid and trisomic mice and two slides for each condition).

3.10 BRDU IMMUNOHISTOCHEMESTRY

One out of 20 sections from the DG and SVZ of P2 animals was processed for BrdU immunohistochemistry. Sections were processed as previously described (Lowry, O.H et al; 1951) and incubated overnight at 4°C with a primary antibody anti-BrdU (1:100, Roche Applied Science). Detection was performed with a horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody (1:200; Jackson Immunoresearch) and DAB kit (Vector Laboratories). Sections were counterstained with hematoxylin. Bright field images where taken with a Leitz Diaplan microscope equipped with a motorized stage and a Coolsnap-Pro digital camera (Media Cybernetics, Silver Spring, MD). The sampled sections covered the whole rostrocaudal extent of the DG and a region of the SVZ that extended from the rostral pole of the lateral ventricle and stretches for ~900 μm in the caudal direction. The total number of BrdU positive cells was estimated by multiplying the number counted in the series of sampled sections by the inverse of the section sampling fraction (1/20).

3.11 PTCH1 IMMUNOHISTOCHEMESTRY AND QUANTIFICATION *IN VIVO*

One out of 12 sections, in P2 animals, and 2–3 sections of the series of 4–5 μm thick sections of human fetuses were stained using an anti-Ptch1 rabbit polyclonal antibody (1:50; ab39266 Abcam). Sections were retrieved with citrate buffer (pH

6.0) at 98°C for 40 min before incubation with the antibody and processed as previously described (Contestabile, Fila et al. 2007). Sections were incubated with Cy3-conjugated anti-rabbit (1:200; Jackson Laboratories) secondary antibody. Image processing and analysis were carried out using the NIS Elements AR software (Nikon). To quantify Ptch1 expression, we normalized the intensity of the signal from the SVZ of mice and hippocampal region of mice and fetuses to that of the corpus callosum and fimbria, respectively.

3.12 WESTERN BLOTTING

Collected neurospheres were lysated in ice-cold lysisbuffer. (1% Triton-X100, 150 mM NaCl, 1 mM EDTA and 20 mM Tris pH 7.4) supplemented with 1mM PMSF and 1% proteases and phosphatases inhibitors cocktail (Sigma). Samples were then incubated in ice for 10 min and clarified by centrifugation at *10,000 x g* for 10 min at 4 °C. (Contestabile, Fila et al. 2009). Protein concentration was estimated by the Lowry method (Lowry, Rosebrough et al. 1951). Proteins (30 μg) were subjected to electrophoresis on an 8% sodium dodecyl sulphate (SDS)-polyacrylamide gel and transferred to a Hybond ECL nitrocellulose membrane (Amersham Life Science). and equal loading of protein in each lane was assessed by brief staining of the blot with 0.1% Ponceau S. Membranes were blocked for 1 h in 5% milk, 0.1% Tween-20 in Tris-buffered saline (150 mM NaCl, 10 mM Tris-HCl, pH 8.0) and incubated overnight at 4 °C with the following primary antibodies: anti-Ptch1 rat monoclonal (1:50; R&D systems) and anti- β-actin (1:2000; Sigma) antibodies. Membranes were washed, incubated with HRP-conjugated anti-rabbit (1:1000 dilution; Amersham Biosciences) and specific reactions revealed with the ECL Western blotting detection reagent (Amersham Biosciences). Densitometric analysis of digitized images was performed with Scion Image software (Scion Corporation, Frederick, MD, USA) and intensity for each band was normalized to the intensity of the corresponding β-actin band.

For AICD detection, nuclear extracts from neurospheres were prepared. For the preparation of nuclear extracts cellswere allowed to swell and lysed in hypotonic buffer (Hepes, 10mM , NaCl, 50 mM , EDTA, 1 mM , Nonidet P-40, 0.1%,

dithiothreitol,1 mM , phenylmethylsulfonyl fluoride, 1 mM , pH 8) for10 min at 4 °C. After centrifugation, nuclei were extracted withhypertonic salt buffer (Hepes, 20 mM , NaCl, 420 mM , EDTA, 1mM , dithiothreitol, 1 mM , glycerol, 10%, phenylmethylsulfonylfluoride, 1 mM , pH 8). Cells extracts (40 μg) were subjected to electrophoresis on a 4–12% NuPAGE Bis-Tris gel (Invitrogen) and transferred to a nitrocellulose membrane. The membrane was processed for antigen-retrieval as previously described (Ryan and Pimplikar 2005). The blot was incubated with the anti-C-terminal APP rabbit primary antibody (1:8000; A8717 Sigma-Aldrich).

3.13 QUANTITATIVE REAL-TIME PCR

Total RNA was isolated from neurosphere cultures with Tri- Reagent (Sigma) according to the manufacturer's instructions. cDNA synthesis was achieved with 1.0 μg of total RNA using the iScript^{TM} cDNA Synthesis Kit according to the manufacturer's instructions. We used the primers that gave efficiency close to 100% (Table 4). Realtime PCR was performed using a SYBR Premix Ex Taq kit (Takara, Shiga, Japan) according to the manufacturer's instructions in an iQ5 real-time PCR detection system (Bio-Rad). Fluorescence was determined at the last step of every cycle. Real-time PCR assay was done under the following universal conditions: 2 min at 50°C, 10 min at 95°C, 50 cycles of denaturation at 95°C for 15 sec, and annealing/extension at 60°C for 1 min. Relative quantification was performed using the ΔΔCt method.

Symbol	*Description*	*Accession number*	*Forward primer*	*Reverse primer*
PPIA	Peptidylprolyl isomerase A	NM_008084	CACTGTCGCTTTTCGCCGCTTG	TTTCTGCTGTCTTTGGAACTTTGTCTGC
Gli1	GLI-Kruppel family member GLI1	NM_010296	CCAGAGTCCAGCGGTTCAAGAG	GTGGCGAATAGACAGAGGTAGGG
Gli2	GLI-Kruppel family member GLI2	NM_001081125	GTTCCAAGGCCTACTCTCGCCTG	CTTGAGCAGTGGAGCACGGACAT
Gli3	GLI-Kruppel family member GLI3	NM_008130	AGCAAGCAGGAGCCTGAAGTCAT	GTCTTGAGTAGGCTTTTGTGCAA
Mycn	v-myc myelocytomatosis viral related oncogene	NM_008709	GGTGGCTGCTCCTGCTCGTG	TCCTCTTCATCTTCCTCCTCGT
Bmi1	Bmi1 polycomb ring finger oncogene	NM_007552	GATGGACTGACGAATGCTGGA	TGTGAGGGAACTGTGGGTGAG
FoxM1	Forkhead box M1	NM_008021	CACTTGGATTGAGGACCACTT	GTCGTTTCTGCTGTGATTCC
Shh	Sonic hedgehog	NM_009170	TTGCTTCCTCGCTGCTGGT	ATGATGGCCGTCCTCATCC
Ptch1	Patched homolog 1	NM_008957	CTGCGGCAAGTTTTTGGTTG	AGGGCTTCTCGTTGGCTACAAG
Smo	Smoothened homolog	NM_176996	CTGACTTTCTGCGTTGCAC	CCAATGCTGCCCACGAAG
App	Amyloid precursor protein	NM_007471	GCAGCAGAACGGATATGAG	GATGGGTAGTGAAGCAATGG

Table4*: List of the primers for real-time PCR expression analysis used in the present study.*

3.14 ANTISENSE EXPERIMENTS

To silence the expression of Ptch1, experiments were performed by using an antisense oligonucleotide (5′-tcCTCCC AGTTTCCCAGTca-3′) and, as a control, a sense oligonucleotide (5′-tgACTGGGAAACTGGGAGga-3′). Both oligonucleotides (Sigma) were phosphorothioated to make them more resistant to the RNAase attack. Dissociated neurospheres were transfected by directly adding either phosphorothioate Ptch1 antisense or sense oligonucleotides into culture medium. Oligonuclotides were daily added (concentration in the culture medium: 10 μM).

3.15 METHYLCYTOSINE IMMUNOPRECIPITATION (mCIP)

The mCIP method was adapted from previous studies (Keshet, Schlesinger et al. 2006; Zhang, Du et al. 2006). The DNA was obtained from 4 x 10^6 cells using Blood and Cell DNA culture midi kit (Qiagen) according to the manufacturer's instructions. Immunoprecipitation was performing using 15 μg of sonicated DNA with 5 μg of the mouse anti-methylcytosine monoclonal antibody (Calbiochem NA81) in 600 μl of buffer FB (10 mM Tris–HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA) at 4°C overnight. A fraction of the recovered DNA was used for real-time PCR to determine the amount of the methylated Ptch1 promoter. Primer pairs for

real-time PCR analysis were designed to amplify two CpG islands (regions c and e; Fig. 23) already described (Takai and Jones 2002) and two non-CpG islands, as control regions (regions a and d; Fig. 24) of the mouse Ptch1 promoter. We additionally amplified the CpG-rich region described by Ecke et al. (Ecke, Petry et al. 2009) (region b; Fig. 24; see Table 5).

3.16 CHROMATIN IMMUNOPRECIPIATATION (ChIP)

Chromatin Immunoprecipitation (ChIP) is a type of immunoprecipitation experimental technique used to investigate the interaction between proteins and DNA in the cell. It aims to determine whether specific proteins are associated with specific genomic regions, such as transcription factors on promoters or other DNA binding sites. ChIP was performed as previously described (Weinmann and Farnham 2002). Briefly, protein and associated chromatin in a cell lysate were temporarily bonded by incubation with 1% formaldehyde for 30 min, the DNA-protein complexes (chromatin-protein) were then sheared by sonication and DNA fragments associated with proteinswere selectively immunoprecipitatedusing 5 µg of anti-Acetyl-Histone H3 (06-599, Upstate) antibody. A preimmune serum was used as a negative control to determine the baseline of the nonspecific background. Dual crosslinking ChIP, a new variant of ChIP which allows the detection of chromatin components not directly binding to DNA, was performed as previously described (Liu, Tee et al. 2007) using 5 µg of the anti C-terminal APP antibody (A8717 Sigma-Aldrich) or IgG (sc-2027, Santa Cruz) as a negative control. The immunoprecipitated DNA samples were analyzed by real-time PCR to determine the amount of the histone H3 acetylation of the Ptch1 and Smo promoters and AICD binding to the Ptch1 and Smo promoters (Table 5 and 6).

Ptch1 Promoter			
Amplicon	***Primer name***	***Sequence***	***Position from start site***
a	aF	TTCCTTACCTCCTCCCTGAAACC	-4929
	aR	GCCAATCTGCCACCATCTTACC	-4763
b	bF	AGAGGGAAACAGTACACGCTTAGG	-3332
	bR	GCTGGTTGTGTGTATGGCATCG	-3244
c	cF	GCCGCCTTCAGGAATGTACTACG	-2536
	cR	CCCAGCCCTCATCACAGTGC	-2427
d	dF	CCTGGGTGGTCTCTCTACTTTGG	- 918
	dR	GCTGTCAGATGGCTTGGGTTTC	- 798
e	eF	AAGCAGCAGACAAGTGAAGGTTG	+ 257
	eR	CGGACCTCAGACAGCCCTTTC	+ 332

Table 5*: List of the primers for real-time PCR Methylcytosine, Acetyl-histone H3 immunoprecipitation and AICD Dual ChIP crosslinking analysis used in the present study.*

Smo Promoter			
Amplicon	***Primer name***	***Sequence***	***Position from start site***
a	aF	CGTCTTGCCCTTCATGCTCTC	-3423
	aR	GGACTGTAGGATGCTGGAAGAAC	-3232
b	bF	GCAAGACATTGTGGATGTTGATAC	-2461
	bR	CCTCCAGTACAAGGACCTTATCC	-2374
c	cF	CCCTGACATACCTGAGCGATAAAG	-1129
	cR	TTACCTAGAACCCTGGTGGAGAAG	- 943
d	dF	ACAAAGAGCCCGAGTAAGGATGG	+ 561
	dR	GATTGCCTCAAGGTCTGAACACTG	+ 673

Table 6*: List of the primers for real-time PCR Acetyl-histone H3 immunoprecipitation and AICD Dual ChIP crosslinking analysisused in the present study.*

3.17 STATISTICAL ANALYSIS

Results are presented as the mean ± standard error (SE) of the mean. Statistical significance was assessed by two-way analysis of variance (ANOVA), followed by Bonferroni's post hoc test or by the two-tailed Student's t-test. A probability level of $P < 0.05$ was considered to be statistically significant.

4. RESULTS

4.1 Neuronal precursor cultures from Ts65Dn mice exhibit the same proliferation impairment as the *in vivo* condition

The first aim of our study was to evaluate whether neuronal precursors (NPCs) from the subventricular zone and from the dentate gyus of trisomic mice exhibit the same proliferation defects observed in vivo. Neural stem and progenitor cells are maintained and expanded as spherical aggregates termed "neurospheres". Primary cultures from both euploid and Ts65Dn mice formed typical neurospheres (Fig. 14A). The growth kinetics of NPCs from the SVZ was monitored over time. While at day 1 in vitro (DIV1) there was no difference in the morphology and number of neurospheres of Ts65Dn and euploid mice; the diameter of trisomic neurospheres was significantly smaller than that of the euploid counterpart starting from DIV3 (Fig. 14A and B), suggesting defective proliferation. In agreement with this finding, trisomic neurospheres exhibited a reduced BrdU incorporation rate versus the control counterpart (-25%; Fig. 14C). Furthermore, proliferation rate through BrdU incorporation was evaluated in NPCs from the hippocampus of trisomic and euploid mice. Results showed a reduced BrdU staining in trsisomic neurospheres just like NPCs from the SVZ (-30%; Fig. 14D).

In order to establish whether the reduced growth and number of BrdU-positive cells observed in trisomic neurospheres could be attributed to an increased cell death, we estimated cleaved caspase-3 immunostaining and pyknotic appearance of the nuclei of dying cells (data not shown), but no difference in apoptotic cell death was observed between control and trisomic neurospheres derived from both the SVZ and hippocampus.

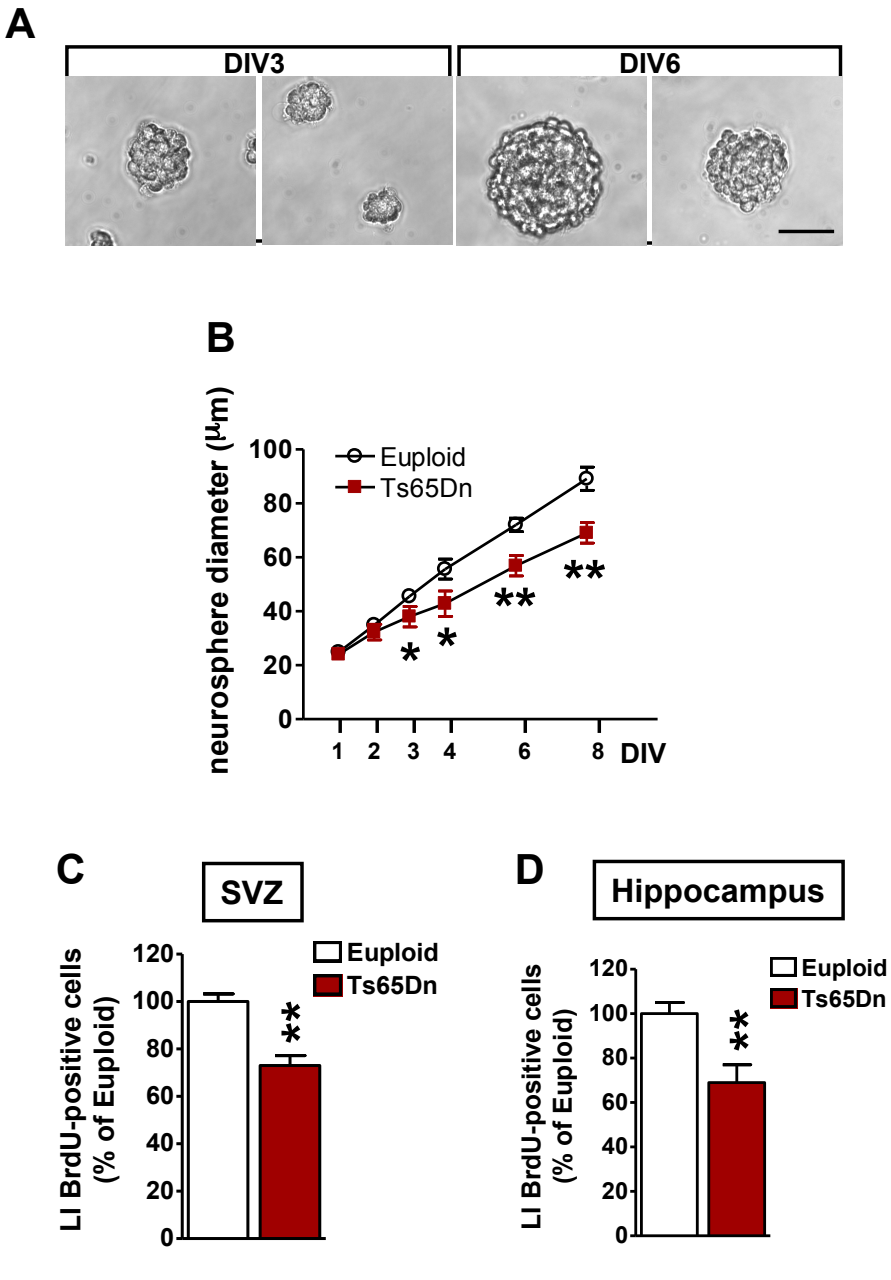

A
DIV3
DIV6
B
neurosphere diameter (μm)
Euploid
Ts65Dn
DIV
C
SVZ
LI BrdU-positive cells (% of Euploid)
Euploid
Ts65Dn
D
Hippocampus
LI BrdU-positive cells (% of Euploid)
Euploid
Ts65Dn

Figure 14:. **Cell proliferation in neurospheres from euploid and Ts65Dn mice.**
(A) *Phase-contrast images of free-floating neurospheres at day 3 (DIV3) or day 6 (DIV6) in vitro from P2 euploid or Ts65Dn mice. Scale bar: 50 mm.* ***(B)*** *Mean diameter of neurospheres derived from P2 euploid or Ts65Dn mice (n=4 per group) at different DIV.* ***(C-D)*** *Cell proliferation in neurospheres from the SVZ and hippocampus of euploid and Ts65Dn mice. LI, defined as percentage of BrdU-positive cells over total cell number, was determined for euploid and trisomic neurospheres. At DIV4, BrdU (10 mM) was added for the last 16 h and thereafter cells were processed for BrdU immunocytochemistry. Results are expressed as percentage of control values and were obtained from neurosphere cultures derived from euploid*

We recently obtained evidence that in fetuses with DS as well as in the Ts65Dn mouse model cell proliferation is impaired most likely due to elongation of the cell cycle (Contestabile, Fila et al. 2007; Contestabile, Fila et al. 2009).The total duration of the cell cycle (Tc) and the duration of the S phase (Ts) can be estimated by BrdU-cumulative LI (Kornack and Rakic 1998). According to this protocol, successive cohorts of cells entering the S phase are labeled with BrdU at consecutive time intervals (typically, each 2 hr). Maximum LI corresponds to the growth fraction (GF) and the time at which the LI reaches a maximum corresponds to Tc-Ts. Then, we analyzed the we evaluated total cell cycle length (TC), S-phase length (TS) and GF by BrdU-cumulative labeling in NPCs from Ts65Dn mice (Fig. 15). Cell cycle analysis revealed that in control neurospheres, the cell cycle length was 29.56 h. In contrast, in Ts65Dn mice, the cell cycle length was 59.02 h, which corresponds to a difference of +100%. The length of the S-phase showed a small but significant increase in trisomic versus control neurospheres Trisomic neurospheres had a GF similar to the euploid counterpart, indicating no difference in the relative size of the population of proliferating NPCs.

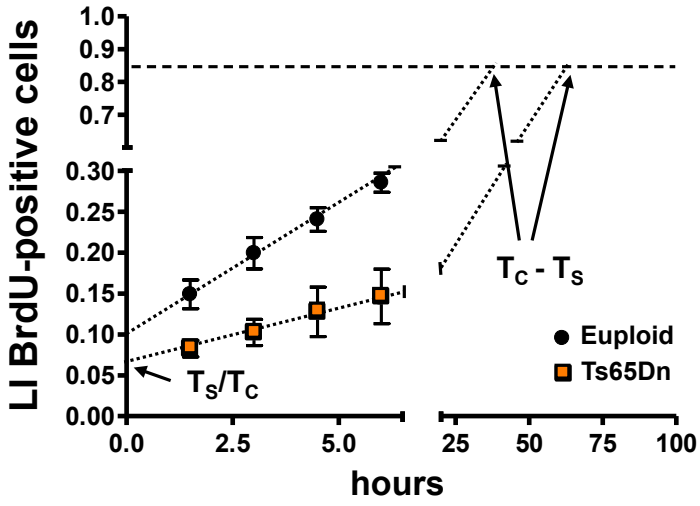


Figure 15: Cell cycle analysis of neurospheres from euploid and Ts65Dn mice. *Cumulative labelling indexes in control (black circles) and trisomic (orange squares) neurospheres as function of time after BrdU administration (time 0). Neurospheres, at the fourth passage in culture, were incubated with 10 μM BrdU for increasing time intervals (1.5, 3.0, 4.5, 6.0, 24, 48 and 72 h) and processed for BrdU immunofluorescence cytochemistry. Parameters used for cell cycle length measurement are indicated in Methods.*

After DNA synthesis, this takes place during the S phase of cell cycle, cells pass through the G2 phase before undergoing division in the M phase. Since the G2 phase is a critical step for cell cycle progression, we have sought to obtain information on the relative size of the population in the G2 versus M phase of cell cycle, by labeling the proliferating cells with an antibody against phosphorylated-histone-H3 (pHH3). This marker is expressed during late G2 and mitosis (anaphase to telophase) and its nuclear pattern allows one to discriminate cells that are in the G2 and M phases of cell cycle (see Methods; (Hendzel, Wei et al. 1997). We found that trisomic neurospheres had a larger percentage of NPCs in G2 and a similar percentage of cells in the M-phase compared with controls (Fig. 16A-B). Evaluation of the length of the G2- and M-phases (see Materials and Methods) indicated a notable elongation (+159%) of the G2-phase in trisomic neurospheres compared with controls, while the length of the M-phase was unchanged. There are no specific markers for the G1-phase of the cell cycle. Thus, we estimated its length by subtracting from the total cell cycle length the summed length of the remaining phases (S, G2, M). We found that in trisomic neurospheres, the duration of the G1-phase was significantly larger (+112%) than controls.

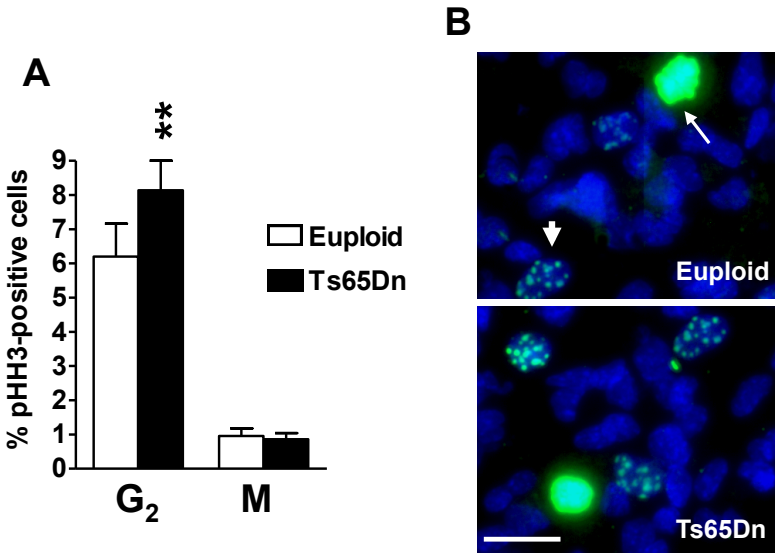


Figure 16: ***(A)*** *Number of cells in G_2- and M-phases of the cell cycle, expressed as percentage of total number of cells positive for pHH3, evaluated in euploid and trisomic neurospheres.* ***(B)*** *Images of pHH3-positive cells in euploid and Ts65Dn neurospheres. Cells in G_2 exhibit a discontinuous pHH3 nuclear staining (arrowhead), cells in the early M-phase exhibit a homogeneously condensed pattern (arrow).*

4.2 Neuronal precursor cultures from Ts65Dn mice exhibit the same defective neuronal differentiation as the *in vivo*condition

Recent evidence shows that not only neuron generation but also differentiation is heavily compromised in DS (Contestabile, Fila et al. 2007; Contestabile, Fila et al. 2009; Guidi, Ciani et al. 2011). We attempted to determine whether NPCs from Ts65Dn mice have defective neuronal differentiation similarly to the in vivo condition (Contestabile, Fila et al. 2007). For this purpose we compared the number of neurons and astrocytes produced by trisomic versus euploid neurospheres. After 7 days in culture conditions that favor differentiation (Reynolds and Weiss 1992), we found that the number of neurons (β-tubulin III-positive cells) generated from trisomic neurosphereswas notably smaller compared with controls (Fig. 17A-C), while the number ofastrocytes (GFAP-positive cells) was larger (Fig. 17B-C).

These results indicate that cultures of trisomic NPCs are a suitable model to study the mechanisms underlying neurogenesis impairment in DS because they exhibit a reduced proliferation rate, elongation of the cell cycle and impaired acquisition of a neuronal phenotype, similarly to the in vivo condition (Chakrabarti, Galdzicki et al. 2007; Contestabile, Fila et al. 2007; Contestabile, Fila et al. 2009; Bianchi, Ciani et al. 2010)

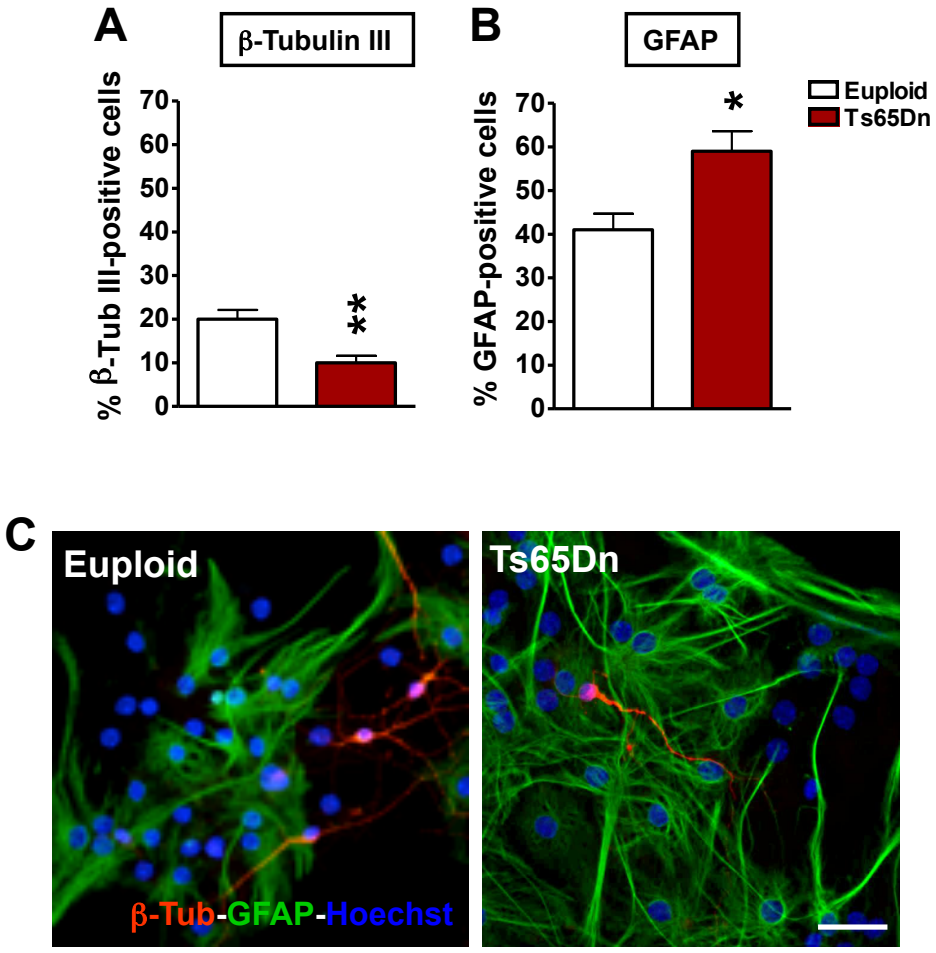


Figure 17: Differentiation of NPCs from euploid and Ts65Dn mice. **A,B**: *Percentages of β-Tubulin III (A) and GFAP (B) positive cells after 7 days of differentiation. *P<0.05, **P<0.01 (two tailed t-test). **(C)**: Representative double-fluorescence images of cells dissociated from neurospheres and immunopositive for β-tubulin III (red) and GFAP (green). Cell nuclei were stained by Hoechst dye (blue).*

4.3 Deranged expression of genes belonging to the Shh pathway in neural precursors from Ts65Dn mice

It has recently been shown that the reduced proliferation of cerebellar granule cell precursors from Ts65Dn mice is related to an attenuated response to Shh (Roper, Baxter et al. 2006), suggesting an alteration in the molecular cascade of this pathway in DS.

As first step, we performed a gene expression study, by quantitative real time PCR (RT–qPCR), to establish whether genes known to be involved in the Shh pathway exhibited an altered expression in trisomic NPCs. We found that the Gli family members Gli1, Gli2 and Gli3, which are transcription factors functioning as Shh effectors, showed a significant down-regulation (-42, -46 and -60%, respectively) in trisomic neurospheres (Fig. 18A).

Looking at the expression of Gli target genes, we found that MycN and Bmi1 were greatly down-regulated (-70 and -40%, respectively) in trisomic neurospheres (Fig. 18B).Additionally, the expression of FoxM1, an indirect Gli target that promotes cell cycle progression at the G2/M-phase transition, was also down-regulated (Fig. 18B). These observations clearly indicate derangement of the Shh pathway in NPCs from Ts65Dn mice.Activation of the Shh pathway starts with the binding of Shh to its receptor Patched1 (Ptch1), which releases the pathway activator Smoothened (Smo) by the Ptch1-dependent inhibition, leading to activation of GLI transcription factors.We next examined the expression of the membrane associated receptors Smo and Ptch1 and the Shh ligand in euploid and trisomic neurospheres. Whereas no significant differences were observed in the expression of Shh and Smo, we found a notable up-regulation of Ptch1 in trisomic neurospheres (Fig. 18C).

Western blot and immunocytochemistry analyses showed a significantly higher expression of Ptch1 in trisomic neurospheres also at the protein level (Fig. 19A and C). Similar results were obtained in trisomic neurospheres derived from the hippocampus (Fig. 19B) suggesting that an increased expression of Ptch1 may be the common molecular denominator of the Shh pathway derangement in trisomic NPCs.

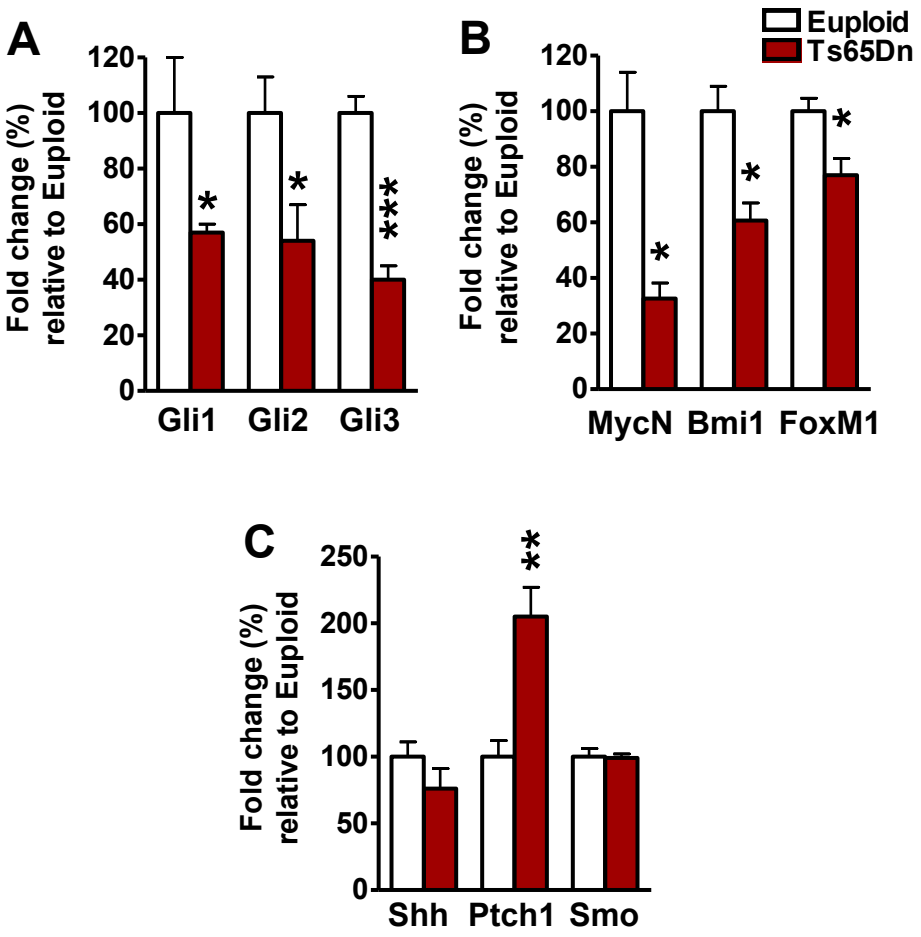


Figure 18: Expression of Shh pathway genes in neurospheres from euploid and Ts65Dn mice. ***(A-C)*** *Quantification by RT–qPCR of Gli1, Gli2 and Gli3* ***(A)****; MycN, Bmi1 and FoxM1* ***(B)****; Shh, Ptch1 and Smo* ***(C)*** *expression in neurospheres from Ts65Dn (n=6) and euploid (n=6) mice, given as percentage of the control condition. *P<0.05, **P<0.01, ***P<0.001 (two tailed t-test).*

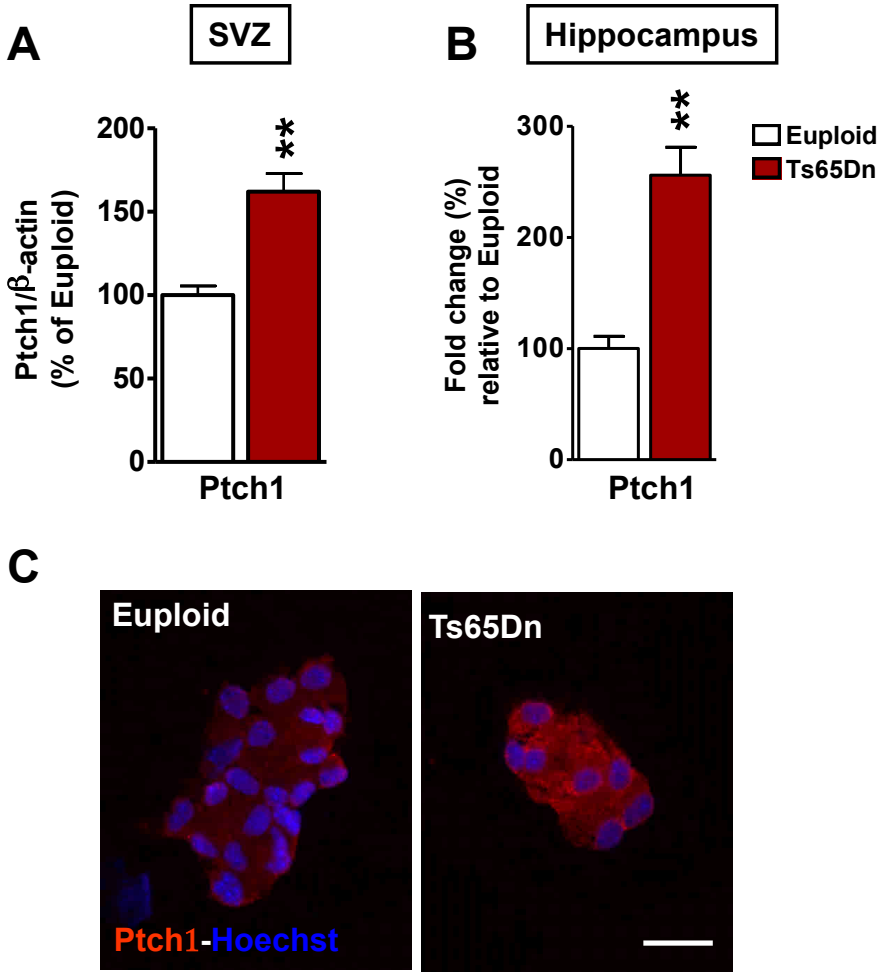


Figure 19: ***(A-B)*** *Western blot analysis of Ptch1 expression in neurospheres from the SVZ and hippocampus of Ts65Dn (n=4) and euploid (n=4) mice. Protein expression was normalized to β-actin content. Values represent mean±SE. * P< 0.05, ** P< 0.01, *** P< 0.001 (two-tailed t-test).* ***(C)*** *Representative images of neurospheres from an euploid and a Ts65Dn mouse processed for Ptch1 fluorescence immunocytochemistry. Cell nuclei were stained by Hoechst dye (blue).*

4.4 Neural precursors from Ts65Dn mice do not respond to Shh

Starting from the recent evidence that cerebellar granule cell precursors from Ts65Dn mice do not respond to the Shh stimulus (Roper, Baxter et al. 2006), we decided to evaluate also the response of NPCs from the SVZ to the Shh.

Administration of Shh for 72 h induced a proliferation increase in neurospheres from control mice, but no increase in neurospheres from Ts65Dn mice (Fig. 20A), indicating that trisomic NPCs from the SVZ are not responsive to Shh similarly to cerebellar granule cell precursors. In control neurospheres, treatment with Shh in the presence of cyclopamine, an antagonist of Smo, prevented the proliferation increase induced by Shh and, additionally, caused a decrease (-20%) in cell proliferation compared with the untreated condition (Fig. 20A). This suggested a basal activation state of the Shh pathway, probably due to paracrine production of Shh, as previously reported (Osterberg and Roussa 2009). In contrast, co-treatment with Shh and cyclopamine had no effect on cell proliferation in trisomic neurospheres. A series of small molecule agonists, that cross the blood–brain barrier, triggers Shh pathway activity by binding to and activating the Smo component of the Shh response pathway (Frank-Kamenetsky, Zhang et al. 2002); (Chen, Taipale et al. 2002). One of these molecules, SAG (smoothened agonist) is an agonist for Smo that relieves the inhibitory effect of Ptch1 on Smo (Chen, Taipale et al. 2002). Incubation of neurospheres with SAG enhanced cell proliferation in euploid (+23%) and trisomic (+56%) NPCs (Fig. 20A). The latter finding clearly indicates that the Shh pathway downstream to Smo is functional in trisomic NPCs, which suggests that its impairment is upstream to Smo. As expected, co-exposure of cells to SAG and cyclopamine prevented the proliferation increase induced by SAG alone in both euploid and Ts65Dn neurospheres (Fig. 20A).

To further confirm that the Shh pathway is impaired in trisomic NPCs upstream to Smo, we examined Gli1, whose expression is dependent on Shh pathway activation. While both Shh and SAG treatments significantly increased Gli1 expression in control neurospheres, in trisomic neurospheres SAG but not Shh treatment increased Gli1 expression (Fig. 20B). These data suggest that NPCs

from Ts65Dn mice are unable to respond to Shh due to up-regulation of Ptch1, which by inhibiting Smo, makes the Shh pathway functionally inactive.

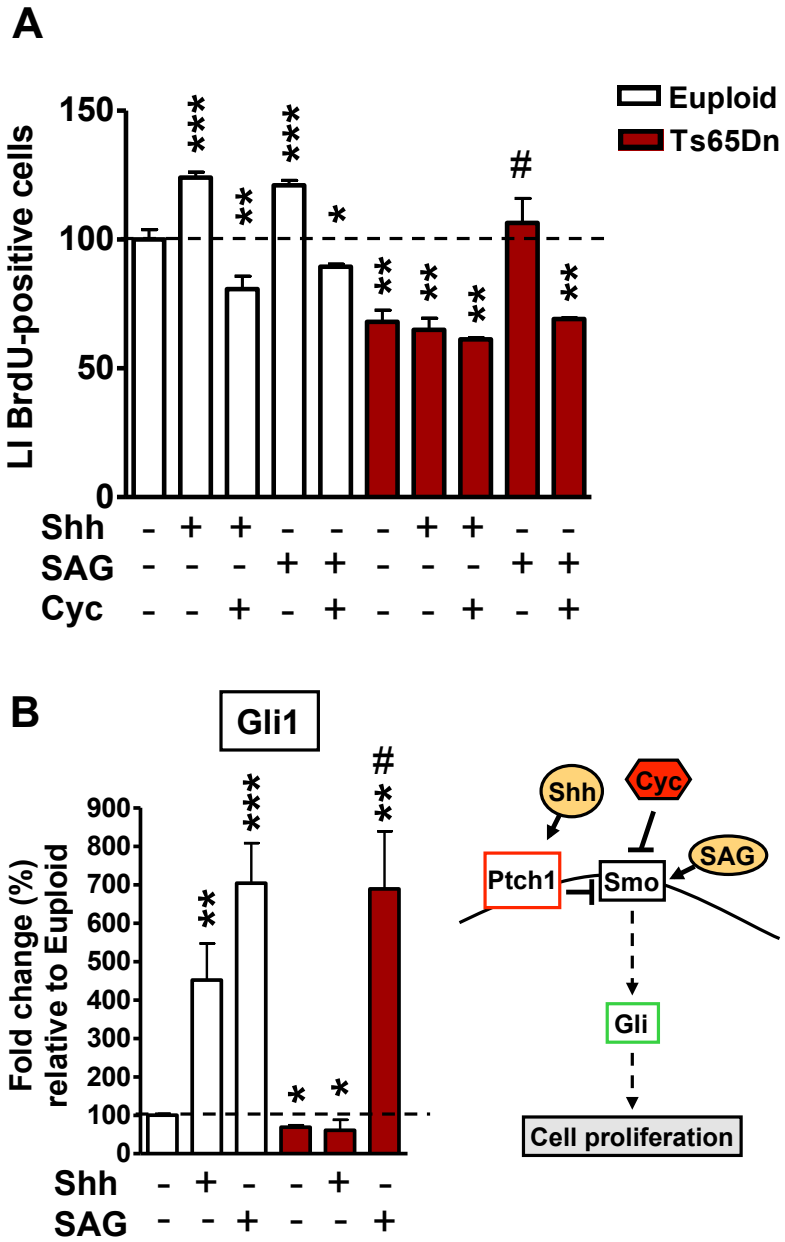


Figure 20: ***Response to Shh in neurospheres from euploid and Ts65Dn mice***. *(**A**) LI was determined for neurospheres from euploid (n=3) and Ts65Dn (n=3) mice. Neurospheres were treated with Shh (3 μg/ml), cyclopamine (Cyc; 10 μg/ml), SAG (250 nM), Shh plus cyclopamine or SAG plus cyclopamine for 72 h starting from DIV1. (**B**) Quantification by RT–qPCR of Gli1 expression in neurospheres treated with Shh (3 μg/ml) or SAG (250 nM) for 24 h starting at DIV4 (three Ts65Dn and three euploid mice). Data are given as percentage of the euploid untreated condition. The scheme on the right shows that Shh drives the Shh pathway by removing the inhibition exerted by Ptch1 on Smo, and the negative and positive effect exerted by Cyc and SAG, respectively, on Smo. Values represent mean+SE. The asterisk indicates a significant difference in euploid and trisomic neurospheres versus euploid untreated neurospheres. The # indicates a significant difference of trisomic treated neurospheres versus trisomic untreated neurospheres. ∗ P<0.05;*

4.5 Silencing of Ptch1 expression restores proliferation in neural precursors from Ts65Dn mice

According to the previous experiments, we found that NPCs from the trisomic NPCs were not able to respond to the Shh stimulus because of an alteration in the Shh pathway, upstream to Smo, due to an aberrant overexpression of Ptch1.

In this sense we further investigate about the role of Ptch1 in the impaired proliferation of NPCs from Ts65Dn mice, reducing its expression by using an antisense oligonucleotide (AS) directed against it. Antisense oligonucleotides are single strands of DNA or RNA that are complementary to a chosen sequence. Antisense DNA can be used to target a specific, complementary RNA, preventing protein translation. AS treatment reduced Ptch1 expression in both euploid and trisomic neurospheres (-60 and -40%, respectively) and, in trisomic neurospheres, Ptch1 expression became similar to that of the untreated euploid counterpart (Fig. 21A). Along with the reduction in Ptch1 expression, there was an increase in cell proliferation in both euploid and trisomic neurospheres (+20% and +39%, respectively) (Fig. 21B).Comparison of AS-treated trisomic neurospheres with untreated euploid neurospheres showed no proliferation differences (Fig. 21B), indicating that inhibition of Ptch1 expression had completely restored cell proliferation in trisomic NPCs. Treatment with a sense oligonucleotide, as a negative control, had no effect on Ptch1 expression and cell proliferation (data not shown).

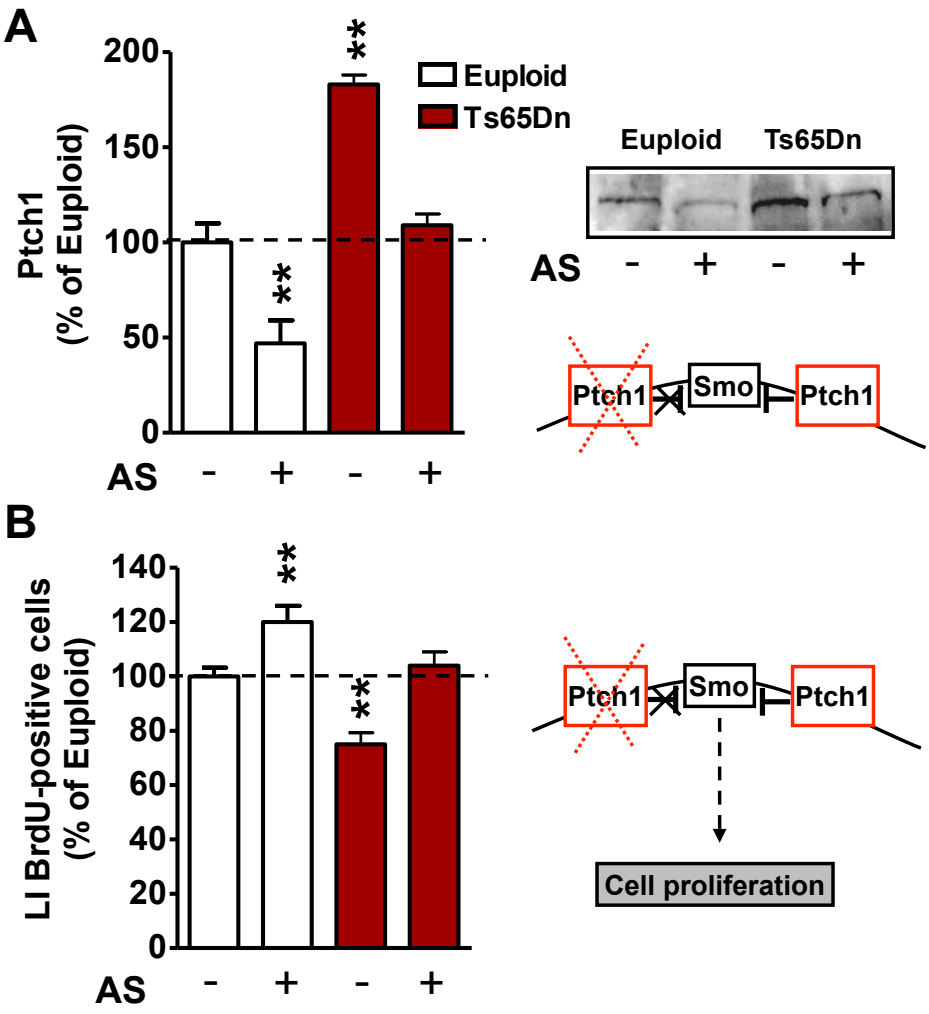


Figure 21: Effect of Ptch1 silencing on cell proliferation in neurospheres from euploid and Ts65Dn mice. (***A***) *Quantification of Ptch1 immunofluorescence in neurospheres from Ts65Dn and euploid mice after 24 h exposure to a Ptch1 antisense oligonucleotide (10 μM) starting at DIV3 (left panel). Representative example of a western blot showing the Ptch1 band in cell extracts from trisomic and euploid neurospheres after 24 h exposure to a Ptch1 antisense oligonucleotide (right panel). The partial silencing of Ptch1 by antisense oligonucleotides is shown in the scheme on the right. (**B**) LI, defined as percentage of BrdU-positive cells over total cell number, was determined for euploid and trisomic neurospheres treated with Ptch1 antisense oligonucleotides (10 μM) for 72 h after DIV1. The asterisks indicate a significant difference of euploid and trisomic neurospheres versus euploid untreated neurospheres. ** P<0.01, *** P<0.001 (Bonferroni's test after ANOVA).*

We next examined whether inhibition of Ptch1 expression made trisomic NPCs responsive to Shh. After treatment with AS, trisomic neurospheres became responsive to Shh and underwent a proliferation increase similarly to the euploid counterpart (Fig. 22: L plus AS). This finding demonstrates that inhibition of Ptch1 overexpression completely restores the response of trisomic NPCs to the Shh-mitogenic stimulus. Addition of a higher concentration of Shh to trisomic NPCs increased cell proliferation to the same level as treated-euploid NPCs (Fig. 22: H), suggesting that stimulation of the Shh pathway with a higher dose of Shh can overcome the inhibitory effect exerted by Ptch1 overexpression on Smo and therefore the mitogenic deficit in trisomic NPCs.

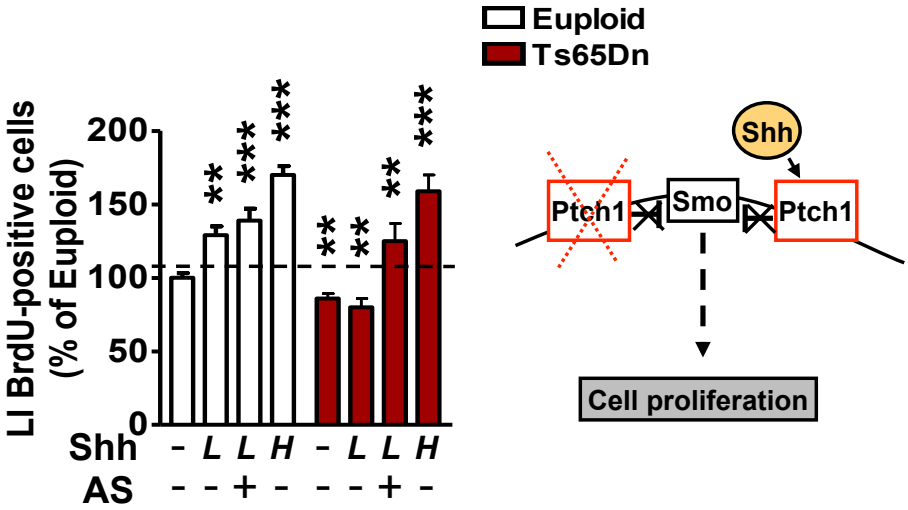


Figure 22*: (C) LI determined for euploid and trisomic neurospheres treated with either Shh alone (3 µg/ml; L and 6 µg/ml; H) or Shh (3 µg/ml; L) plus Ptch1 antisense oligonucleotides (10 µM) for 72 h after DIV1. Data are given as percentage of the euploid untreated condition, are expressed as mean±SE (four euploid and four Ts65Dn mice).*
*The asterisks indicate a significant difference of euploid and trisomic neurospheres versus euploid untreated neurospheres. ** P<0.01, *** P<0.001 (Bonferroni's test after ANOVA).*

4.6 Ptch1 promoter is highly acetylated in neural precursors from Ts65Dn mice

Gene expression is finely regulated by epigenetic mechanisms of methylation and acetylation. These epigenetic mechanisms have an important regulatory role in gene expression and act by inhibiting and favoring gene transcription, respectively (Vaissiere, Sawan et al. 2008).There is evidence that epigenetic modification of the *PTCH1* promoter significantly affect *PTCH1* gene expression in various disease conditions (Cretnik, Musani et al. 2007; Pritchard and Olson 2008). For this reason, we sought to determine whether DNA methylation and/or histone acetylation can regulate the activity of the Ptch1 promoter in NPCs. In this sense, we treated neurospheres with either 5-Aza-2′-Deoxycytidine (AZA), an inhibitor of DNA methyltransferase or Trichostatin A (TSA), an inhibitor of histone deacetylase. While AZA treatment induced a small increase (2–3-folds) in Ptch1 expression, TSA treatment highly increased (20–22-folds) Ptch1 expression in both control and trisomic neurospheres (Fig. 23). This suggests that DNA methylation of the Ptch1 promoter is not a major determinant of Ptch1 regulation, while histone acetylation appears to be an important epigenetic mechanism that regulates Ptch1 expression in NPCs.

We next attempt to establish whether trisomic neurospheres exhibited a different Ptch1 promoter methylation and/or acetylation status versus euploid neurospheres. To this purpose, we employed methylcytosine immunoprecipitation (mCIP) and acetyl-histone H3 immunoprecipitation (chromatin immunoprecipitation (ChIP)) assays. In the promoter region of Ptch1close to the start site, there are two CpG islands (Fig. 24: **c**, **e**) and a CpG-rich region (Fig. 23: **b**) (Takai and Jones 2002). The relative enrichment of methylated DNA in the CpG islands (**c, e**) and in the CpG-rich region (**b**), normalized to the enrichment of two control regions (non-CpG-rich regions; **a, d**) is shown in Figure 24 (upper histograms). It can be noted that, unlike the CpG-rich region, the CpG islands of the Ptch1 promoter were unmethylated, both in control and trisomic NPCs. In the CpG-rich region of trisomic NPCs, the methylation status was slightly, though not significantly, higher than the euploid counterpart (Fig. 24: b). On the contrary, we found that

the histone H3 acetylation was stronger in all these regions (a–e) in trisomic versus euploid NPCs (Fig. 24). More specifically, hyper-acetylation occurred at those promoter regions proximal to the transcription start site (c, d and e in Fig. 24). These data indicate that in trisomic NPCs the Ptch1 promoter is highly acetylated and provide a plausible explanation for why Ptch1 is overexpressed in trisomic NPCs. No difference in the acetylation status of the Smo promoter was observed between euploid and trisomic neurospheres (Table 7) suggesting that the increased acetylation in trisomic neurospheres is peculiar for the Ptch1 promoter.

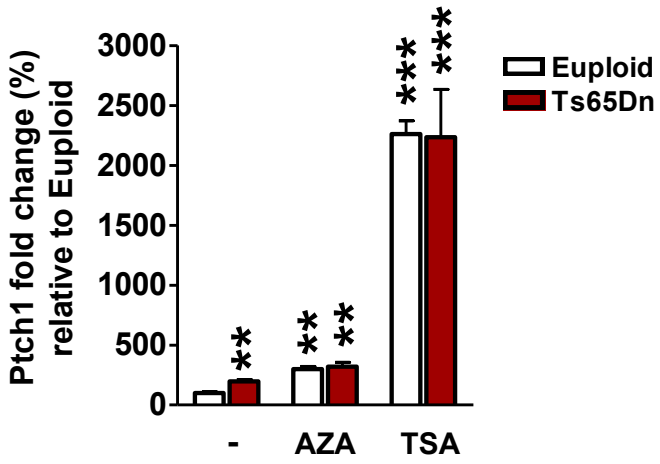


Figure 23: Methylation and acetylation status of the Ptch1 promoter in neurospheres from euploid and Ts65Dn mice.
*(**A**) Quantification by RT–qPCR of Ptch1 expression in neurospheres from Ts65Dn (n . 4) and euploid (n . 4) mice treated with either 5-Aza-2'-deoxycytidine (AZA, 5 μM) or TSA (150 nM) for 72 h after DIV1. Data, given as percentage of the euploid untreated condition, are expressed as mean+SE. The asterisks indicate a significant difference between euploid and trisomic neurospheres versus euploid untreated neurospheres. **** P<0.01, ***** P<0.001 (Bonferroni's test after ANOVA).*

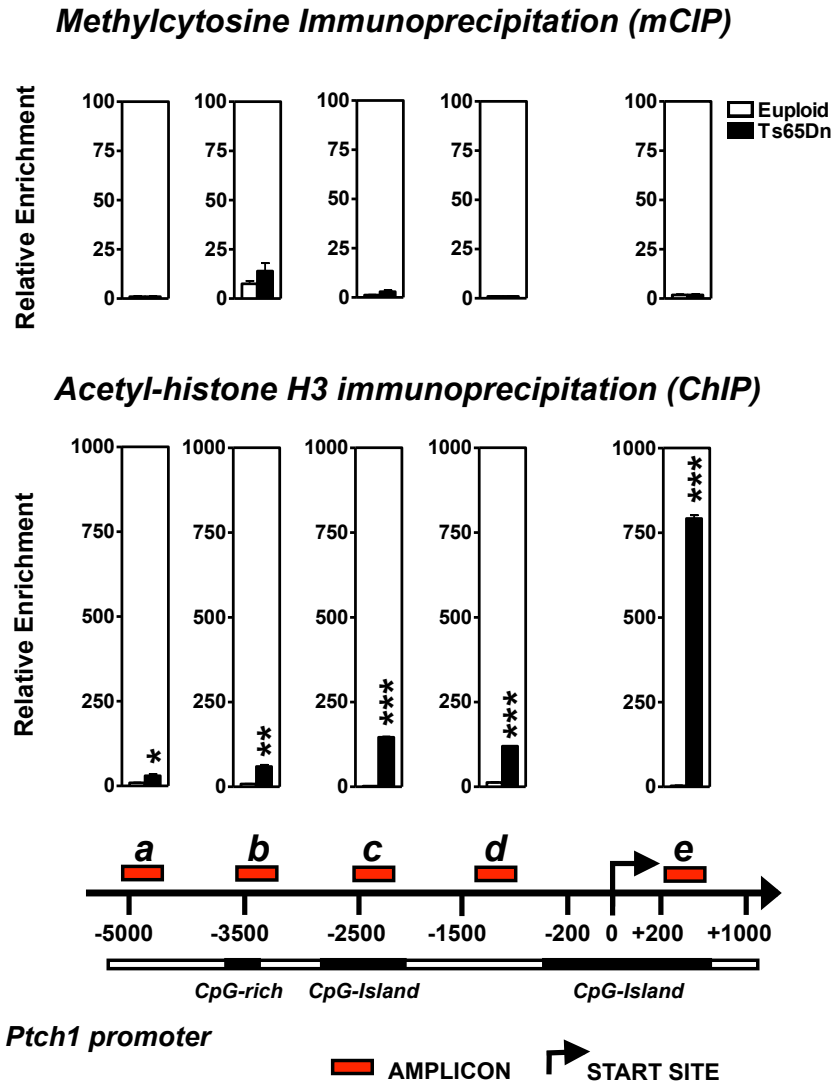

Methylcytosine Immunoprecipitation (mCIP)
Relative Enrichment
100
75
50
25
0
Euploid
Ts65Dn
Acetyl-histone H3 immunoprecipitation (ChIP)
Relative Enrichment
1000
750
500
250
0
*
**
***
***
***
a
b
c
d
e
-5000
-3500
-2500
-1500
-200
0
+200
+1000
CpG-rich
CpG-Island
CpG-Island
Ptch1 promoter
AMPLICON
START SITE

Figure 24: ***Detailed methylation and acetylation status of the Ptch1 promoter in neurospheres from euploid and Ts65Dn mice.***
The mCIP assay (upper panels) was performed with an antimethylcytosine antibody and primers targeting amplicons a–e in neurospheres at DIV4 from trisomic (n . 4) and euploid (n . 4) mice. Fold enrichment of the Ptch1 gene promoter by anti-methylcytosine antibody was calculated by dividing the PCR product from amplicons b, c or e by the PCR product from amplicons a and d that represent non-CpG control regions. The ChIP assay (middle panels) was performed using an anti-Acetyl-Histone H3 and the same primers used for the mCIP. Fold enrichment of a given DNA region immunoprecipitated with the anti-Acetyl-Histone H3 antibody was calculated as the ratio between the enrichment obtained with the specific antibody compared with pre-immune serum. Values represent mean±SE. ∗ P<0.05, ∗∗ P<0.01, ∗∗∗ P<0.001 (two-tailed t-test).
The lower panel shows a schematic representation of the mouse Ptch1 gene promoter. The diagram includes: transcription start site (black arrow); target regions of PCR primers for quantitative ChIP assay (amplicons a–e: gray boxes); CpG-rich region and CpG islands (black boxes)

Acetyl-histone H3 immunoprecipitation (ChIP)

	Amplicon	**a**	**b**	**c**	**d**
Euploid	(n=3)	12.8±0.2	20.9±1.5	123±11	344±120
Ts65Dn	(n=3)	12.4±0.3	14.3±5.0	126±9.0	522±44

AICD immunoprecipitation (ChIP)

	Amplicon	**a**	**b**	**c**	**d**
Euploid	(n=4)	1.0±0.19	2.07±1.2	1.11±0.33	1.72±0.78
Ts65Dn	(n=4)	1.0±0.12	0.79±0.53	0.70±0.62	1.51±0.21
Euploid + DAPT	(n=3)	1.0±0.42	0.84±0.48	0.37±0.57	0.51±0.86
Ts65Dn +DAPT	(n=3)	1.0±0.33	0.96±0.28	2.21±0.64	1.55±0.44

Table 7: *Acetyl-histone H3 and AICD immunoprecipitation analysis of the Smo promoter*

4.7 Triplicated amyloid precursor protein increases Ptch1 expression through AICD

In Ts65Dn mice, there is an extra copy of the gene coding for the amyloid precursor protein (APP), which led to a greater expression of this protein in the Ts65Dn brains. (Holtzman, Santucci et al. 1996).

APP undergoes extensive post-translational modification including proteolytic processing to generate peptide fragments. APP is commonly cleaved by proteases of the secretase family; α-secretase, β-secretase and γ-secretase. It has been recently shown that the APP intracellular domain (AICD), that is generated from the APP protein through sequential proteolysis by α- and γ-secretases, up-regulates PTCH1 expression in neuroblastoma cells (Raychaudhuri and Mukhopadhyay). AICD has been reported to form a complex with Fe65 and the histone acetyltransferase Tip60 and cause transcriptional activation by increasing the acetylation status of the chromatin (Cao and Sudhof 2001; Kim, Kim et al. 2004). Quantification of the APP gene in trisomic NPCs showed that its expression was very close to the theoretical 1.5-fold increase expected from the presence of one extra copy of APP (Fig. 25A). In parallel with these findings, AICD had significantly increased levels in the nucleus of trisomic NPCs (Fig. 25B).

We next examined the potential interaction between AICD and the Ptch1 promoter, by ChIP analysis. Crosslinked chromatin extracts from trisomic and euploid neurospheres, immunoprecipitated with an antibody against AICD, showed the presence of AICD binding to the three regions of the Ptch1 promoter with highest acetylation (Fig. 24C: c–e). In contrast, in euploid neurospheres, the levels of chromatin extracts immunoprecipitated with AICD were similar to the background (=1), indicating the absence of AICD binding to the Ptch1 promoter

(Fig. 24C). We treated neurospheres with DAPT, an inhibitor of γ-secretase, in order to reduce AICD formation and found that this treatment reduced the binding of AICD to the Ptch1 promoter in trisomic neurospheres (Fig. 24C). In contrast, the binding of AICD to the Smo promoter was similar in euploid and trisomic neurospheres under basal conditions and after treatment with DAPT (Table 7). Treatments with DAPT reduced Ptch1 expression in trisomic neurospheres (Fig. 26D). The same results were obtained with Compound E, another inhibitor of γ-secretase (Fig. 26D). These data provide evidence for a relationship between AICD levels and Ptch1 expression in trisomic neurospheres.

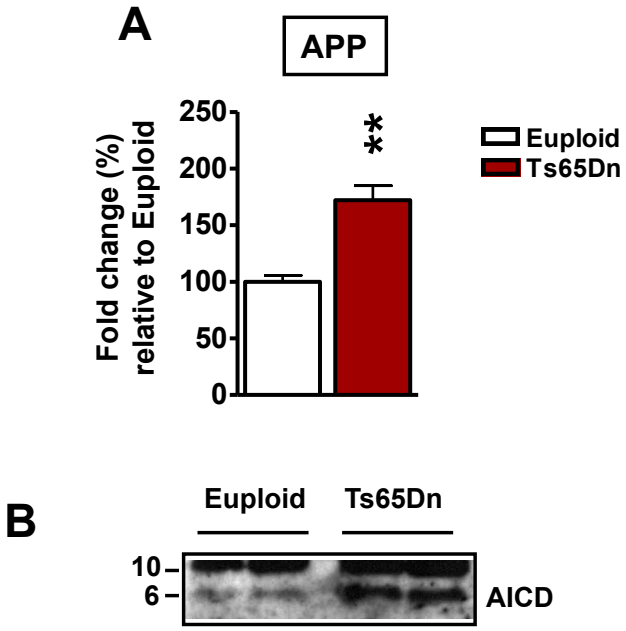


Figure 25: APP and AICD expression in neurospheres from euploid and Ts65Dn mice. (*A) Quantification by RT–qPCR of APP expression in trisomic and euploid neurospheres at DIV4. Values represent mean+SE. ** P , 0.01 (two-tailed t-test).*

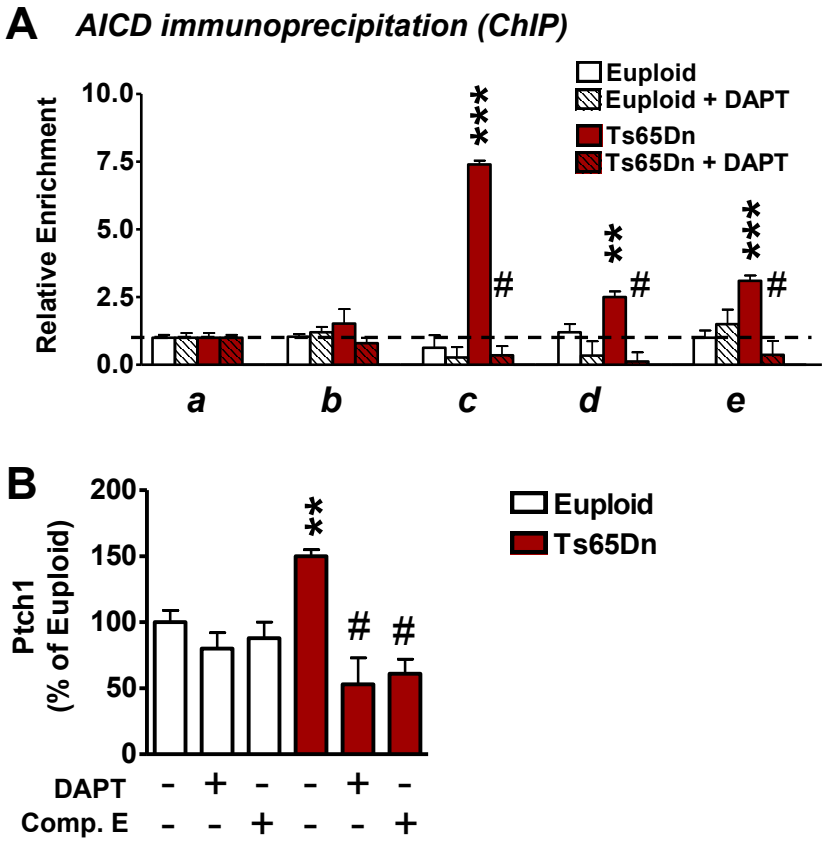
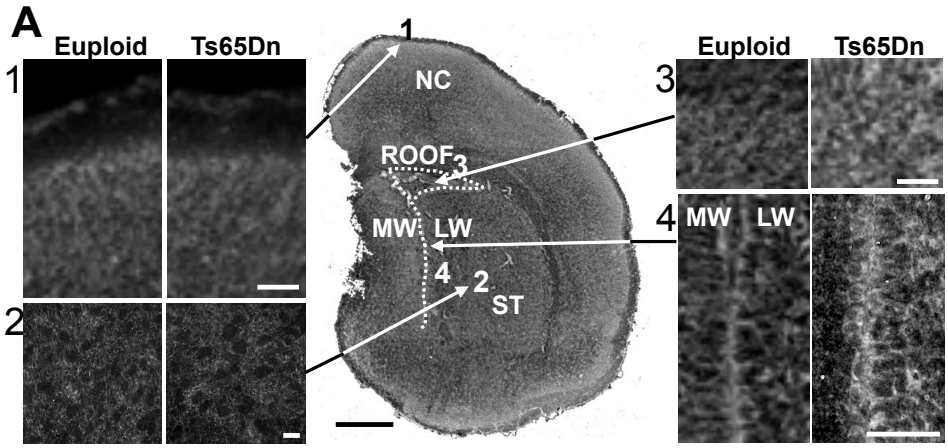
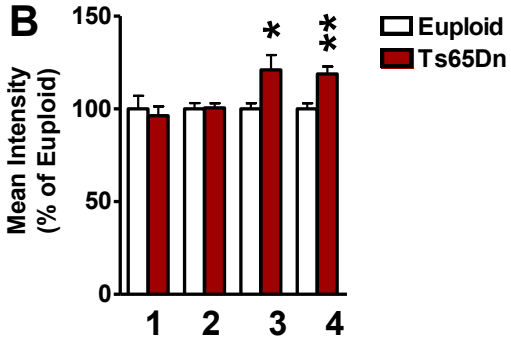


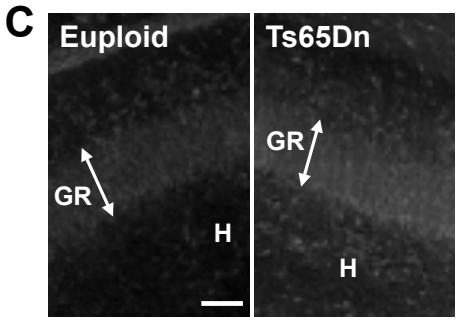
Figure 26: AICD binding to Ptch1 promoter in neurospheres from euploid and Ts65Dn mice. *(A) ChIP assay using an anti-C-terminal APP antibody which recognizes AICD and the same primers used for the mCIP (amplicons a–e are the same as Fig. 24) in neurospheres from trisomic (n = 4) and euploid (n = 4) mice in the presence of absence of the γ-secretase inhibitor DAPT (10 μM; 48 h). Fold enrichment of a given DNA region of the mouse Ptch1 gene promoter was calculated as the ratio between the enrichment obtained with the specific antibody compared with a non-specific IgG (control). (**B**) Quantification of Ptch1 immunofluorescence in neurospheres from Ts65Dn and euploid mice after 48 h exposure to the γ-secretase inhibitor DAPT (10 μM) or Compound E (Comp. E; 1 nM), starting at DIV1. The asterisk indicates a significant difference of euploid and trisomic neurospheres versus euploid untreated neurospheres. The # indicates a significant difference of trisomic treated neurospheres versus trisomic untreated neurospheres. * P , 0.05; ** P , 0.01; *** P , 0.001 (Bonferroni's test after ANOVA).*

4.8 *In vivo* Ptch1 overexpression in the trisomic brain

We next sought to establish whether the reduced proliferation rate of trisomic NPCs was accompanied by a high expression of Ptch1also in the in vivo condition. We found that neonate (P2) Ts65Dn mice had remarkably fewer proliferating cells (BrdU-positive cells) in the SVZ (euploid: 22260±1979, Ts65Dn: 9106±2271; $P<0.01$) and in the hippocampal dentate gyrus (DG; euploid: 6320±2616, Ts65Dn: 2154±670; $P<0.05$) compared with control mice, as previously observed in P15 adult trisomic mice (Bianchi, Ciani et al. 2010; Bianchi, Ciani et al. 2010). We first analysed Ptch1 expression in whole brain homogenates derived from neonate Ts65Dn mice and found no difference vs euploid mice (data not shown). However, in view of the small number of precursor cells compared with the number of neurons and glial cells forming the brain, a possible up-regulation of Ptch1 confined to neuronal precursors may be undetectable in whole brain homogenates. Consequently, we analyzed Ptch1 expression by fluorescence immunohistochemistry. Consistently, with the up-regulation of Ptch1 observed in vitro (Fig. 19A, B and C), we found that in the SVZ and DG of Ts65Dn mice, Ptch1 had a considerably higher expression compared with that of euploid mice (Fig. 27A–D). No difference in Ptch1 expression was found in the striatum and prefrontal cortex of Ts65Dn versus euploid mice (Fig. 27A and B). These data suggest that overexpression of Ptch1 in trisomic mice takes place specifically in proliferating neural precursors.


A
Euploid
Ts65Dn
1
2
3
4
NC
ROOF
MW
LW
ST


B
Mean Intensity
(% of Euploid)
150
100
50
0
1
2
3
4
Euploid
Ts65Dn


C
Euploid
Ts65Dn
GR
H

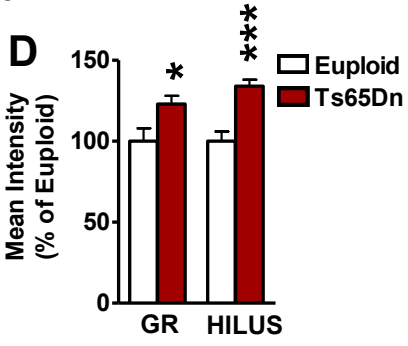

D
Mean Intensity
(% of Euploid)
150
100
50
0
GR
HILUS
Euploid
Ts65Dn

Figure 27: Ptch1 expression in Ts65Dn mice. *(**A**) Examples of Ptch1 fluorescence immunohistochemistry at the level of the rostral part of the lateral ventricle of a P2 euploid and a Ts65Dn mouse. Images were taken in the regions labeled with the corresponding numbers in the section shown in the middle (1, neocortex; 2, striatum; 3, roof of the lateral ventricle; 4, lateral wall of the lateral ventricle). Calibrations: low magnification image = 200 µm; higher magnification images = 40 µm. (**B**) Quantification of Ptch1 expression in euploid and Ts65Dn mice. (**C**) Examples of Ptch1 fluorescence immunohistochemistry at the level of the hippocampal DG (left panels). Scale bar: 60 µm. (**D**) Quantification of Ptch1 expression in the granule cell layer (GR) and hilus (SGZ plus hilus proper) of euploid and Ts65Dn mice. Data (in B and D), given as percentage of the euploid condition, are expressed as mean±SE (three euploid and three Ts65Dn mice). ∗ P<0.05, ∗∗ P<0.01, ∗∗∗ P<0.001 (two-tailed t-test). GR, granule cell layer; H, hilus; I, layer one; II layer two; LW, lateral wall of the lateral ventricle; ML, medial wall of the lateral ventricle; NC, neocortex; ROOF, roof of the lateral ventricle; ST, striatum.*

Recently, we found that human fetuses with DS had remarkably fewer proliferating cells in all germinal zones of the hippocampal region (Contestabile, Fila et al. 2007; Guidi, Bonasoni et al. 2008). To establish whether, similarly to the mouse model, fetuses with DS exhibit PTCH1 overexpression, we examined the expression pattern of PTCH1 in the ventricular zone of the hippocampus and parahippocampal gyrus (PHG) (Fig. 28A). At the examined ages (17–21 weeks), the ventricular zone of the hippocampus and PHG was formed by several rows of actively dividing cells (Guidi, Bonasoni et al. 2008). Immunohistochemical analysis showed that PTCH1 had a larger expression in the ventricular zone of the hippocampus (+50%) and PHG (+88%) of fetuses with DS (Fig. 28B), indicating that a defect in PTCH1 expression is present also in neuronal precursor cells of human subjects with DS.

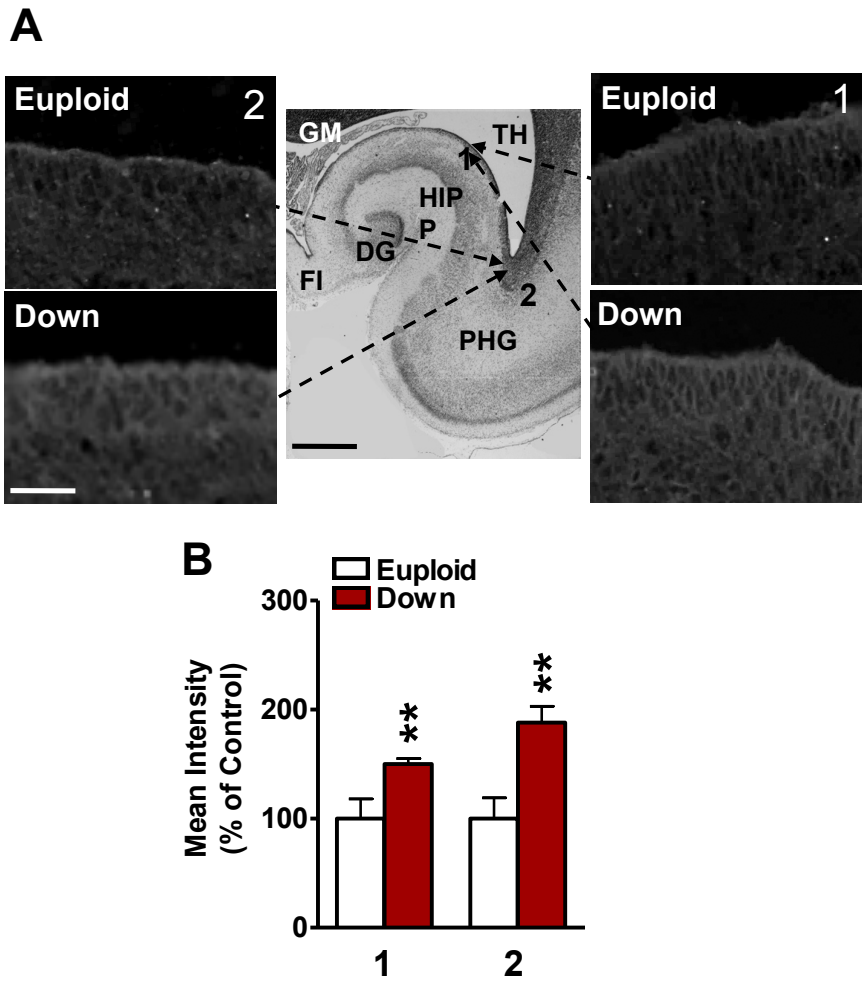


Figure 28: PTCH1 expression in control and DS human fetuses. *(A) Examples of PTCH1 fluorescence immunohistochemistry at the level of the hippocampal region of a control (GW 19) and a DS (GW 19) fetuses. Images were taken in the regions labeled with the corresponding numbers in the Nissl-stained section shown in the middle. Calibrations: low magnification image = 1000 µm; higher magnification images = 50 µm. DG, dentate gyrus; FI, fimbria; GM, germinal matrix; HIPP, hippocampus; PHG, parahippocampal gyrus; TH, temporal horn of the lateral ventricle. (**B**) Quantification of PTCH1 expression in control and DS fetuses. Data, given as percentage of the control condition, are expressed as mean±SE (three control and three DS fetuses). ** P< 0.01 (two-tailed t-test).*

5. DISCUSSION

5.1 Cultured trisomic NPCs exhibit the same neurogenesis defects as the in *vivo* condition.

Analysis of cultures of NPCs from Ts65Dn mice showed that they exhibited a significantly reduced proliferation rate compared to the euploid counterpart. Furthermore, we demonstrated that it is due to an elongation of the cell cycle, with a particularly prominent elongation of the G1- and G2-phases. Finally, similarly to previous observation in vivo (Contestabile, Fila et al. 2007; Contestabile, Fila et al. 2009), trisomic NPCs exhibited an impaired acquisition of a neuronal phenotype.

These findings indicate that cultures of NPCs from the Ts65Dn mouse replicate the same proliferation/differentiation defects observed in vivo (Contestabile, Fila et al. 2007; Contestabile, Fila et al. 2009), suggesting that the in vitro system faithfully recapitulates the molecular mechanisms that underlie neurogenesis impairment in DS. Our findings are in line with recent studies that have demonstrated proliferation and differentiation impairment in cultures of embryonic NPCs from the Ts1Cje mouse model (Moldrich, Dauphinot et al. 2009) and from fetuses with DS (Bahn, Mimmack et al. 2002; Bhattacharyya, McMillan et al. 2009)

5.2 Impairment of the Shh pathway characterizes trisomic NPCs

Accumulating evidence has highlighted the importance of Shh signaling for neuronal precursor proliferation. Shh treatment of cultured neural stem cells derived from the SVZ and hippocampus has been shown to increase proliferation of multipotent neural stem cells (Lai, Kaspar et al. 2003; Palma, Lim et al. 2005). Additionally, while viral delivery of Shh increases proliferation of cells in the hippocampal subgranular zone, conditional knockout of Smo (a membrane protein that mediates the Shh effects) results in a decrease in neural precursor proliferation and neurosphere formation (Lai, Kaspar et al. 2003; Machold,

Hayashi et al. 2003). Previous research demonstrated that cerebellar granule cell precursors of Ts65Dn mice had a deficit in the Shh-induced mitogenic response versus the euploid counterpart (Roper, Baxter et al. 2006). Due to the very wide brain expression of the Shh pathway, we hypothesized that trisomic NPCs from different neurogenic regions may exhibit a similar Shh response deficit, which would explain the widespread neurogenesis impairment that characterizes the DS brain. We indeed found a deficit in the Shh-induced mitogenic response in NPCs derived from both the SVZ and hippocampus of Ts65Dn mice. The response deficit to Shh in cerebellar granule cells (Roper, Baxter et al. 2006), SVZ and hippocampal precursors confirms our hypothesis that Shh pathway impairment may be a common mechanism that underlies the proliferation deficit of trisomic neuronal precursors. A recent work in the Ts65Dn mouse demonstrated that neural crest progenitors giving origin to the mandible have a deficient mitotic response to Shh (Roper, VanHorn et al. 2009). In addition, recent evidence in human fetuses with DS shows that aberrant lymphatic endothelial cells exhibit an increased Shh expression (de Mooij, van den Akker et al. 2009). These findings suggest that the trisomic condition is characterized by a widespread alteration of the Shh pathway that may involve peripherical tissues.

5.3 Ptch1 overexpression underlies impairment of Shh-induced mitogenic response of trisomic NPCs

The molecular and intracellular interactions that transduce the mitogenic response to Shh are not completely understood but are believed to include the nuclear translocation of cytoplasmic Gli1 with increased transcription of MycN, cyclin D, Ptch1 and Gli1 itself (Ding, Fukami et al. 1999; Kenney, Cole et al. 2003). We found here that genes known to be involved in the Shh pathway exhibited an altered expression in trisomic NPCs. While most of the genes downstream to Shh signaling (including the three Gli proteins and MycN) were down-regulated, there was a large increase in Ptch1 expression. A defining characteristic of the Shh pathway is the antagonistic relationship between Shh and Ptch1. Activation of the Shh pathway starts with the binding of Shh to its receptor Ptch1, which releases

Smo by the Ptch1-dependent inhibition. A recent study has demonstrated, by examining the effects of Ptch1 overexpression in transgenic mice, that a balance between Shh and Ptch1 is necessary for normal brain development (Goodrich, Jung et al. 1999). An excess of Ptch1 attenuates the induction of Shh target genes and causes abnormal neural tube patterning. This indicates that Ptch1 overexpression is able to oppose Shh signaling and reveals an important role for Ptch1 in brain development and growth. On the contrary, loss-of-function mutations or transcriptional repression of Ptch1 leads to constitutive activation of Shh signaling pathway and result in an aberrant increase in cell proliferation (Izraeli, Lowe et al. 2001). We found that either treatment with SAG, an agonist of the Shh pathway that acts downstream to Ptch1 or inhibition of Ptch1 expression by antisense oligonucleotides, can overcome the mitogenic deficit in trisomic NPCs. These results demonstrate that an excess of Ptch1 is the key determinant of the reduced transcription of Shh target gene and proliferation deficits in trisomic NPCs. The notably increased Ptch1 expression in trisomic NPCs explains why Shh levels that were sufficient to activate the pathway in euploid NPCs failed to produce a similar effects in trisomic NPCs. Interestingly, recent studies have demonstrated that by increasing Shh concentration, it is possible to restore the Shh-mitogenic response of trisomic granule cell and neuronal crest precursors (Roper, Baxter et al. 2006; Roper, VanHorn et al. 2009). This is in line with our findings, because in the presence of an increased number of Ptch1 molecules (and hence a strong inhibition of the pathway), a corresponding increase in Shh levels is required to activate the pathway in trisomic precursor cells. In line with data obtained in NPCs from the Ts65Dn mouse model, we found up-regulation of PTCH1 in neuronal precursors of human fetuses with DS. Interestingly, a very recent study (Derwinska, Smyk et al. 2009) identified PTCH1 duplication in a family with microcephaly and mild developmental delay. These patients display many characteristics similar to DS such as hypotonia, developmental delay, flat occipit, broad facies, inner epicanthal folds, brachydactyly, loose joints, heart defects and delayed myelination. According to all this evidence, deregulation of PTCH1 expression may represent a

key factor that underlies impairment of neural cell proliferation in individuals with DS and, most likely, also different facets of the somatic DS phenotype.

5.4 Mechanisms underlying Ptch1 overexpression in trisomic NPCs

Cleavage of the APP by γ-secretase generates the APP intracellular domain (AICD) peptide. Recent evidence shows that AICD is involved in the regulation of gene transcription and that PTCH1 is one of the genes that are regulated by AICD (Kim, Kim et al. 2004; Muller, Meyer et al. 2008; Raychaudhuri and Mukhopadhyay 2010). We found increased levels of AICD in trisomic NPCs, which is in agreement with the overexpression of the trisomic gene APP, and an increased binding of AICD to the Ptch1 promoter. Inhibition of AICD formation reduced the binding of AICD to the Ptch1 promoter and reduced Ptch1 expression. These findings strongly suggest that AICD overexpression underlies Ptch1 up-regulation in trisomic NPCs. It has been recently shown that, in addition to genetic mutations in PTCH1 (Evangelista, Tian et al. 2006), epigenetic silencing of PTCH1 promoter by DNA methylation and histone deacetylation contributes to brain tumor formation (Pritchard and Olson 2008; Ecke, Petry et al. 2009)). We found that while Ptch1 expression was almost unaffected by demethylation (by AZA), it was enhanced by inhibition of deacetylation (by TSA), suggesting that acetylation is a major epigenetic mechanism involved in the regulation of Ptch1 expression in NPCs. The covalent modification of core histones by acetylation leads to a decondensed nucleosomic structure that allows access of transcription factors to the DNA (Jenuwein and Allis 2001). The finding that in trisomic NPCs, the promoter of Ptch1 was hyper-acethylated suggests that this hyper-acetylation may underlie Ptch1 overexpression. AICD has been reported to form a complex with Fe65 and the histone acetyltransferase Tip60 and cause transcriptional activation by increasing the acetylation status of the chromatin (Cao and Sudhof 2001; Kim, Kim et al. 2004). It may, therefore, be hypothesized that overproduction of AICD in trisomic NPCs may be involved in the hyper-acetylation status of the Ptch1 promoter. Further experiments are

needed to clear this issue.

The role of triplicated genes in the neurological phenotype of DS is still poorly understood. Our data suggest that the trisomic gene APP may be a key determinant of impaired neurogenesis in DS, through an AICD-dependent up-regulation of Ptch1 and consequent deregulation of the Shh mitogenic pathway. Interestingly, recent evidence shows that AICD transgenic mice exhibit impaired neurogenesis similarly to trisomic mice (Ghosal, Stathopoulos et al. 2010).

APP is cleaved by different secretases, which may result in functionally distinct outcomes. The amyloidogenic pathway is initiated by β-secretase cleavage of APP to yield the N-terminal soluble APP plus a C-terminal fragment β (CTFβ). CTFβ can then be processed by γ-secretase to generate β-amyloid (Aβ) and a free AICD. The current study shows a key role of AICD in neurogenesis defects in DS. Recent evidence suggests that in addition to Aβ, AICD may be involved in the pathogenesis of Alzheimer's disease. In agreement with APP triplication and, hence, increased levels of Aβ and AICD, most individuals with DS develop Alzheimer's disease after the fourth–fifth decade of life. Compounds that inhibit or modulate γ-secretase, the pivotal enzyme that generates both Aβ and AICD, may be potential therapeutics for the early neurogenesis impairment and late-occurring neurodegeneration in individuals with DS.

6. CONCLUSION

All our studies demonstate that proliferation impairment which charaterize the trisomic brain, is due to an alteration of the Shh pathway. In particular we show that NPcs from Ts65Dn mice exhibit an aberrant overexpression of Ptch-1, Shh inhibitory regulator. Ptch-1 overexpression is promoted by a hyperacetilation status of its promoter caused by the increades levels of AICD, the APP intracellular domain.

At the same time, NPCs from human fetuses with DS are characterized by an overexpression of PTCH1 suggesting that **Ptch1 overexpression might represent the key determinant of derangement of the Shh pathway and, hence, of the reduced proliferation that characterizes the DS brain**.

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