ROLE OF DYRK1A IN HIPPOCAMPAL NEUROPLASTICITY: IMPLICATIONS FOR DOWN SYNDROME

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Als meus pares, a la Marina i al Pol

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ABSTRACT / RESUM

Abstract

Synaptic connections in the brain respond throughout their lives to the activity of incoming neurons, adjusting their biological properties to increment activity- dependent changes. Hippocampal neuronal plasticity disruptions have been suggested as mechanisms underlying cognitive impairments in Down syndrome (DS). However, it remains unknown whether specific candidate genes are implicated in these phenotypes in the multifactorial context of trisomy 21. DYRK1A is a serine/threonine kinase, which overexpression is sufficient to recapitulate hippocampal learning and memory deficits characteristic of DS individuals and trisomic mouse models. In this Thesis we have studied the effects of DYRK1A overexpression on activity-dependent plasticity in the hippocampus. We found that transgenic mice overexpressing Dyrk1A (TgDyrk1A) present hippocampal morphological alterations in CA1 and CA3 that may constrain network connectivity, and therefore are relevant to the structure-function relationship. We also found reduced LTP that may derive from the changes in connectivity and in dendritic occupancy. Dendritic excitability and neuronal morphology are determinants of synaptic efficacy and thus may contribute to the hippocampal learning and memory deficits detected. In addition, we demonstrated important defects in adult neurogenesis in the dentate gyrus including reduced cell proliferation rate, altered cell cycle progression and reduced cell cycle exit leading to premature migration, altered differentiation and reduced survival of newly born cells. Moreover, less proportion of newborn hippocampal TgDyrk1A neurons are activated upon learning, suggesting reduced integration in learning circuits. Some of these alterations were rescued by normalizing DYRK1A kinase using a DYRK1A inhibitor, epigallocatechin-3-gallate. Interestingly, environmental stimulation also normalized DYRK1A kinase overadosage in the hippocampus, and rescued hippocampal morphology, synaptic plasticity and adult neurogenesis alterations in TgDyrk1A mice. We conclude that *Dyrk1A* is a good candidate gene to explain neuronal plasticity deficits in DS and that targeting DYRK1A kinase activity excess either pharmacologically or using environmental stimulation in the adult could correct these defects in DS.

Resum

Les connexions sinàptiques tenen la capacitat de respondre a l'activitat de neurones ajustant les seves propietats biològiques per incrementar els canvis activitat-depenents. Alteracions en la plasticitat neuronal de l'hipocamp s'han suggerit com a mecanismes subjacents als deterioraments cognitius característics en la síndrome de Down (SD). No obstant, es desconeix quins gens específics estan implicats en aquests fenotips en el context de la trisomia del cromosoma 21. DYRK1A és una serina / treonina quinasa, que quan es troba sobreexpressada recapitula el dèficit d'aprenentatge i de memòria depenent de l'hipocamp característic de la SD. En aquesta tesi, s'han estudiat els efectes de la sobreexpressió de DYRK1A en la plasticitat activitat-dependent de l'hipocamp. Hem descobert que ratolins transgènics amb sobreexpressió de Dyrk1A (TgDyrk1A) presenten alteracions morfològiques en les regions CA1 i CA3 de l'hipocamp, una limitació estructural en les connexions neuronals que és rellevant per entendre la relació entre estructura i funció. A més, hem trobat una reducció en la LTP possiblement deguda als canvis en la connectivitat i ocupació dendrítica. L'excitabilitat de les dendrites i la morfologia neuronal són factors determinants de l'eficàcia sinàptica i per tant poden contribuir als dèficits d'aprenentatge i la memòria de l'hipocamp detectats. Hem demostrat defectes importants en la neurogènesi adulta en el gir dentat incloent una reduïda taxa de proliferació cel·lular, alteracions en el cicle cel·lular i reducció de cèl·lules que surten del cicle cel·lular que condueix a una migració precoç de les noves cèl·lules generades i una reducció de la supervivència. D'altra banda, en ratolins TgDyrk1A hi ha menys proporció de neurones generades *de novo* que s'activen amb l'aprenentatge, indicant una menor integració d'aquestes en els circuits implicats en l'aprenentatge. Algunes d'aquestes alteracions han estat rescatades per la normalització de DYRK1A quinasa utilitzant un inhibidor de DYRK1A, epigallocatechin-3-gallate. L'estimulació del medi ambient també normalitza la sobreexpressió de DYRK1A quinasa en l'hipocamp, i rescata la morfologia, la plasticitat sinàptica i les alteracions en la neurogènesi adulta en ratolins TgDyrk1A. Arribem a la conclusió que *Dyrk1A* és un bon gen candidat per explicar els dèficits de plasticitat neuronal en la SD i que tractant l'excés d'activitat de la quinasa DYRK1A farmacològicament o mitjançant l'estimulació ambiental en l'adult podria corregir aquests defectes en la SD.

PRESENTATION

Presentation

The present Doctoral Thesis has been devoted to study the contribution of DYRK1A, a dual specificity serine/threonine kinase to hippocampal intellectual dysfunction in Down syndrome (DS). The trisomic condition in DS suggests that the expression deregulation of some candidate genes could contribute to intellectual disability. Concretely, we focus on *Dyrk1A* because i) it is localized in the DS critical region of human chromosome 21, ii) it is overexpressed in fetal and adult DS hippocampus, iii) the encoded protein plays key roles in cell proliferation and survival during embryogenesis and neuritogenesis and iv) engineered excess or defect of Dyrk1A in mice impairs hippocampal-dependent learning and memory thus indicating its dosage sensitivity.

Hippocampal plasticity disruptions are at the core of cognitive processes and therefore, increasing adult hippocampal neurogenesis and stimulating neuronal plasticity pharmacologically could be a very useful strategy towards preserving its structure and function. Based on these assumptions, we explored whether the overexpression of Dyrk1A is sufficient to impair *in vivo* hippocampal neural plasticity and to produce learning and memory defects. Specifically, we focused our research on hippocampal CA1 and CA3 neuronal morphology and synaptic transmission, and adult neurogenesis in the dentate gyrus. We have used a DYRK1A kinase inhibitor epigallocatechin-3-gallate (EGCG), to normalize kinase activity and rescue learning and memory phenotypes.

In addition, the ability of trisomic neurons to undergo neuroplasticity-related adaptations to the environment is reduced, as we demonstrated in our previous work in trisomic mice. However, early intervention programs are the only effective therapy to ameliorate, though transiently, the cognitive impairments in DS individuals. Thus, we explored whether Dyrk1A overdosage modulated the effects of environmental enrichment (EE), a neuroplasticity *in vivo* model, on hippocampal neuronal plasticity and cognition and the long-term stability of the phenotypes upon EE discontinuation. Finally, we focused our research on targeting DYRK1A kinase activity excess pharmacologically together with environmental stimulation to evaluate a new putative therapy for DS neuroplasticity defects.

During the present Doctoral Thesis we had the opportunity to be part of several international and national scientific collaborations with well-known research groups in

the neuroscience field. Concretely, to study the putative therapeutical effects on cognition of EGCG in DS individuals and trisomic mice we collaborate with Dr. Rafael de la Torre (IMIM), Dr. Jean Maurice Delabar (Université Paris Diderot) and Dr. Yann Herault (IGBMC). This collaboration gave rise to a paper, which is in press in *Molecular Nutrition and Food Research*. Moreover, we collaborated with Dr. Inma Ballesteros (UCLM) to analyze the apical dendritic complexity of CA1 pyramidal neurons using Lucifer yellow injections (manuscript in preparation). We also collaborate with Dr. Manel Esteller (IDIBELL) to perform a methylation array to evaluate whether DYRK1A kinase activity was involved in the epigenetic regulation of promoter methylation of some specific genes (ongoing). My work gave rise to a publication in *Neurobiology of Disease* and to another manuscript that is in preparation and will be submitted to *Journal of Neuroscience*. Moreover, we published a review highlighting the relation between adult neurogenesis and intellectual disabilities. I had the specific support of a predoctoral fellowship of the Center for Genomic Regulation, and my project was funded by grants of the Jerome Lejeune Foundation, and the Spanish Ministry of Science. During my PhD I had the opportunity to present my work in three national and eight international meetings, and I have participated also in other activities of the laboratory.

Hence, we have a global vision of the problem from different scientific points of view in an international environment to elucidate the functional and structural organization of the cognitive networks in Dyrk1A overexpressing mice that underlie the DS neuropathology.

List of scientific publications

1. **Pons-Espinal M**, Martinez de Lagran M, & Dierssen M. **Environmental enrichment rescues Dyrk1A activity and hippocampal adult neurogenesis in TgDyrk1A.** Neurobiology of Disease. 2013. *In press.*

2. De la Torre R, De Sola S, **Pons-Espinal M**, Martínez de Lagran M, Farré M, Fitó M, Benejam B, Langohr K, Rodriguez J, Pujadas M, Duchon A, Bizot JC, Cuenca A, Janel N, Covas M, Blehaut H, Herault Y, Delabar JM, Dierssen M. **Epigallocatechin-3-gallate, a Dyrk1A inhibitor, rescues cognitive deficits in Down syndrome mouse models and in humans.** Molecular Nutrition and Food Research. 2013. *In press.*

3. **Pons-Espinal M,** Martinez de Lagran M, & Dierssen M. **Functional implications of hippocampal adult neurogenesis in intellectual disabilities.** Amino Acids. 2013; 45(1):113-131. Review.

4. **Pons-Espinal M**, Gener T, Ballesteros-Yanez I, Sanchez-Vives MV, Martinez de Lagran M & Dierssen M. **Dyrk1A overdosage impairs long-term hippocampal structural plasticity effects of environmental enrichment** *(In preparation)*

INDEX

Índex

INTRODUCTION

1. INTRODUCTION

1.1. NEURONAL PLASTICITY

Throughout most of the twentieth century, the neuroscience community assumed that the central nervous system (CNS) of mammals became structurally stable soon after birth. Nowadays, it is widely accepted that experience can modify many aspects of the brain function and structure not only during the period of active brain growth, but also during adult life.

Neuronal plasticity can be defined as the capacity of neurons and neural circuits in the brain to change structurally and functionally in response to experience. It is crucial for the development of brain and behavior, and it is at the basis of brain's performance such as learning and memory. During early windows of development genetic programs are shaping neural circuits. However, the vertebrate brain continues its maturation long after birth, and the interplay between genes and environment ensures that early sensory experiences have a significant influence on how the brain is organized and can functionally impact the way the mature brain works.

The first study demonstrating the importance of sensory experience for brain development was performed by Hubel and Wiesel showing that vision shapes the synaptic organization of visual cortex during a critical period in postnatal early life (Hubel 1982). However, since then, several studies have reinforced the idea that experience-dependent regulation is also present in the adult brain (Maguire, Gadian et al. 2000; Schneider, Fries et al. 2002; Bermudez, Lerch et al. 2009; Rutter 2012).

Nowadays, although it is assumed that the brain is intrinsically plastic, the level of neural plasticity diminishes as brain's development proceeds, which would suggest greater adaptability of the organism to variable conditions early in life, and efficient neural connections for known conditions in adulthood. Adult brain plasticity seems more restricted and qualitatively different than developmental plasticity. Though the exact molecular and cellular mechanisms underlying these differences are not completely elucidated, some authors have pointed to structural and functional neuronal "brakes" that stabilize the neural networks after development (Bavelier, Levi et al. 2010). Thus, environmental and pharmacological manipulations targeting this "brakes"

may reactivate brain plasticity in the adulthood to a similar extent as during the developmental critical period.

1.2. CELLULAR MECHANISMS UNDERLYING ADULT NEURONAL PLASTICITY

Although the cellular and molecular mechanisms underlying adult neuronal plasticity are not yet completely understood, murine and human neuroimaging studies have revealed changes in grey and white matter volumes of some specific brain regions such as cortex and hippocampus upon experience (Boyke, Driemeyer et al. 2008; Zatorre, Fields et al. 2012). Neuronal changes underlying these variations may include neurogenesis, synaptogenesis and changes in neuronal morphology. In the white matter, changes in the number of axons, axon diameter, branching and trajectories have been found (De Paola, Holtmaat et al. 2006; Galimberti, Gogolla et al. 2006). Moreover, changes in glial cell size and number, and angiogenesis have also been described (Sirevaag and Greenough 1991).

1.2.1. Activity-dependent hippocampal structural plasticity

The hippocampus, located in the temporal lobe of the brain, is involved in the encoding, storage and recall of memories. Moreover, it plays a crucial role in integrating spatio-temporal and sensory information from individual's life experiences and keeps episodes in their correct context.

The hippocampus consists mainly of three sequential synaptic pathways (perforant, mossy fiber, and Schaffer collateral pathways). The main input to the hippocampus (perforant pathway) arises from the layer II of the entorhinal cortex (EC) and projects to the granule cells of the dentate gyrus (DG). From the granule cells of the DG connections are made onto area CA3 of the hippocampus through mossy fibers. CA3 pyramidal neurons send connections to CA1 pyramidal cells via the Schaffer collateral pathway targeting the apical CA1 dendrites located in the *stratum radiatum*. The final output from the CA1 subfields passes though the subiculum to finally target layer V of the EC. Although the trisynaptic pathway is the most accepted, the connectivity is

known to be much more complex since cells from the layer III of the EC in fact target CA1 subfield directly, and cells in layer II of the EC project to CA3 (the temporo-ammonic pathway) without passing through the DG (Amaral 1993). See Figure 1.

The hippocampus is not a homogenous structure and exhibits differences in connectivity and function, as well as in gene expression, along the longitudinal or septotemporal axis (Bannerman, Rawlins et al. 2004; Leonardo, Richardson-Jones et al. 2006). Functionally, whereas the dorsal hippocampus participates in working and spatial memories, the ventral hippocampus is primarily involved in emotional memory and anxiety. In this Thesis we are interested in activity-dependent neuronal plasticity underlying spatial learning and recognition memory, and for this reason we have restricted our studies in the dorsal hippocampus.

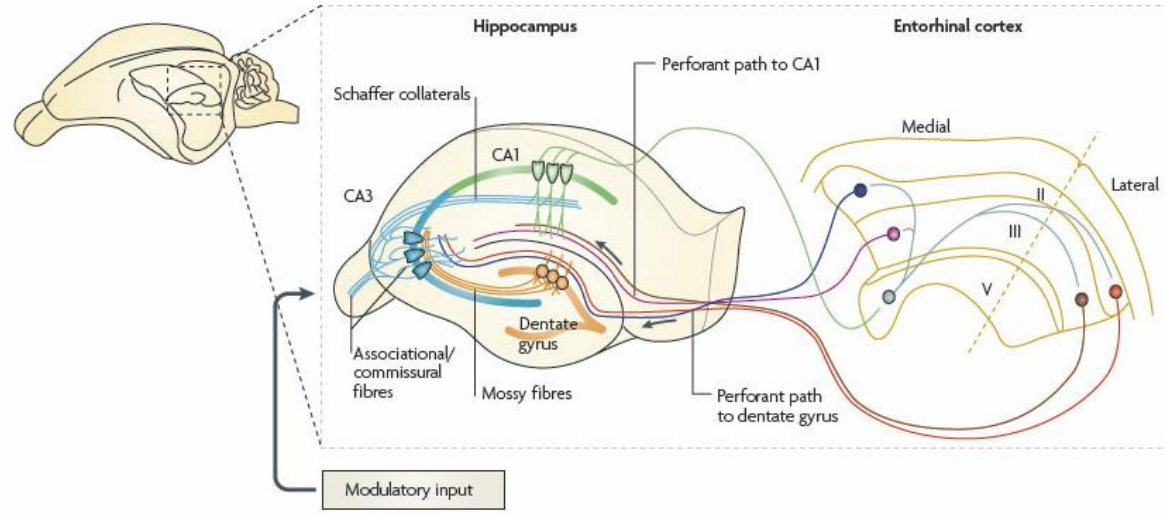

Figure 1. Basic anatomy of the hippocampus. Schematic representation showing a transversally cut section of the hippocampus, its major inputs from the entorhinal cortices, and the basic intrahippocampal connectivity. (Neves, 2008)

Whereas genetic factors contribute crucially to brain function, early-life events exert long-lasting influence on neuronal function. The observation from clinical studies in humans and animal models that disturbances in early-life environment can result in severe and permanent deficits in memory and emotional capacities (O'Connor, Rutter et al. 2000), suggested that postnatal-experience-driven changes in neuronal structure and synapses, would determine the individuals' behavior later in life. Specifically certain early-life events influence populations of hippocampal neurons acutely, and impact the function and integrity of the hippocampus long-term. During brain's development,

synapses and dendrites of hippocampal neurons undergo continuous rearrangements to establish the basic neuronal connections. Previous studies suggested that early experience influences the development of cognitive functions, probably as a consequence of changes in neuronal morphology and synapses functionality during early life. Specifically in the hippocampus, rearing rats after weaning in complex or impoverished environments produced opposite changes in dendritic branching complexity (Walsh 1981), and synapse densities (Joseph 1999) of pyramidal neurons probably affecting the numbers and topologies of functional connections between neurons. Moreover, postnatal stressful or traumatic experiences, such as maternal separation, have long-lasting effects on the number of excitatory synaptic inputs of pyramidal neurons in the hippocampus that can affect cognitive capacities during later life (Poeggel, Helmeke et al. 2003; Ovtscharoff, Helmeke et al. 2006). Conversely, increased maternal care improves cognition and reduces sensitivity to stress in the adult (Escorihuela, Fernandez-Teruel et al. 1995). These adult offspring of high licking and grooming mothers exhibit longer dendritic branch length and increased spine density in CA1 neurons and enhanced LTP in the Schaffer collateral pathway (Champagne, Bagot et al. 2008).

During adulthood, the morphology of mature hippocampal neurons can also change in response to environmental modifications, experience or upon learning and memory. For instance, rearing animals under environmental enriched (EE) conditions significantly increased dendritic length and branching in CA1 pyramidal cells in adult mice (Bindu, Alladi et al. 2007; Beauquis, Roig et al. 2010). Dendritic branching of CA3 and CA1 pyramidal neurons also increases upon self-rewarding experience (Ramkumar, Srikumar et al. 2008) in the adulthood. In the hippocampal DG granule neurons increases dendritic length and branching upon EE (Faherty, Kerley et al. 2003) and spatial learning (Lemaire, Tronel et al. 2012). Moreover, the connectivity between the different hippocampal subfields can also be influenced. For example, long-term potentiation (LTP) (Adams, Lee et al. 1997) or spatial learning (Ramirez-Amaya, Escobar et al. 1999), induce sprouting of axons of the mossy fiber pathway (connections between the DG and CA3 region). However, the causal relationship of these cellular changes and learning has been questioned since analogous changes have also been reported after physical exercise in the absence of learning (Toscano-Silva, Gomes da Silva et al. 2010). Besides, it has recently been shown that the infrapyramidal mossy fiber tract

changes its morphology not only upon external environmental stimuli but also through intrinsic mechanisms related to the levels of newly generate cells in the adult (Romer, Krebs et al. 2011).

In addition to modifications in dendritic branching and length, EE conditions increased synapse density in CA1 pyramidal neurons of adult mice (Rampon, Tang et al. 2000) and in the DG (Liu, He et al. 2012). Similar modifications have also been described upon spatial learning (Moser, Trommald et al. 1994).

In humans, MR-based morphometry studies have provided new evidences of the *in vivo* capacity of the human brain to achieve functional and structural reorganizations during adulthood with highly and unexpected levels of plasticity (for review see (Draganski and May 2008)). For example spatial navigation performance has been correlated with higher hippocampal size of taxi drivers (Maguire, Gadian et al. 2000). However, since the majority of the experiments were performed in a single time point, the effect of independent environmental and genetic factors that could also contribute to these effects has hampered the interpretation of the results. Recent longitudinal studies have highlighted that upon complex learning tasks the hippocampal grey matter expanded significantly and dendrites from cortical regions increased the complexity (Jacobs, Batal et al. 1993).

1.2.2. Neurogenesis in the adult brain

In the last decades, a relevant type of adult cellular plasticity has been detected, by which new neurons are continuously generated from specific niches integrating in the preexisting neuronal network and affecting different functional domains including cognition.

Neurogenesis, the process of generating functional neurons from precursors, was traditionally believed to occur only during embryonic and perinatal stages in mammals. However, Altman and Das provided the first anatomical evidence of newly generated dentate granule cells in the postnatal rat hippocampus (Altman and Das 1965). The proof of functional integration of the newly formed neurons in the adult CNS was shown some years later in songbirds (Paton and Nottebohm 1984). We now know that adult neurogenesis occurs throughout life in almost all mammals examined (Eriksson, Perfilieva et al. 1998). Some authors postulate that it can take place in the neocortex

(Gould, Reeves et al. 1999), substantia nigra (Zhao, Momma et al. 2003), striatum (Luzzati, De Marchis et al. 2007) and amygdala (Bernier, Bedard et al. 2002), but in those structures it is very rare and its functional consequences have not yet been established. By contrast, there are two well-defined regions involved in adult neurogenesis: the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the hippocampal DG (see Figure 2). New neurons generated in SVZ migrate through the rostral migratory stream to the olfactory bulb to become interneurons (Kempermann and Gage 2000). The functional significance of newborn neurons in the olfactory bulb involves maternal behavior (Bridges and Grattan 2003), pheromone-related behaviors such as mating and social recognition (Feierstein, Lazarini et al. 2010), and odor discrimination (Gheusi, Cremer et al. 2000; Schellinck, Arnold et al. 2004), while its role in olfactory associated memories is controversial (reviewed in (Lazarini and Lledo 2011)).

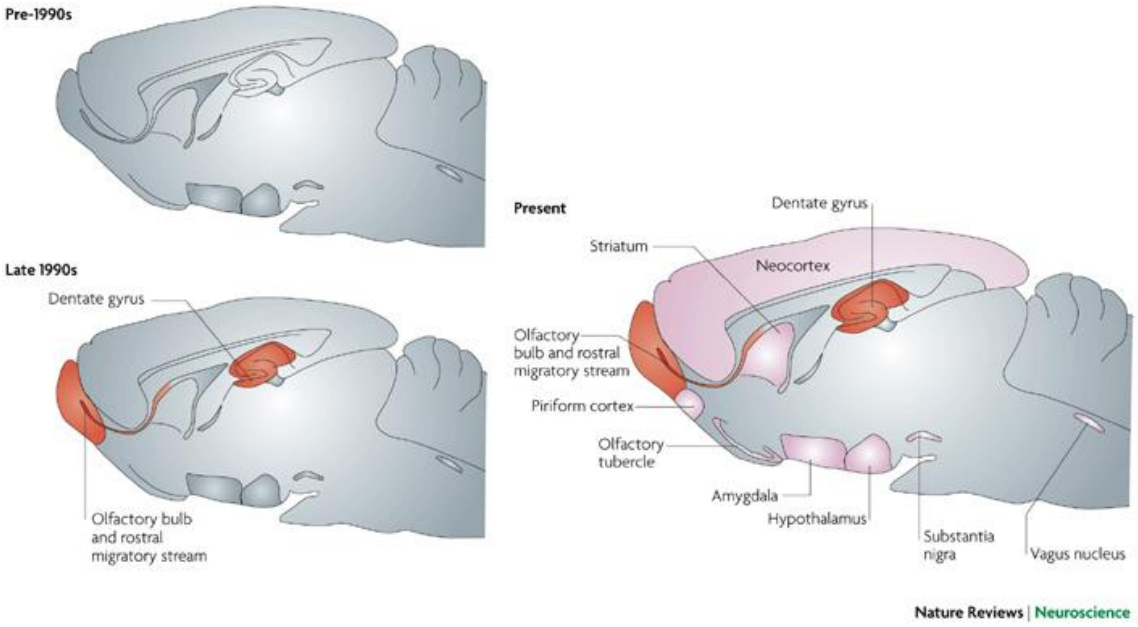

Figure 2. Neurogenic regions in the adult brain. Depicted in red are the brain areas in which adult neurogenesis is mainly studied. In pink, brain areas where adult neurogenesis is very low. In grey, non-neurogenic regions (Gould 2007).

In the hippocampus, newly born neurons in the SGZ migrate shortly into granule cell layer (GCL) of the DG to mature into granule cells and be integrated in the preexisting network (Cheng, Li et al. 2011). Hippocampal stem cells (also known as radial precursors) divide asymmetrically to produce one new stem cell and one neuronal

precursor cell (also known as transient amplifying stem cell*)*, which return to a quiescent state to support a continuous self-renewal (Lugert, Basak et al. 2010). Indeed, this quiescent state of stem cells has been proposed as a way to maintain the size of the pool and to prevent mutations (Rossi, Seita et al. 2007). Radial precursors, even though expressing glial fibrillary acidic protein (GFAP), differ from astrocytes by their morphology, the expression of nestin and Sox2 proteins, and their ability to generate neurons. The transient amplifying stem cells are highly proliferative cells that have lost their glial characteristics and start to express more neuronal traits. Neuroblasts, characterized by expressing doublecortin, NeuroD and Prox1 proteins, represent the last stage of proliferating progenitor cells showing a more limited proliferative rate. After exiting cell cycle, immature postmitotic neurons migrate into GCL, differentiating into dentate granule cells (Cheng, Li et al. 2011). Within the first week of maturation, newborn neurons rapidly extend their axons towards the CA3 region but these projections become stable around the fourth week of birth (Gu, Arruda-Carvalho et al. 2012). Almost at the same time, at around 10 days of maturation, the apical dendrites reach the inner part of the molecular layer, growing to the middle and edge part of the layer at around days 14 and 21, respectively, while they begin to develop dendritic spines (Zhao, Teng et al. 2006; Toni, Laplagne et al. 2008). Interestingly, even though at one month newly generated neurons have acquired a mature morphological phenotype (Zhao, Zhou et al. 2010), they are functionally immature both in their firing properties and excitatory synapses composition (Laplagne, Kamienkowski et al. 2007; Spampanato, Sullivan et al. 2012). Indeed, dendrites continue to mature along the molecular layer and the afferent spines develop until they become functionally integrated by about eight weeks (Zhao, Teng et al. 2006).

One critical aspect is that while large numbers of new neurons are born in the DG, only a fraction of these cells survive. Concretely, within the first two weeks of birth, half of the newborn neurons die. Indeed, there are two critical periods for newborn neuron survival: one between the transient amplifying stem cell and neuroblast stages (Sierra, Encinas et al. 2010) and a second one at the stage of integration of the immature neuron (Tashiro, Sandler et al. 2006).

Although the majority of one month-old surviving cells differentiate towards a neuron fate at expenses of glial fate (Steiner, Kronenberg et al. 2004), adult neural stem cells (NSC) in the SGZ have the potential to give rise to both neurons and astrocytes (Suh,

Consiglio et al. 2007). Newly formed astrocytes promote proliferation, neuronal differentiation and integration of newly formed neurons in the DG (Song, Stevens et al. 2002; Barkho, Song et al. 2006). Recent evidence points that, once activated, some hippocampal adult neural stem cells leave the pool of stem cells and differentiate directly to mature hippocampal astrocytes (Encinas, Michurina et al. 2011), contributing to the neuronal differentiation of other neighbor adult NSCs (See Figure 3).

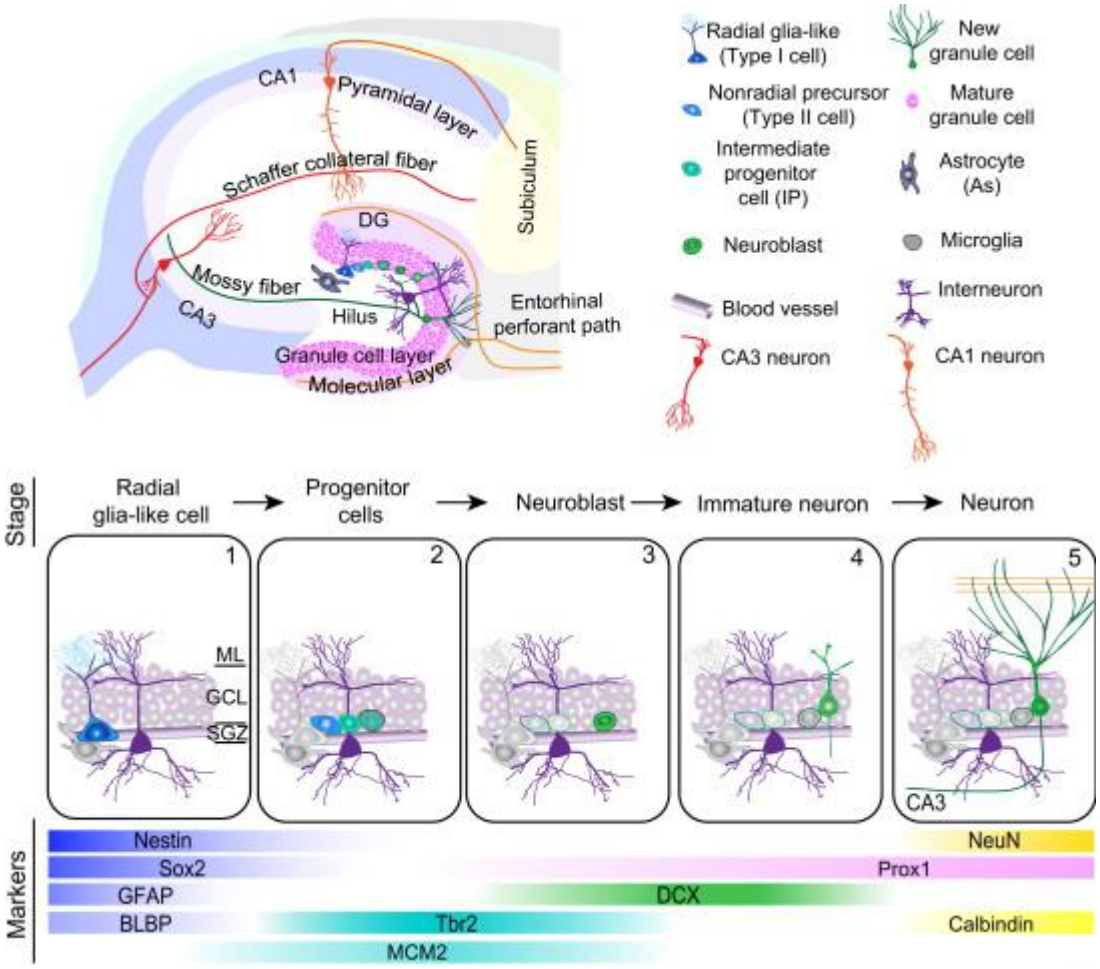

Figure 3. Adult Neurogenesis in the dentate gyrus of the hippocampus. Summary of five developmental stages during adult hippocampal neurogenesis: (1) activation of quiescent radial glia-like cell in the subgranular zone (SGZ); (2) proliferation of non radial precursor and intermediate progenitors; (3) generation of neuroblasts; (4) integration of immature neurons; and (5) maturation of adult-born dentate granule cells. The expression of stage-specific markers, sequential process of synaptic integration, and critical periods regulating survival and plasticity are shown. ML: molecular layer; GCL: granule cell layer; SGZ: subgranular zone; GFAP: glial fibrillary acidic protein; BLBP: brain lipid-binding protein; DCX: doublecortin; NeuN: neuronal nuclei (Ming and Song 2011).

In summary, hippocampal adult neurogenesis is a very dynamic complex cellular process with multiple steps finely tuned by molecular mechanisms. However, the system allows activity-dependent changes in almost every stage, from proliferation of neuronal precursors (Kronenberg, Reuter et al. 2003; Lugert, Basak et al. 2010; Bonaguidi, Wheeler et al. 2011) to integration (Kitamura, Saitoh et al. 2010; Stone, Teixeira et al. 2011) and activation of newly formed neurons in the preexisting network (Zhao, Deng et al. 2008). The possibility of changing newly formed cells in the adult brain upon environmental stimuli, add a new level of structural plasticity. Hence, not only preexisting neurons in the adult are able to be shaped, newly formed neurons also respond to new environmental demands, allowing the brain to refine the new computational pieces of the system, probably helping to encode and store new external information.

1.2.2.1. The role of adult hippocampal neurogenesis in cognitive functions

It is generally assumed that dendritic and synaptic reorganization of mature neurons contribute to hippocampal function, but several evidences support the idea that newly born cells might also contribute to adult brain function. It has been proposed that the addition of new cells in the hippocampal neuronal network would replace older dying cells contributing to maintain the correct number of cells. However, this is unlikely to occur since newly formed cells are not enough to replace the high cell death occurring in the adult hippocampus (Dayer, 2003). Hence, it also has been proposed that newly formed cells would refine and modulate the preexisting network contributing to certain hippocampal-dependent functions. In support to this idea, newly born neurons must undergo considerable maturation before becoming completely integrated in the synaptic network (Carlen, Cassidy et al. 2002). Indeed, during their maturation, newly formed cells present unique properties being structurally plastic and possessing differential electrophysiological characteristics (Ge, Pradhan et al. 2007). These features support the view that immature neurons might exert a substantial influence on hippocampal properties and function. However, although a myriad of studies have suggested a role of hippocampal adult neurogenesis in cognitive functions, other authors did not find any relation, being nowadays a field of intense debate. The apparent discrepancies in the literature probably derive from differences in many parameters, such as the timing, duration and cell types ablated, the paradigms of training and behavioral tests, and the animals (age, sex, and genetic background) used.

Adult hippocampal neurogenesis involvement in learning and memory

The first evidence of a possible contribution of adult neurogenesis to cognitive function came from correlation studies that associated proliferation and/or survival rates of newly generated cells with behavioral performance of hippocampal-dependent learning tasks. For example, performance of mice in an associative learning test, the trace eyeblink conditioning, improved as proliferation (Lemaire, Aurousseau et al. 1999) and survival (Leuner, Mendolia-Loffredo et al. 2004) of newly formed cells in the DG increased. By contrast, similar experiments after Morris Water Maze (MWM), an extensively used hippocampal-dependent task, have shown highly controversial results. While some authors found a good association between spatial learning and the number of newly generated neurons in the hippocampus of different mouse strains (Kempermann and Gage 2002) and in old rats (Drapeau, Mayo et al. 2003), others did not find any correlation (Merrill, Karim et al. 2003). In view of the discrepancy observed in correlation studies, some authors have used direct approaches such as pharmacologically abolishing adult neurogenesis and studying its effect on cognition. For example, Shors and colleagues found a significant impairment in the trace eyeblink conditioning but not in the MWM upon administration of the antimitotic drug MAM (Shors, Miesegaes et al. 2001). However, this also caused severe side effects due to inhibition of cell proliferation in the whole animal obscuring the interpretation of the results. Other neurogenesis ablation technique commonly used is the x-ray irradiation of the hippocampus. Raber and colleagues showed significant spatial learning impairments in the Barnes maze (a non aversive hippocampal-dependent spatial learning paradigm) in two-month old x-ray irradiated rats, but, again, could not affect the MWM performance (Raber, Rola et al. 2004). However, in younger rats irradiation produced a significant impaired spatial learning in the MWM suggesting an age-dependent sensitivity (Rola, Raber et al. 2004). However, in these experiments irradiation's effects are not restricted to DG, and can also affect post-mitotic neurons causing a substantial inflammatory response. One possible strategy to overcome this lack of specificity might be the generation of inducible transgenic mice that selectively target neuronal precursors that could help to identify the function of specific cell populations on hippocampal-dependent learning and memory. Although nowadays there are few studies using specific methods, the generation of transgenic mouse models fusing the nestin promoter with thymidine kinase to specifically ablate dividing tk-expressing cells upon

delivery of ganciclovir (GCV), has added another layer of specificity to the system. Inducible nestin-tk transgenic mice targeting precursor cells present reduced proliferation and survival of newly formed cells, and have shown impaired long-term retention in the MWM and extinction memory in the contextual fear conditioning test (Deng, Saxe et al. 2009; Pan, Chan et al. 2012). Also in this line, Dupret and colleagues found that the specific ablation of adult-born hippocampal neurons using another inducible transgenic mice, overexpressing the proapoptotic protein Bax under nestin promoter, showed impairment in complex spatial learning tasks in the MWM. Conversely, simple forms of spatial learning or other forms of learning that depend on the hippocampus to a lesser degree, such as novelty exploration or contextual fear conditioning, remain unchanged (Dupret, Revest et al. 2008). Similarly, impairment in spatial memory was observed using inducible transgenic mouse expressing the DTA toxin in nestin positive cells (Imayoshi, Sakamoto et al. 2008).

Since the specific ablation of neuronal precursors represents only a small subpopulation of all dentate granule cells there is a high probability that compensatory mechanisms of granule cells generated earlier during the development could be occurring. To overcome this limitation, recently a new ablation method targeting mature newly-generated neurons has been used. Interestingly, the ablation of these adult-formed mature neurons after training in the MWM impairs spatial memory (Arruda-Carvalho, Sakaguchi et al. 2011), suggesting that adult-born granule neurons could also be involved in hippocampal-dependent spatial memory processes deriving from the use of complex strategies to learn and retain the learned information (for review see (Aimone, Deng et al. 2011). Finally, studies analyzing the expression of immediate early genes have demonstrated that newly-generated neurons are activated in response to spatial learning (Kee, Teixeira et al. 2007; Snyder, Radik et al. 2009) and memory retrieval (Epp, Scott et al. 2011) also support the involvement of these cells in hippocampal dependent cognitive functions.

It has been proposed that learning would likewise modulate the survival and integration of newly born neurons in the preexisting networks leading to a two-way relationship between the generation of new neurons in the adult hippocampus and cognitive processes. In this line, hippocampal-dependent learning tasks such as MWM have been shown to increase the number of surviving neurons in the DG (for review see (Leuner, Gould et al. 2006). However, the influence of spatial learning on newborn neurons only

occurs in a critical window of time, between one and two-weeks after cell birth when newborn cells begin to differentiate to neurons, receiving inputs from other parts of the brain and sending signals to the CA3 region. Concretely, it has been reported that the early learning phase of the MWM increases neuronal survival and dendritic complexity only in one week-old neurons but does not modify cell proliferation (Dobrossy, Drapeau et al. 2003; Dupret, Fabre et al. 2007; Tronel, Fabre et al. 2010) whereas the late learning phase, in which animals have already learned the task, increases the survival of cells generated before learning while decreasing the survival of cells born during the early phase of learning through apoptosis. Finally, in the last phases of the learning, enhanced cell proliferation provides the hippocampus with a new pool of young neurons (Dupret, Fabre et al. 2007; Anderson, Sisti et al. 2011). In accordance with these results, learning increases the survival of cells generated before the task in aged rats (Drapeau, Montaron et al. 2007).

The continuous integration of new neurons in the preexisting granule cells network of the DG is probably changing the structural organization where the preexisting information is stored and could thus provide new networks for new memories (for review see (Lledo, Alonso et al. 2006). Kitamura and colleagues found that the ablation of hippocampal adult neurogenesis attenuates the loss of the hippocampal dependency of remote contextual fear memory indicating that memory still resides in the hippocampus of neurogenesis-deficient mice and reinforced the idea that newly-generated adult neurons contribute to memory consolidation to other extra-hippocampal brain regions (Kitamura, Saitoh et al. 2009). In support of this assumption, the blockade of DG neurogenesis in adult male rats by inhibiting Wnt signaling impaired the long-term retention of spatial memory in the water maze task (Jessberger, Clark et al. 2009).

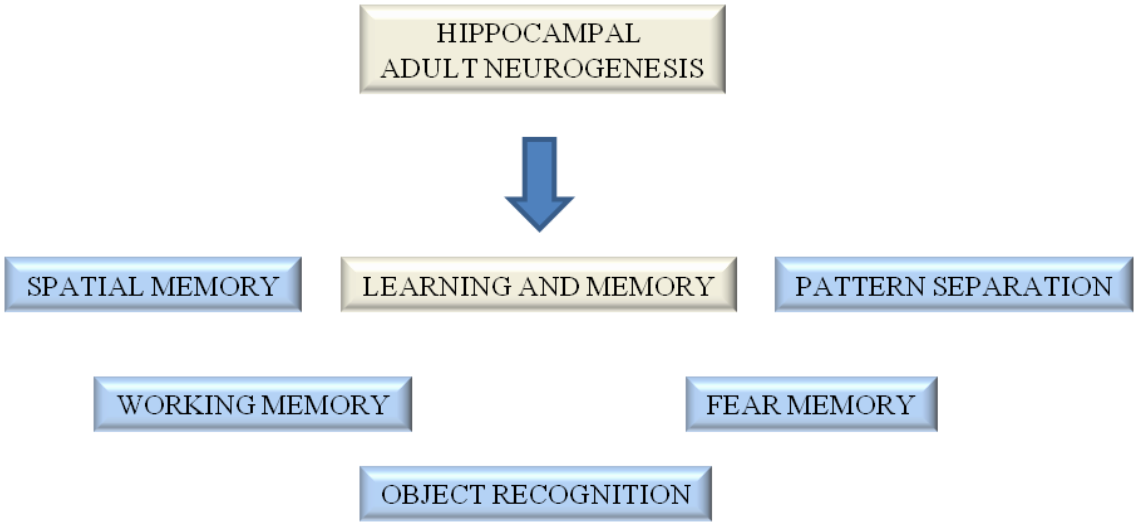

Figure 4. Involvement of hippocampal adult neurogenesis in learning and memory. In blue, hippocampal-dependent specific learning and memory tasks where adult neurogenesis is involved (Adapted from (Lafenetre, Leske et al. 2011).

1.3. INTELLECTUAL DISABILITY AS NEURONAL PLASTICITY DISORDERS

Having a flexible and prolonged period of postnatal development makes the vertebrate's brain more able to adapt to the environmental demands, but also more vulnerable to environment. Neurodevelopmental disorders are disabilities associated primarily with neurological dysfunction and they are caused by genetic but also environmental insults (Ehninger, Li et al. 2008), suggesting that neural plasticity is possibly a key mechanism contributing to cognitive impairment. Intellectual disability is considered a developmental disorder characterized by significant impairment of cognitive functioning and adaptive skills with onset before the age of 18 and ranks first among chronic conditions that cause major limitations to live a normal, independent life.

Neural mechanisms underlying intellectual disability may include defects in the formation of neuronal networks but also defects in properties of brain plasticity that are believed to be important for information processing and adaptive resources during adult life. One of better-established features of intellectual disabilities refers to dendritic abnormalities that have been considered a patognomonic sign. However, adult neurogenesis impairment should not be discarded. Here we focus on Down syndrome, a

neurodevelopmental disorder characterized by impaired hippocampal neuronal plasticity, as a model to study these cellular mechanisms underlying intellectual disabilities.

1.4. DOWN SYNDROME

Down syndrome (DS, OMIM 190685) is the most prevalent genetic cause of intellectual disability (Hassold and Jacobs 1984) affecting 1 each 800-1000 newborn children all over the world (Roizen and Patterson 2003). The prevalence of DS increases with maternal age being 9 in 1000 live born at maternal age of 40 (Hook 1983).

DS is the most common autosomal aneuploidy that involves the total or partial trisomy of the human chromosome 21 (HSA21). Depending on the cytogenetic origin, trisomy 21 is divided into different types. In about 95% of DS persons, trisomy results from meiotic non-disjunction of the chromosome 21 pair. About 4% of persons with DS have 46 chromosomes, one of which is carrying the Robertsonian translocation between chromosome 21q and the long arm of one of the other acrocentric chromosomes (usually chromosome 14 or 22). 1% of DS cases are mosaicisms, with cell populations showing either a normal or a trisomic 21 karyotipe; in this case, the phenotype may be milder than that of the typical trisomy 21 (Patterson 1987; Nadal, Mila et al. 1996).

DS individuals present a wide range of phenotypic abnormalities with variable penetrance including cardiovascular, skeletal, and motor alterations. Intellectual disability, muscle hypotonia and early development of the neuropathology of Alzheimer disease by the age of 40 are present to some extent in all affected individuals (Sabbagh, Fleisher et al. 2011).

1.4.1. Neuropathology and neuropsychological aspects in Down syndrome

The most limiting feature in DS is intellectual disability that affects 100% of individuals. DS individuals typically display an average Intelligence Quotient (IQ) of 50 (ranging from 40 to 70) (Vicari 2004). The main problem of using only IQ to define the degree and quality of intellectual disability is that it is a composite measure of cognition

and may mask specific deficits because of its nonspecific nature. Therefore, the Arizona Cognitive Test Battery has been developed to specifically assess the Down syndrome cognitive phenotype (Edgin, Mason et al. 2010).

Neuropsychological tests have revealed specific deficits in learning and memory, language or executive functions in DS individuals that lead to mild to profound impairment in intellectual functioning (reviewed by (Lott and Dierssen 2010). The presence of severe cognitive impairments in DS points towards structural and cellular alterations in brain structures involved in the processing and storage of information, such as the cortex and the hippocampus. At the macroscopic level, post-mortem observations and magnetic resonance (MR) analysis have revealed reduced brain volumes (microcephalic brains) with disproportionately smaller volumes in the hippocampus, prefrontal cortex and cerebellum in DS individuals (Aylward, Li et al. 1999; Pinter, Eliez et al. 2001). However, whereas no significant differences are found at birth in skull shape brain weight or proportion of cerebral lobes in individuals with DS, (Schmidt-Sidor, Wisniewski et al. 1990), gross differences appear in the first few months of life and include reduced growth of frontal lobes, narrowing of the superior temporal gyrus and diminished size of the brainstem and cerebellum (Nadel 2003). Importantly, hippocampal volume continues to decrease with age in DS individuals (Teipel, Schapiro et al. 2003) and was found to be inversely correlated with the degree of cognitive impairment (Smigielska-Kuzia, Bockowski et al. 2011).

The observation of reduced hippocampal volume in DS individuals in MRI studies (Pinter, Brown et al. 2001) together with functional impairments in the hippocampal activation of DS individuals analyzed by fMRI studies when visuo-spatial performance was assessed (Jacola, Byars et al. 2011), pointed that the hippocampus is one of the main brain structures underlying cognitive impairments in DS. Indeed, neuropsychological tests revealed important deficits in cognitive functions that depend on the hippocampus (Pennington, Moon et al. 2003). DS children (16 to 30 months old) show impairments on the delayed recall probes for the place-learning tasks, DS adolescents present deficits in episodic memory (Carlesimo, Marotta et al. 1997) and adult DS individuals show long-term memory deficits and impairments in recognizing pictures and remembering their locations (Caltagirone, Nocentini et al. 1990; Pennington, Moon et al. 2003) a result that is consistent with hippocampal dysfunction. Also, neuropsychological investigations requiring the use of spatial information in

problem solving indicate that deficits in hippocampal-mediated learning and memory processes are hallmarks of DS (Nadel 2003).

1.4.2. Neuronal structure in Down syndrome

Microscopic studies in DS individuals have revealed changes in the number, morphology and distribution of different cellular populations together with an abnormal dendritic growth during brain's development (reviewed in (Cramer and Galdzicki 2012). Until gestational week 22 the overall cortical structure of DS fetuses appears normal, with no significant differences with respect to typically developing fetuses in the numbers of neurons and glial cells of the neocortex (Ulfig, Tietz et al. 1999), or in the number and dendritic development of differentiated neurons of the visual cortex (Takashima, Becker et al. 1981). However, by gestational week 30 the neocortex of DS fetuses showed altered lamination and granular neuronal densities in different neocortical layers (Golden and Hyman 1994) along with reduced levels of neurotransmitters such as serotonin, GABA and dopamine (Whittle, Sartori et al. 2007) neurogenesis, glial differentiation, neurite branching and synaptic contacts in cortical layers (Ruediger and Bolz 2007). At 4 months of age, pyramidal neurons of DS babies show a relative increase in dendritic tree complexity, but during the first year of life the complexity is gradually reduced, producing a clear branching deficit that persists until adulthood (Becker, Mito et al. 1991). Therefore, the area occupied by these neurons is smaller as a result of reduced dendritic complexity and this would lead to altered connectivity between different brain areas. Also, in the cortex of 19 months-old children dendritic spine numbers are reduced and spine morphology is altered being abnormally enlarged (Marin-Padilla 1976). Consistent with alterations in cortical dendritic morphology and number of spines, synaptogenesis is also affected in DS children as shown by reduced contact zones (Wisniewski 1990) and decreased synaptic markers such as synaptophysin and SNAP-25 (Downes, Robson et al. 2008) that remain deficient in adulthood (Takashima, Iida et al. 1994). In the hippocampus, structural alterations appear between 17 to 21 weeks of gestation as shown by altered morphology (Sylvester 1983). Moreover, DS fetuses between 17-21 weeks of gestation show reduced number of neurons in the hippocampal DG along with reduced proliferation of newly formed cells, increased apoptosis and altered cell cycle progression (Contestabile, Fila et al. 2007; Guidi, Bonasoni et al. 2008).

In adult DS individuals neurons and dendritic spines in the CA1 and CA3 pyramidal neurons are also reduced in the hippocampus, (Ferrer and Gullotta 1990) probably affecting the functional neuronal connections between the hippocampus and other extra-hippocampal brain regions. These alterations in the connectivity, however, do not affects equally all cell populations, and as a result, the balance between excitatory and inhibitory neurotransmission is disturbed in DS (Zhang and Sun 2011). A significant deficit of glutamate and a non-significant 27% reduction in the levels of GABA were found in the hippocampus of adult DS subjects (Reynolds and Warner 1988). Moreover, in the parahippocampal gyrus of adult DS individuals the levels of glutamate were also reduced (Risser, Lubec et al. 1997). In summary, the overall neuronal architecture and synaptic communication alterations in the cortex and hippocampus beginning during brain's development in DS fetuses and persisting into adulthood would affect the flow of information between brain regions and the storage of information, being one important mechanisms to explain learning and memory deficits.

1.4.3. Neurogenesis in Down syndrome

The neuroanatomical abnormalities in the hippocampus of 17-21 weeks gestation DS fetuses points to neurodevelopmental impairment, such as embryonic neurogenesis. DS is caused by a trisomy, and thus, aberrant copies of single genes could alter the timing of cell cycle and the proliferating rate of neuronal precursors (Mittwoch and Wilkie 1971). Several studies in DS fetuses have identified alterations in embryonic hippocampal neurogenesis (see Table 2). Indeed, at 17–21 weeks of gestation, neurogenic cell proliferation is significantly impaired, as demonstrated by reduced numbers of dividing cells in the DG (−65%) with increased G2 cell cycle length. These results may suggest that a longer time spent by prolifering cells in G2 could result in a longer cell cycle and, hence, reduced proliferation rate (Contestabile, Fila et al. 2007). In addition to reduced proliferation, Guidi and colleagues found reduced number of neurons in the DS hippocampus at 17–21 weeks of gestation caused by increased number of cycling cells differentiating into glia at expenses of neuronal phenotype and increased levels of apoptosis suggesting impaired neuronal fate programs leading to aberrant differentiation (Guidi, Bonasoni et al. 2008). Alterations in cellular differentiation have also been described in *in vitro* studies showing that NPCs isolated from DS fetal brains give rise to reduced numbers of neurons (Bahn, Mimmack et al.

2002; Esposito, Imitola et al. 2008) and defects in their ability to give rise to GABAergic interneurons (Bhattacharyya, McMillan et al. 2009).

Besides alterations embryonic neurogenesis, disruptions in adult neurogenesis could also underlie cognitive impairments in DS (for review see (Pons-Espinal, de Lagran et al. 2013). However, these alterations are difficult to be indentified in humans. To date only one study in epileptic patients undergoing hippocampus resection indicate that low proliferation and differentiation capacities of adult hippocampal stem cells correlate with memory dysfunction (Coras, Siebzehnrubl et al. 2010). Given that nowadays, no information is available regarding adult neurogenesis in the hippocampus in DS individuals, our knowledge derives from experiments performed in animal models of DS.

1.4.4. Genetic dependence of neuronal phenotypes

Different hypothesis have been proposed to explain how trisomy of HSA21 produces DS phenotypes. The *amplified developmental instability hypothesis* proposes that trisomy of HSA21 causes an unspecific alteration in developmental homeostasis that leads to DS phenotypes (Roizen and Patterson 2003). However, another hypothesis to explain the impact of gene dosage imbalance is the *gene dosage effect* hypothesis, which relays on the idea that DS phenotype result from the overexpression of a subset of genes located on the triplicate chromosome (Antonarakis, Lyle et al. 2004). The smallest chromosomal segment among DS individuals who present a common feature is named as Down syndrome critical region (DSCR) that extends 5 Mb located on the distal third of the long arm (region 21q22) and contains around 33 genes. This specific chromosomal region has been reported to be sufficient in determining the short stature, joint hyperlaxity, hypotonia, mental retardation and 9 anomalies of the face, hand and foot typical in DS (Delabar, Theophile et al. 1993). However, DS individuals with significant intellectual disabilities bearing rare segmental trisomies of various regions of HSA21 were not trisomic for DSCR genes (Korbel, Tirosh-Wagner et al. 2009; Lyle, Bena et al. 2009). This question has been addressed in DS mouse models. Belichenko and colleagues have shown that three copies of DSCR genes are sufficient for the impaired performance in the novel object recognition, a hippocampal-dependent task (Belichenko, Belichenko et al. 2009). However, other studies demonstrated that this region when triplicated was not sufficient to explain neither the craniofacial skeleton

dismorphologies (Olson, Richtsmeier et al. 2004) nor hippocampal-dependent learning performance in the MWM or hippocampal LTP (Olson, Roper et al. 2007). Moreover, when DSCR was subtracted from the partial trisomic Ts65Dn (containing a triplicated copy of DSCR) by mating this mice with another model (Ms1Rhr) bearing a monosomie of this region, and the hippocampal-dependent learning impairments in the MWM in Ts65Dn were rescued (Olson, Roper et al. 2007). Taken together, these studies suggest that triplication of DSCR is necessary but not sufficient to cause a defect in the MWM.

Several studies analyzing DS cases resulting from partial trisomies of HSA21 and the development of partial trisomic mouse models have provided insight on the causative role of dosage-sensitive genes on DS phenotypes. In fact, these studies have confirmed the role of single dosage-sensitive genes on different phenotypes, but have also demonstrated that many of these DS features are due to increased copies of multiple HSA21 genes and their interactions with genes located in other chromosomes (Lana-Elola, Watson-Scales et al. 2011). Therefore, recently it has been proposed a *genome instability hypothesis* in which the additive effect of multiple HSA21 and non-HSA21 genes that are affected by the dosage imbalance is combined with changes in functional regulation of mRNAs or other non-coding and epigenetic elements. In this line, meta-analysis of heterogeneous DS data sets identified 324 genes with consistent gene expression deregulation: 77 on HSA21 and 247 on non-HSA21 regions (Vilardell, Rasche et al. 2011). However, other levels of regulation involving copy number alterations of functional non-traditional genomic elements such as non-coding genes should not be discarded. In fact, recent evidences indicate that some microRNAs (miR99a, let-7c, miR-125b-2, miR-155 and miR- 802) encoded by genes harbored on HSA21, are overexpressed in DS hippocampus (Kuhn, Nuovo et al. 2008), and therefore regulating the expression of certain mRNAs. In addition, epigenetic modifications involving DNA methylation and histone modifications should also be taking into consideration since DNA methylation is altered in trisomy 21 blood samples (Loudin, Wang et al. 2011). Indeed, some HSA21 genes such as *Dyrk1A* are associated with SWI/SNF chromatin remodeling complex affecting the expression of histone deacetylases (Lepagnol-Bestel, Zvara et al. 2009). In addition, increased oxidative stress from overexpression of various HSA21 genes could modulate DNA methylation at the CpG sites (Donkena, Young et al. 2010). In this line, DNA methyltransferase3L

(DNMT3L), located in HSA21, when overexpressed affects the interaction with another DNA methyltransferase (DNMT3A) producing an increase in methylation activity. Therefore, since DNMT3A methylates the promoter of several genes involved in neurogenesis and synaptic plasticity regulation, the altered DNMT3A activity would affect the expression of these genes with important consequences (Wu, Coskun et al. 2010).

1.5. MOUSE MODELS OF DOWN SYNDROME

The long arm of HSA21 contains approximately 552 genes, being around 161 of them coding-protein genes. Among them, 157 genes are orthologous to genes localized in syntenic regions of three mouse chromosomes: MMU16, MMU17, and MMU10 (Hattori, Fujiyama et al. 2000; Sturgeon and Gardiner 2011). Based on these homologies, several mouse models that are trisomic for different sets of HSA21 genes have been developed (see Figure 5). The first attempt to create a mouse model of DS was to develop a mouse, named Ts16, which was trisomic for the entire MMU16 (Gropp, Kolbus et al. 1975), However, this model does not resemble the DS aneuploidy because MMU16 presents syntenies with regions of HSA3, HSA8, HSA16, and HSA21; thus, it bears triplicates of many genes that are not in trisomy in DS. Therefore, segmental trisomic mouse models were generated that have the advantage that they are viable and some of them, such as the Ts65Dn, recapitulate some phenotypes observed in DS individuals. However, their limitation is the underlying genetic complexity and the impossibility to analyze single gene contribution to DS pathogenesis. Conversely, the transgenic mouse models, overexpressing one or few genes, allow a direct association between the overexpression of a specific gene with a certain phenotype, but their limitation is the loss of interactions with other genes that would be triplicated in DS contributing to the complexity of the phenotypes due to combinatorial effects of various deregulated genes.

1.5.1. Trisomic mouse models

The Ts16 showed a delay in neocortical development (Haydar, Blue et al. 1996; Cheng, Haydar et al. 2004). Furthermore, Ts16 embryos die *in utero*, making it impossible to test phenotypes in young and adult mice.

The next approach was the generation of mouse models containing partial trisomies of MMU16. The Ts65Dn, bearing a partial trisomy of a segment of MMU16 (extending from the *Mrp139* to the *Znf295* genes), is the most commonly used and widely studied mouse model of DS (Davisson, Schmidt et al. 1993). Importantly, the Ts65Dn recapitulates most of the cognitive as well as neuromorphological and electrophysiological alterations of DS individuals. Similar to DS, Ts65Dn mice show impairment in performing hippocampal-dependent tasks, such as spontaneous alterations in the T-maze, contextual fear conditioning, novel object recognition (Fernandez and Garner 2008), and spatial memory in the Morris water maze test (Reeves, Irving et al. 1995; Escorihuela, Vallina et al. 1998).

Hippocampal hypocellularity and reduced volume of the DG have been reported in postnatal and adult Ts65Dn (Kurt, Kafa et al. 2004; Rueda, Mostany et al. 2005; Lorenzi and Reeves 2006). Ts65Dn mice show alterations in embryonic and postnatal development (Contestabile, Fila et al. 2007; Chakrabarti, Galdzicki et al. 2007), adult neurogenesis deficits and structural plasticity impairments (Clark, Schwalbe et al. 2006; Bianchi, Ciani et al. 2010; Chakrabarti, Scafidi et al. 2011). In addition, these mice present impaired LTP in CA1 and DG hippocampal subfields that has been related to alterations in the properties of inhibitory neurotransmission with increased efficiency in the GABAergic system compared to the excitatory neurotransmission system (Kleschevnikov, Belichenko et al. 2004; Belichenko, Kleschevnikov et al. 2009).

Similar alterations during embryonic and postnatal development have been described in Ts1Cje, a mouse model containing a smaller segment of the MMU16 in trisomy (from *Sod1* to *Znf295* genes) than the Ts65Dn (Sago, Carlson et al. 1998) but not including functional *Sod1*. This strain also presents impaired hippocampal-dependent tasks (Sago, Carlson et al. 2000), reduced embryonic and adult neurogenesis in the DG (Ishihara, Amano et al. 2010), as well as abnormal CA1 hippocampal synaptic plasticity as shown by reduced LTP and increased LTD (Siarey, Villar et al. 2005).

Because some phenotype alterations in the Ts1Cje are less pronounced than in the Ts65Dn, it was proposed that the larger the segment in trisomy is, the more pronounced

phenotype is expressed. However, other mouse models such as the Tc1 in which the entire human HSA21 has been triplicated (O'Doherty, Ruf et al. 2005) did not support this idea. The milder phenotype of Tc1 mice is due to mosaicism and internal deletions in HSA21q, which leave significant genes such as *Itsn1* and *Rcan1* in disomy (Gardiner, Fortna et al. 2003; O'Doherty, Ruf et al. 2005). In fact, a mouse model bearing the whole orthologous HSA21 genes in trisomy was recently generated which showed similar impaired cognitive deficits and reduced hippocampal LTP as partial trisomic mice (Yu, Li et al. 2010). The conclusion of all these studies was that, although overdosed genes in the DSCR would contribute to certain DS structural and functional phenotypes, whole dosage-sensitive chromosomal domains (including coding-protein genes and non-coding elements) would determine the final phenotype indicating the extreme complexity of the genetic basis of DS.

In the above mentioned mouse models, large chromosomal regions are triplicated, thus precluding narrowing down the genetic dependence of the phenotypes. To overcome this problem, a library of mouse models containing small segmental regions in trisomy of approximately 2 Mb of HSA21 in a YAC (Yeast Artificial Chromosome) were created to allow the study of the contribution of specific set of genes. In this Thesis, it is important to highlight the TgYAC152F7 which contains 5 triplicated genes including *Dyrk1A* and shows similar cognitive impairments as trisomic DS mouse models but increased brain volume (Branchi, Bichler et al. 2004; Rachidi, Lopes et al. 2007; Sebrie, Chabert et al. 2008).

1.5.2. Transgenic mouse models

To study the role of particular genes underlying DS phenotypes, several mouse models overexpressing a single gene have been created in order to develop putative new therapeutic targets, such as TgSIM2 (Ema, Ikegami et al. 1999), TgS100beta (Gerlai, Friend et al. 1993), TgSOD1 (Gahtan, Auerbach et al. 1998), TgAPP (Harris-Cerruti, Kamsler et al. 2004) and TgDyrk1A (Altafaj, Dierssen et al. 2001) (see below). However, we will focus on the models that overexpress our main target: *Dyrk1A.*

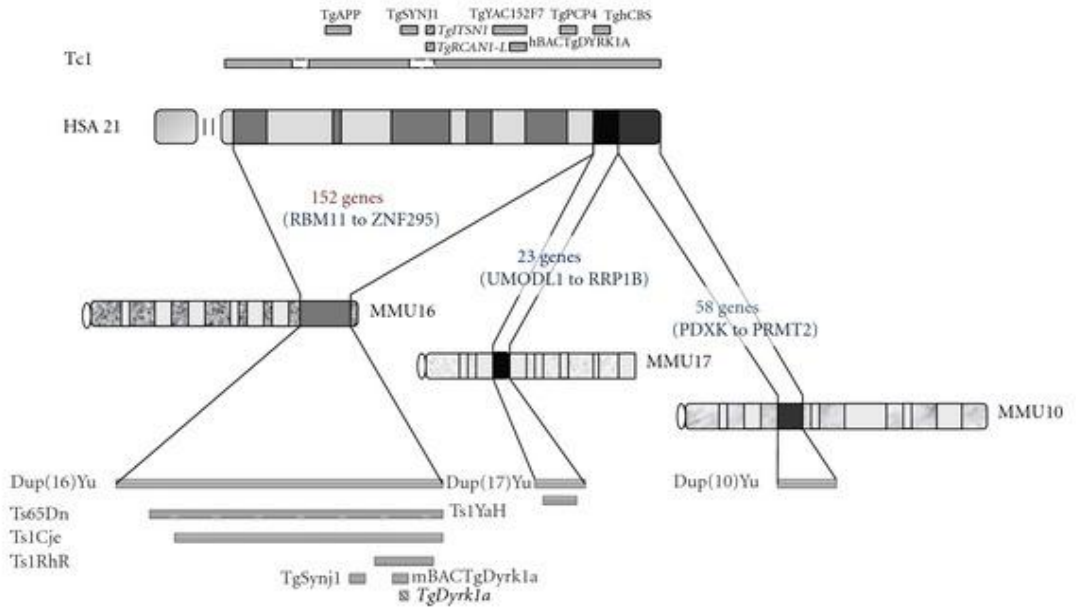

Figure 5. Schematic representation of HSA21 and syntenic regions of MMU16, MMU17 and MMU10 showing different trisomic and transgenic mouse models for different sets of genes orthologous to those of HSA21 (Creau 2012).

1.6. *DYRK1A*, A CANDIDATE GENE FOR DOWN SYNDROME'S RELATED NEURONAL PLASTICITY

Dyrk1A (Dual specificity Yak1 Related Kinase) is a DS candidate gene overexpressed in fetal and adult DS brain (Guimera, Casas et al. 1996; Guimera, Casas et al. 1999) that plays important roles during the brain development but also in the adult. *Dyrk1A* gene is located in the 21q22.13 within the DSCR (Hattori, Fujiyama et al. 2000). The full-length cDNA of *Dyrk1A* is 5.2 kb and is composed of at least 14 exons spanning 150 kb.

1.6.1. DYRK1A protein

The *DYRK1A* gene encodes two main protein isoforms of 763 and 754 amino acids. DYRK1A is a protein kinase of approximately 90kDa that belongs to DYRK family as a part of the CMGC group of kinases (named after the initials of some members), which also include cyclin-dependent kinases (CDKs), mitogen-activated protein kinases (MAPKs), glycogen synthase kinases (GSK) and CDK-like kinases (For review see (Aranda, Laguna et al. 2010). DYRK1A is a dual-specificity protein kinase that

autophosphorylates on tyrosin (Tyr) residues and phosphorylates substrates only on serine and threonine residues (Becker, Weber et al. 1998). Autophosphorylation of Tyr 312/321 in the activation loop is required for the full catalytic activity (Himpel, Panzer et al. 2001; Becker and Sippl 2011). The kinase domain is located centrally in the primary structure of the protein and contains four leucines (Leu) probably forming a Leucin-zipper domain for DNA binding. DYRK1A shares with the other DYRKs a conserved motif situated N-terminal to the kinase domain and known as DYRK homology (DH)-box. It also contains a functional, bipartite nuclear localization signal (NLS) N-terminal to the DH-box and a second NLS between subdomains X and XI within the kinase domain. Also, DYRK1A contains a C-terminal PEST motif involved in protein signaling degradation, and a polyhistidine tract that acts as a nuclear speckle-targeting signal (see Figure 6).

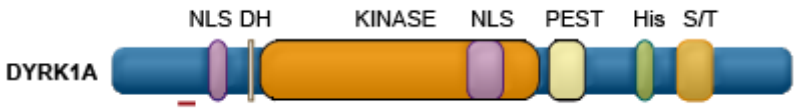

Figure 6. Schematic representation of DYRK1A protein. NLS: nuclear localization signal. DH: DYRK-homology box. PEST: Pest motif. His: Polyhistidine extension. S/T: Serine and Threonine-rich region. The line shows show alternatively spliced segment of 9 amino acids that give rise to the two main isoforms.

1.6.2. DYRK1A subcellular localization

DYRK1A can be located in the nucleus and in the cytoplasm leading to an extensive distribution through the cell that indicates a high functional versatility. Although the majority of DYRK1A subcellular localization studies have been done in cellular lines, some studies in mouse tissues showed that DYRK1A in neurons is distributed along the soma, dendrites and synapses (Hammerle, Elizalde et al. 2008). Indeed, several DYRK1A substrates (localized in different subcellular regions) are involved in alternative splicing regulation, cell cycle progression of progenitor cells, neuronal differentiation, endocitic machinery and vesicles recycling, synaptic transmission and neurodegeneration processes (see Table 1).

Substrate	Subcellular localization	Function	Reference
Amphiphysin	C	Synaptic vesicles	(Murakami, 2006)
APP (Amyloid Precursor Protein)	M	Synapse related	(Ryoo, 2008)
Arip4 (Androgen receptor interacting protein 4)	N	Transcription regulation induced by steroid hormones	(Sitz, 2004)
ASF (alternative splicing factor)	N	Regulation of alternative splicing	(Shi, 2008)
Caspase 9	C	Regulation of apoptosis	(Laguna, 2008; Seifert, 2008; 2009)
CREB (cAMP responsive element binding protein)	N	Neuronal differentiation	(Yang, 2001)
Cyclin D1	N/C	Cell cycle regulation	(Yabut, 2010)
CRY2 (Cryptochrome 2)	N	Circadian rhythm	(Kurabayashi, 2010)
DNM1 (Dynamin 1)	C	Membrane trafficking	(Chen-Hwang, 2002; Huang, 2004)
Endophilin 1	M	Membrane trafficking	(Murakami, 2009)
E2F1	N	Cell cycle regulation and neuronal differentiation	(Maenz, 2008)
FKHR (Forkhead)	N	Transcription regulation through insulin	(Woods, 2001; von Groote-Bidlingmaier, 2003)
GLI1 (Glioma-associated oncogen 1)	N	Transcription regulation involved in SHH signaling	(Mao, 2002; Morita, 2006)
GSK-3 (Glycogen synthase kinase 3)	C	Multiple cellular processes	(Scales, 2009; Woods, 2001; Skurat, 2004)
Hip1 (Huntingtin interacting protein 1)	C	Accessory protein of the clathrin-mediated endocytosis pathway	(Kang, 2005)
MAP1B	M	Microtubule-associated protein	(Scales, 2009)
Munc18-1	N/C	Synaptic vesicle fusion	(Park, 2012)
NFAT (Nuclear Factor of activated T-cells)	N/C	Transcription regulation	(Arron, 2006; Gwack, 2006)
Notch	M	Cell-cell signaling for cell differentiation	(Fernandez-Martinez, 2009)
NRSF/REST (neuron-restrictive silence factor)	N	Transcriptional repressor involved in proliferation and neuronal differentiation	(Canzonetta, 2008)
p53	N/C	Cell cycle regulation and apoptosis	(Park, 2010)
p120-catenin	N/C	Componentof the Wnt signaling	(Hong, 2012)
Presenilin1	M	Catalytic subunit of gamma-secretase responsible for the cutting of APP	(Ryu, 2010)
SEPT4 (septin 4)	N/C	Synaptic vesicles	(Sitz, 2008)
SIRT1	N/C	Cell death	(Guo, 2010)
SPRY2 (sprouty2)	C	Proliferation and neuronal differentiation regulated by FGF	(Aranda, 2008; Ferron, 2010)
STAT3 (Signal transducer and activator of transcription 3)	N	Transcription activator	(Matsuo, 2001; Weichmann, 2003)
SJI1 (synaptojanin 1)	C	Synapse related	(Adayev, 2006)
α-synuclein	C	Vesicle trafficking	(Kim, 2006)
Tau	C	Microtubules regulation	(Kimura, 2007; Woods, 2001; Ryoo, 2008)
N-WASP	N/C	Cytoskeletal regulator involved in actin nucleation	(Park, 2012)
14-3-3	N/C	Mitogenic signal transduction, cell death and cell cycle control	(Kim, 2004; Alvarez, 2007)

Table 1. DYRK1A substrates adapted from (Tejedor and Hammerle 2011). N: nucleus. C: cytosol. M: membrane.

1.6.3. DYRK1A functions in the Central Nervous System

DYRK1A is highly and ubiquitously expressed during vertebrate's embryonic brain development while in adult mouse brain its expression is reduced and restricted to certain regions such as cortex, hippocampus, cerebellum, deep motor nuclei, hipotalamic nuclei and olfactory bulb (Marti, Altafaj et al. 2003). In the adult human brain DYRK1A has a similar expression pattern. Some of the brain regions where Dyrk1A is expressed are highly affected in DS such as cortex, cerebellum and hippocampus. Conversely, in humans, Dyrk1A expression has not been detected in the olfactory bulb or the cerebellum (Wegiel, Kuchna et al. 2004). The differential expression along life, with a ubiquitous pattern during the CNS development that gets more restricted in the adult suggests that DYRK1A may have different functions during the animals' live. The fact that DYRK1A is expressed in adult brain regions involved in cognition such as the hippocampus and cortex (Marti, Altafaj et al. 2003), phosphorylates proteins involved in synaptic plasticity and neuronal differentiation and produces cognitive alterations when overdosed in mouse models (Altafaj, Dierssen et al. 2001; Ahn, Jeong et al. 2006), reinforces the idea that DYRK1A could participate in cognitive processes.

1.6.4. Dyrk1A involvement in structural plasticity

Trisomic mouse models show alterations in hippocampal structural plasticity with reduced dendritic complexity and spines, but also in synaptic plasticity mechanisms underlying learning and memory impairments. Dyrk1A is overexpressed in these trisomic mice and it is know to phosphorylate proteins involved in synaptic transmission, thus being proposed as an important candidate gene contributing to this altered plasticity in DS.

Several studies demonstrate that Dyrk1A contributes to neuritogenesis *in vitro* both in cortical cultures with DYRK1A knockdown (Scales, Lin et al. 2009) or in primary cortical cultures derived from Tg152F7 mice that overexpresses Dyrk1A (Lepagnol-Bestel, Zvara et al. 2009), and *in vivo* in the heterozygous Dyrk1A mice (Dyrk1A $^{+/-}$)

(Benavides-Piccione, Dierssen et al. 2005) and Dyrk1A overepressing mice (TgDyrk1A) (Martinez de Lagran, Benavides-Piccione et al. 2012). In fact, mice overexpressing Dyrk1A (BACTgDyrk1A) showed alterations in hippocampal LTD and LTP (Ahn, Jeong et al. 2006), altogether suggesting a putative role of Dyrk1A in the regulation of synaptic and structural plasticity in the adult.

Among the plausible underlying mechanisms, DYRK1A has been shown to interact with REST/NRSF chromatin-remodeling complex to deregulate genes such as *Elmo2* or *L1cam* related to neurite outgrowth (Lepagnol-Bestel, Zvara et al. 2009). Moreover, DYRK1A has been shown to modify splicing-associated transcripts involved in synaptic function such as TRKBT1 transcript with consequences on BDNF expression crucial for neuritogenesis (Toiber, Azkona et al. 2010). In addition, DYRK1A has been reported to modulate the distribution of microtubules respect to actin filaments affecting the dynamic properties of axonal growth (Martinez de Lagran, Benavides-Piccione et al. 2012). Although the exact mechanisms are not yet understood, DYRK1A contributes to microtubule dynamics by priming GSK3b phosphorylation of MAP1B (Scales, Lin et al. 2009) and phosphorylating Tau (Kimura, Kamino et al. 2007; Ryoo, Jeong et al. 2007), and interacts with N-WASP compromising actin polymerization (Park, Sung et al. 2012).

1.6.5. Dyrk1A involvement in neurogenesis

Dyrk1A has been suggested to be critical for the sequential events required for proper neuronal development during embryogenesis (Aranda, Laguna et al. 2010; Tejedor and Hammerle 2011) affecting both proliferation and differentiation processes (Dierssen and de Lagran 2006). The *Drosophila* orthologue, the minibrain (*mnb*) gene is required for normal postembryonic neurogenesis and mutant *Drosophila* flies display a size reduction in specific brain areas such as optical lobes due to the abnormal spacing of neuroblasts in the outer proliferation center of larval brain that leads to reduced neuronal progeny (Tejedor, Zhu et al. 1995). The homozygous Dyrk1A mice are lethal, and present a large reduction of the embryo size due to a delay of structural development and a decrease of the number of postmitotic neurons. Heterozygous Dyrk1A mice also have a general delay in the development and present reduced brain size (Fotaki, Dierssen et al. 2002).

During the development of the CNS DYRK1A is expressed in four sequential phases. It is transiently expressed in preneurogenic progenitors, it shows cell cycle-regulated expression in neurogenic progenitors, transient expression in recently born neurons and persistent expression in late differentiating neurons (Hammerle, Elizalde et al. 2008). Several authors have implicated Dyrk1A in cell cycle regulation of NPCs mainly over G1-S phase transition. Concretely, DYRK1A has been shown to induce p53 phosphorylation at Ser-15 leading to a robust induction of p53 target genes such as $p21^{CIP1}$ and when overexpressed, it impairs G0/G1-S phase transition, resulting in attenuated cell proliferation in human embryonic stem cells (Park, Oh et al. 2010). In the same line, transient overexpression of Dyrk1A by *in utero* electroporation inhibits cell cycle progression in the developing mouse neocortex due to nuclear export and degradation of cyclin D1 (Yabut, Domogauer et al. 2010). Another mechanism for Dyrk1A-induced cell cycle arrest has been proposed where DYRK1A is able to upregulate at the transcriptional level, the expression of cyclin-dependent kinase inhibitor $p27^{KIP1}$ in the embryonic chick spinal cord and mouse telencephalon (Hammerle, Ulin et al. 2011). Finally, DYRK1A could also regulate neuronal differentiation through inhibition of NOTCH signaling (Hammerle, Ulin et al. 2011) and/or perturbation of NRSF/REST levels, deregulating pluripotency and embryonic stem cell fate (Canzonetta, Mulligan et al. 2008) or through positive modulation of p120-catenin protein levels, affecting the expression of certain Wnt target genes involved in development (Hong, Park et al. 2012).

In spite of the clear role of DYRK1A in embryonic neurogenesis only one previous article has been published regarding adult neurogenesis. DYRK1A is symmetrically and asymmetrically distributed during adult neural stem cell divisions in the mitotic phase, modulating the self-renewal of those derived from the subependimal zone (Ferron, Pozo et al. 2010).

Embryonic neurogenesis					
Model	**Specie**	**Age**	**Type of study**	**Type of neurogenesis alteration**	**Reference**
Ts16	mouse	E16	*In vivo*	Delay in neocortical neuronal development	Haydar, 1996
Ts16	mouse	E14	*In vivo*	Reduced neocortical precursors and increased apoptosis Increased S phase duration and reduced cell cycle exit in neocortical VZ	Haydar, 2000
Ts16	mouse	E12.5-14.5	*In vivo*	Delay in neocortical neuronal development	Cheng, 2004
Ts1Cje	mouse	E14.5	*In vitro*	Reduced proliferating rate and increased cell cycle duration, glia differentiation and apoptosis of NPC	Moldrich, 2009
Ts1Cje	mouse	E14.5	*In vivo*	Reduced cell cycle exit and cortical neurogenesis	Ishihara, 2010
Ts65Dn	mouse	E14.5-18.5	*In vivo*	Increased S phase and reduced cell cycle progression, cell cycle exit and number of generated neurons	Chakrabarti, 2007
Ts65Dn	mouse	P2	*In vivo*	Reduced cell proliferation and survival and increased differentiation of glia cells Higher number of proliferating cells in the G2 phase at expenses of the M phase	Contestabile, 2007
DS	human	fetuses (8–18 w)	*In vitro*	NPC give rise to reduced number of neurons	Bahn, 2002
DS	human	fetuses (17-21 w)	*In vivo*	Reduced number of dividing cells with reduced cells in S phase and increased proliferating cells in the G2 phase at expenses of the M phase	Contestabile, 2007
DS	human	fetuses (17-21 w)	*In vivo*	Reduced cell proliferation and number of differentiated neurons Increased differentiation of glia cells and apoptosis	Guidi, 2008
DS	human	fetuses (19-21 w)	*In vitro*	NPC give rise to reduced number of neurons and increased a more glial-predominant progenitor phenotype	Esposito, 2008
DS	human	fetuses (13-18 w)	*In vitro*	NPC give rise to reduced GABAergic interneurons in the cortex	Bhattacharya, 2009
mnb-KO	*Drosophila*	larvae	*In vivo*	Abnormal spacing of neuroblasts leading to reduced neuronal progeny	Tejedor, 1995
Mnb/Dyrk1A overexpression	Chick	Stage 12	*In vivo*	Reduced cell proliferation, increased cell cycle exit and increased neuronal differentiation	Hämmerle, 2011
	mouse	E14.5	*Electroporation ex vivo*		
Dyrk1A inhibition	Chick	Stage 12	*In vivo*	Increased cell proliferation and apoptosis	
	mouse	E14.5	*Electroporation ex vivo*		
Dyrk1A-KO	mouse	E9.5-11.5	*In vivo*	Reduced postmitotic neurons	Fotaki, 2002
BACTg Dyrk1A	mouse	E14.5	*In vivo*	Reduced cell proliferation through increased p53 phosphorylation and $p21^{CIP1}$	Park, 2010

Dyrk1A overexpression	mouse	E14.5	*In utero electroporation*	Reduced cell proliferation and cell cycle progression by promoting the nuclear export and degradation of cyclin D1 in the neocortex Premature neuronal differentiation	Yabut, 2010
Dyrk1A overexpression	human ES		*In vitro*	Impaired G0/G1-S phase transition	Park, 2010
Kinase-inactive DYRK1A	Human cell line		*In vitro*	Reduced ability of cells to enter quiescence by disrupting DREAM assembly	Litovchick, 2011

Adult neurogenesis					
Model	**Specie**	**Age**	**Type of study**	**Type of neurogenesis alteration**	**Reference**
Ts1Cje	mouse	3 m	*In vivo*	Reduced cell proliferation and number of neuroblasts	Ishihara, 2010
Ts1Cje	mouse	3 m	*In vivo*	No differences in the number of NSC in SVZ	Hewitt, 2010
Ts1Cje	mouse	3 m	*In vitro*	Reduced numbers of neural progenitors, neuroblasts and differentiated neurons Increased number of astrocytes	
Ts65Dn	mouse	13-15 m	*In vivo*	Reduced cell proliferation	Rueda, 2005
Ts65Dn	mouse	2.5 m	*In vivo*	Reduced cell proliferation and survival	Clark 2006
Ts65Dn	mouse	P6 and P30	*In vivo*	Reduced mitotic cells	Lorenzi & Reeves, 2006
Ts65Dn	mouse	P15	*In vivo*	Reduced cell proliferation, survival, neurogenesis and gliogenesis No differences in apoptosis	Bianchi, 2010
Ts65Dn	mouse	12 m	*In vivo*	Reduced cell proliferation in SVZ	Bianchi, 2010
Ts65Dn	mouse	P18	*In vivo*	Reduced cell proliferation, survival, neurogenesis and gliogenesis	Chakrabarti, 2011
Ts65Dn	mouse	2-3 m	*In vivo*	Reduced cell proliferation	Belichenko, 2011
YAC152F7	mouse	P16-18	*In vivo*	Increased neuronal size in the DG Increased levels of cyclin B1	Brachi, 2004
Dyrk1A-HET	mouse	3 m	*In vivo*	Reduced number of stem cells, progenitor cells (not neuroblasts) and life-long maintenance of adult progenitors Defects in self-renewal of adult neural stem cells in SEZ	Ferron, 2010

Table 2. Representative embryonic and adult neurogenesis studies related to Down syndrome. For each study we detail the model and the specie used, the age of the sample, the type of study and a brief description of the neurogenesis alterations observed. VZ: ventricular zone. NPC: neuronal progenitor cell. NSC: neural stem cells. SVZ: subventricular zone. DG: dentate gyrus. SEZ: subependimal zone. E: embryonic day; P: postnatal day; w: weeks; m: months.

1.6.6. Dyrk1A overexpressing mice (TgDyrk1A)

To study the specific contribution of the single overexpression of *Dyrk1A* on DS phenotypes, two transgenic mouse models have been generated. The BACDYRK1A mouse model contains exclusively the human *DYRK1A* gene in a Bacterial Artificial Chromosome (BAC). These mice show learning and memory impairments as well as synaptic plasticity alterations that are associated with LTP and LTD modifications (Ahn, 2006) and hyperphosphorylation of tau (Ryoo, Jeong et al. 2007) among other alterations associated with DS pathology.

In this Thesis, we have used a transgenic mouse model overexpressing Dyrk1A (TgDyrk1A) developed by the group of Cristina Fillat (CEK, Centro Ester Koplowitz) to evaluate the impact of Dyrk1A overexpression on neuronal plasticity in adult mice. This model overexpresses the cDNA of rat *Dyrk1A* under the control of the inducible sheep metallothionein-Ia (*sMT-Ia*) promoter. However, previous work demonstrated that the levels of *Dyrk1A* overexpression were more similar to those observed in DS when the transgene was not induced (Toiber, Azkona et al. 2010). The study of DYRK1A expression in the CNS revealed an overall increase in the brain (Marti, Altafaj et al. 2003). Behavioral analysis showed that TgDyrk1A mice present a delayed acquisition of mature locomotor activity, reduced motor coordination during neurodevelopment and maintained to some extend in the adult, impaired motor learning and altered visuospatial learning and reference memory in the adult (Altafaj, Dierssen et al. 2001; Martinez de Lagran, Altafaj et al. 2004). These behavioral alterations have suggested a dysfunction in some brain structures involved in learning and memory (cortex, hippocampus and cerebellum) and motor function (cerebellum, striatum and motor cortex) in TgDyrk1A mice. Moreover, pyramidal neurons in layer III of the secondary motor cortex presented reduced dendritic length and branching accompanied with fewer spines (Martinez de Lagran, Benavides-Piccione et al. 2012).

1.7. THERAPEUTIC STRATEGIES FOR INTELLECTUAL DISABILITIES

Given that neurodevelopmental disorders leading to intellectual disability show important alterations in neuronal plasticity, the development of therapeutic approaches targeting molecular pathways involved in plasticity are one of the major efforts that scientists are making. Brain development in neurodevelopmental disorders comprises a sequence of critical periods and abnormalities occurring during early development that have been considered irreversible in adulthood. However, nowadays several reports suggest that is possible to reverse certain structural and functional deficits associated with these disorders in adults by genetic or pharmacological manipulations (Bavelier, Levi et al. 2010). Specifically, reactivation of neuronal plasticity in the adult to a juvenile-like plasticity has been proposed as therapeutical strategy to ameliorate some abnormal symptoms in intellectual disabilities.

1.7.1. Pharmacological therapies for neuronal plasticity disorders

Until now, most of the therapeutic strategies for DS individuals have been focused on ameliorating general symptoms using unspecific nutrition-based approaches in order to restore some metabolic alterations. However, nowadays the scientific community is making a huge effort in investigating new therapies targeting specific DS-derived neuropathological features to ameliorate specific cognitive dysfunctions. To this end, the use of mouse models has become a fundamental tool to understand the basis of these treatments before treating humans. Hence, basically every proposed treatment for DS individuals has developed from results in Ts65Dn mice. One of the main efforts has been focused on developing pharmacological therapies based on neurotransmitter replacement to restore the altered neurotransmitter signaling. For example, treatment with L-DOPS (a specific β1 adrenergic agonist) corrected the deficiency in norepinephrine-mediated signaling between locus coeruleus and hippocampus restoring learning and memory deficits in Ts65Dn mice (Salehi, Faizi et al. 2009). Moreover, inhibitors of acetylcholine esterase reversed hippocampal-dependent cognitive deficits in 4-months-old Ts65Dn mice but not older ones (Chang and Gold 2008). Given that trisomic mice present huge alterations on hippocampal-dependent synaptic plasticity (assessed by LTP) due to an excitatory-inhibitory imbalance that could underlie the

cognitive impairments, the use of pharmacological treatments targeting GABAergic and glutamatergic system has become very important. In this line, using inhibitors of γ-aminobutyric acid A (GABA-A) receptors (Kleschevnikov, Belichenko et al. 2004; Belichenko, Kleschevnikov et al. 2007) showed significant improvements in the novel object recognition task (Fernandez, Morishita et al. 2007) and Morris water maze paradigm (Rueda, Florez et al. 2008) in adult Ts65Dn mice. Recently, it has been demonstrated that treating Ts65Dn mice with a selective GABA-A α5 inverse agonist or GABA-B receptor antagonist significantly reverse hippocampal LTP, neuromorphologycal and cognitive deficits (Kleschevnikov, Belichenko et al. 2012; Martinez-Cue, Martinez et al. 2013). The use of memantine, an antagonist of N-methyl D-aspartate (NMDA) receptors, has also been reported to improve learning and memory alterations in trisomic mice in different behavioral paradigms (Lockrow, Boger et al. 2010; Rueda, Llorens-Martin et al. 2010). Another group of drugs are designed to affect the altered brain morphology in trisomic mice usually by stimulating proliferation of neuronal populations. For example, fluoxetine and lithium increase neurogenesis in the DG and improves learning and memory (Clark, Schwalbe et al. 2006; Bianchi, Ciani et al. 2010; Contestabile, Greco et al. 2013) and SAG 1.1 (an agonist of sonic hedgehog) restores the cerebellar volume and granular cell number reductions postnatally (Roper, Baxter et al. 2006).

In the context of DS, one possible strategy is to normalize the expression/activity levels of proteins related to plasticity which overexpression may produce deleterious effects of different plasticity-related cellular events. Hence, the specific normalization of triplicated genes in transgenic mice could also help to understand the concrete functional consequence of overexpressing this gene and, when normalized in a complete trisomic context serves as a proof of concept that some specific phenotypes would directly depend on the expression of this gene. As an example, APP is triplicated in DS individuals and trisomic mice causing an increase in Aβ peptides that leads to cognitive impairment. Targeting APP-derived Aβ peptides through the administration of DAPT, an inhibitor of γ-secretase mediating Aβ production, reduces the levels of these peptides in trisomic mice improving learning and memory (Netzer, Powell et al. 2010). Here, it is important to highlight recent advances on targeting Dyrk1A expression or activity. Ortiz-Abalia and colleagues demonstrated for the first time that the normalization of Dyrk1A expression in the striatum of TgDyrk1A mice, through the injection of an

adeno-associated virus type 2-mediated Dyrk1A RNA inhibitor (AAVshDyrk1A), rescued motor alterations in these animals (Ortiz-Abalia, Sahun et al. 2008). More recently, Altafaj and colleagues demonstrated that the normalization of Dyrk1A expression in the hippocampus of Ts65Dn mice rescued cognitive impairments in the MWM (Altafaj, Martin et al. 2013). In the same line, harmine (a specific DYRK1A kinase activity inhibitor) is able to rescue neuritogenesis alterations in cortical cultures from TgDyrk1A mice (Martinez de Lagran, Benavides-Piccione et al. 2012). However, the use of harmine *in vivo* in animal models of DS, is not really useful because of its toxicity and its inhibitory activity on monoamine oxidase-A (MAO-A) (Kim, Sablin et al. 1997). Nowadays, several lines of investigation are focused on the discovery of novel molecules that inhibit DYRK1A kinase activity more effectively, such as INDY since it also reversed tau-phosphorylation and rescued the repressed NFAT signaling in *vitro* induced by Dyrk1A overexpression (Ogawa, Nonaka et al. 2010). Some years ago, Bain and colleagues described the properties of 30 inhibitors, tested with 25 kinases, one of which was epigallocatechin-3-gallate (EGCG) a natural component from the green tea extract. EGCG was found to be a DYRK1A kinase inhibitor with an apparent IC50 of 0.33 µM (Bain, McLauchlan et al. 2003). The inhibition of DYRK1A kinase by EGCG was also described in NIH3T3 cells and found a mechanism involving a non competitive inhibition with ATP (Adayev, Chen-Hwang et al. 2006). Interestingly, when EGCG was given to pregnant BACTgDyrk1A females, it prevented the alterations in brain volume and cognitive deficits of their pups (Guedj, Sebrie et al. 2009).

1.7.2. Environmental enrichment, as a neuronal plasticity enhancer in intellectual disability disorders

One of the strategies to promote *in vivo* neuroplasticity in rodents is the use of an enriched environment (EE) that provides animals increased physical activity, learning experiences and social interaction (Rosenzweig and Bennett 1996). Many studies have shown that EE enhances learning and memory (Nithianantharajah and Hannan 2006) through the induction of biochemical, morphological and functional changes in the adult brain due to posttranslational modifications affecting protein activity or to changes in the expression of genes involved in neuronal structure, synaptic signaling and plasticity (Rampon, Jiang et al. 2000). Among them, brain-derived neurotrophic factor (BDNF), widely implicated in activity-dependent regulation of adult neurogenesis and dendritic

morphology, and increases its expression in the hippocampus upon EE exposure (Kuzumaki, Ikegami et al. 2011).

In the context of DS, early intervention programs in DS children are the only effective strategy to improve cognitive capabilities. However, despite resulting in positive results, those are limited and temporary (Mahoney, Perales et al. 2006; Bonnier 2008). In mice we showed that postweaning EE rescued behavioral performance and learning abilities of Ts65Dn mice, improving hippocampal dependent memories (Martinez-Cue, Baamonde et al. 2002). Among the plausible mechanisms of this improvement are modifications in G-protein-associated signal transduction systems (Baamonde, Martinez-Cue et al. 2011) and regulation of excitatory–inhibitory imbalance by reversing the increased release of GABA from synaptosomes in Ts65Dn mice (Begenisic, Spolidoro et al. 2011).

EE also modulates hippocampal adult neurogenesis by increasing the number of surviving newly formed cells (Kempermann, Kuhn et al. 1997; van Praag, Kempermann et al. 2000) and reducing spontaneous apoptotic cell death by 45% (Young, Lawlor et al. 1999) in wild type mice. The impact of EE on neurogenesis is different depending on the age that is applied. For example, some studies show that in contrast to postweaning enrichment, preweaning enrichment had no lasting measurable effect on adult neurogenesis (Kohl, Kuhn et al. 2002). However, the rescuing EE effects on adult neurogenesis have been described both in young and aged mice with diseases that are accompanied by hippocampal neuronal loss and abnormal neurogenesis like Alzheimer's disease (AD). For example, postweaning EE rescued adult neurogenesis impairment in two-months-old Ts65Dn mice (Chakrabarti, Scafidi et al. 2011) but also in twelve-months-old aged mice with beta-amyloid pathology (Berardi, Braschi et al. 2007).

Therefore, it appears possible to regulate aberrant neuronal plasticity features without pharmacological intervention and achieve similar behavioral outcomes without the concerns associated with non-specific actions or adverse side effects of drugs.

HYPOTHESIS AND OBJECTIVES

2. HYPOTHESIS AND OBJECTIVES

2.1. HYPOTHESIS

Neurodevelopmental disorders leading to intellectual disability, such as Down syndrome (DS; trisomy HSA21) are characterized by reduced brain plasticity which consequences are limited cognitive and adaptive resources during adult life. Since DS is caused by a trisomy of the chromosome 21, hundreds of genes could contribute to the phenotype making DS a very complex syndrome to decode. Accumulating data suggest that overexpression of the dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A (*DYRK1A*), a dose-sensitive gene in HSA21, is critical in the intellectual deficit. Here we propose that the *in vivo* overexpression of *DYRK1A* is key in the adult hippocampal neuronal plasticity and is contributing to cognitive impairments in DS individuals and trisomic mouse models through this disrupted plasticity.

2.2. OBJECTIVES

The main objective of this work is to elucidate the impact of *in vivo* Dyrk1A overexpression on the activity-dependent adult neuronal plasticity in the hippocampus using a mouse model overexpressing Dyrk1A (TgDyrk1A). We have also studied if altered plasticity due to Dyrk1A overexpression could underlie DS-related learning and memory impairments.

Specific objectives have been:

1. To evaluate the involvement of DYRK1A on structural and synaptic plasticity in CA3-CA1 and adult neurogenesis in the dentate gyrus in adult TgDyrk1A mice.
2. To analyze the effects of normalizing DYRK1A kinase activity using epigallocatechin-3-gallate (EGCG), on hippocampal neuronal plasticity impairments in adult TgDyrk1A mice.
3. To investigate the impact of environmental enrichment, an *in vivo* model of activity-dependent neuroplasticity, on hippocampal plasticity in adult TgDyrk1A mice.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. EXPERIMENTAL DESIGN

3.1.1. Characterization of hippocampal-dependent cognition and adult hippocampal neuronal plasticity in TgDyrk1A mice

The main objective in this Thesis was to investigate the effect of Dyrk1A overexpression on hippocampal-dependent learning and memory, and to evaluate the hippocampal neuronal plasticity mechanisms underlying cognition in TgDyrk1A mice. We analyzed two cellular plasticity mechanisms in the hippocampus. First, we tested the possibility that morphological parameters such as hippocampal layering, and structural dendritic complexity and dendritic spine density of hippocampal pyramidal neurons may be affected in Dyrk1A overexpressing mice. In collaboration with Imma Ballesteros (UCLM) we also analyzed in depth the dendritic branching of CA1 region (see results in Annex III). Moreover, we investigated the electrophysiological properties of the Schaffer collateral pathway (see results in Annex III) in collaboration with Thomas Gener (CRG). Second, we evaluated whether adult neurogenesis in the dentate gyrus (DG) could also be affected in TgDyrk1A mice.

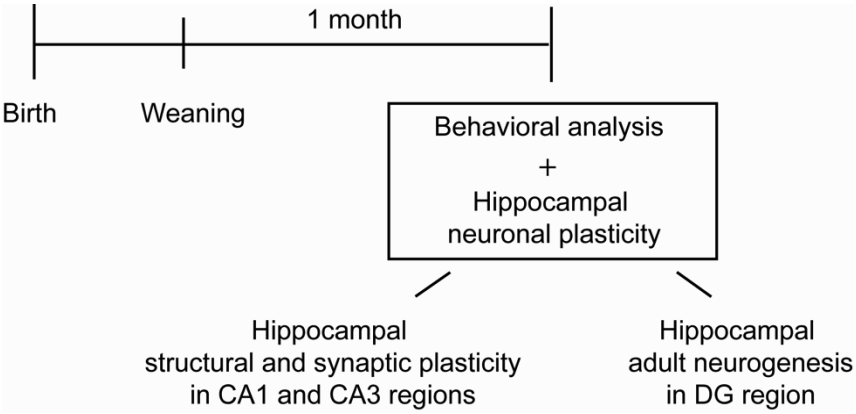

Figure 1. Conceptual diagram. Schematic representation showing the main features of analysis used in this Thesis.

In order to test the specific involvement of DYRK1A kinase on *in vivo* hippocampal-dependent learning and memory performance and neuronal plasticity changes in TgDyrk1A mice we used epigallocatechin-3-gallate (EGCG), a DYRK1A kinase inhibitor, to normalize its activity to wild type's levels. To this end, wild type and

TgDyrk1A mice received during one month EGCG (see details below) or water. First, we checked whether EGCG was inhibiting DYRK1A kinase activity in the hippocampus of wild type and TgDyrk1A mice following the protocol described in section 3.2.5. Second, we evaluated the effect of one month treatment with EGCG on hippocampal-dependent learning and memory in the MWM and NOR. We also used an elevated plus maze (EPM), to examine whether EGCG had an anxiogenic effect. Detailed protocols are explained in section 3.2.3. We then asked if DYRK1A activity normalization could rescue the morphological alterations observed. To this end we evaluated the effect of EGCG on hippocampal structural morphology double transgenic mice (Thy-YFP/TgDyrk1A), which received water or EGCG during one month (section 3.2.4.2). Finally, we checked the involvement of DYRK1A kinase activity in adult neurogenesis, in wild type and TgDyrk1A mice reared during one month with EGCG treatment by analyzing cell proliferation and cell cycle progression as detailed in section 3.2.4.5.

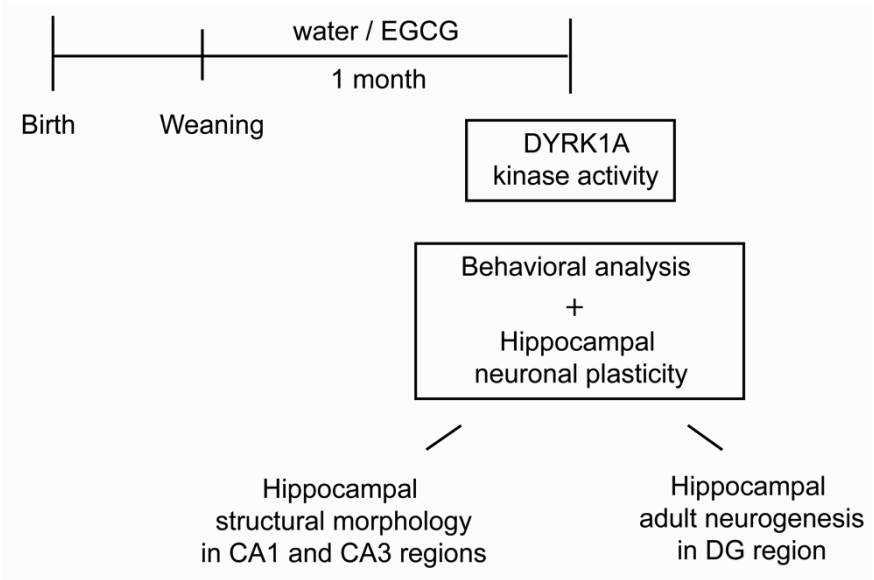

Figure 2. Experimental schedule. Experimental schedule used to analyze the effect of one month of EGCG treatment on DYRK1A kinase activity from hippocampal extracts, hippocampal-dependent learning and memory and structural plasticity of wild type and TgDyrk1A mice.

3.1.2. Effect of environmental enrichment, a non-pharmacological treatment to promote neuronal plasticity in TgDyrk1A mice

Our second aim was to determine the *in vivo* impact of Dyrk1A overexpression on activity-dependent neuronal plasticity and cognition upon physiological conditions where neuronal plasticity is enhanced. To this end, we used environmental enrichment (EE) as an *in vivo* neuroplasticity model that promotes social and physical interactions and stimulate different cognitive domains of the brain. TgDyrk1A mice and their control littermates were reared during one month under non-enriched (NE) or enriched (EE) conditions as explained in detail in section 3.2.2.2.

To evaluate the effect of EE on hippocampal-dependent cognition, NE and EE mice were tested in the MWM, NOR and EPM paradigms (section 3.2.3). To analyze the effect of one month of EE on hippocampal structural morphology, double transgenic mice (Thy-YFP/TgDyrk1A) and their control littermates were also reared during one month under NE or EE conditions and thereafter we analyzed the length of the different hippocampal strata, YFP+ pyramidal cell numbers and area of CA1, CA3 and DG subfields and spine density as described in section 3.2.4.2, 3.2.4.3 and Annex III. We also evaluated the effects of EE on activity-dependent regulation of adult neurogenesis (section 3.2.4.5). Concretely, we studied proliferation rate and cell cycle exit, migration, survival and differentiation, and activation of newly generated cells. Finally, we examined whether the neuronal plasticity and cognitive effects of EE in TgDyrk1A mice were through changes in DYRK1A kinase activity in the hippocampus (section 3.2.5).

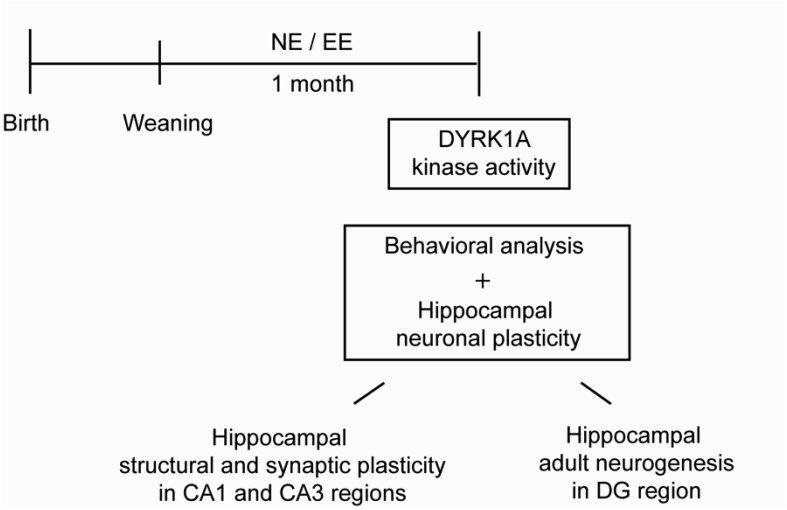

Figure 3. Experimental schedule. Representation of the experimental schedule used to analyze the effect of one month of EE rearing conditions on DYRK1A kinase activity from hippocampal extracts, hippocampal-dependent learning and memory and neuronal plasticity of wild type and TgDyrk1A mice.

To determine the impact of Dyrk1A overexpression on the maintenance of EE effects on hippocampal plasticity, TgDyrk1A mice and their control littermates were reared during one month under EE conditions followed by four months under NE conditions. We investigated the long-term effects of EE on DYRK1A kinase activity (section 3.2.5), hippocampal-dependent learning and memory in the MWM and NOR (section 3.2.3), hippocampal structural plasticity modifications using Thy-YFP/TgDyrk1A mice (section 3.2.4.2), and synaptic plasticity in the Schaffer collateral pathway (Annex III).

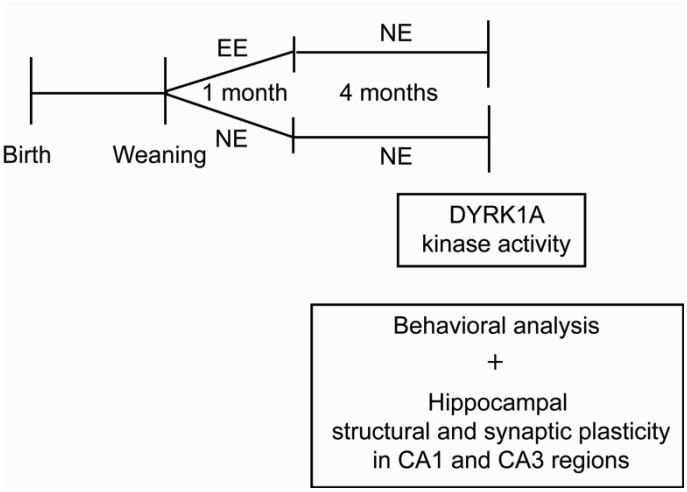

Figure 4. Experimental schedule. Representation of the experimental schedule used to analyze the long-term effects of EE on DYRK1A kinase activity from hippocampal extracts, hippocampal-dependent learning and memory and neuronal plasticity of wild type and TgDyrk1A mice.

Finally, we evaluated whether the combination of EE and EGCG treatments could have synergic effects on hippocampal-dependent learning and memory in TgDyrk1A mice. Wild type and transgenic mice NE or EE received wither water or EGCG treatment during one month and were analyzed for their performance in the MWM, NOR and EPM.

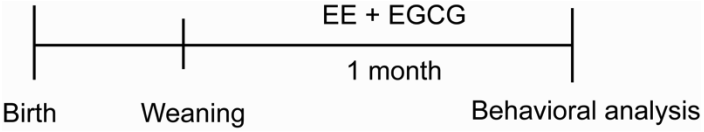

Figure 5. Experimental schedule. Representation of the experimental schedule used to analyze the effect of one month treatment of EE and EGCG on cognition in wild type and TgDyrk1A mice.

3.2. METHODS

3.2.1. Animal models and genotyping

Transgenic mice overexpressing Dyrk1A (TgDyrk1A) were generated in the laboratory of Cristina Fillat (CEK, Centro Ester Koplowitz) as previously described (Altafaj, Dierssen et al. 2001). Briefly, the transgene was microinjected into fertilized eggs that were flushed out from the oviducts of superovulated C57BL6/SJL mice and viable embryos were re-implanted in the oviducts of pseudopregnant mice. The transgene was constructed using the full-length cDNA of *Dyrk1A* from rat that shares high sequence homology with the corresponding mouse cDNA (Song et al., 1996), under the control of the heterologous sheep metallothionein-Ia (*sMT-Ia*) promoter, which is inducible by $ZnSO_4$. In this work the transgene was not induced because we aimed at moderate levels of *Dyrk1A* overexpression that are more similar to those observed in DS (Toiber, Azkona et al. 2010). Mice used in this Thesis, were obtained by crossing transgenic males with C57BL6/SJL female mice.

To study morphological features of the hippocampus, we obtained double transgenic mice (Thy-YFP/TgDyrk1A) crossing TgDyrk1A male mice with *Thy1-Yellow Fluorescent Protein (YFP)* female mice (strain B6.Cg-Tg(Thy1-YFPH)2Jrs/J nº 003782; The Jackson Laboratories). Thy-YFP wild type and Thy-YFP TgDyrk1A mice expressed YFP sparsely in subset of pyramidal neurons allowing measuring the different layers of the hippocampus and quantifying the number of YFP+ neurons in pyramidal layers (dorsal CA1, CA3 and DG subregions). In all cases, the non-transgenic TgDyrk1A littermates served as control. Since our previous experiments showed that Ts65Dn males had a poor response to enriched rearing due to the subdominant behavior of this strain (Martinez-Cue, Baamonde et al. 2002) that may cause stress, the present experiments only used females.

In basal and non-enriched conditions, mice were reared in conventional cage (20 x 12 x 12 cm height, Plexiglas cage) in groups of 2-3 animals and maintained under the same 12 hours (h) light-dark cycle (8:00 to 20:00) in controlled environmental conditions of humidity (60%) and temperature (22 ± 1ºC) with free access to food and water. All animal procedures were approved by the local ethical committee (Comité Ético de Experimentación Animal del PRBB (CEEA-PRBB); procedure numbers MDS-08-

1060P1 and JMC-07-1001P1 and JMC-07-1001P2-MDS MDS), and met the guidelines of the local (law 32/2007) and European regulations (EU directive n° 86/609, EU decree 2001-486) and the Standards for Use of Laboratory Animals n° A5388-01 (NIH). The CRG is authorized to work with genetically modified organisms (A/ES/05/I-13 and A/ES/05/14).

TgDyrk1A genotyping was performed by amplifying genomic DNA obtained from the mice tail. *In vitro* DNA amplification was performed by polymerase chain reaction (PCR) technique using the oligonucleotides DyrkF (5'-GTCCAAACTCATCAATCTATC-3') and DyrkR (5'-CTTGAGCACAGCACTGTTG-3').

3.2.2. Treatments

3.2.2.1. Epigallocatechin-3-gallate (EGCG) treatment

To determine if the structural plasticity and hippocampal-dependent cognitive phenotypes in TgDyrk1A mice were related to DYRK1A kinase activity, TgDyrk1A and wild type mice were treated for one month with epigallocatechin-3-gallate (EGCG) dissolved in drinking water (EGCG concentration: 90 mg/mL for a dose of 2-3 mg per day). EGCG solution was prepared freshly every 3 days from a green tea leaf extract [Mega Green Tea Extract, Decaffeinated, Life Extension®, USA; EGCG content of 326.25 mg per capsule].

3.2.2.2. Environmental enrichment

To evaluate the impact of *in vivo* Dyrk1A overexpression on hippocampal cognition and activity-dependent structural plasticity upon enhanced physiological conditions female TgDyrk1A and wild type mice were randomly reared under either NE or EE conditions for 30 days after weaning (21 days of age). Mice under NE conditions were reared in conventional cages (20 x 12 x 12 cm height, Plexiglas cage) in groups of 2-3 animals. The EE consisted in a spacious (55 x 80 x 50 cm height) Plexiglas cage with toys, small houses, tunnels and platforms. Wheels were not introduced in the cages to avoid the effect of physical exercise on adult neurogenesis. The arrangement was changed every 3 days to keep novelty conditions. We also stimulated social interactions, housing 6-8 mice per cage. All groups of animals were maintained under the same 12 h light-dark

cycle (8:00 to 20:00) in controlled environmental conditions of humidity (60%) and temperature (22 ± 1ºC) with free access to food and water.

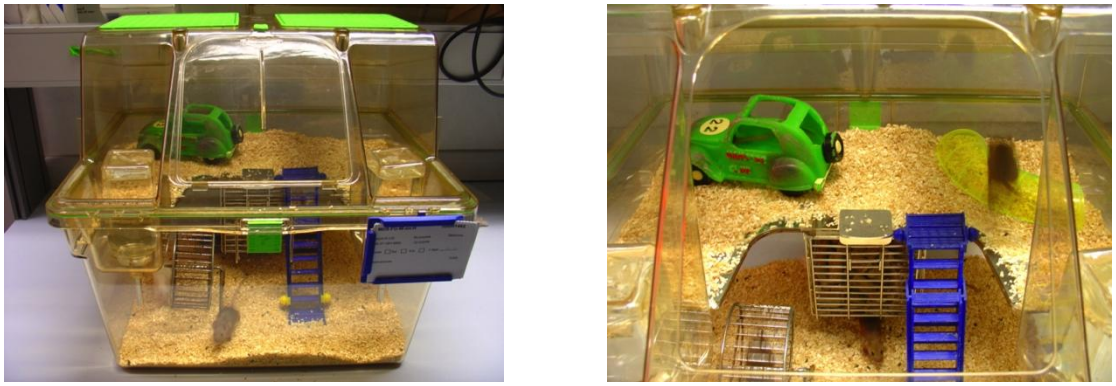

Figure 6. Environmental enrichment cages. Representative photographs showing mice reared under environmental enrichment.

3.2.3. Behavioural analysis

We evaluated hippocampal-dependent learning and memory in adult TgDyrk1A mice under different conditions. Behavioural paradigms such as the Morris Water Maze and Novel Object Recognition were performed in wild type and TgDyrk1A mice in control conditions and just after receiving either one-month of EE or EGCG treatments, or both. Additionally, the Elevated Plus Maze test was used to analyze possible anxiety-like behaviours. To determine the long-term effects of EE, behavioural tests were performed in six-month-old mice.

3.2.3.1. Visuo-spatial learning and memory: Morris Water Maze (MWM)

The Morris Water Maze (MWM), widely used to assess hippocampal-dependent visuo-spatial learning and memory, consisted of a circular pool (diameter, 1.70 m; height, 0.6 m) filled with tepid water (19°C) opacified by the addition of non-toxic white paint. A white escape platform (12 cm diameter, height 24 cm) made of Plexiglas was located 1 cm below the water surface in a fixed position (22 cm away from the wall). White curtains with affixed black patterns to provide an arrangement of spatial cues surrounded the maze. The test was performed under low non-aversive lighting conditions (50 lux). 12-20 mice per experimental group were tested.

The whole procedure is divided into 10 sessions:

Pre-training session (PT)

On the first day, the platform was visible and located on the centre of the pool, which allowed the mice getting familiarized and acquired procedural aspects of the task.

Acquisition sessions (A)

Visuo-spatial learning was assessed during 5 or 6 consecutive days (4 trials per day) or until the mice reached the best (defined by the time to reach the platform in the cued session) or a stable performance level using a hidden platform situated in the north-east (NE) quadrant of the pool.

Removal session

To assess reference memory a probe test was performed 24 h after last acquisition session. The platform was removed and mice were allowed to swim for 60 seconds (s). In this part of test, only one trial session was given and mice were situated in the south-west (SW) quadrant of the pool.

Cued session (C)

Following the probe test, a cued session using the visible platform (elevated 1 cm above the water) and a visible cue (black flag) to indicate its position, was performed to test motivation, motor and visual abilities.

Reversal session (RV)

To measure cognitive flexibility, three reversal learning sessions were performed for 3 consecutive days. In this case, the platform was hidden and located in the opposite quadrant of the pool (SW).

In all trials, mice were placed facing the outer edge of the pool and placed at one of the starting locations in random order. Mice were allowed to swim until they located the platform. Mice failing to find the platform within 60 s were placed on it for 20 s. After each trial, all mice were returned to their home cage at the end of every trial.

All the trials were recorded and traced with an image tracking software (System Motor Activity Record and Tracking, SMART, Panlab) connected to a video camera placed above the pool. Escape latencies, distance travelled, swimming speed, percentage of time spent in each quadrant of the pool and time in the centre and periphery were

recorded for posterior data analysis. Performance in the probe test was quantified comparing the amount of time spent in the target zone versus the average of the three other equivalent zones of the pool. To more accurately analyze learning strategy we used a in-house designed software, jTracks, that allows to representing the frequency of stay in a group of mice (Arque, de Lagran et al. 2009).

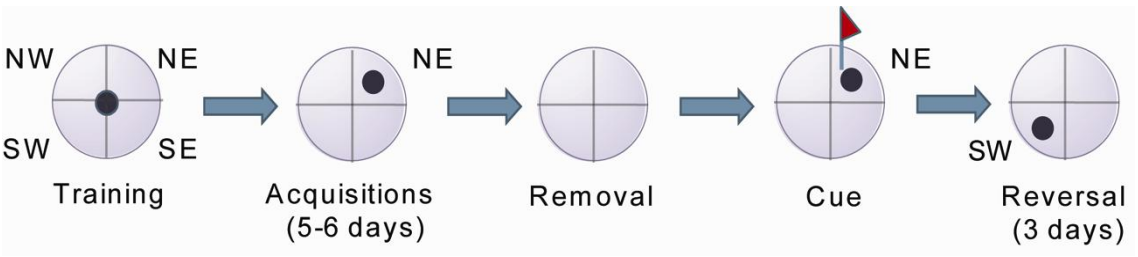

Figure 7. Experimental schedule of the MWM. Representation of the experimental outline used in the MWM to test visuospatial learning, reference memory and cognitive flexibility.

3.2.3.2. Short-term recognition memory: Novel Object Recognition (NOR)

The novel object recognition test is based on the innate tendency of rodents to preferentially explore novel objects over familiar ones. This test has been widely used to assess recognition memory in rodents. Mice were placed into an open-field (70 cm wide x 70 cm long x 30 cm high) made of red metacrylate and surrounded by curtains. The task was performed under non-aversive low lighting conditions (50 lux). An overhead camera connected to the video-tracking software (SMART, Panlab) was used to monitor the animal's behavior. To eliminate odor cues, the arena and the objects were thoroughly cleaned with 10% odorless soap and dried. Moreover, the position of the objects in the familiarization and test session was counterbalanced between animals. Sniffing the objects was used as the measure of exploration that was registered manually by an experimenter blind to genotype and treatment.

The experimental schedule included three sessions:

Habituation

The first day, mice were allowed to explore the arena during 5 minutes (min). Total distance, speed and the percentage of time spent in centre and periphery were calculated. Thereafter, an object was placed in the centre of the arena to habituate the animals. The object exploration was recorded for 5 min.

Familiarization

On the second day, the animals had to explore during 10 min two identical objects placed in the center of the apparatus. Mice exploring less than 20 s were discarded.

Test

One hour after the familiarization session, mice were allowed to explore for 5 min the same arena but one of the familiar objects had been changed for a new one. The exploration time for the familiar (TF) and the new object (TN) during the test phase was recorded. Memory was operationally defined by the percentage of discrimination index for the novel object (DI) as the time spent investigating the novel object minus the time spent investigating the familiar one in the testing period [% Discrimination Index, DI = [(TN – TF) / Total Exploration Time] x 100].

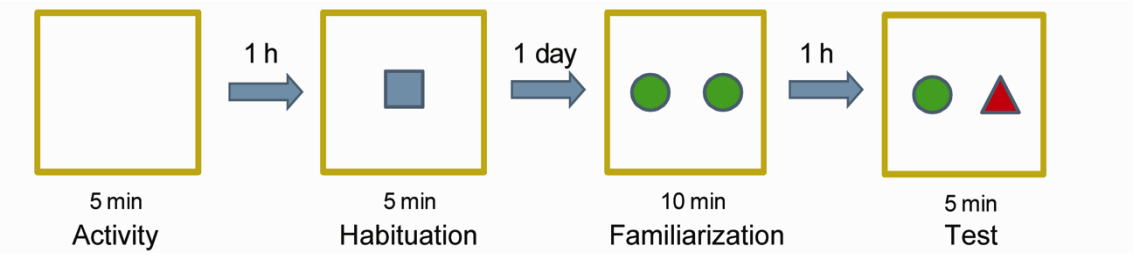

Figure 8. Experimental schedule of the NOR. Representation of the experimental outline used in the NOR to test locomotor activity (in the open field) and short-term recognition memory.

3.2.3.3. Anxiety: Elevated Plus Maze (EPM)

The elevated plus maze is a widely used behavioural assay to assess anxiety in rodents. The apparatus consists of four methacrylate arms (30 cm long and 8 cm wide) two of which are open and two enclosed (20 cm high walls) arranged to form a plus shape and elevated 50 cm. Mice were placed in the intersection of the four arms and the behavior is recorded for 5 min. Behavior in this task reflects a conflict between the mice's preference for protected areas (closed arms) and their innate motivation to explore novel environments. Total distance travelled, percentages of time spend in the center of the apparatus and closed/open arms were recorded.

Figure 9. Elevated Plus Maze apparatus. Representation of the apparatus used to analyze anxiety.

3.2.4. Histological analysis

Mice were anaesthetized by means of carbon dioxide and perfused intracardially with 0.1M phosphate buffered saline (PBS), pH = 7.4, followed by chilled 4% paraformaldehyde (PFA; Sigma). The brains were removed from the skull and postfixed in the same fixative at 4ºC overnight. After rinsing in PBS, brains were cryoprotected in 30% sucrose and stored at 4°C. 40 μm coronal sections were obtained using a cryostat (CM3050S, Leica Microsystems) and stored at -20ºC in cryoprotective solution (30% ethylenoglycol, 30% glycerol, 40% PBS).

3.2.4.1. Immunofluorescence and immunohistochemistry

The immunofluorescence and immunohistochemistry staining was performed in one of every three section covering the entire dorsal hippocampus (Bregma, -1.06 to -2.18 mm, Paxinos and Franklin, 2001). Before staining, sections were extensively washed with 0.1M PBS and permeabilized with 0.2% PBS-T (PBS-Triton X-100) at room temperature (RT). The immunostaining procedure consisted on the incubation of free-floating sections for 1 h at RT with blocking solution containing 0.2% PBS-T, 0.2% gelatin, 10% fetal bovine serum (FBS). Then, sections were incubated with primary antibodies in a solution containing 0.2% PBS-T, 0.2% gelatin and 5% FBS overnight at 4°C and subsequently incubated for 1 h with the corresponding secondary fluorescent antibody (1/500, Alexa 488, 555, 594 or 647nm, Molecular Probes). Sections were mounted and cover slipped with mowiol reagent.

For immunohistochemistry experiments using peroxidase detection, sections were preincubated in 3% H_2O_2, 10% methanol in PBS 0.1M for 20 min to block endogenous peroxidase and visualized using the corresponding biotinylated secondary antibodies

(1/150, Vector Laboratories) followed by 2 h of incubation with ABC kit system (Vectastain, Vector Laboratories). Diaminobenzidine (DAB) chromogen was used to visualize the positive cells. Finally, sections were washed in PBS 0.1M, mounted on slides, dehydrated with sequential solutions containing increasing concentration of ethanol and coverslipped with DPX.

3.2.4.2. Morphological analysis of the hippocampus

To assess morphological features of the hippocampus, Thy-YFP/TgDyrk1A mice were sacrificed and 150 µm coronal brain sections were obtained using a vibratome (VT1000S, Leica Microsystems), washed extensively with 0.1M PBS, mounted and coverslipped with mowiol reagent.

Images were acquired with a confocal microscope (SPE; Leica Microsystems) using 10x objective with a sequential line scan at 1024 × 1024 pixel resolution. Serial optical sections were acquired with a 3-µm step size. Confocal image stacks were imported into Image J software (Macbiophotonics) and an average projection was generated. The width of several layers of dorsal DG and CA1 hippocampal subregions was calculated using Image J software according to the stereotaxic coordinates adopted from a mouse brain atlas (Bregma, -1.06 to -2.18 mm, Paxinos and Franklin, 2001). Briefly, four measures per hippocampal section were analyzed using the straight line selection tool in Image J in 5 sections per animal. Area of CA1 and CA3 subregions was measured by using the Polygon tool in Image J. Mean cell number of Thy+ cells in CA1 and CA3 subfields were also calculated using the Cell Counter Plugin in Image J software.

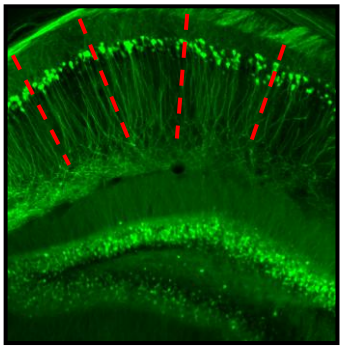

Figure 10. Representative confocal micrograph of Thy-YFP/TgDyrk1A mice. Representation of the method used to analyze the length of each hippocampal strata in the Image J. Four measures per hippocampal section (red lines) were analyzed using the straight line selection tool in Image J in 5 sections per animal.

3.2.4.3. Characterization of dendritic spine density in hippocampal pyramidal neurons

Four 2 months-old TgDyrk1A and wild type mice per group were used to study apical dendritic spine density in the stratum radiatum of pyramidal CA1 cells. Intracellular injections of Lucifer yellow (LY) were performed in dorsal CA1 pyramidal neurons. Animals were perfused with 4% PFA and 150 μm coronal sections were obtained with the vibrotome, mounted and coverslipped with mowiol reagent. Cell injection methodology was previously described in detail (Elston, Benavides-Piccione et al. 2001). Briefly, cells were injected with LY by continuous current. Only pyramidal neurons whose entire apical dendritic arbor was completely filled were included in the analysis. Images for the dendritic spine analysis were acquired with a confocal microscope (SPE; Leica Microsystems) using 63x oil objective plus 5 times magnification with a sequential line scan at 1024 × 1024 pixel resolution. Serial optical sections were acquired with a 0.1 μm step size and z-projected for the analysis with the aid of Image J software.

Dendritic spines on labeled CA1 pyramidal cells were measured by counting the number of spines in each segment 30 μm from the soma in a total of 20 μm dendritic length. Spines were counted on 8-10 apical projecting dendrites per animal, randomly taken from different cells. Spine density was calculated by expressing the average number of spines in a 10 μm portion of the dendrite. Spines were assigned to several morphological categories: type A, thin spines without a clear differentiated head; type B, long neck with small round head; and type C, short neck with round large head. Representative images were obtained using the Huygens Essential software for confocal images deconvolution followed by the Imaris software for spines reconstruction.

3.2.4.4. Characterization of excitatory-inhibitory balance in *stratum radiatum* CA1 region

Free floating brain sections were immunostained overnight at 4ºC with the primary antibodies mouse anti-vesicular glutamate transporter 1 (VGLUT1) (1:200, clone 317G6, Synaptic Systems) to analyze excitatory puncta and guinea pig anti-vesicular GABA transporter (VGAT) (1:200, cytoplasmatic domain, Synaptic Systems) to evaluate inhibitory puncta. The following day, slices were incubated with the

corresponding secondary fluorescence antibodies (1:1000, Alexa 488 and 555, Invitrogen) 1 h at RT protected from light.

Pictures were captured in the stratum radiatum of CA1 hippocampal subfield using a confocal microscope with 63x objective and 5x magnification (SPE; Leica Microsystems). For each region, all pictures were captured with identical confocal settings for laser' power, gain and offset levels. Images were imported into Image J and for each image the negative control background intensity was subtracted to each channel, converted into binary data and threshold, to outline immunopositive puncta. Number and size of VGLUT1 and VGAT puncta per field were quantified using 'analyze particle' function of the software.

3.2.4.5. Characterization of adult neurogenesis in the hippocampus of TgDyrk1A mice

To evaluate whether Dyrk1A overexpression was responsible of hippocampal adult neurogenesis impairments in DS, we studied proliferation rate, cell cycle progression, cell cycle exit, neuronal migration, differentiation, survival and integration in the pre-existing circuitry of the DG. Newly generated cells were identified using 5'-bromo-2'-deoxyuridine (50 mg/kg BrdU intraperitoneal injection; Sigma) and proliferation rate was analyzed in mice receiving a single BrdU injection and sacrificed 2 h later. Cell cycle exit index was evaluated 24 h after a single BrdU injection.

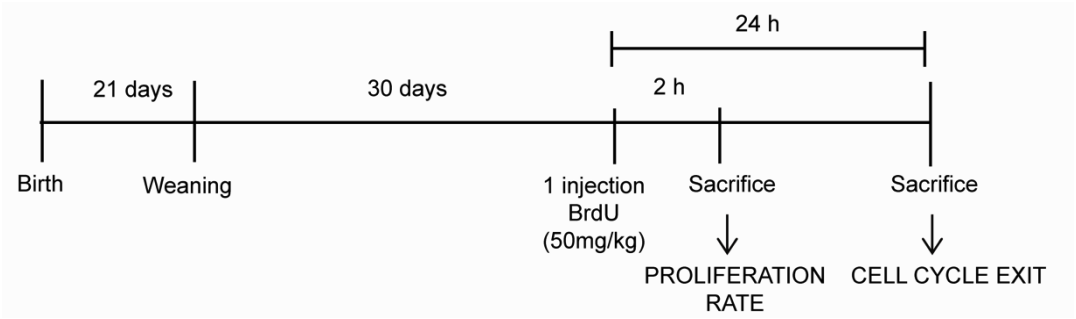

Figure 11. Experimental schedule. Representation of the experimental schedule used to analyze proliferation rate and cell cycle exit.

To examine the migrating behavior, survival and differentiation of newly generated cells mice received four BrdU injections every 2 h and were sacrificed 24 h, 1 week or 1 month after the last BrdU injection.

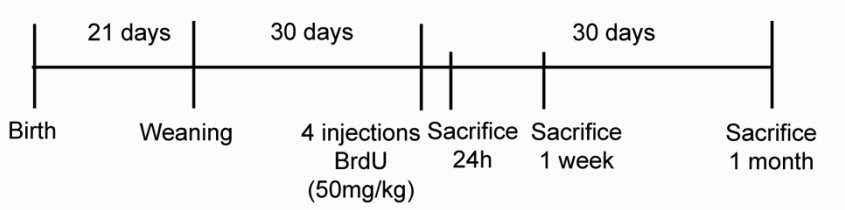

Figure 12. Migration survival and differentiation. Representation of the experimental schedule used to analyze migration, survival and differentiation of newborn cells in the DG.

Specific immunofluorescence and immunohistochemistry protocols were used for the following stainings:

BrdU detection

After washing sections with 0.1M PBS and 0.5% PBS-T at RT, sections were pretreated with 0.1N HCL at 4°C during 10 min, 2N HCL at 37°C for 30 min and 0.1M borate buffer pH = 8.5 to denaturate DNA. After extensive washings with 0.5% PBS-T, sections were blocked during 2 h with 0.5% PBS-T, 0.2% gelatin, 10% FBS and 0.2M Glycine, followed by incubation with the anti-BrdU antibody in a solution containing 0.5% PBS-T, 0.2% gelatin and 5% FBS overnight at 4ºC.

Dyrk1A, Ki67, Sox2, pH3 and caspase3 immunostaining

Sections were washed in 0.1M PBS followed by 30 min with 0.2% PBS-T and incubated with citrate buffer 10mM pH = 6 treatment during 10 min at 95°C. After that, sections were washed extensively with 0.1M PBS and 0.2% PBS-T, and incubated with blocking solution containing 1% Bovine Serum Albumin (BSA) and 5% goat serum in 0.2% PBS-T during 1 h. Finally, sections were incubated with the corresponding primary antibody in a solution containing 0.2% PBS-T, 1% BSA and 2.5% goat serum overnight at 4ºC.

Double detection of ki67 and BrdU

Sections were washed in 0.1M PBS and 0.2% PBS-T and treated with citrate buffer 10mM pH = 6 treatment during 10 min at 95°C. After washing sections with 0.2% PBS-T during 30 min, they were incubated with HCL and borate buffer pretreatment to denature DNA as described before and blocked with 0.2% PBS-T, 0.2% gelatin and 10% FBS. Next, anti-ki67 and anti-BrdU antibodies were added together in a solution containing 0.2% PBS-T, 0.2% gelatin and 5% FBS.

Double detection of BrdU and doublecortin (DCX)

Double detection was obtained by sequential staining. First, sections were washed extensively with 0.1M PBS and 0.2% PBS-T followed by incubating them during 1 h with blocking solution containing 0.2% PBS-T, 0.2% gelatin and 3% donkey serum. Then, sections were incubated with DCX primary antibody in a solution contacting 0.2% PBS-T, 0.2% gelatin and 1% donkey serum overnight at 4ºC followed by the corresponding secondary antibody during 1 h. After that, sections were washed with 0.2% PBS-T and were pretreated with BrdU treatment followed by incubating them with BrdU primary antibody.

Primary antibodies used are listed in Supplementary Table 1 (Annex II).

3.2.4.5.1. Characterization of cellular types of the subgranular neurogenic niche

Schematic representation of the markers used to identify the different cellular types of the DG neurogenic niche is depicted in Figure 38A (Results section). Progenitor precursors were identified using an antibody against Sox2 that is expressed in *type 2a* cells. *Type 1* population was distinguished using Sox2+ GFAP+ double staining. To identify proliferating cells, we used an antibody against ki67, an endogenous marker expressed during G1, S, G2 and M cell cycle phases and the proportion of proliferating progenitor cells, was with double immunostaining of Sox2+ ki67+.

Cell cycle exit was analyzed by quantifying the percentage of BrdU+ ki67- cells respect to total BrdU+ cells 24 h after a single BrdU injection. An antibody against pH3 was used to discriminate proliferating cells in either the G2 or M phases based on their nuclear pattern [(Hendzel, Wei et al. 1997); Figure 43C in Results section] and doublecortin (DCX) antibody was used to study the dendritic morphology of immature newborn neurons.

Neuronal differentiation of one week surviving cells was evaluated using double immunostaining of BrdU+ DCX+ and BrdU+ calretinin+ for immature neurons. The cell fate of the surviving cells was identified using BrdU+ NeuN+ and BrdU+ GFAP+ for neuronal and glial population respectively, in a group of mice sacrificed one month after the last BrdU injection. Finally, neuronal integration of surviving cells in the

preexisting network was determined using triple immunostaining of BrdU+ NeuN+ cFos+, considering cFos as an activity-dependent early immediate gene.

The optical fractionator was used for stereological counting of BrdU+ and total granule cell numbers, in one hemi-hippocampus of the DG (Bregma, -1.06 to -2.18 mm, (Paxinos and Franklin 2001) from 4-8 animals per group using an OLYMPUS BX51 microscope (Olympus) with the aid of CAST-GRID software package. Given that in the rest of the experiments the density of the positive cells was relatively low, 2-μm step size confocal stacks obtained with Confocal TCS SP5 microscope (Leica, 40x) were merged using Image J software for counting. Final cell number was corrected after checking along z-stack that none overlapping cells were previously counted.

Morphological analysis of the dendritic tree of DCX+ cells was performed using Neuroexplorer (Neurolucida software) in an OLYMPUS BX51 microscope (Olympus). A total of 80 neurons (5 animals per experimental group) from the DG-free blade (infrapyramidal) were traced and, dendritic length and number of nodes were measured.

3.2.4.5.2. Activation of newly generated cells after spatial learning

To test the capability of newborn neurons to be activated upon learning, mice received four BrdU injections and one month later a MWM protocol (four acquisitions of six trials per day and a probe test 24 h the last acquisition session) was performed (Kee, Teixeira et al. 2007). One hour after the probe session animals were sacrificed and brain sections were immunostained with BrdU, cFos and NeuN antibodies.

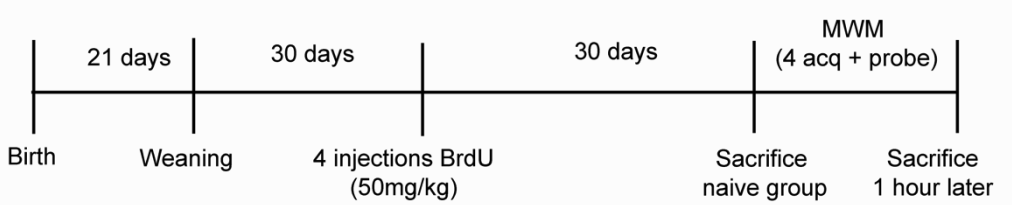

Figure 13. Experimental schedule. Representation of the experimental schedule used to analyze the activity-dependent integration of surviving cells in the DG without learning stimuli or after the MWM.

3.2.5. DYRK1A kinase activity

Brains were removed and the hippocampus was dissected and quickly frozen in liquid nitrogen. Hippocampal tissue was homogenyzed in 400 μl of Hepes lysis buffer

(100mM Hepes pH 7.4, 4mM EDTA, 300mM NaCl, 2% NP40) with 7x protease inhibitors (*Complete*) and phosphatase inhibitors (2mM Na_3VO_4, 30mM PPiNa, 25mM NaF). After homogenization, samples were kept in ice for 15 min, centrifuged for 20 min at 13200 rpm at 4ºC and the supernatant was collected for protein quantification. The protein concentration of lysate supernatants was determined using the BCA Protein Assay (Pierce) reagents, according to the manufacturer's protocol. Absorption was measured at 562nm with a Versa max microplate reader (Molecular devices).

Mouse anti-Dyrk1A antibody (Abnova, 3 μg/sample) was immobilized on glutathione-Sepharose beads (GE Healthcare) during 1 h at RT and centrifuged at 0.8g during 2 min at RT to eliminate the supernatant. After that, 1mg of protein extract was added to the beads and incubated overnight at 4ºC. The recovered samples were washed extensively with Hepes washing buffer (100 mM Hepes pH 7.4, 4 mM EDTA, 300mM NaCl, 0.1% NP40) and analyzed by immunoblotting and *in vitro* kinase assays.

For immunoblotting analysis, 500 μg of the purified protein in the immunocomplexes were incubated with 35 μl of lading buffer at 98ºC during 15 min and resolved by 7.5% SDS-PAGE, transferred on a nitrocellulose membrane and incubated with anti-Dyrk1A antibody (1/1000) overnight at 4°C. After that, membranes were incubated for 1 h at room temperature with the corresponding secondary antibody. Detection was performed using ECL (GE Healthcare) and determined with LAS-3000 image analyzer (Fuji PhotoFilm). Protein quantification was performed using Image Gauge software version 4 (Fuji PhotoFilm).

To determine the catalytic activity of DYRK1A, 500 μg of the purified protein were incubated for 20 min at 30°C in 30 μl of phosphorylation buffer containing 2mM of specific DYRK1A peptide [DYRKtide-RRRFRPASPLRGPPK; (Himpel, Tegge et al. 2000)], 1mM ATP, [γ-^{32}P]ATP (2 μCi/sample) and kinase buffer (50 mM Hepes pH 7.4, 10 mM $MgCl_2$, 10 mM $MnCl_2$ and 1 mM DTT). 5 μl of reaction aliquots were dotted onto P81 Whatman paper for every sample every 10 min in a total of 4 times. After washing extensively with 5% phosphoric acid, counts were determined in a liquid scintillation counter. Relative kinase activity was obtained normalizing against the amount of DYRK1A protein present in the immunocomplexes previously quantified.

3.2.6. Statistical analysis

Data were expressed as mean + S.E.M. We used the *Student t* test analysis when two independent variables were compared. Results were considered significant when $p<0.05$. For the statistical analysis of four groups containing two independent variables, the multivariate analysis of variance (MANOVA) was applied. Bonferroni was used for *post-hoc* analysis when a significant, or a trend to, genotype x treatment interaction was found ($p<0.09$). Whenever the interaction between genotype and treatment was not significant, the Genotype effect or the Treatment effect was considered statistically significant when $p<0.05$. For those experiments with repeated events giving consecutive data (curves) were compared using MANOVA repeated measures. Paired sample t-test was used to analyze the probe session of the Morris Water Maze. All the analyses were performed using the statistical package SPSS for Windows, version 12.0.

Symbol			
Significance	**Genotype effect**	**Treatment effect**	**Comparison between groups (Bonferroni)**
p<0.05	ω	π	*
p<0.01	ωω	ππ	**
p<0.001	ωωω	πππ	***

RESULTS

4. RESULTS

4.1. IMPACT OF *DYRK1A* OVEREXPRESSION ON HIPPOCAMPAL-DEPENDENT LEARNING AND MEMORY IN ADULT TGDYRK1A MICE

As described in the Introduction, DYRK1A is expressed brain regions involved in cognition such as the hippocampus and cortex (Marti, Altafaj et al. 2003) and phosphorylates several proteins involved in synaptic plasticity and neuronal differentiation. In the first part of the Thesis we evaluated the impact of *Dyrk1A* overexpression on hippocampal learning and memory in TgDyrk1A mice, as a proof that the hippocampus was affected in these mice during adulthood. Second, we analyzed whether normalization of DYRK1A kinase overactivation was sufficient to rescue the observed cognitive phenotype by using epigallocatechin-3-gallate (EGCG), a DYRK1A kinase inhibitor. Finally, we characterized whether non-pharmacological treatments such as environmental enrichment (EE) that enhance neuronal plasticity, could rescue hippocampal-dependent learning and memory impairments in TgDyrk1A mice.

4.1.1. Deficits in hippocampal-dependent learning and memory in adult TgDyrk1A mice

The experiments were carried out on a standard Morris Water Maze (MWM) and in the Novel Object Recognition (NOR) paradigms.

4.1.1.1. Visuo-spatial learning and memory: Morris Water Maze (MWM)

In the first experiment, we analyzed the performance of adult wild type and TgDyrk1A mice in the Morris Water Maze (MWM) paradigm. During the acquisition sessions, TgDyrk1A mice presented a significant learning impairment in the last sessions of the test as shown by the shift to the right of the learning curve compared to wild type mice (Figure 1A, TG vs. WT *t* test, A5: t (1,27) = -2.35, $p<0.05$. A6: t (1,27) = -2.14, $p<0.05$). Moreover, when analyzed the learning strategy of the animals TgDyrk1A mice showed a reduced preference for the target zone as shown in the depicted heat maps representing the spatial distribution of activity of wild type and transgenic mice (Figure 1B). These deficits were specific, since no differences were detected between genotypes

in the pre-training session, when procedural learning was assessed, or in the cued session, in which motivation and motor capabilities are the only constrains to reach the platform (Figure 1A). These data resemble the impaired performance of Ts65Dn mice (Martinez-Cue, Baamonde et al. 2002) reinforcing the role of Dyrk1A overexpression in DS related visuo-spatial learning impairment (Altafaj, Dierssen et al. 2001).

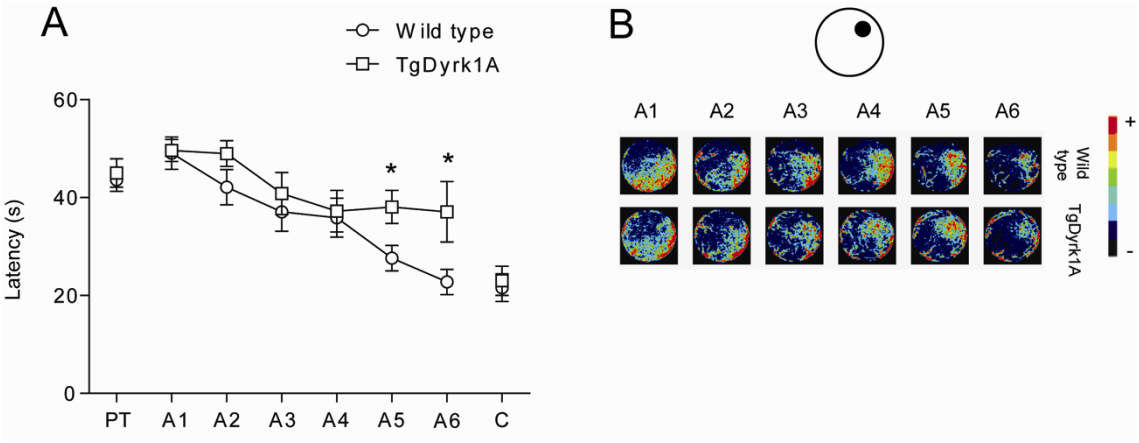

Figure 1. Visuo-spatial learning in adult TgDyrk1A mice. (A) Latency to reach the escape platform in the pre-training (PT) session, along the learning sessions (A1-A5) and cued (C) session in wild type (WT) and transgenic (TG) mice. (B) Color-coded histograms representing the spatial distribution of activity of wild type and transgenic mice in the maze during acquisition sessions. Color scale is depicted on the right of the histogram, where red color corresponds to the most visited zones and black color to the less or non-visited zones during learning sessions (A1-A6). WT n = 12; TG n = 16. Data are represented as mean ± SEM. Student *t* test for independent samples * $p<0.05$. PT: Pre-training. A: Acquisition. C: Cued.

In the reference memory (probe) trial, all groups spent a significantly higher percentage of time in the trained zone (Figures 2A and 2B). However, the preference for the target zone was less marked in TgDyrk1A as compared to wild type mice (Figure 2A, WT paired *t* test, $t\ (1,12) = 3.60$, $p<0.001$; TG paired *t* test, $t\ (1,16) = 2.26$, $p<0.05$) indicating poorer reference memory in TgDyrk1A mice.

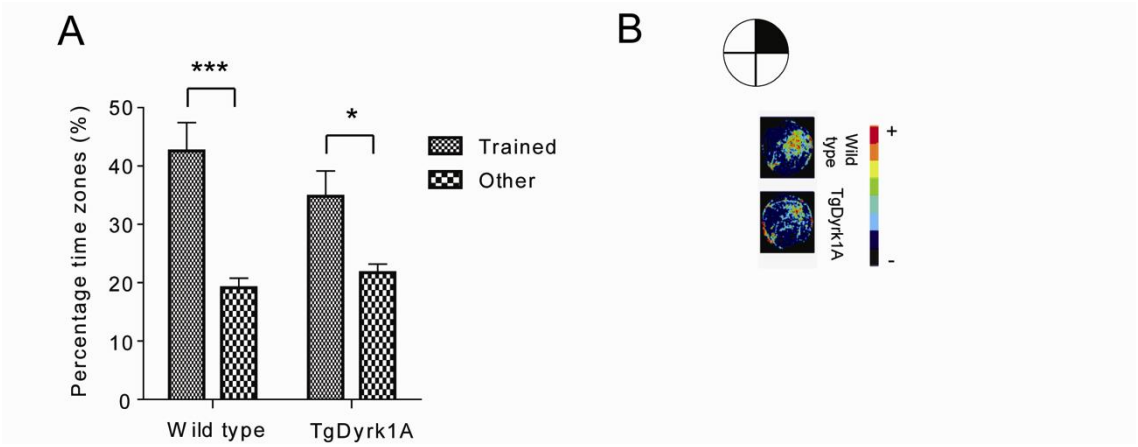

Figure 2. Reference memory in TgDyrk1A mice. (A) Percentage of time that mice spent in the trained compared to the non-trained quadrants during the probe test. (B) Color-coded histograms representing the spatial distribution of activity of wild type and transgenic mice during the probe session. WT n = 12; TG n = 16. Data were represented as mean ± SEM. Student *t* test for paired samples * p<0.05; *** p<0.001.

To test cognitive flexibility, during 3 days mice had to learn a new platform position. TgDyrk1A mice spent more time to reach the platform in the new position along days (Figure 3A, one-way ANOVA repeated measures F (1,27) = 6.36, p<0.01) and in the third reversal session they did not present preference for the new platform position compared to wild type mice (Figure 3C, WT paired *t* test, *t* (1,13) = 4.03, p<0.01; TG paired *t* test, *t* (1,16) = 0.54, p=0.59). This result is also shown in the color-coded plots (Figure 3B) and indicates an impairment in cognitive flexibility.

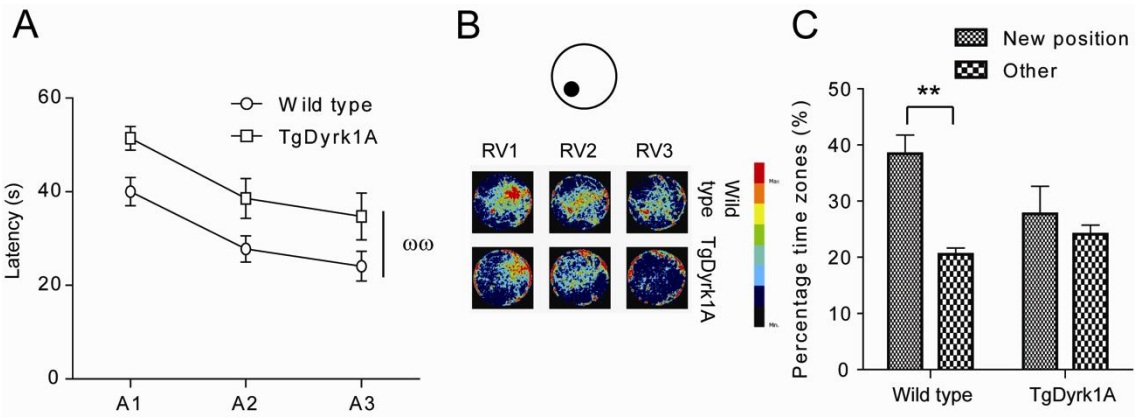

Figure 3. Cognitive flexibility in TgDyrk1A mice. (A) Latency to reach the escape platform in the new position along the reversal sessions (RV1-3) in wild type (WT) and transgenic (TG) mice. (B) Color-coded histograms representing the spatial distribution of activity of wild type and transgenic mice during reversal session. (C) Percentage of time spent in the new quadrant compared to the other quadrants during the last reversal session (RV3). WT n = 12; TG n = 16. Data were represented as mean ± SEM. One-way ANOVA repeated measures genotype effect ωω p<0.01. Paired *t* test ** p<0.01. RV: Reversal.

4.1.1.2. Short-term recognition memory: Novel Object Recognition (NOR)

In the next experiment, we tested whether other hippocampal-dependent cognitive tasks could be also affected by Dyrk1A overexpression. TgDyrk1A and their wild type littermates were trained in the novel object recognition (NOR) paradigm, used to test cortico-hippocampal-dependent tasks in mice. In the familiarization session, in which mice were allowed to explore two identical objects (Figure 4A), total time of exploration was similar in both genotypes (Figures 4B). To analyze the discrimination ability between the two objects, we calculated the discrimination index (see Material and methods section 3.2.3.2.). TgDyrk1A mice showed a significant impairment in object recognition compared to wild types (Figure 4D, TG *vs.* WT *t* test, $t(1,25) = 3.67$, $p<0.001$), with no differences in total exploration time (Figure 4C).

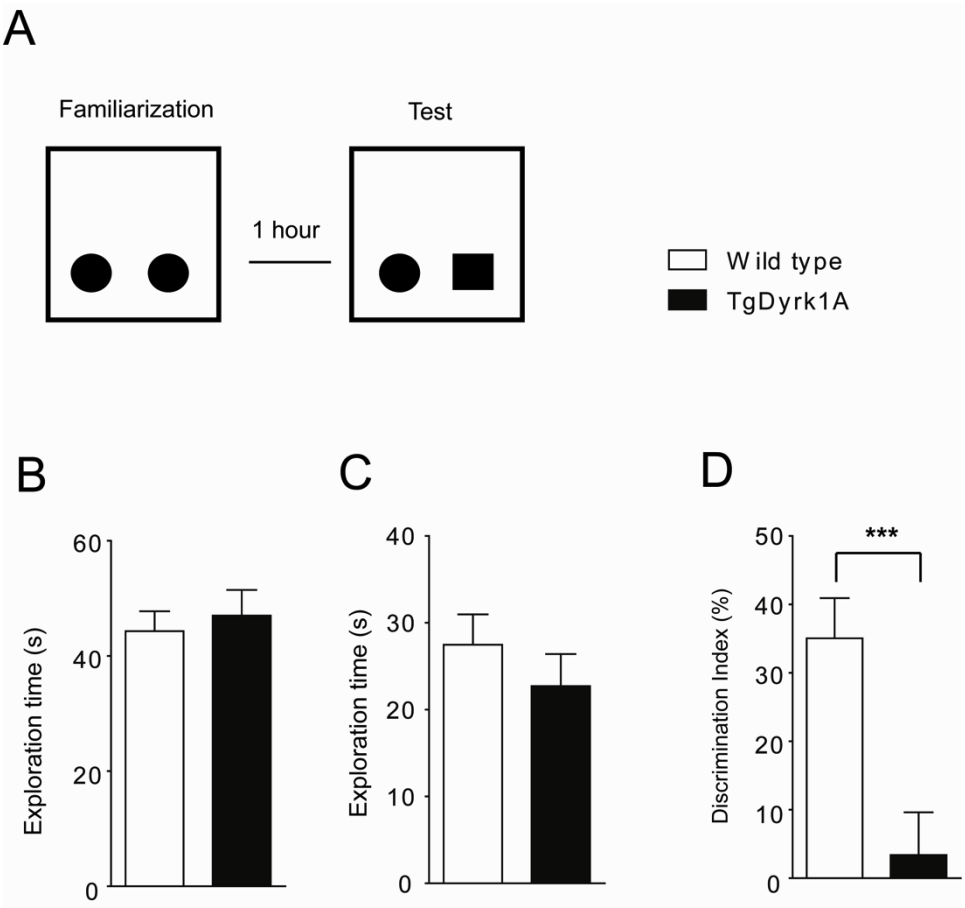

Figure 4. Novel Object Recognition test in TgDyrk1A mice. (A) Schematic representation of the NOR paradigm. (B) Average time of exploration during the familiarization session in the NOR. (C) Average time of exploration during the test session in the NOR. (D) Discrimination index during the test session in the NOR. Data are expressed as ± SEM. WT n = 15; TG n = 12. Student *t* test for independent samples *** $p<0.001$.

4.1.2. Epigallocatechin-3-gallate (EGCG), a DYRK1A kinase inhibitor, rescues hippocampal-dependent learning and memory deficits in adult TgDyrk1A mice

Epigallocatechin-3-gallate (EGCG), a major polyphenolic constituent of green tea is a potent and selective inhibitor of DYRK1A kinase activity (Bain, McLauchlan et al. 2003; Adayev, Chen-Hwang et al. 2006). It is thus a good tool to demonstrate the specific involvement of DYRK1A in hippocampal-dependent learning and memory, and could serve as a promising therapeutic tool.

We evaluated whether one month treatment with a green tea extract containing 45% EGCG could rescue the hippocampal-dependent cognitive impairments in adult TgDyrk1A mice to demonstrate a DYRK1A kinase-dependence.

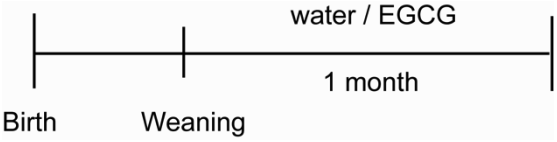

Figure 5. Experimental schedule. Representation of the experimental schedule used to analyze the effect of one month of treatment of EGCG in mice.

4.1.2.1. EGCG normalizes DYRK1A kinase activity *in vivo* in the hippocampus of TgDyrk1A mice

First, we examined whether EGCG was able to inhibit DYRK1A kinase activity in the hippocampus. DYRK1A kinase activity was measured from hippocampal extracts analyzing the incorporation of radioactive phosphate in a specific DYRK1A peptide. In basal, non-treated conditions, relative DYRK1A kinase activity was 1.8 fold higher in TgDyrk1A hippocampus compared to wild type ones (Figure 6, non treated TG *vs.* non treated WT two-way ANOVA genotype-treatment interaction $F(1,32) = 5.41$ $p<0.05$; Bonferroni as *post-hoc* $p<0.01$). One month of EGCG treatment normalized the levels of DYRK1A kinase activity in TgDyrk1A hippocampus (Figure 6, EGCG treated TG *vs.* non treated TG two-way ANOVA genotype-treatment interaction $F(1,32) = 5.41$ $p<0.05$; Bonferroni as *post-hoc* $p<0.01$) without affecting wild type mice.

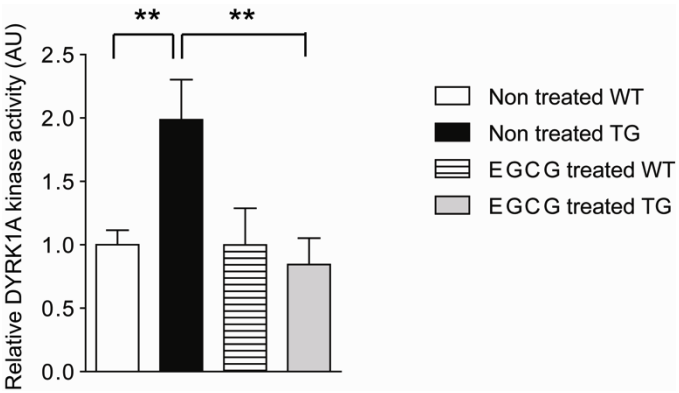

Figure 6. Relative DYRK1A kinase activity upon one month of EGCG. (A) Relative DYRK1A kinase activity in the hippocampus. Non-treated WT n = 8; non-treated TG n = 8; EGCG WT n= 8; EGCG TG n= 8. Data are represented as mean ± SEM. Two-way ANOVA with Bonferroni as *post-hoc* ** $p<0.01$.

4.1.2.2. EGCG increases locomotion and rescues hippocampal-dependent learning and memory impairments in TgDyrk1A mice

4.1.2.2.1. Effects of EGCG on locomotor activity: Open field

To characterize the effects of DYRK1A inhibition on behavior, we first evaluated if one month of treatment with EGCG affected locomotor activity. We used a non-aversive open field that mice were allowed to explore during 5 minutes. EGCG significantly increased the distance traveled (Figure 7A, two-way ANOVA genotype-treatment interaction $F(1,70) = 0.82$, $p=0.36$; two-way ANOVA treatment effect $F(1,70) = 38.14$, $p<0.001$) and the speed (Figure 7B, two-way ANOVA genotype-treatment interaction $F(1,70) = 0.45$, $p=0.50$; two-way ANOVA treatment effect $F(1,70) = 30.06$, $p<0.001$) in both genotypes, suggesting that EGCG treatment led to a general activation in both genotypes.

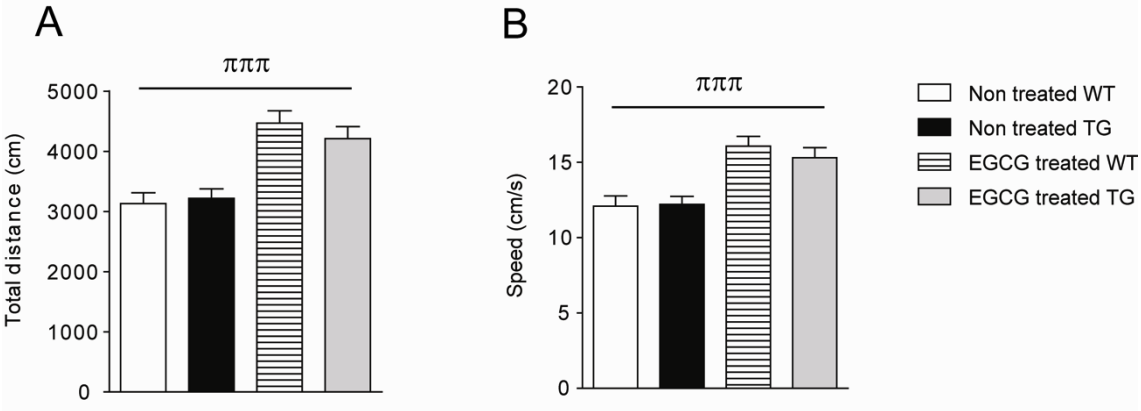

Figure 7. Distance traveled and speed in the open field using a DYRK1A kinase activity inhibitor. (A) Total distance traveled in wild type (WT) and TgDyrk1A (TG) mice reared without treatment or

under EGCG during one month. (B) Mean speed in the open field. Data are expressed as ± SEM. Non-treated WT n = 20; non-treated TG n = 19; EGCG treated WT n = 18; EGCG treated TG n = 19. Two-way ANOVA treatment effect πππ □p<0.001.

We also evaluated whether EGCG altered the ratio between the time spent in the center respect to the periphery as a measure of anxiety in this test. EGCG significantly increased the percentage of time in center in wild type mice (Figure 8A, EGCG treated WT *vs*. non treated WT two-way ANOVA genotype-treatment interaction F(1,70) = 5.70, p<0.05; Bonferroni as *post-hoc* p<0.05) without modifying the levels in TgDyrk1A mice, suggesting that EGCG reduced the anxiety-like behavior in wild type mice. Moreover, EGCG significantly reduced the latency to the periphery in TgDyrk1A mice (Figure 8B, EGCG treated TG *vs.* non treated TG two-way ANOVA genotype-treatment interaction F(1,70) = 4.94, p<0.05; Bonferroni as *post-hoc* p<0.01) without affecting wild type mice.

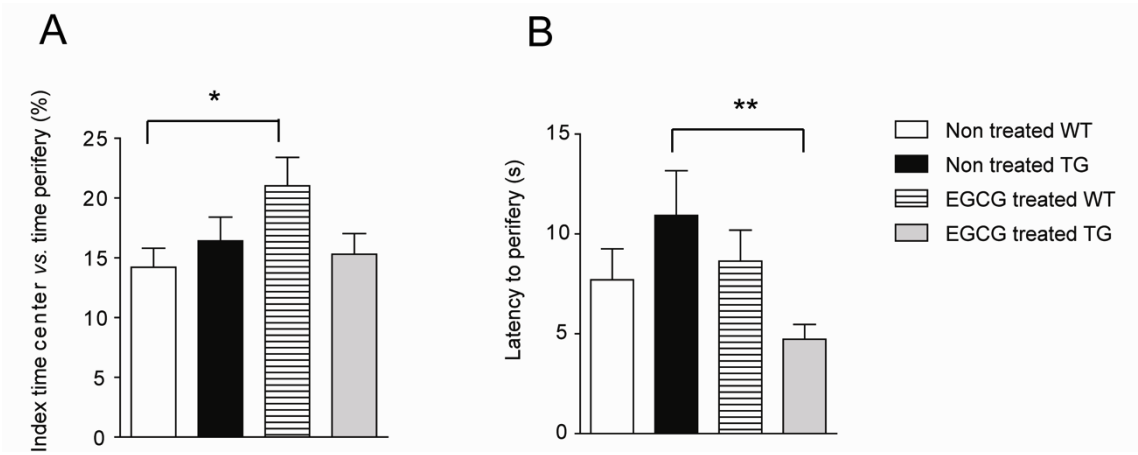

Figure 8. Index center periphery and latency to periphery in the open field using a DYRK1A kinase activity inhibitor. (A) Percentage of time in the center *vs.* the time spent in the periphery of the apparatus in wild type (WT) and TgDyrk1A (TG) mice reared without treatment or under EGCG during one month. (B) Latency to the periphery in the open field. Data are expressed as ± SEM. Non-treated WT n = 20; non-treated TG n = 19; EGCG treated WT n = 18; EGCG treated TG n = 19. Two-way ANOVA Bonferroni as *post-hoc* * p<0.05; ** p<0.01.

4.1.2.2.2. Effects of EGCG on anxiety-related behavior: Elevated Plus Maze

EGCG significantly increased the total distance traveled in the apparatus in both genotypes (Figure 9A, two-way ANOVA genotype-treatment interaction F(1,47) = 0.63, p=0.43; two-way ANOVA treatment effect F(1,47) = 5.28, p<0.05), confirming that EGCG increased the locomotor activity. However, no differences were detected in the percentage of time spent in the closed arms or in the center of the maze between groups

(Figure 9B). Instead, TgDyrk1A mice spent less time in the open arms independently of the treatment conditions compared to wild types (Figure 9B, two-way ANOVA genotype-treatment interaction F(1,47) = 0.40, p=0.52; two-way ANOVA genotype effect F(1,47) = 3.78, p<0.05), indicating that TgDyrk1A mice show increased anxiety-like behavior, and EGCG does not modify anxiety levels in this behavioral test.

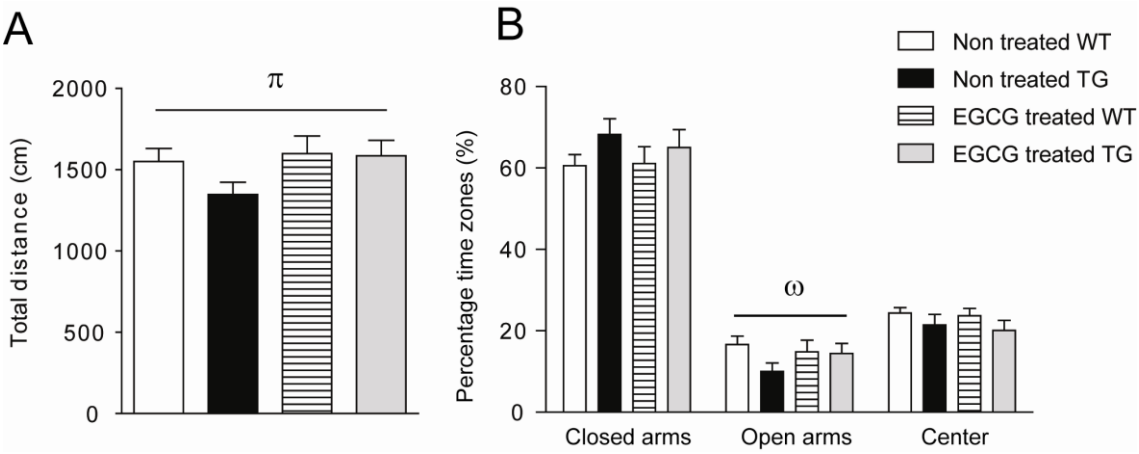

Figure 9. Effect of EGCG on the Elevated Plus Maze test. (A) Total distance travelled in wild type (WT) and TgDyrk1A (TG) mice reared without treatment or under EGCG during one month. (B) Percentage of time that mice spend in the closed and open arms, as well as in the center of the apparatus. Data are expressed as ± SEM. Non-treated WT n = 14; non-treated TG n = 13; EGCG treated WT n = 7; EGCG treated TG n = 13. Two-way ANOVA genotype effect ω p<0.05; treatment effect π p<0.05.

4.1.2.2.3. Effects of EGCG on visuo-spatial learning and memory: Morris Water Maze (MWM)

In the MWM, no differences were observed between treated and untreated groups in the pre-training nor in the cued session, indicating that EGCG did not affect procedural learning nor motivation. In contrast, during the acquisition sessions, one month of EGCG treatment rescued hippocampal-dependent learning deficits in TgDyrk1A mice (Figure 10A, two-way ANOVA repeated measures genotype-treatment interaction F (1,55) = 2.45, p = 0.125; two-way ANOVA repeated measures treatment effect F(1,55) = 6.80, p<0.05) without modifying the performance of wild type mice (Figure 10A). During learning sessions EGCG significantly increased swimming speed in TgDyrk1A mice (Figure 10C, EGCG treated TG *vs.* non treated TG two-way ANOVA repeated measures genotype-treatment interaction F (1,55) = 6.16, p <0.01; Bonferroni as *post-hoc* p<0.001), confirming the EGCG hyperactivity detected in the open field and EPM. However, EGCG also improved the strategy to find the hidden platform in TgDyrk1A

mice (Figure 10B), indicating that the increased locomotor activity was not the cause of improvement of visuo-spatial learning induced by EGCG.

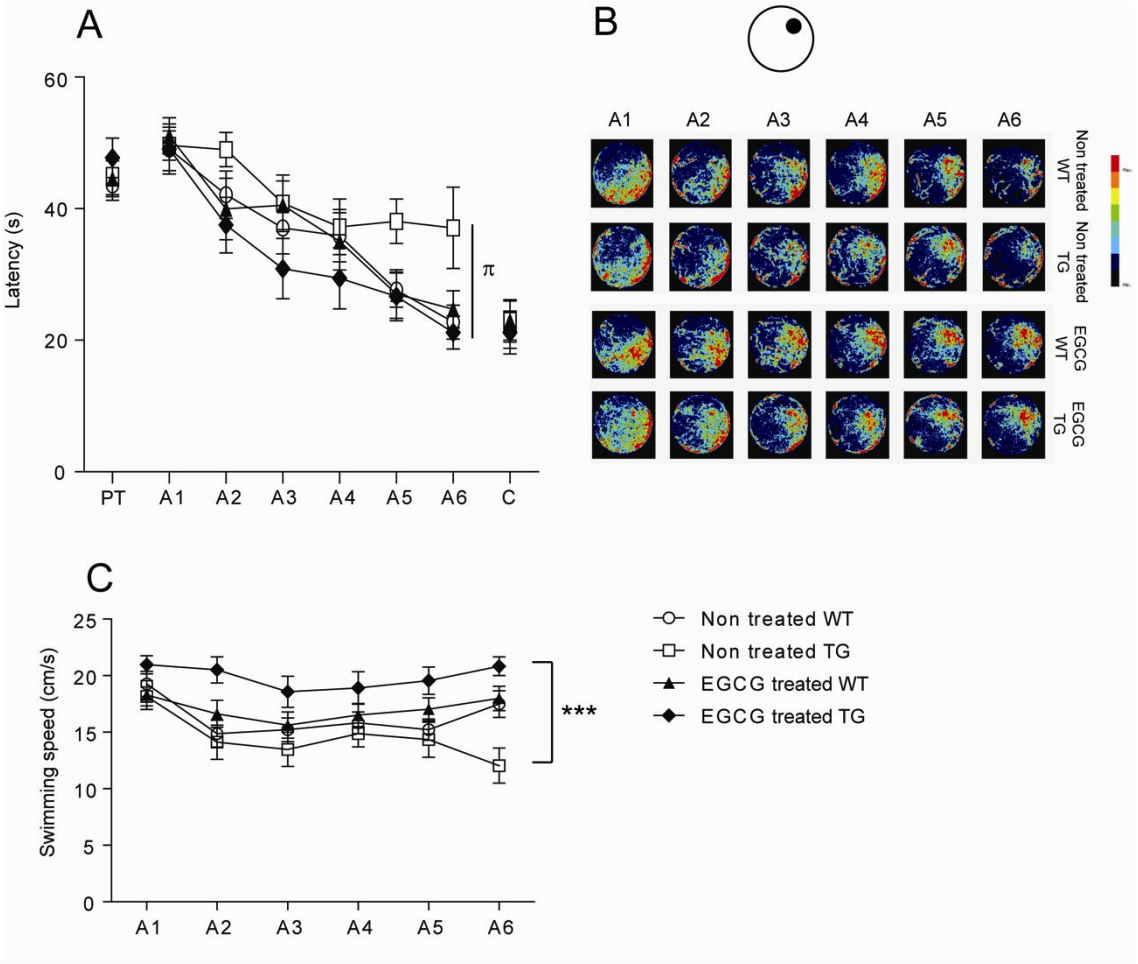

Figure 10. Visuo-spatial learning performance using a DYRK1A kinase inhibitor. (A) Latency to reach the escape platform during the pre-training session, along the learning sessions (A1-A6) and cued session in wild type (WT) and transgenic (TG) mice with non treatment or EGCG treatment. (B) Color-coded histograms representing the spatial distribution of activity of wild type and transgenic mice in the maze. Color scale is given on the right of the histogram, where red color corresponds to the most visited zones and black color to the less or non-visited zones (J-tracks plots) during learning sessions (A1-A6). (C) Swimming speed during the acquisition sessions. Non-treated WT = 12; non-treated TG n = 16; EGCG treated WT n = 16; EGCG treated TG n= 12. Data were represented as mean ± SEM. Two-way ANOVA repeated measures treatment effect π $p<0.05$. Bonferroni as *post-hoc* *** $p<0.001$. PT: Pre-training. A: Acquisition. C: Cued.

In the probe trial all groups spent a significantly higher percentage of time in the trained zone (Figure 11A). Interestingly, one month of EGCG treatment increased the preference for the target zone in TgDyrk1A (Figures 11A and 11B, EGCG treated TG

paired *t* test, *t* (1,14) = 3.68, p<0.01) but not in wild type mice (Figures 11A and 11B, EGCG treated WT paired *t* test, *t* (1,16) = 3.85, p<0.01).

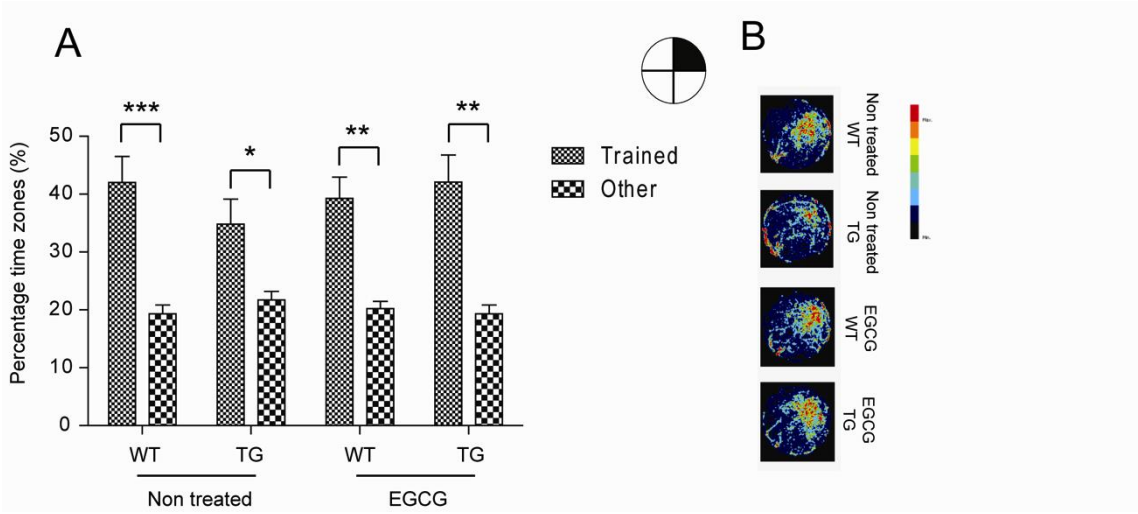

Figure 11. Reference memory using a DYRK1A kinase inhibitor. (A) Percentage of time spent in the trained compared to the non-trained quadrants during the probe test in non treated and EGCG conditions (B) Color-coded histograms representing the spatial distribution of activity of wild type and transgenic mice during probe session. Non-treated WT = 12; non-treated TG n = 16; EGCG treated WT n = 16; EGCG treated TG n= 12. Data were represented as mean ± SEM. Student *t* test for paired samples * p<0.05; ** p<0.01; *** p<0.001.

In the reversal learning sessions EGCG significantly increased the latency to reach the platform in wild type mice (Figure 12A, EGCG treated WT *vs.* non treated WT two-way ANOVA genotype-treatment interaction F(1,59) = 4.28, p<0.05; Bonferroni as *post-hoc* p<0.05) while not improving the performance of TgDyrk1A mice. Interestingly, in the last reversal session (RV3) EGCG significantly increased the percentage of time TgDyrk1A mice spent in the new platform position (Figures 12B and 12C, EGCG treated TG paired *t* test, *t* (1,14) = 2.51, p<0.05) reaching non treated wild types levels. However, in the same session, EGCG-treated wild types did not show preference for the new platform position (Figure 12B and 12C, EGCG treated WT paired *t* test, *t* (1,16) = 0.60, p=0.553), thus indicating impaired cognitive flexibility.

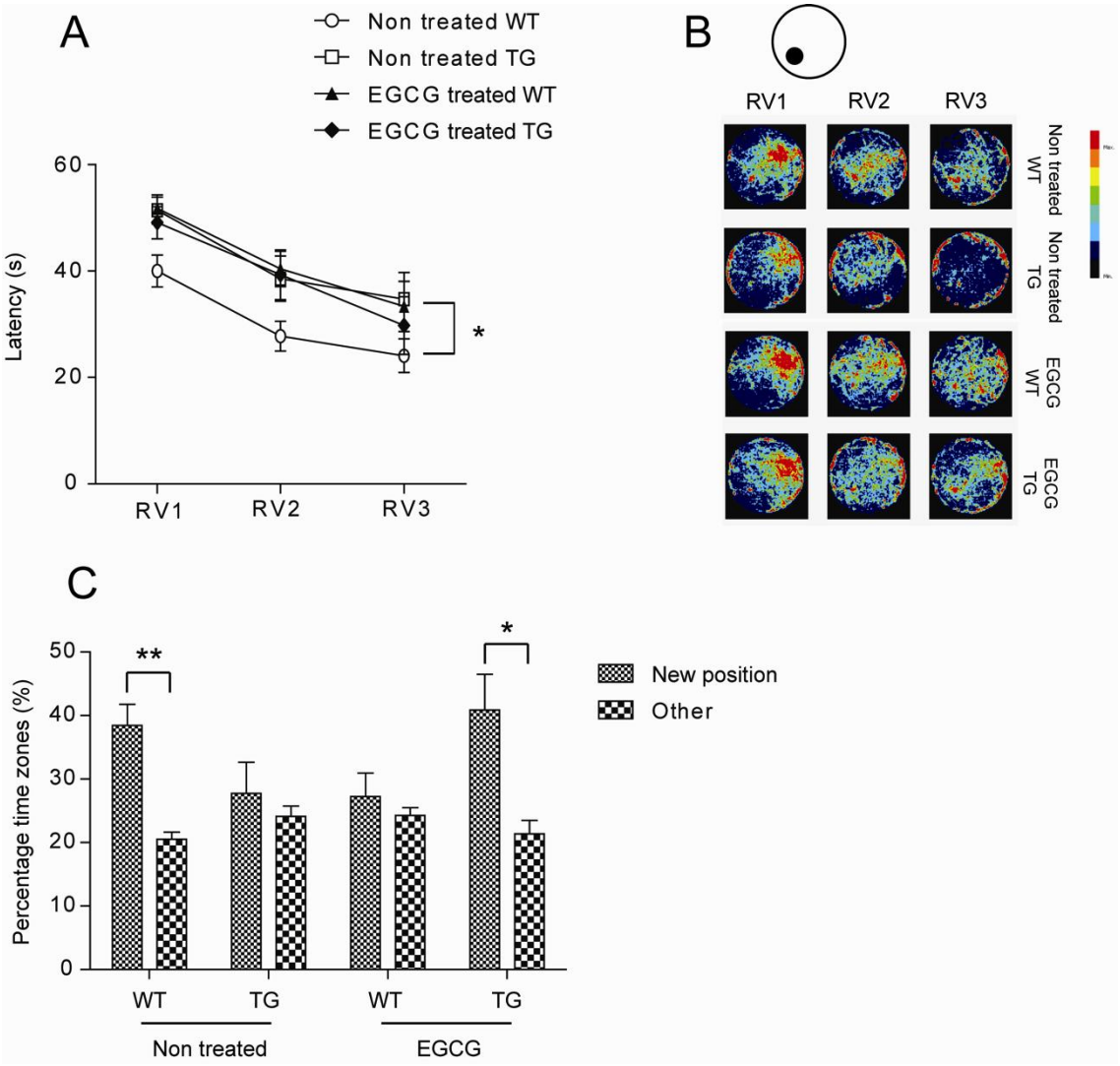

Figure 12. Cognitive flexibility using a DYRK1A kinase inhibitor. (A) Latency to reach the escape platform in the new position along the reversal sessions (RV1-3) in wild type (WT) and transgenic (TG) mice without treatment or EGCG treatment. (B) Color-coded histograms representing the spatial distribution of activity of wild type and transgenic mice during reversal session. (C) Percentage of time spent in the new quadrant compared to the other quadrants during the last reversal session (RV3). Non-treated WT = 12; non-treated TG n = 16; EGCG treated WT n = 16; EGCG treated TG n= 12. Data were represented as mean ± SEM. Two-way ANOVA repeated measures Bonferroni as *post-hoc* * $p<0.05$. RV: Reversal. Student *t* test for paired samples * $p<0.05$; ** $p<0.01$.

4.1.2.2.4. Effects of EGCG on short-term recognition memory: Novel Object Recognition (NOR)

We next performed the NOR to analyze if one month of EGCG was also able to rescue hippocampal-dependent object recognition impairments. During the familiarization phase, we observed a significant increase in the total time of exploration in EGCG

treated wild type but not TgDyrk1A mice (Figure 13A, EGCG treated WT *vs.* non treated WT two-way ANOVA genotype-treatment interaction $F(1,61) = 5.62$, $p<0.05$; Bonferroni as *post-hoc* $p<0.01$). During the test phase, TgDyrk1A mice showed a significantly reduced exploration time independently of the treatment received (Figure 13B, two-way ANOVA genotype-treatment interaction $F(1,58) = 1.02$ $p=0.31$; two-way ANOVA genotype effect $F(1,58) = 5.25$ $p<0.05$). Importantly, when we analyzed the discrimination index we found that one month of EGCG treatment improved significantly object discrimination in TgDyrk1A mice (Figure 13C, EGCG treated TG *vs*. non treated TG two-way ANOVA genotype-treatment interaction $F(1,58) = 14.98$ $p<0.05$; Bonferroni as *post-hoc* $p<0.01$), reaching the levels of non-treated wild type mice. Conversely, EGCG treated wild types presented a significant impairment in object discrimination compared to non-treated mice (Figure 13C, EGCG treated WT *vs.* non treated WT two-way ANOVA genotype-treatment interaction $F(1,58) = 14.98$ $p<0.05$; Bonferroni as *post-hoc* $p<0.01$).

Taken together, these results suggested that Dyrk1A could play an important role in the functioning of the cortico-hippocampal connectivity involved in cognitive tasks. Thus, changes in DYRK1A kinase activity dosage affect the performance in visuo-spatial learning and memory and object discrimination.

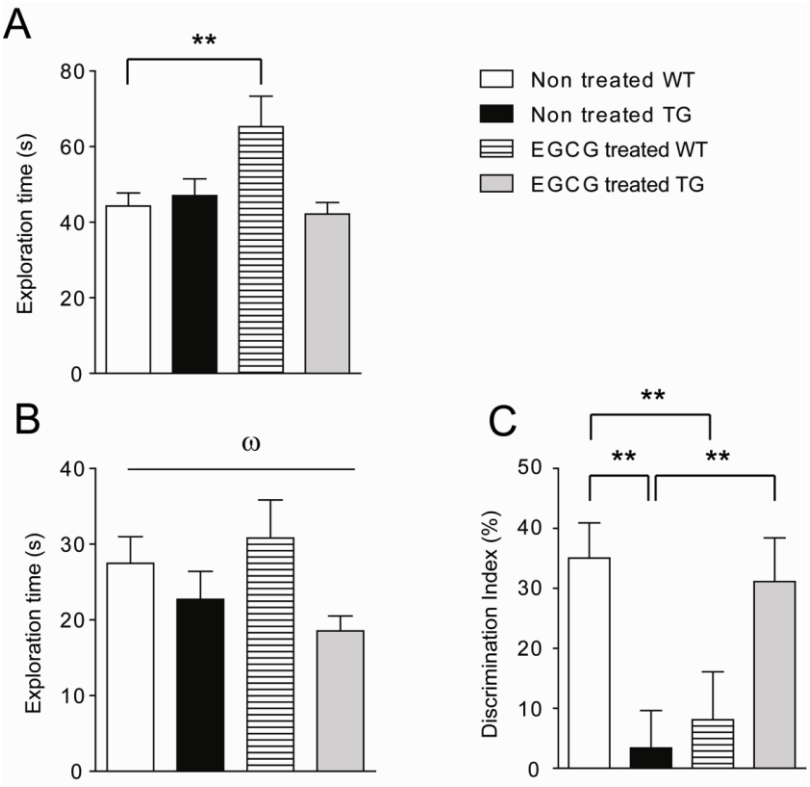

Figure 13. Novel Object Recognition using a DYRK1A kinase inhibitor. (A) Average time of exploration during the familiarization session in wild type (WT) and TgDyrk1A (TG) mice reared without treatment or EGCG during one month. (B) Average time of exploration during the test session. (C) Discrimination index during the test session in the NOR. Data are expressed as ± SEM. Non-treated WT n = 15; non-treated TG n = 12; EGCG treated WT n = 15; EGCG treated TG n = 16. Two-way ANOVA genotype effect ω $p<0.05$; Bonferroni as *post-hoc* ** $p<0.01$.

4.1.3. Environmental enrichment rescues hippocampal-dependent learning and memory impairments in TgDyrk1A mice

In order to evaluate whether complex environments promoting neuroplasticity could also rescue hippocampal-dependent learning and memory deficits in TgDyrk1A mice, we analyzed the effect of one month of environmental enrichment (EE) on different behavioral paradigms in wild type and TgDyrk1A mice reared under either non-enriched (NE) or enriched (EE) conditions for 30 days.

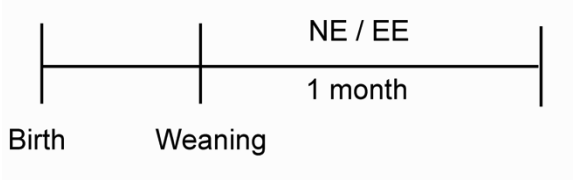

Figure 14. Experimental schedule. Representation of the experimental schedule.

4.1.3.1. Effects of EE on locomotor activity: Open Field

EE did not modify the total distance traveled (Figure 15A) or the speed (Figure 15B) in any experimental group, indicating that EE rearing conditions did not alter the locomotor activity.

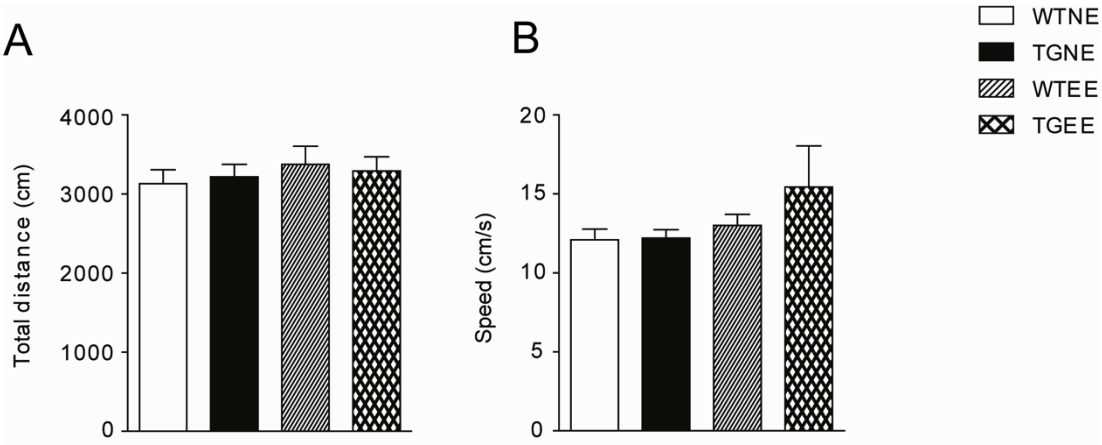

Figure 15. Distance traveled and speed in the open field in TgDyrk1A mice under NE or EE conditions. (A)Total distance traveled in wild type (WT) and TgDyrk1A (TG) mice under non enriched (NE) or environmental enriched (EE) conditions. (B) Mean speed in the open field. Data are expressed as ± SEM. WTNE n = 20; TGNE n = 19; WTEE n = 22; TGEE n = 23.

Conversely, the percentage of time spend in the center of the arena was significantly reduced in TgDyrk1A mice under EE conditions (Figure 16A, TGEE *vs.*TGNE two-way ANOVA genotype-treatment interaction $F(1,70) = 6.72$, $p<0.05$; Bonferroni as *post-hoc* $p<0.05$) indicating that TgDyrk1A mice reared under EE conditions have more anxiety-like behavior. EE also significantly reduced the time to the periphery in both genotypes (Figure 16B, two-way ANOVA genotype-treatment interaction $F(1,70) = 1.49$, $p=0.22$; two-way ANOVA treatment effect $F(1,70) = 20.22$, $p<0.001$).

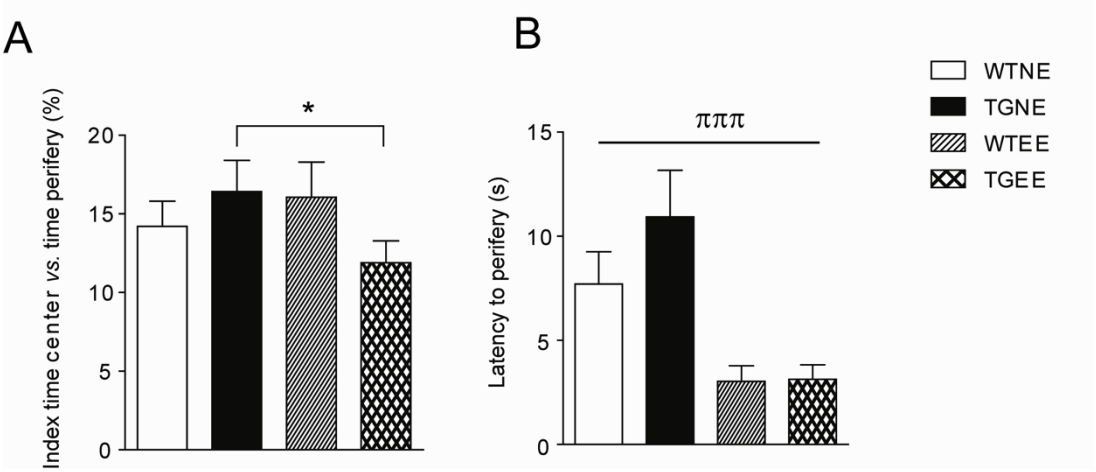

Figure 16. Index center periphery and latency to periphery in the open field in TgDyrk1A mice under NE or EE conditions. (A) Percentage of time in the center vs. the time spend in the periphery of the apparatus in wild type (WT) and TgDyrk1A (TG) mice under non enriched (NE) or environmental enriched (EE) conditions. (B) Latency to the periphery in the open field. Data are expressed as ± SEM. WTNE n = 20; TGNE n = 19; WTEE n = 22; TGEE n = 23. Two-way ANOVA treatment effect πππ p<0.001; Bonferroni as *post-hoc* * p<0.05.

4.1.3.2. Effects of EE on anxiety-like behavior: Elevated Plus Maze

A significant reduction in the total distance travelled was again observed in TgDyrk1A mice in NE conditions that was not modified by EE (Figure 17A, two-way ANOVA genotype-treatment interaction F(1,58) = 0.86, p=0.35; two-way ANOVA treatment effect F(1,58) = 3.78, p<0.05). We did not found significant EE effects in the percentage of time spent in the closed arms between groups. Also, enrichment did not rescue the anxiety-like behavior in TgDyrk1A, as shown by the reduced percentage of time spent in open arms as compared to wild types (Figure 17B, two-way ANOVA genotype-treatment interaction F(1,58) = 2.75, p=0.1; two-way ANOVA genotype effect F(1,58) = 3.60, p=0.06). One month of EE significantly reduced the percentage of time spend in the center of the apparatus (Figure 17B, two-way ANOVA genotype-treatment interaction F(1,58) = 0.67, p=0.41; two-way ANOVA treatment effect F(1,58) = 6.82, p<0.05).

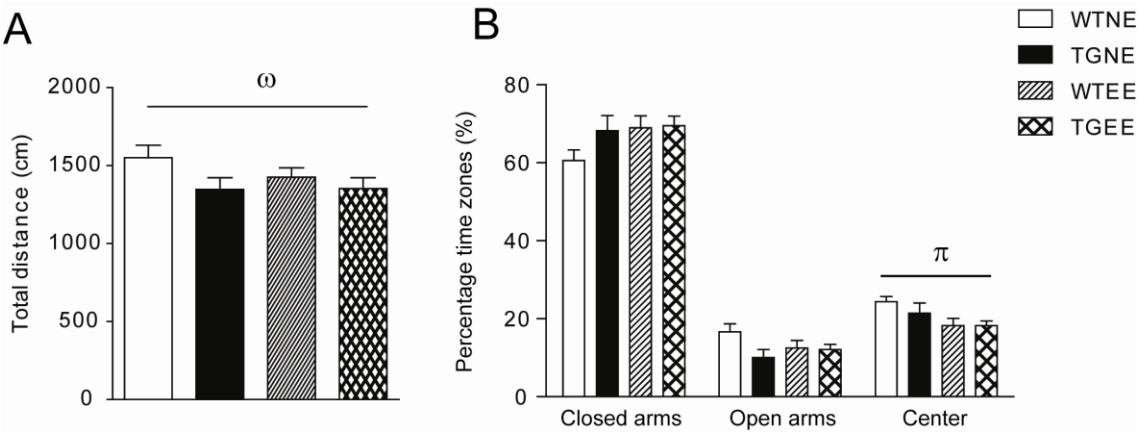

Figure 17. Effect of EE on the Elevated Plus Maze test in TgDyrk1A mice. (A) Total distance travelled in wild type (WT) and TgDyrk1A (TG) mice under non enriched (NE) or environmental enriched (EE) conditions. (B) Percentage of time that mice spend in the closed and open arms, as well as in the center of the apparatus. Data are expressed as ± SEM. WTNE n = 14; TGNE n = 13; WTEE n = 15; TGEE n = 16. Two-way ANOVA genotype effect ω p<0.05; treatment effect π p<0.05.

4.1.3.3. Effects of EE on visuo-spatial learning and memory: Morris Water Maze (MWM)

We investigated whether EE was able to improve the performance of hippocampal-dependent tasks, and to rescue the altered cognitive phenotype in TgDyrk1A mice. During the acquisition sessions, EE significantly improved learning as shown by the reduction of the escape latency (time to reach the hidden platform) in both genotypes (Figure 18A, two-way ANOVA repeated measures genotype-treatment interaction $F(1,55) = 0.015$, $p = 0.902$; two-way ANOVA repeated measures treatment effect $F(1,55) = 10.977$, $p < 0.01$). Since EE mice swam faster than the NE counterparts (Figure 18C, two-way ANOVA repeated measures genotype-treatment interaction $F(1,55) = 0.023$, $p=0.880$; two-way ANOVA repeated measures treatment effect $F(1,55) = 8.99$, $p<0.01$) as was previously described (Wood, Carta et al. 2010), we analyzed the learning strategy of the animals to ensure the cognitive dependence of the improved performance. EE increased the preference for the target zone in wild type and TgDyrk1A mice as shown in the depicted heat maps (Figure 18B). Importantly, enriched TgDyrk1A mice showed a more focused swimming pattern form the third acquisition session similar to wild type mice, suggesting that EE was able to rescue visuo-spatial learning deficits in adult TgDyrk1A mice (Figure 18B). Interestingly, in the cued session, EE reduced the latency to reach the platform in both genotypes (Figure 18A, two-way ANOVA genotype-treatment interaction $F(1,55) = 0.05$, $p=0.817$; two-way ANOVA treatment effect $F(1,55) = 15.89$, $p<0.01$).

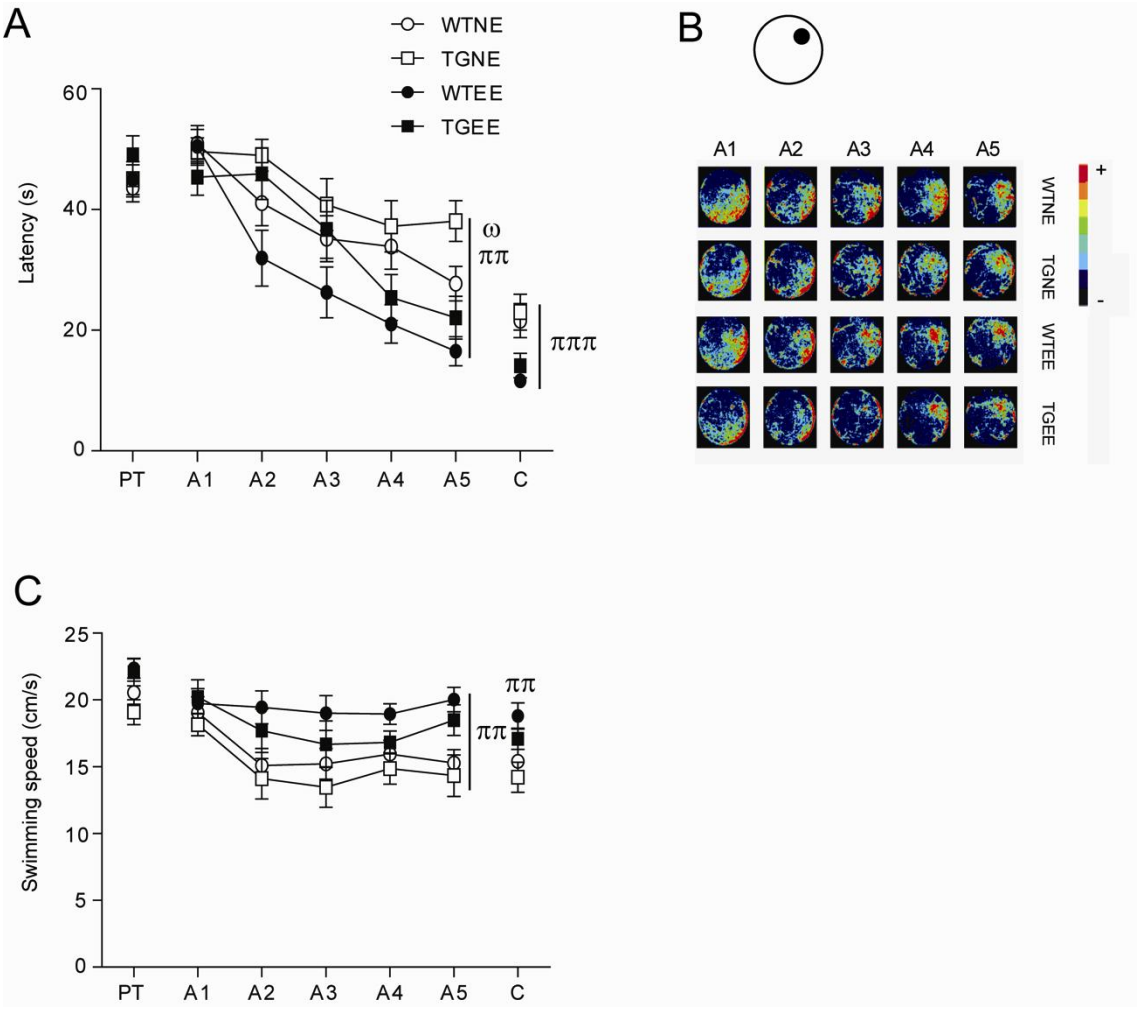

Figure 18. Visuo-spatial learning in TgDyrk1A mice under normal and enriched rearing conditions. (A) Latency to reach the escape platform along the pre-training, learning sessions (A1-A5) and cued session in wild type (WT) and transgenic (TG) mice reared under non-enriched (NE) or enriched (EE) conditions. (B) Color-coded histograms representing the spatial distribution of activity of wild type and transgenic mice in the maze. Color scale is given on the right of the histogram, where red color corresponds to the most visited zones and black color to the less or non-visited zones (J-tracks plots) during learning sessions (A1-A5). (C) Swimming speed during the pre-training, acquisition and cued sessions. WTNE = 12; TGNE n = 16; WTEE n = 16; TGEE n= 12. Data were represented as mean ± SEM. Two-way ANOVA repeated measures genotype effect ω $p<0.05$; treatment effect π $p<0.05$;ππ $p< 0.01$. PT: Pre-training. A: Acquisition. C: Cued.

In the probe trial, EE improved reference memory in both genotypes (Figure 19A, WTEE paired *t* test, t (1,16) = 5.55, $p<0.0001$; TGEE paired *t* test, t (1,12) = 3.37, $p<0.01$). Importantly, enriched TgDyrk1A mice were as efficient as NE wild type littermates in the probe test (Figure 19B), as shown by a more focalized searching strategy in the target quadrant as compared to NE-TgDyrk1A.

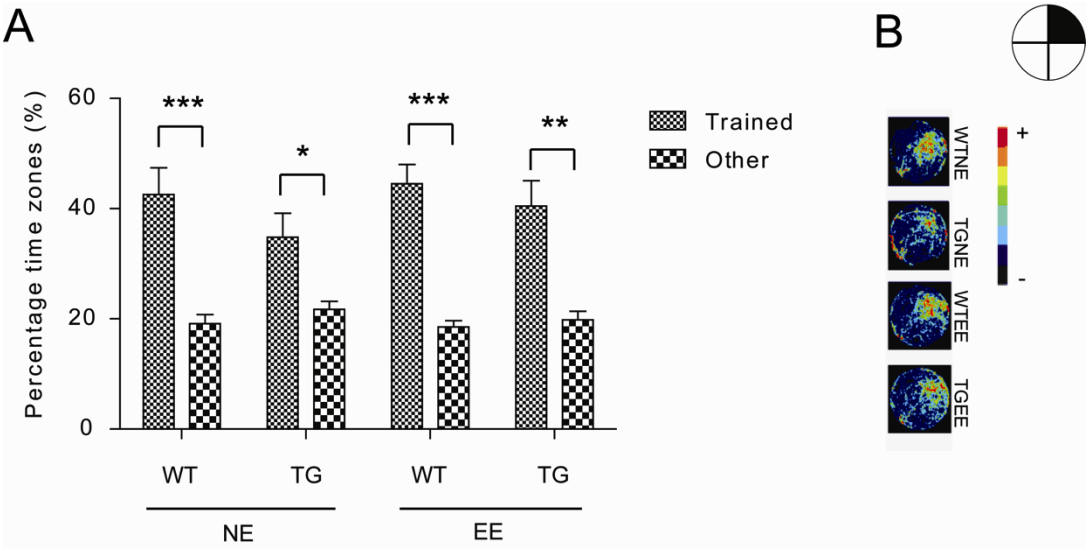

Figure 19. Reference memory in TgDyrk1A mice under normal and enriched rearing conditions. (A) Percentage of time spent in the trained compared to the non-trained quadrants during the probe test in enriched and non-enriched conditions (B) Color-coded histograms representing the spatial distribution of activity of wild type and transgenic mice during probe session. WTNE = 12; TGNE n = 16; WTEE n = 16; TGEE n= 12. Data were represented as mean ± SEM. Student *t* test for paired samples * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

In the reversal sessions, EE decreased the time to reach the platform in both genotypes (Figure 20A, two-way ANOVA repeated measures genotype-treatment interaction $F(1,57) = 1.06$, $p=0.3$; two-way ANOVA repeated measures treatment effect $F(1,57) = 6.12$, $p<0.05$) and increased the percentage of time in the new quadrant in the third reversal session (Figures 20B and 20C, WTEE paired *t* test, $t(1,16) = 5.20$, $p<0.001$; TGEE paired *t* test, $t(1,12) = 3.10$, $p<0.01$), thus achieving a complete recover of the altered cognitive flexibility in TgDyrk1A mice.

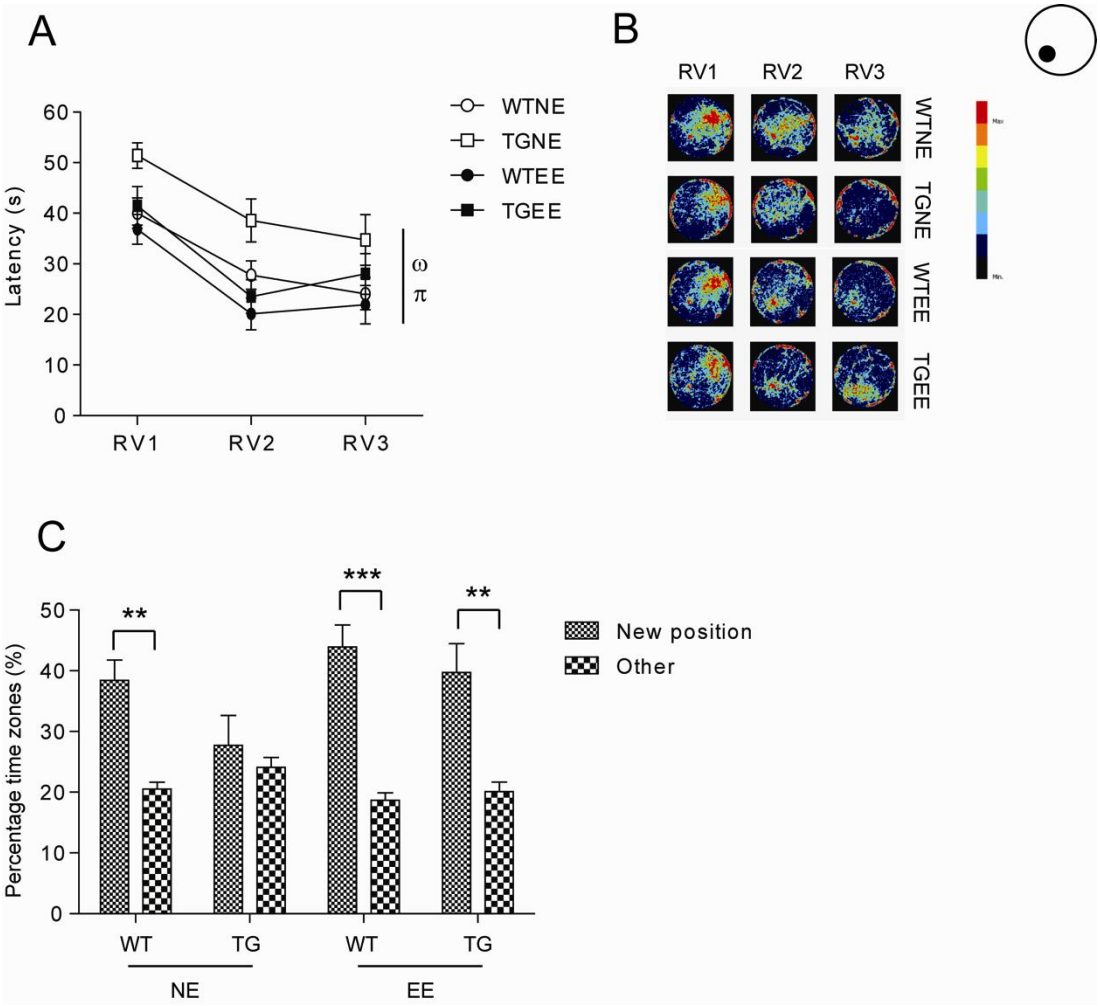

Figure 20. Cognitive flexibility in TgDyrk1A mice under normal and enriched rearing conditions. (A) Latency to reach the escape platform in the new position along the reversal sessions (RV1-3) in wild type (WT) and transgenic (TG) mice reared under non-enriched (NE) or enriched (EE) conditions. (B) Color-coded histograms representing the spatial distribution of activity of wild type and transgenic mice during reversal session. (C) Percentage of time spent in the new quadrant compared to the other quadrants during the last reversal session (RV3) in enriched and non-enriched conditions WTNE = 12; TGNE n = 16; WTEE n = 16; TGEE n= 12. Data were represented as mean ± SEM. Two-way ANOVA repeated measures genotype effect ω $p<0.05$; treatment effect π $p<0.05$. Student *t* test for paired samples ** $p<0.01$; *** $p<0.001$. RV: Reversal.

4.1.3.4. Effects of EE on short-term recognition memory: Novel Object Recognition (NOR)

EE did not modify exploration in the familiarization (Figure 21B) or the test phases (Figure 21C), in either genotype. However, EE rearing significantly rescued the short-term memory alterations observed in TgDyrk1A mice (Figure 21D, TGEE *vs.* TGNE two-way ANOVA genotype-treatment interaction $F(1,56) = 7.56$, $p<0.01$; Bonferroni as

post-hoc: p<0.001), as shown by the increased discrimination index, without affecting wild type performance.

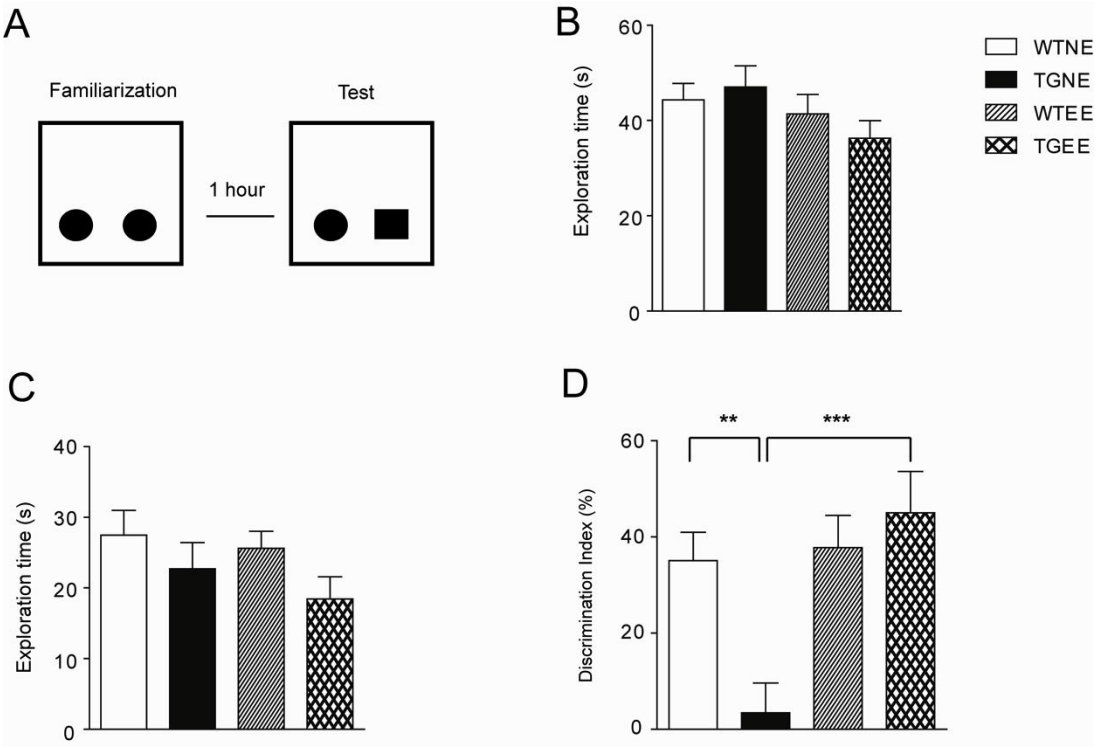

Figure 21. Effect of EE on the Novel Object Recognition test in TgDyrk1A mice. (A) Schematic representation of the NOR paradigm. (B) Average time of exploration during the familiarization session in the NOR in wild type (WT) and TgDyrk1A (TG) mice under non enriched (NE) or environmental enriched (EE) conditions. (C) Average time of exploration during the test session in the NOR. (D) Discrimination index during the test session in the NOR. Data are expressed as ± SEM. WTNE n = 15; TGNE n = 12; WTEE n = 14; TGEE n = 15. Two-way ANOVA Bonferroni as *post hoc* ** p<0.01; *** p<0.001.

4.1.4. Environmental enrichment normalizes DYRK1A kinase activity in TgDyrk1A hippocampus

It is well established that EE enhances cognition through the change in the expression and/or activity of several proteins involved in learning and memory, but no data regarding its effect on Dyrk1A have been previously published. Since we had demonstrated that normalizing DYRK1A kinase with EGCG rescued hippocampal-dependent learning impairments (see above), the rescuing effects of EE could be driven, at least in part, by changes in DYRK1A kinase activity. Thus, we measured DYRK1A kinase activity from hippocampal extracts of NE and EE animals. One month of EE was able to normalize the increased levels of DYRK1A kinase activity in TgDyrk1A

hippocampus (Figure 22, TGEE *vs.* TGNE Bonferroni as *post-hoc:* p<0.05) without affecting wild type mice.

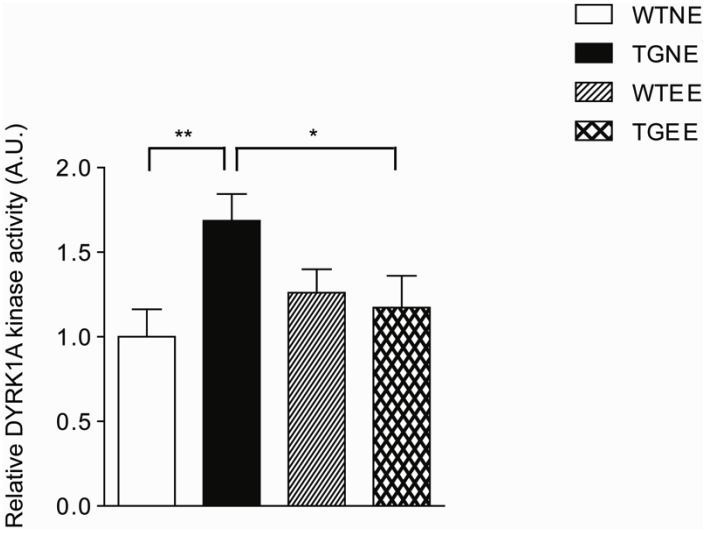

Figure 22. Effect of EE on relative DYRK1A kinase activity in TgDyrk1A mice. Relative DYRK1A kinase activity in the hippocampus in wild type (WT) and TgDyrk1A (TG) mice under non enriched (NE) or environmental enriched (EE) conditions. WTNE = 12; TGNE n = 12; WTEE n = 12; TGEE n= 12. Data are represented as mean ± SEM. Two-way ANOVA Bonferroni as *post-hoc* * p<0.05; ** p<0.01.

4.1.5. Combining environmental enrichment and EGCG enhances visuospatial learning and memory but impairs recognition memory in TgDyrk1A mice

As described above, both environmental stimuli and EGCG are able to rescue hippocampal-dependent learning and memory impairments in Dyrk1A overexpressing mice. Thus, we evaluated whether the combination of both treatments during one month might have synergistic effects.

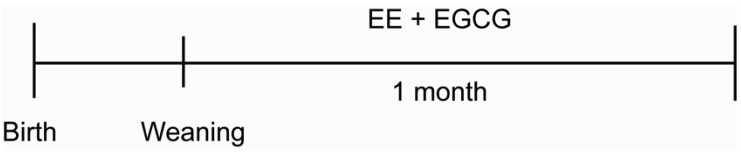

Figure 23. Experimental schedule. Representation of the experimental schedule used to analyze the effect of one month EGCG and EE treatment in mice.

4.1.5.1. Effects of combined EE and EGCG on locomotor activity: Open Field

We first evaluated whether the combination of EE and EGCG modulates locomotor activity using a non-aversive open field. Total distance was increased in mice receiving combined EE and EGCG treatments compared to non treated or EE mice (Figure 24A, two-way ANOVA genotype-EE-EGCG interaction $F(1,135) = 2.97$, $p<0.05$), suggesting that the increased locomotor activity is mainly due to EGCG. Although a similar effect was detected in both genotypes, in wild type EE-EGCG treated mice the hyperactivity was less pronounced than in those only receiving EGCG (Figure 24A, EE and EGCG treated WT vs. EGCG treated WT two-way ANOVA genotype-EE-EGCG interaction $F(1,135) = 2.97$, $p<0.05$, Bonferroni as *post-hoc* $p<0.05$). Similarly, speed was also increased in EE-EGCG treated mice but this effect was again due to EGCG (Figure 24B, two-way ANOVA genotype-EE-EGCG interaction $F(1,135) = 1.81$, $p=0.16$; two-way ANOVA EGCG effect $F(1,135) = 4.91$, $p<0.05$).

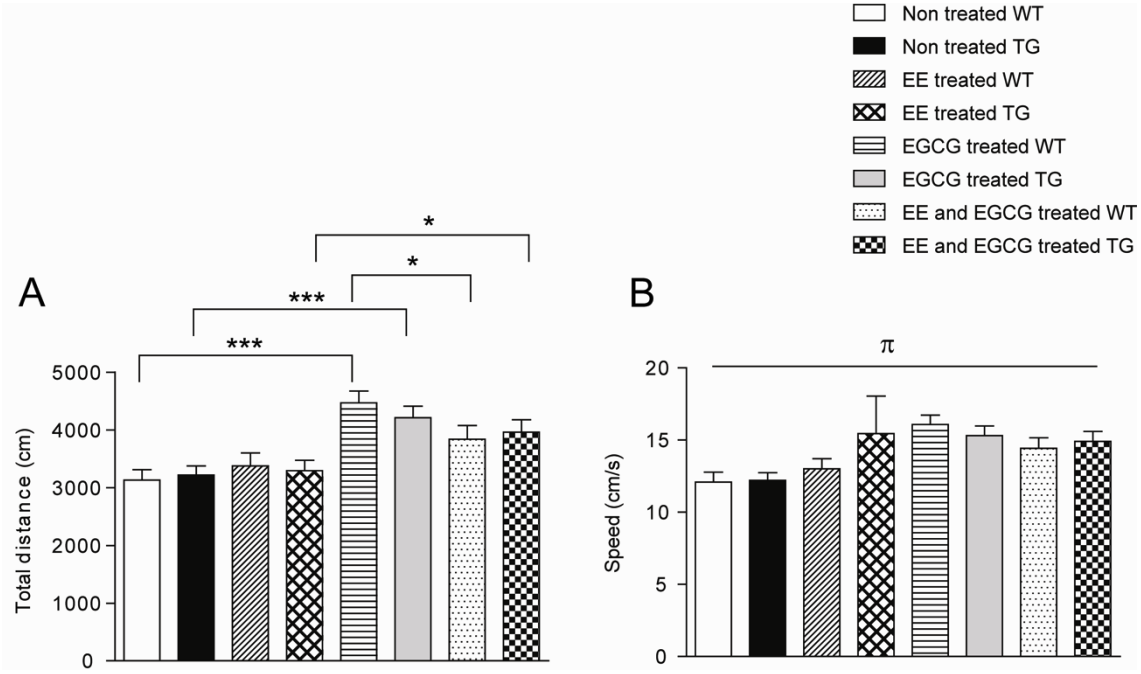

Figure 24. Distance traveled and speed in the open field using EE and EGCG. (A)Total distance traveled in wild type (WT) and TgDyrk1A (TG) mice reared without treatment, reared under EE alone, reared with EGCG only or EE and EGCG during one month. (B) Mean speed in the open field. Data are expressed as ± SEM. Non-treated WT n = 17; non-treated TG n = 16; EE treated WT n = 18; EE treated TG n = 19; EGCG treated WT n = 18; EGCG treated TG n = 19; EE and EGCG treated WT n = 17; EE and EGCG treated TG n = 13. Two-way ANOVA Bonferroni as post-hoc * $p<0.05$; *** $p<0.001$. Two-way ANOVA EGCG effect π $p<0.05$.

On the other hand, the anxiolytic effects of EGCG alone in wild types, where lost when combining EE and EGCG treatments (Figure 25A, EE and EGCG treated WT vs.

EGCG treated WT two-way ANOVA genotype-EE-EGCG interaction F (1,135) = 3.73, p<0.05; Bonferroni as *post-hoc* p<0.05). Regarding the latency to the periphery the combination of both treatments had similar effects as EE alone (Figure 25B, two-way ANOVA genotype-EE-EGCG interaction F (1,135) = 1.93, p=0.14; two-way ANOVA EE effect F (1,135) = 38.95, p<0.001); two-way ANOVA EGCG effect F (1,135) = 7.19, p<0.01).

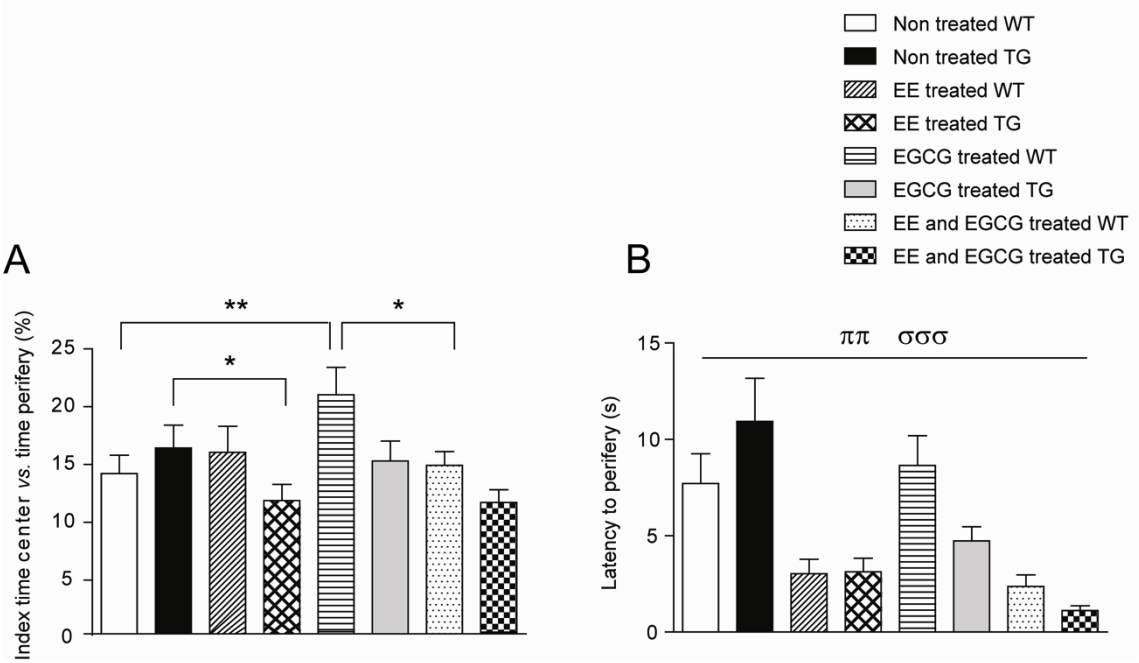

Figure 25. Index center periphery and latency to periphery in the open field using EE and EGCG. (A) Percentage of time in the center *vs.* the time spend in the periphery of the apparatus in wild type (WT) and TgDyrk1A (TG) mice reared without treatment, reared under EE alone, reared with EGCG only or EE and EGCG during one month. (B) Latency to the periphery in the open field. Data are expressed as ± SEM. Non-treated WT n = 17; non-treated TG n = 16; EE treated WT n = 18; EE treated TG n = 19; EGCG treated WT n = 18; EGCG treated TG n = 19; EE and EGCG treated WT n = 17; EE and EGCG treated TG n = 13. Two-way ANOVA Bonferroni as posthoc * p<0.05; ** p<0.01. Two-way ANOVA EGCG effect ππ p<0.01. Two-way ANOVA EE effect σσσ p<0.001.

4.1.5.2. Effects of combined EE and EGCG on anxiety: Elevated Plus Maze

Mice treated with EE-EGCG showed similar levels of locomotor activity as compared to mice treated with EGCG alone (Figure 26A, two-way ANOVA genotype-EE-EGCG interaction F(1,87) = 1.57, p=0.21; two-way ANOVA EGCG effect F(1,87) = 21.79, p<0.001), indicating that the combination of both treatments did not produce an additional increase in mice's activity. Mice receiving combined EE and EGCG showed similar percentage of time spend in the closed arms (Figure 26B, two-way ANOVA

genotype-EE-EGCG interaction F(1,87) = 2.25, p=0.11; two-way ANOVA EGCG effect F(1,87) = 16.10, p<0.001) and in the open arms (Figure 26B, two-way ANOVA genotype-EE-EGCG interaction F(1,87) = 1.00, p=0.37; two-way ANOVA EGCG effect F(1,87) = 13.17, p<0.001) as compared to mice that were only treated with EGCG, indicating that EE was counteracting the anxiolytic effects of EGCG treatment.

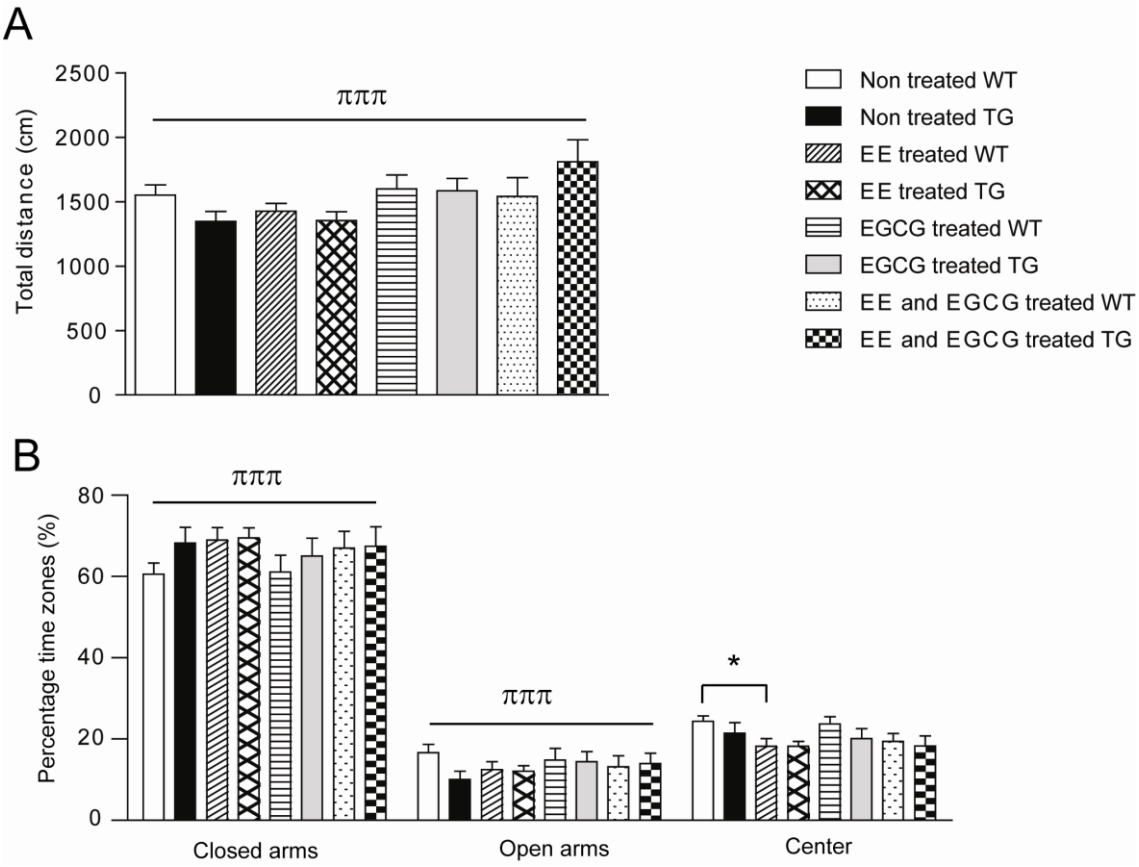

Figure 26. Effect of EE and EGCG on the Elevated Plus Maze test. (A) Total distance travelled in wild type (WT) and TgDyrk1A (TG) mice reared without treatment, reared under EE alone, reared with EGCG only or EE and EGCG during one month. (B) Percentage of time that mice spend in the closed and open arms, as well as in the center of the apparatus. Data are expressed as ± SEM. Non-treated WT n = 14; non-treated TG n = 13; EE treated WT n = 15; EE treated TG n = 16; EGCG treated WT n = 7; EGCG treated TG n = 13; EE and EGCG treated WT n = 8; EE and EGCG treated TG n = 8. Two-way ANOVA EGCG effect ππ p<0.01, πππ p<0.001. Two-way ANOVA Bonferroni as posthoc * p<0.05.

4.1.5.3. Effects of combined EE and EGCG on visuo-spatial learning and memory: Morris Water Maze (MWM)

We previously have shown that EE and EGCG given separately significantly improved the performance in TgDyrk1A mice. When analyzing the combination of EE and EGCG, wild type mice did not improve learning as compared to wild types that only

received EE (Figures 27A and 27B). This suggested that the effects of the combined treatment are due to EE since EGCG alone had no effects in wild types. In contrast, there is a trend to reduce the time to reach the platform in TgDyrk1A mice reared with EE and EGCG as compared to transgenic mice that only received EE or EGCG (Figures 27A and 27B, two-way ANOVA p=0.08). Interestingly, TgDyrk1A mice receiving both EGCG and EE where improved to EE wild type levels. These results suggested that both treatments produce some synergistic effects on hippocampal-dependent visuo-spatial learning task in TgDyrk1A. Thus, the pharmacological normalization of DYRK1A kinase activity would enhance the effects of EE in this genotype.

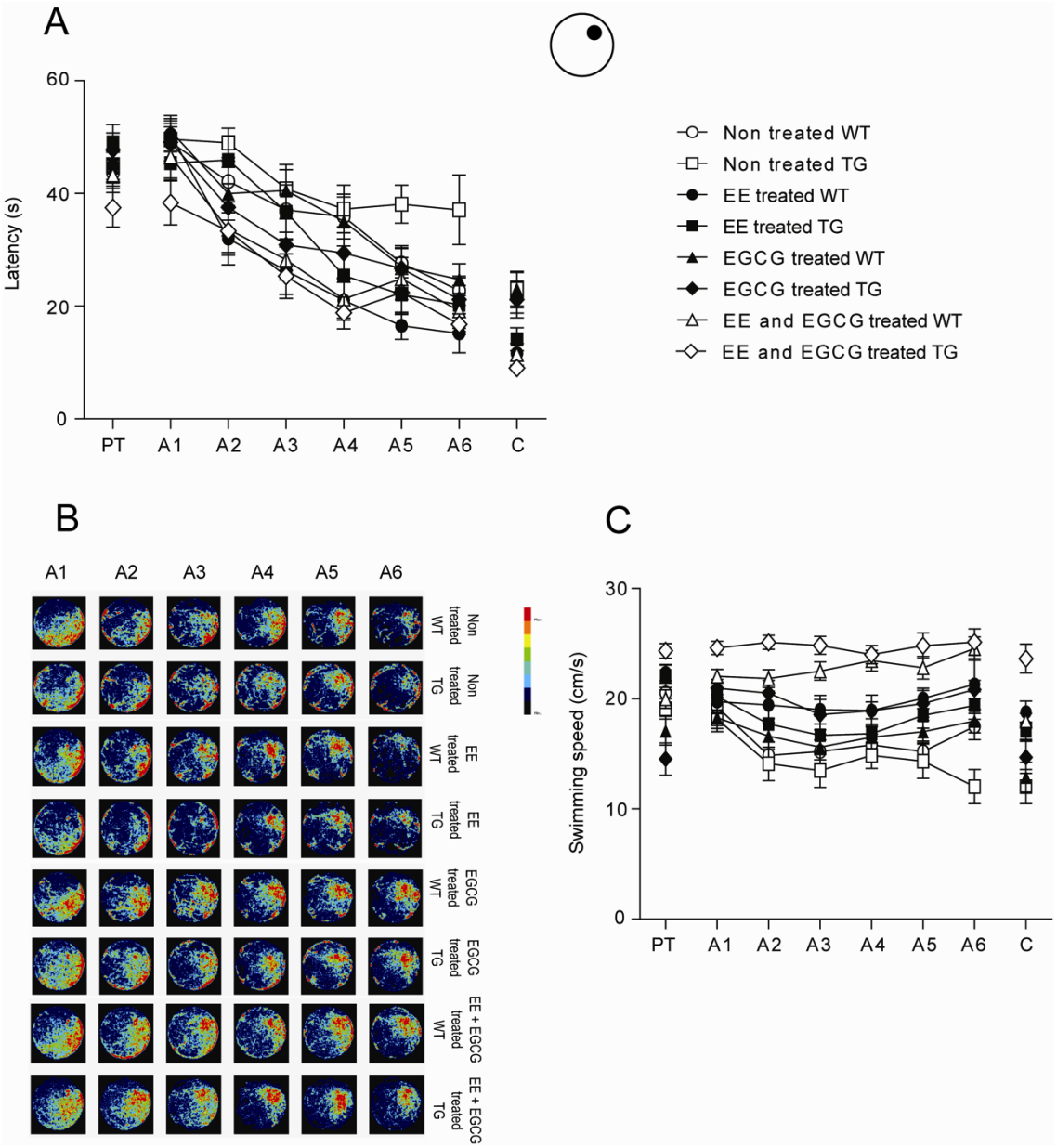

Figure 27. Visuo-spatial learning performance using EE and EGCG. (A) Latency to reach the escape platform along the learning sessions (A1-A6) in wild type (WT) and transgenic (TG) mice reared without treatment, reared under EE alone, reared with EGCG only or EE and EGCG during one month. (B) Color-coded histograms representing the spatial distribution of activity of wild type and transgenic mice in the maze. Color scale is given on the right of the histogram, where red color corresponds to the most visited zones and black color to the less or non-visited zones (J-tracks plots) during learning sessions (A1-A6). (C) Swimming speed during pre-training, acquisitions and cued sessions. Non treated WT = 12; Non treated TG n = 16; EE treated WT n = 16; EE treated TG n = 12; EGCG treated WT n = 16; EGCG treated TG n= 14; EE and EGCG treated WT n = 17; EE and EGCG treated TG n = 12. Data were represented as mean ± SEM. Two-way ANOVA repeated measures treatment effect π $p<0.05$. A: Acquisition.

In the probe test assessing reference memory we previously showed that either EE or EGCG significantly improved the reference memory impairment in TgDyrk1A mice without modifying the performance in wild type mice. When combined, EE and EGCG did not affect the performance of wild type mice as compared to single treatments (Figures 28A and 28B, EE-EGCG treated WT paired *t* test, $t\ (1,17) = 4.05$, $p<0.001$). Conversely, in TgDyrk1A mice EE and EGCG significantly increased the preference for the trained quadrant when compared to transgenic animals that received only one treatment (Figures 28A and 28B, EE-EGCG treated TG paired *t* test, $t\ (1,12) = 7.01$, $p<0.001$), reinforcing the concept that EE and EGCG produce synergic effects in visuospatial memory tasks in TgDyrk1A mice.

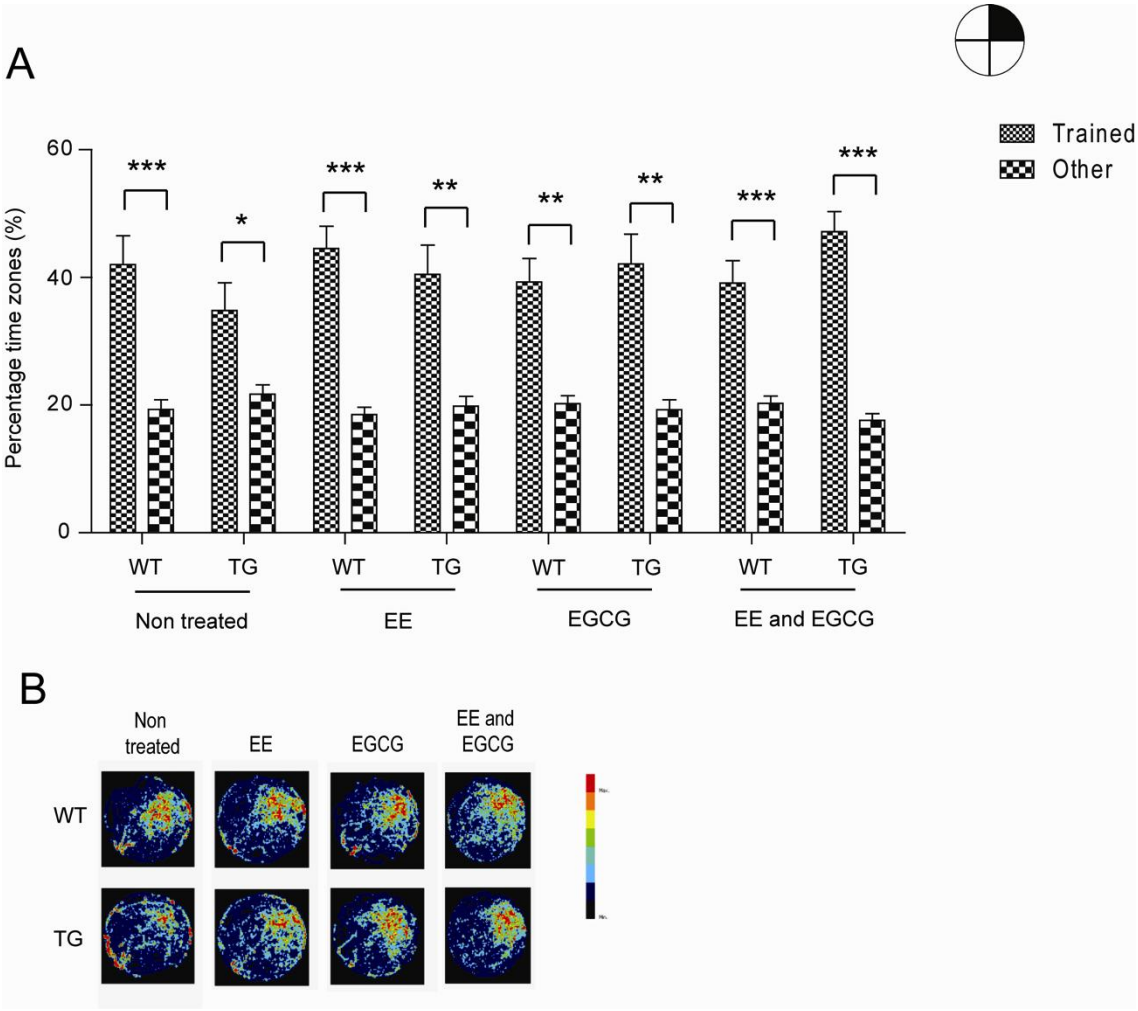

Figure 28. Reference memory using EE and EGCG. (A) Percentage of time spent in the trained compared to the non-trained quadrants during the probe test in wild type (WT) and transgenic (TG) mice reared without treatment, reared under EE alone, reared with EGCG only or EE and EGCG during one month. (B) Color-coded histograms representing the spatial distribution of activity of wild type and transgenic mice during probe session. Non treated WT = 12; Non treated TG n = 16; EE treated WT n = 16; EE treated TG n = 12; EGCG treated WT n = 16; EGCG treated TG n= 14; EE and EGCG treated WT n = 17; EE and EGCG treated TG n = 12. Data were represented as mean ± SEM. Student *t* test for paired samples * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Finally, we evaluated whether the combination of EE and EGCG could affect cognitive flexibility in the reversal sessions. EE and EGCG produced opposite effects when administered separately in wild type mice, showing improved cognitive flexibility with EE and impairment with EGCG. In the case of TgDyrk1A mice, both treatments given separately rescued the cognitive flexibility impairments, reaching the levels of untreated wild type mice.

In wild type mice, the combined treatment with EE and EGCG reduced the time to

reach the hidden platform and increased the percentage of time spend in the new position (Figures 29B and 29C, EE-EGCG treated WT paired *t* test, t (1,17) = 5.78, $p<0.001$) as compared to untreated mice, but to the same levels of wild type treated with EE alone (Figure 29A). Interestingly, TgDyrk1A mice receiving both EE and EGCG presented a significant improvement in the time to reach the platform (Figure 29A) and a marked preference for the new quadrant (Figures 29B and 29C, EE-EGCG treated TG paired *t* test, t (1,12) = 10.13, $p<0.001$), indicating a synergistic effect.

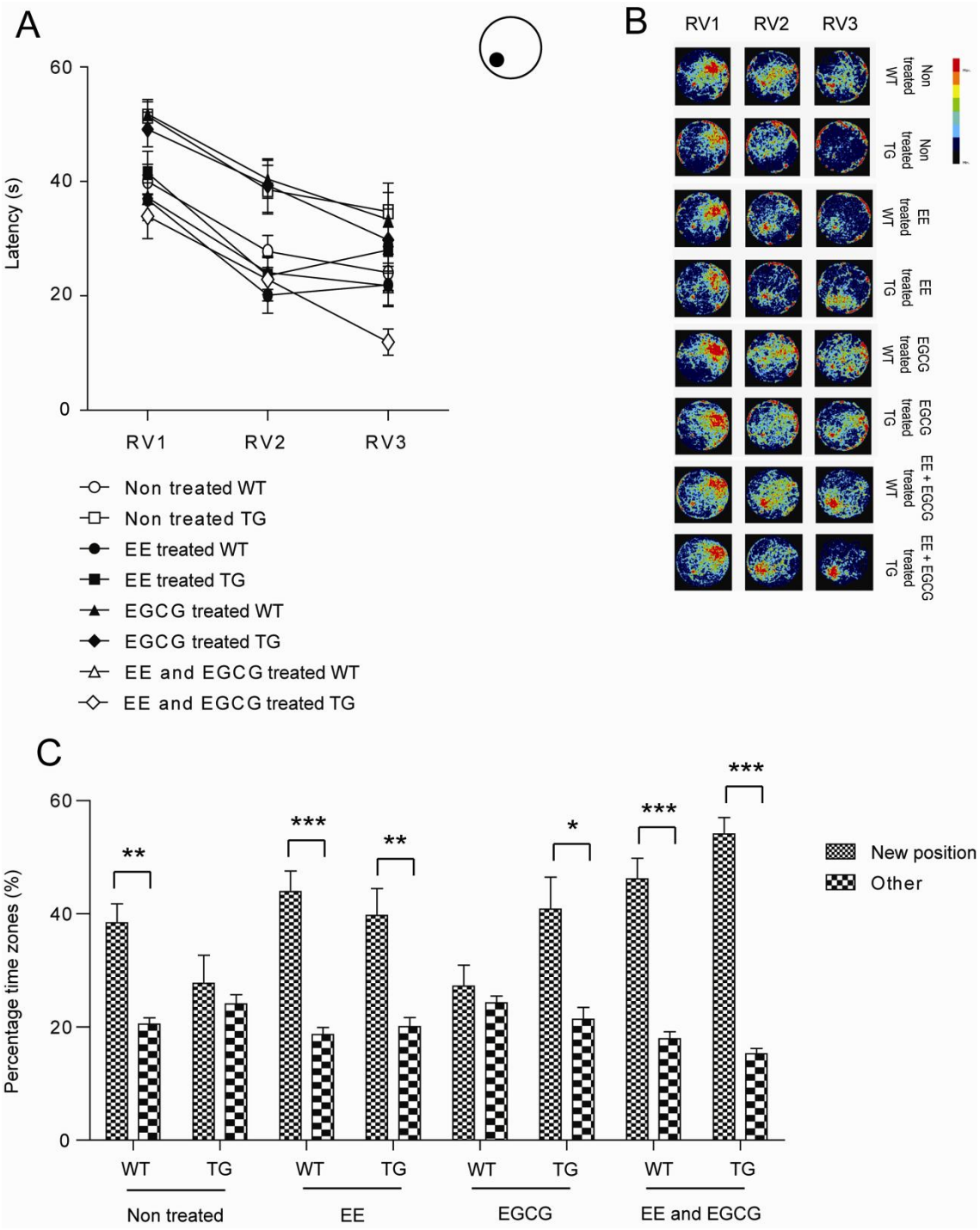

Figure 29. Cognitive flexibility using EE and EGCG. (A) Latency to reach the escape platform in the new position along the reversal sessions (RV1-3) in wild type (WT) and transgenic (TG) mice reared without treatment, reared under EE alone, reared with EGCG only or EE and EGCG during one month. (B) Color-coded histograms representing the spatial distribution of activity of wild type and transgenic mice during reversal session. (C) Percentage of time spent in the new quadrant compared to the other quadrants during the last reversal session (RV3) in enriched and non-enriched conditions. Non treated WT = 12; Non treated TG n = 16; EE treated WT n = 16; EE treated TG n = 12; EGCG treated WT n = 16; EGCG treated TG n= 14; EE and EGCG treated WT n = 17; EE and EGCG treated TG n = 12. Data

were represented as mean ± SEM. Two-way ANOVA repeated measures Bonferroni as *posthoc* *p<0.05. RV: Reversal. Student *t* test for paired samples * p<0.05; ** p<0.01.

4.1.5.4. Effects of combined EE and EGCG on short-term recognition memory: Novel Object Recognition (NOR)

We previously have shown that one-month of EE or EGCG treatments alone rescued the impaired memory in object discrimination in TgDyrk1A mice. Conversely, both treatments produced different effects in wild type mice.

We next evaluated whether one month of EE and EGCG treatments given together could produce a synergic effect in TgDyrk1A mice. The combination of EE and EGCG conditions not only did not improve but even impaired object discrimination in TgDyrk1A mice as compared to single treatments (Figure 30B, EE-EGCG treated TG vs. EE treated TG two-way ANOVA genotype-EE-EGCG interaction $F(1,115) = 9.55$, $p<0.001$; Bonferroni as *post-hoc*: $p<0.01$).

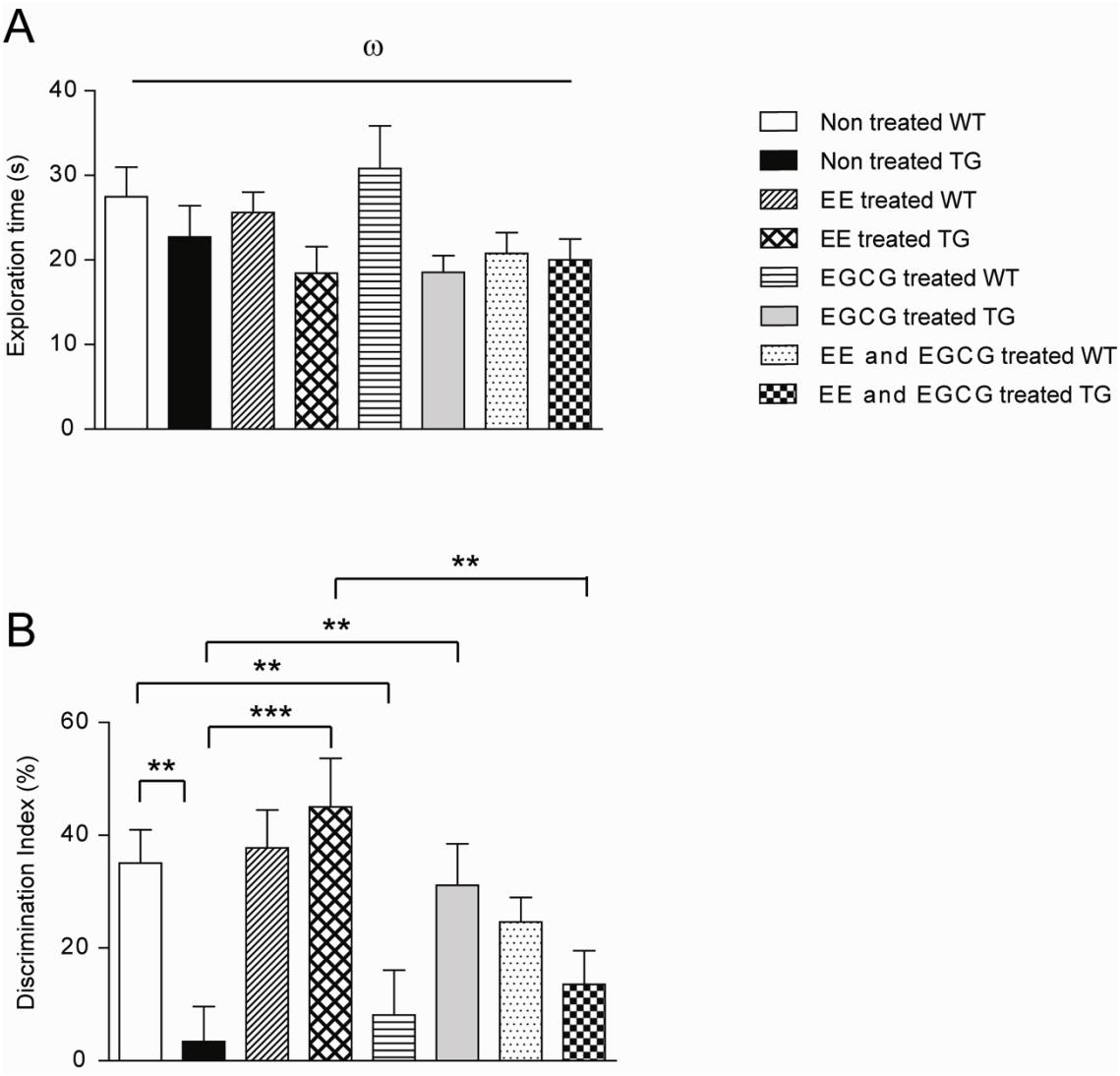

Figure 30. Novel Object Recognition using EE and EGCG. (A) Average time of exploration during the test session in wild type (WT) and TgDyrk1A (TG) mice reared without treatment, reared under EE alone, reared with EGCG only or EE and EGCG during one month. (B) Discrimination index during the test session in the NOR. Data are expressed as ± SEM. Non treated WT n = 15; Non treated TG n = 12; EE treated WT n = 14; EE treated TG n = 15; EGCG treated WT n = 15; EGCG treated TG n = 16; EE and EGCG treated WT n = 16; EE and EGCG treated TG n = 13. Two-way ANOVA genotype effect ω $p<0.05$; Bonferroni as *post-hoc* ** $p<0.01$; *** $p<0.001$.

Taken together, the results obtained in the hippocampal-dependent visuo-spatial learning task (MWM) and recognition memory (NOR) suggested that one month of EGCG treatment and EE conditions alone are able to rescue the cognitive alterations present in TgDyrk1A mice in both paradigms. However, when both treatments are applied, we observed opposite results depending on the task, having positive synergistic effects in the MWM but negative effects in the object recognition task.

4.2. IMPACT OF DYRK1A OVEREXPRESSION ON HIPPOCAMPAL STRUCTURAL AND SYNAPTIC PLASTICITY OF TGDYRK1A MICE

The next question we addressed was whether Dyrk1A overexpression had an impact on the morphology of CA1-CA3 hippocampal layers and on adult neurogenesis in the dentate gyrus (DG). As in the previous experiments, we used EGCG and EE, which normalize DYRK1A kinase activity and cognitive deficits in TgDyrk1A mice, to evaluate whether these treatments also affect neuronal plasticity.

4.2.1. Effect of Dyrk1A overexpression on CA3-CA1 structural and synaptic plasticity of adult TgDyrk1A mice

We focused our analysis on hippocampal CA3-CA1 structural and synaptic plasticity. To analyze the morphology of different hippocampal strata we used double transgenic Thy-YFP/TgDyrk1A mice (Materials and Methods section 3.2.1.) that are suitable for a general structural analysis. However, this technique does not allow a fine grain analysis of the dendritic arborization. Thus, in collaboration with Dr. Imma Ballesteros (UCLM; see results in Annex III) we analyzed in depth the dendritic branching pattern and the dendritic spines of CA1 apical dendrites in the *stratum radiatum* using Lucifer yellow injections. Importantly, the CA1 *stratum radiatum* receives projections both from the perforant and the temporoammonic pathway. We also analyzed hippocampal synaptic plasticity, investigating the electrophysiological properties of the Schaffer collateral pathway in collaboration with Dr. Thomas Gener (CRG; see results in Annex III). Finally, since changes in excitatory-inhibitory neurotransmitters balance have been proposed as one of the possible mechanisms underlying synaptic plasticity modifications (observed in the electrophysiological recordings) in DS, we also evaluated the ratio between VGLUT1 and VGAT puncta in *stratum radiatum* of CA1 subfield.

4.2.1.1. Dyrk1A overexpression alters CA1-CA3 hippocampal gross morphology, spine morphology and synaptic excitatory-inhibitory balance

We first evaluated whether Dyrk1A overexpression affected the structural morphology of the hippocampal subregions CA1, CA3 and dentate gyrus (DG) using double

transgenic Thy-YFP/TgDyrk1A mice (Figures 31A and 31C). Thy-YFP/TgDyrk1A mice showed a significantly reduced width of *stratum oriens* (Figures 31A and 31B, TG *vs.* WT *t* test, t (1,8) = 5.33, $p<0.001$) and *stratum radiatum* in CA1 (Figures 31A and 31B, TG *vs.* WT *t* test, t (1,8) = 12.09, $p<0.001$) as compared to wild types. Interestingly, although no changes in the mean cell number of YFP+ cells in the pyramidal layer of CA1 was observed (Figures 31A and 31D), the area occupied by the cellular pyramidal layer was increased in Thy-YFP/TgDyrk1A compared to their control littermates (Figures 31A and 31E, TG *vs.* WT *t* test, t (1,8) = -3.25, $p<0.01$). These results suggested that CA1 pyramidal cells of Dyrk1A overexpressing mice are more scattered.

Regarding the morphology of CA3, although no differences between genotypes were observed in the area occupied by the cellular pyramidal layer (Figures 31C and 31E), Thy-YFP/TgDyrk1A mice showed a significant reduction in the mean cell number of YFP+ cells compared to wild types (Figures 31C and 31D, TG *vs.* WT *t* test, t (1,8) = 3.94, $p<0.01$). The area of the *stratum oriens and radiatum* could not be measured using this technique.

Finally, no differences in the granular cell layer or molecular layer width of the DG between genotypes were detected (Figure 31F).

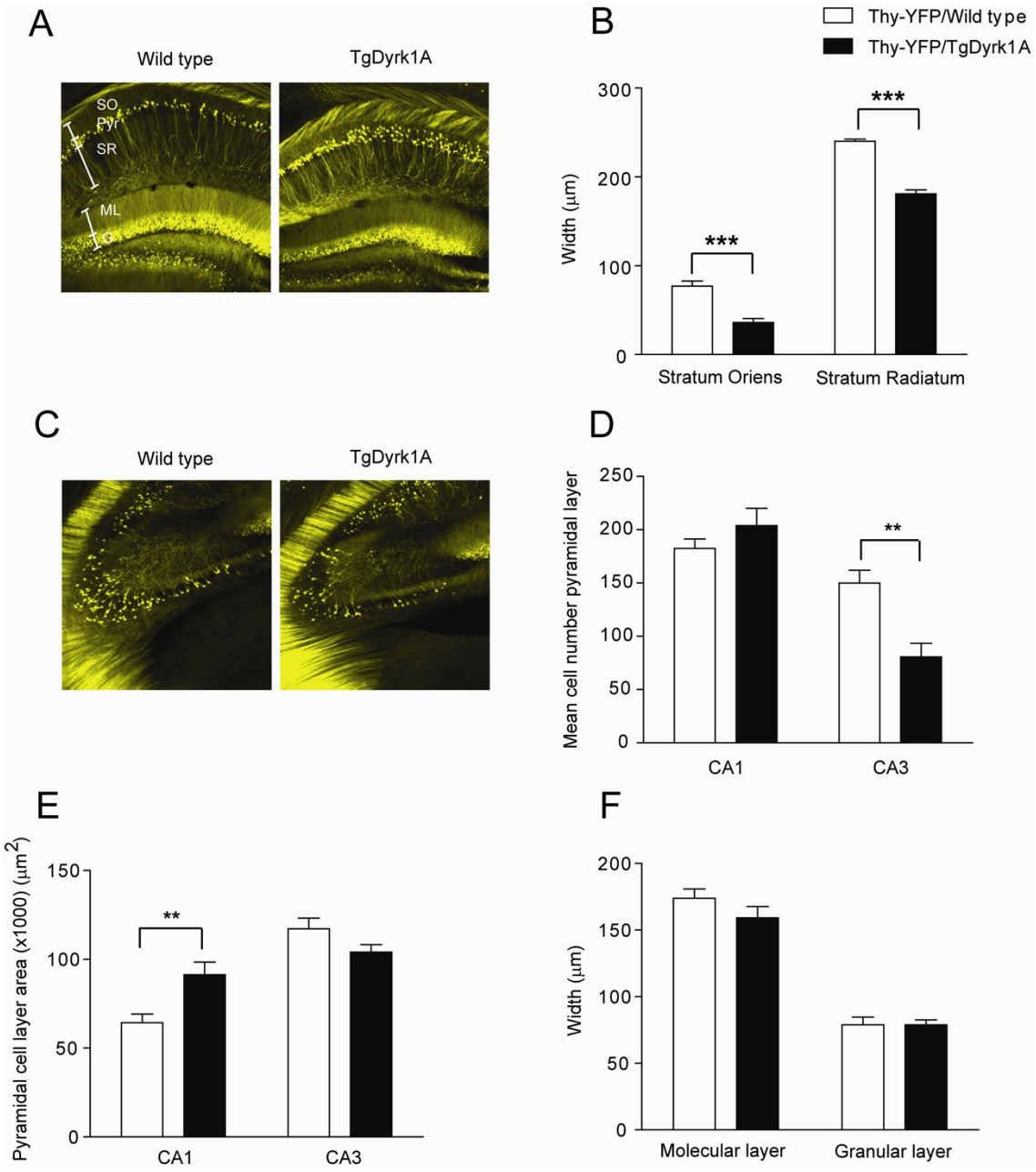

Figure 31. Hippocampal morphology in Thy-YFP/TgDyrk1A mice. (A) Representative confocal micrographs showing Thy-YFP+ cells in the dentate gyrus and CA1 region. (B) Average width of *stratum oriens* and *stratum radiatum* of CA1 region. (C) Representative confocal micrographs of Thy-YFP+ cells in CA3 region. (D) Mean cell number of Thy-YFP+ pyramidal cells in the CA1 and CA3 regions. (E) Area of Thy-YFP+ pyramidal layer in CA1 and CA3 regions. (F) Average width of molecular layer and granular layer in the DG. Data are expressed as ± SEM. Thy-YFP/WT n = 5; Thy-YFP/TG n = 5. Student *t* test for independent samples ** $p<0.01$; *** $p<0.001$.

Next we evaluated whether TgDyrk1A CA1 apical dendrites exhibit any abnormalities is spine density and morphology in Lucifer yellow injected neurons. No differences were observed when the density of spines was analyzed (Figures 32A and 32B) but significant differences were detected in spine morphology in TgDyrk1A. Whereas in

wild type apical dendrites most of the spines presented a mushroom-like protrusion phenotype, known to be more mature spines (Figures 32A and 32D, TG *vs.* WT *t* test, $t(1,46) = 6.62$, $p<0.001$), TgDyrk1A dendrites showed a higher proportion of thin (Figures 32A and 32D, TG *vs.* WT *t* test, $t(1,46) = -6.52$, $p<0.001$), and long spines (Figures 32A and 32D, TG *vs.* WT *t* test, $t(1,46) = -4.52$, $p<0.001$), both of them representing more immature spines, suggesting that an impairment in spine maturation could be occurring in TgDyrk1A dendritic spines.

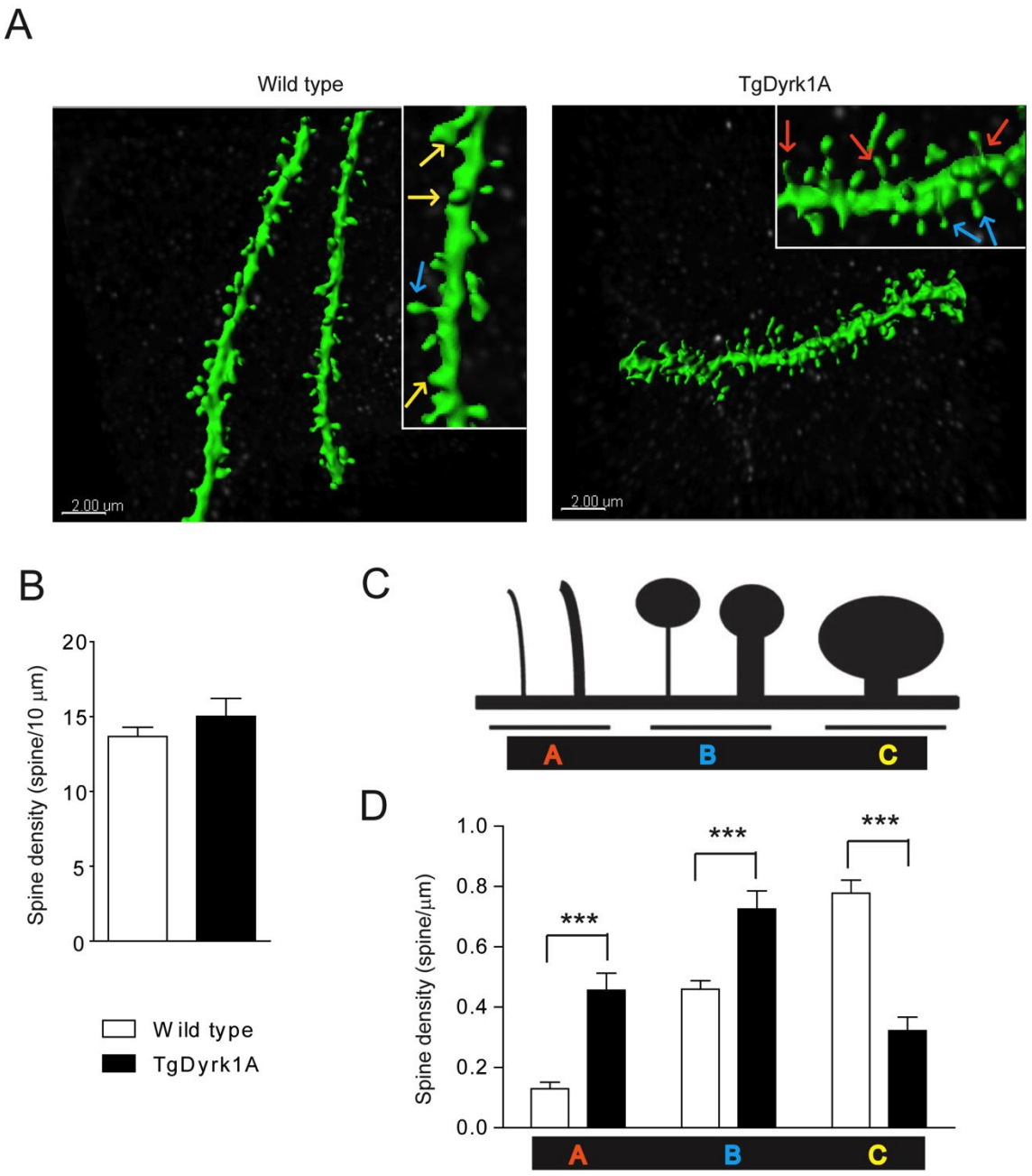

Figure 32. Dendritic spine morphology in CA1 *stratum radiatum* pyramidal neurons. (A) Representative reconstructed confocal images showing CA1 apical dendrites with spines in wild type and TgDyrk1A mice. Scale bar = 2 µm. Right upper insert corresponds to a higher magnification of the

dendrite boxed. Orange, blue and white arrows denote type A, type B and type C spines respectively. (B) Spine density in 10 μm of dendrite. (C) Illustration of the morphological categories used to classify spines. (D) Density of each type of spine. Data are expressed as ± SEM. Number of animals / dendrites analyzed: WT = 4/32; TG = 4/16. Student *t* test for independent samples *** p<0.001.

These abnormalities were accompanied by defects in synaptic plasticity in CA1 and CA3 hippocampal regions, as detected in the electrophysiological experiments (see Annex III). Specifically we detected a reduced LTP in TgDyrk1A mice. Previous results in the literature pointed that impaired synaptic plasticity in DS mouse models is due to an imbalance between excitatory and inhibitory transmission in the hippocampus (Kleschevnikov, Belichenko et al. 2004). Therefore, we also analyzed if an altered ratio between excitatory (VGLUT1+) and inhibitory (VGAT+) puncta in CA1 *stratum radiatum* could contribute to the observed phenotype. In fact, TgDyrk1A mice showed a significant reduced VGLUT1/VGAT ratio compared to wild types (Figures 33A and 33B, TG *vs.* WT Student *t* test, $t(1,12) = 2.50$, $p<0.05$), indicating altered balance towards inhibition in TgDyrk1A *stratum radiatum*.

Our results suggest that altered structure and an overinhibited transmission could underlie LTP defects in TgDyrk1A.

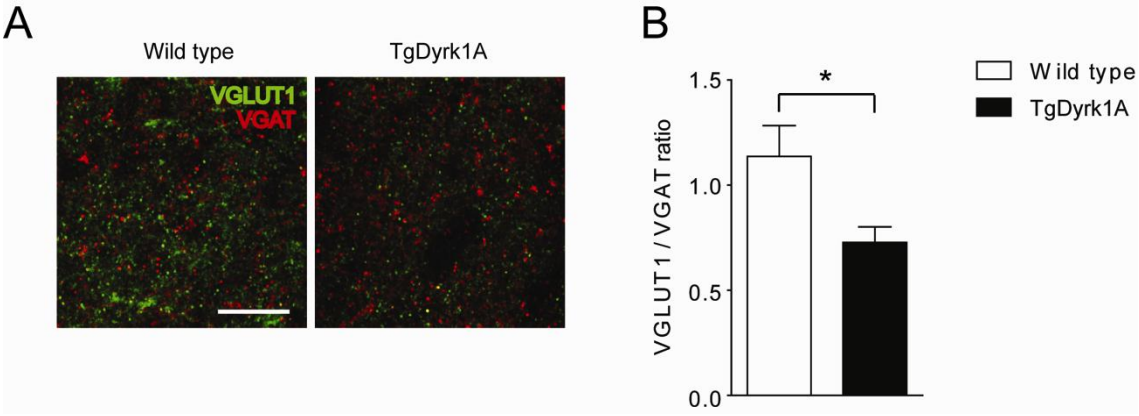

Figure 33. Synaptic excitatory-inhibitory balance in CA1 *stratum radiatum*. (A) Representative confocal images showing VGLUT1+ and VGAT+ puncta in CA1 *stratum radiatum* hippocampal subfield in wild type and TgDyrk1A mice Scale bar = 10 μm. (B) Histograms showing the ratio between VGLUT1+ and VGAT+ puncta. Data are expressed as ± SEM. WT = 7; TG = 7. Student *t* test for independent samples * p<0.05.

4.2.1.2. EGCG rescues hippocampal morphology alterations in CA1 pyramidal cells of TgDyrk1A mice

To investigate the possible rescue of the altered hippocampal morphology by normalizing DYRK1A kinase activity in TgDyrk1A mice, we treated Thy-

YFP/TgDyrk1A mice during one month with EGCG. EGCG increased significantly the width of the CA1 *stratum oriens* (Figures 34A and 34B, EGCG treated TG *vs.* non treated TG two-way ANOVA genotype-treatment interaction $F(1,20) = 28.79$, $p<0.001$; Bonferroni as *post-hoc:* $p<0.001$) and *stratum radiatum* (Figures 34A and 34B, EGCG treated TG *vs.* non treated TG two-way ANOVA genotype-treatment interaction $F(1,20) = 18.28$, $p<0.001$; Bonferroni as *post-hoc:* $p<0.05$) and slightly reduced the area of the pyramidal layer in CA1 in TgDyrk1A mice, almost rescuing the normal hippocampal structure. Conversely, EGCG had opposite effects in wild type mice. In fact, the treatment decreased the width of *stratum oriens* (Figures 34A and 34B, EGCG treated WT *vs.* non treated WT two-way ANOVA genotype-treatment interaction $F(1,20) = 28.79$, $p<0.001$; Bonferroni as *post-hoc:* $p<0.05$) and of *stratum radiatum* (Figures 34A and 34B, EGCG treated WT *vs.* non treated WT two-way ANOVA genotype-treatment interaction $F(1,20) = 18.28$, $p<0.001$; Bonferroni as *post-hoc:* $p<0.001$). It also increased the area of the pyramidal layer in wild types (Figures 34A and 34E, EGCG treated WT *vs.* non treated WT two-way ANOVA genotype-treatment interaction $F(1,20) = 12.18$, $p<0.01$; Bonferroni as *post-hoc:* $p<0.01$) without changes in the mean number of YFP+ cells (Figures 34A and 34D). These results suggested that hippocampal structure of pyramidal cells in CA1 subfield is sensitive to EGCG, suggesting dosage-sensitive effects of DYRK1A in this hippocampal region.

Regarding CA3, EGCG significantly reduced the mean number of YFP+ cells in wild type mice (Figures 34C and 34D, EGCG treated WT *vs.* non treated WT two-way ANOVA genotype-treatment interaction $F(1,20) = 4.70$, $p<0.05$; Bonferroni as *post-hoc:* $p<0.05$) without modifying the number of YFP+ cells in TgDyrk1A mice. No effects of EGCG were detected in the granular cell layer or in the molecular layer of the DG among groups (Figures 34A and 34F).

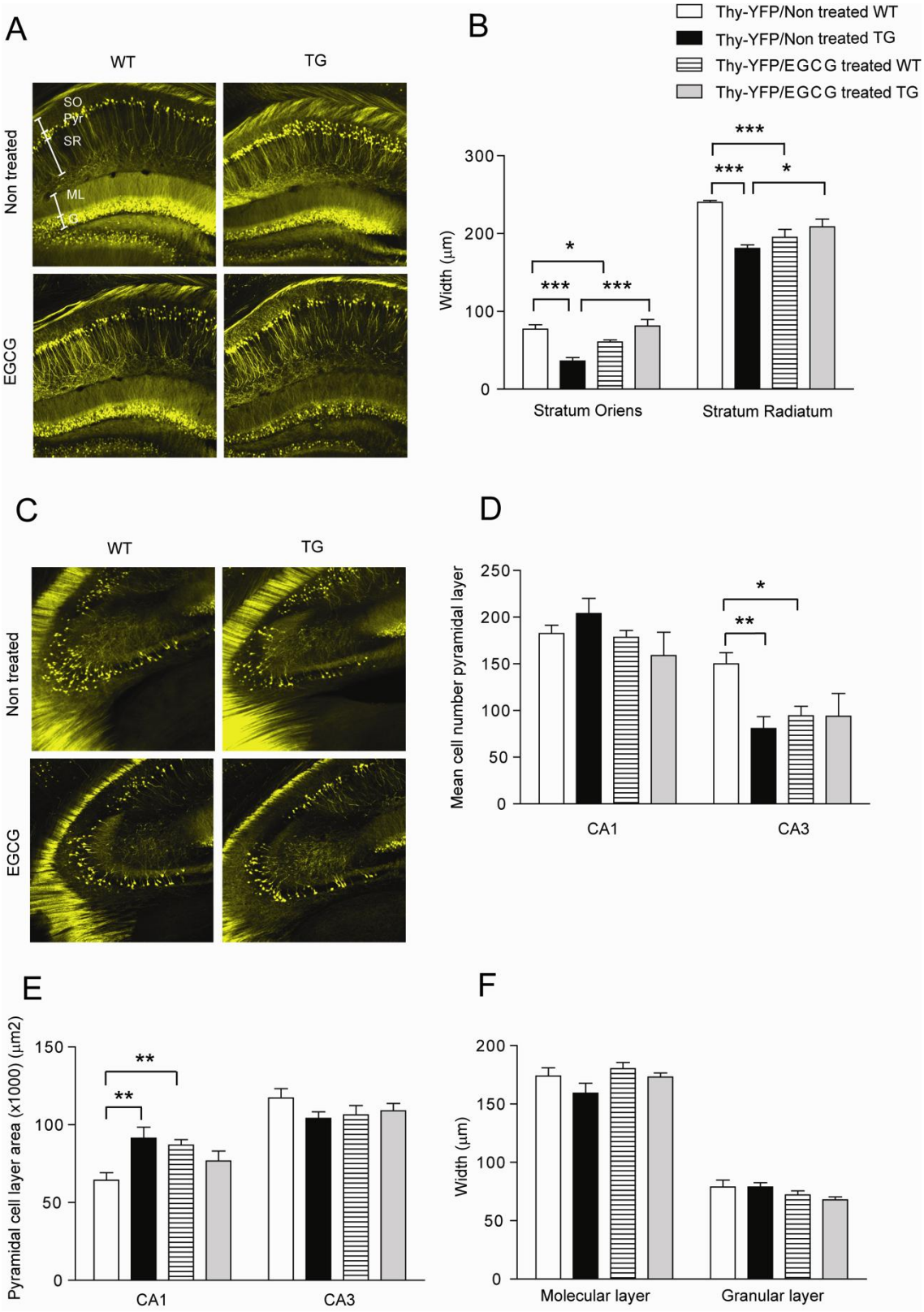

Figure 34. Effect of a DYRK1A kinase inhibitor administration on hippocampal structural plasticity. (A) Representative confocal micrographs showing Thy1-YFP+ cells in the dentate gyrus and CA1 region in wild type (WT) and TgDyrk1A (TG) mice without treatment or EGCG during one month. (B) Average width of *stratum oriens* and *stratum radiatum* in CA1 region. (C) Representative confocal micrographs of Thy1-YFP+ cells in CA3 region. (D) Mean number of Thy1-YFP+ pyramidal cells in the

CA1 and CA3 regions. (E) Area of Thy1-YFP+ pyramidal layer in CA1 and CA3 regions. (F) Dendritic molecular layer and granular layer width in the DG. Data are expressed as ± SEM. Thy-YFP/Non-treated WT n = 5; Thy-YFP/Non-treated TG n = 5; Thy-YFP/EGCG treated WT n = 6; Thy-YFP/EGCG treated TG n = 5. Two-way ANOVA Bonferroni as *post-hoc* * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

These results suggested that one month of EGCG was able to restore most of the morphological alterations in TgDyrk1A mice but impaired the hippocampal morphology in wild type mice, suggesting that CA1 pyramidal neurons are extremely plastic and sensitive to DYRK1A kinase activity dosage.

4.2.1.3. Environmental enrichment normalizes activity-dependent structural and synaptic plasticity in the hippocampus of TgDyrk1A mice

Pyramidal neurons of the hippocampus could also change upon environmental stimuli. Hence, we tested whether the morphology of the hippocampal strata could also respond to EE. One month of EE significantly increased the width of *stratum oriens* (Figures 35A and 35B, TGEE *vs.* TGNE two-way ANOVA genotype-treatment interaction $F(1,20) = 5.51$, $p<0.05$; Bonferroni as *post-hoc:* $p<0.001$) and *stratum radiatum* (Figures 35A and 35B, TGEE *vs.* TGNE two-way ANOVA genotype-treatment interaction $F(1,20) = 10.36$, $p<0.01$; Bonferroni as *post-hoc:* $p<0.01$) in Thy-YFP/TgDyrk1A mice without altering wild type ones. Upon EE, despite the number of Thy+ pyramidal cells did not change, the area of CA1 pyramidal layer was normalized to wild type size in Thy-YFP/TgDyrk1A mice (Figures 35A and 35E, TGEE *vs.* TGNE two-way ANOVA genotype-treatment interaction $F(1,20) = 7.56$, $p<0.05$; Bonferroni as *post-hoc:* $p<0.01$). Conversely, EE did not affect the area of the CA3 pyramidal layer nor the number of YFP+ cells in either genotypes (Figures 35C and 35D, two-way ANOVA genotype-treatment interaction $F(1,20) = 0.869$, $p=0.369$; two-way ANOVA genotype effect $F(1,20) = 18.63$, $p<0.001$).

Finally, one month of EE conditions reduced the granular cell layer width of the DG in both genotypes (Figure 35F, two-way ANOVA genotype-treatment interaction $F(1,20) = 0.304$, $p=0.589$; two-way ANOVA treatment effect $F(1,20) = 6.92$, $p<0.05$).

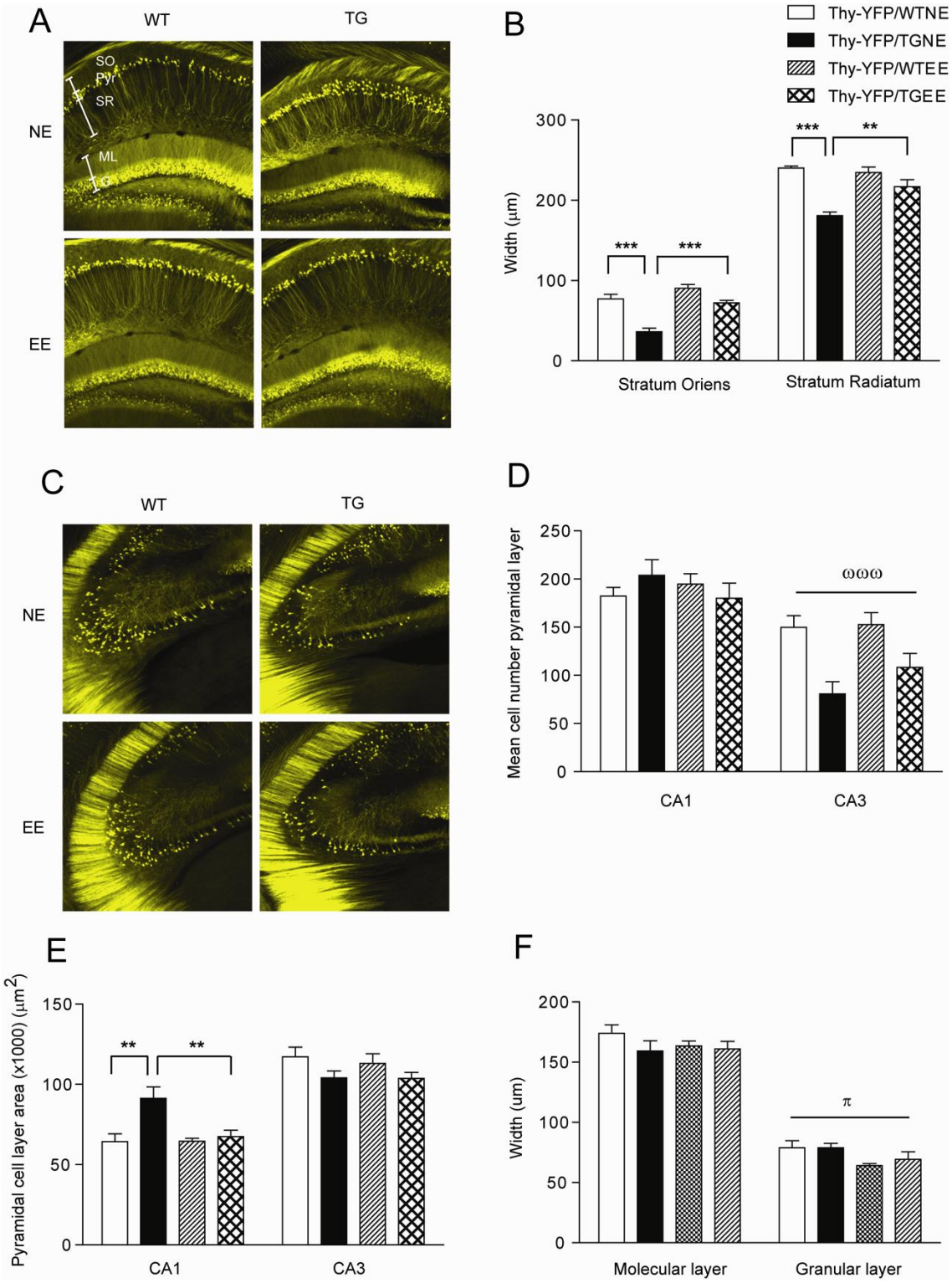

Figure 35. Activity-dependent hippocampal structural plasticity in Thy-YFP/TgDyrk1A mice. (A) Representative confocal micrographs showing Thy-YFP+ cells in the dentate gyrus and CA1 region under non enriched (NE) or environmental enriched (EE) conditions. (B) Average width of *stratum oriens* and *stratum radiatum* of YFP+ pyramidal cells in CA1 region. (C) Representative confocal micrographs of Thy-YFP+ cells in CA3 region. (D) Mean number of Thy-YFP+ pyramidal cells in the CA1 and CA3 regions. (E) Area of Thy-YFP+ pyramidal layer in CA1 and CA3 regions. (E) Dendritic molecular layer and granular layer width in the DG. Data are expressed as ± SEM. Thy-YFP/WTNE n = 5; Thy-

YFP/TGNE n = 5; Thy-YFP/WTEE n = 6; Thy-YFP/TGEE n = 5. Two-way ANOVA Genotype effect ωωω p<0.001; Treatment effect π p<0.05; Bonferroni as *post hoc* ** p<0.01; *** p<0.001.

Regarding dendritic spines, EE significantly increased the density of apical dendritic spines in wild type mice (Figures 36A and 36B, WTEE vs. WTNE two-way ANOVA genotype-treatment interaction F(1,101)=10.89, p<0.001; Bonferroni as *post-hoc* p<0.001). Specifically affecting thin (Figure 36A and 36D, WTEE vs. WTNE two-way ANOVA genotype-treatment interaction F(1,101)=31.44, p<0.001; Bonferroni as *post-hoc* p<0.01) and long spines (with a more immature phenotype) (Figure 36A and 36D, WTEE vs. WTNE two-way ANOVA genotype-treatment interaction F(1,101)=19.74, p<0.001; Bonferroni as *post-hoc* p<0.001) without altering the density of mushroom-like protrusions (mature spines).

In TgDyrk1A, EE reduced the density of thin spines (Figure 36A and 36D, TGEE vs. TGNE Bonferroni as *post-hoc* p<0.001) and increased the density of mushroom-like spines (Figure 36A and 36D, TGEE vs. TGNE two-way ANOVA genotype-treatment interaction F(1,101)=6.85, p<0.001; Bonferroni as *post-hoc* p<0.05) without affecting the total spine density. These results indicated that EE in wild types stimulated spinogenesis, whereas in TgDyrk1A mice, that showed more immature spines, EE stimulated spine stabilization, shifting the balance to increasing the density of mature ones.

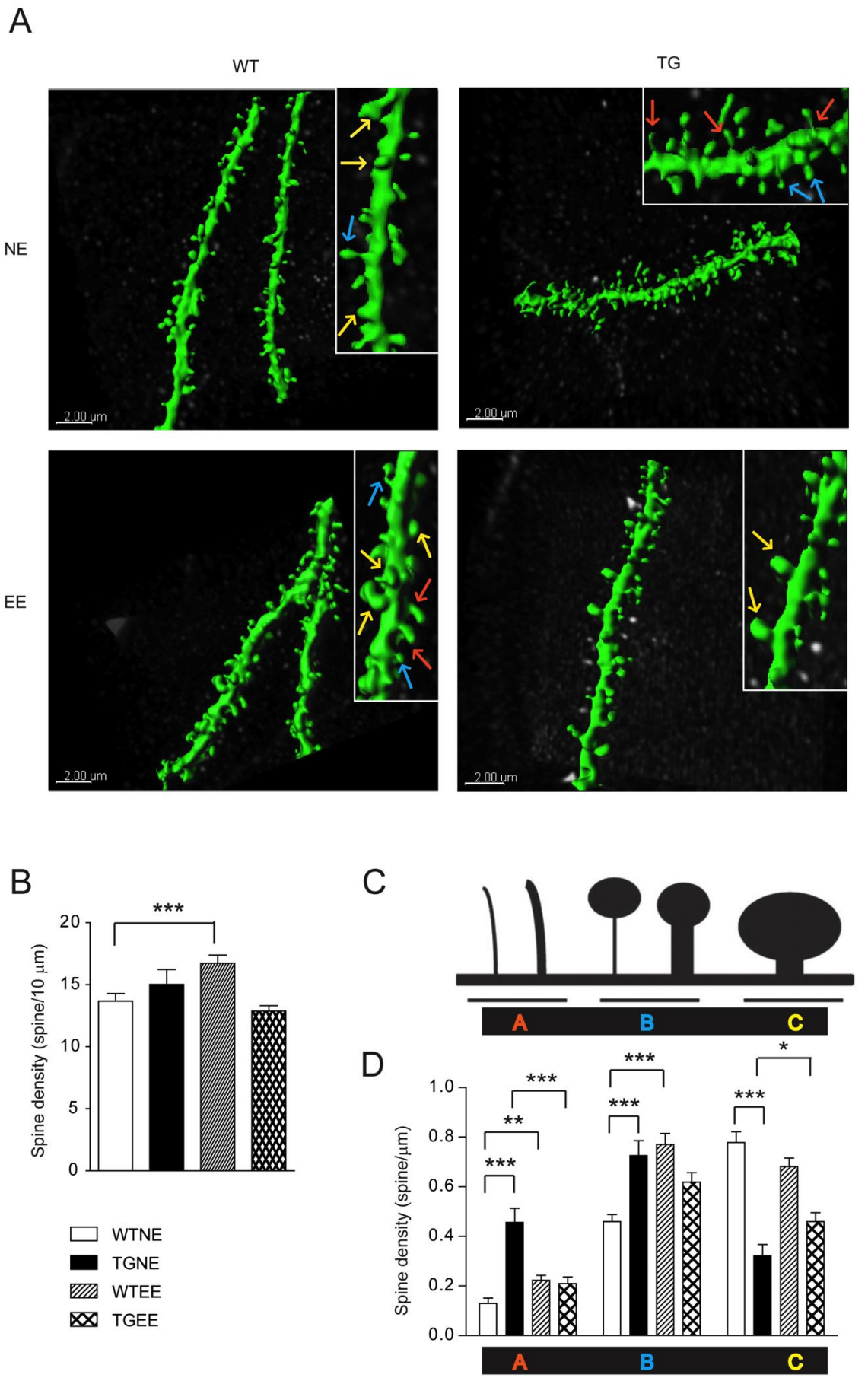
A
WT
TG
NE
EE
2.00 µm
B
***
20
15
10
5
0
Spine density (spine/10 µm)
WTNE
TGNE
WTEE
TGEE
C
A
B
C
D
***
**
***
***
***
***
*
1.0
0.8
0.6
0.4
0.2
0.0
Spine density (spine/µm)
A
B
C

Figure 36. Activity-dependent synaptogenesis in CA1 *stratum radiatum* pyramidal neurons. (A) Representative reconstructed confocal images showing CA1 apical dendrites with spines in wild type (WT) and TgDyrk1A (TG) mice under non enriched (NE) or environmental enriched (EE) conditions during one month. Scale bar = 2 μm. Right upper insert corresponds to a higher magnification of the dendrite boxed. Orange, blue and white arrows denote type A, type B and type C spines respectively. (B) Spine density in 10 μm of dendrite. (C) Illustration of the morphological categories used to classify spines. (D) Density of each type of spine. Data are expressed as ± SEM. Number of animals / dendrites analyzed: WTNE = 4/32; TGNE = 4/16; WTEE = 4/37; TGEE = 4/16. Two-way ANOVA Bonferroni as post-hoc: * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Under NE basal conditions, TgDyrk1A mice recapitulated the excitation inhibition imbalance of DS mouse models in the hippocampus, an alteration that was rescued by EE in the Ts65Dn trisomic mouse model (Begenisic, Spolidoro et al. 2011). Thus, we next analyzed if this would also be the case in our transgenic mouse. To this end we studied the ratio between excitatory (VGLUT1+ puncta) and inhibitory (VGAT+ puncta) in CA1 *stratum radiatum* after one month of EE. In fact, EE increased significantly VGLUT1/VGAT ratio in both genotypes which led a complete recover of the excitatory-inhibitory imbalance in TgDyrk1A mice (Figures 37A and 37B, two-way ANOVA genotype-treatment interaction $F(1,29) = 0.24$, $p=0.62$; two-way ANOVA genotype effect $F(1,29) = 5.03$, $p<0.05$; two-way ANOVA treatment effect $F(1,29) = 4.03$, $p<0.05$).

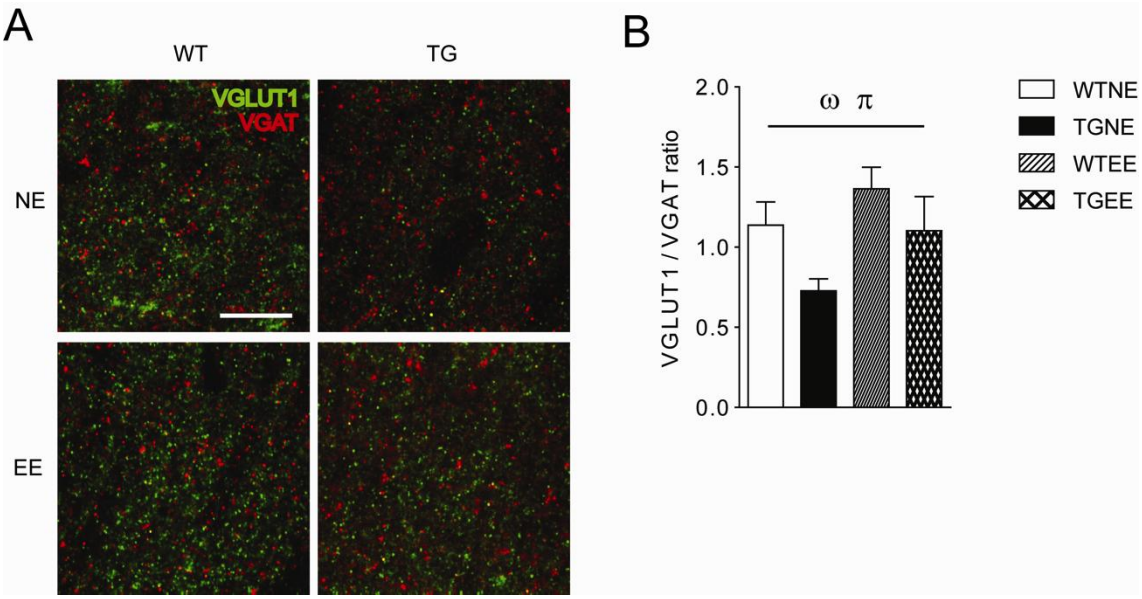

Figure 37. Activity-dependent excitatory-inhibitory balance changes in CA1 *stratum radiatum*. (A) Representative confocal images showing VGLUT1+ and VGAT+ puncta in CA1 *stratum radiatum* hippocampal subfield in wild type (WT) and TgDyrk1A (TG) mice reared during one month under non enriched (NE) or environmental enriched (EE) conditions Scale bar = 10 μm.. (B) Histograms showing

the ratio between VGLUT1+ and VGAT+ puncta. Data are expressed as ± SEM. WTNE = 7; TGNE = 7; WTEE = 8; TGEE = 7. Two-way ANOVA genotype effect ω $p<0.05$; treatment effect π $p<0.05$.

4.2.2. Impact of Dyrk1A overexpression on adult neurogenesis in the dentate gyrus of TgDyrk1A mice

The generation of hippocampal neurons during the adulthood and their integration in the dentate gyrus (DG) is considered one of the mechanisms involved in hippocampal-dependent spatial learning and memory. Given that *Dyrk1A* plays key roles in cell proliferation and survival (Hammerle, Elizalde et al. 2008) during embryogenesis (Aranda, Laguna et al. 2011; Tejedor and Hammerle 2011), in this part of the Thesis, we examined the impact of Dyrk1A overexpression on adult neurogenesis progression in the DG.

4.2.2.1. Dyrk1A is expressed in the adult DG neurogenic zone

Although Dyrk1A expression in adult brain of mice was described several years ago (Marti, Altafaj et al. 2003), its expression in adult neurogenic regions had only been studied in the subventricular zone (Ferron, Pozo et al. 2010). We here demonstrate that in the DG neurogenic region, Dyrk1A was expressed in Sox2 neuronal precursors (Figure 38B) and in proliferating cells positive for ki67 and pH3 (Figures 38C and 38D). Moreover, we detected Dyrk1A expression in cells that expressed BrdU (Figure 38E) and in neuroblasts and immature neurons expressing DCX (Figure 38F). These results suggested that Dyrk1A is expressed during all phases of adult neurogenesis in the DG.

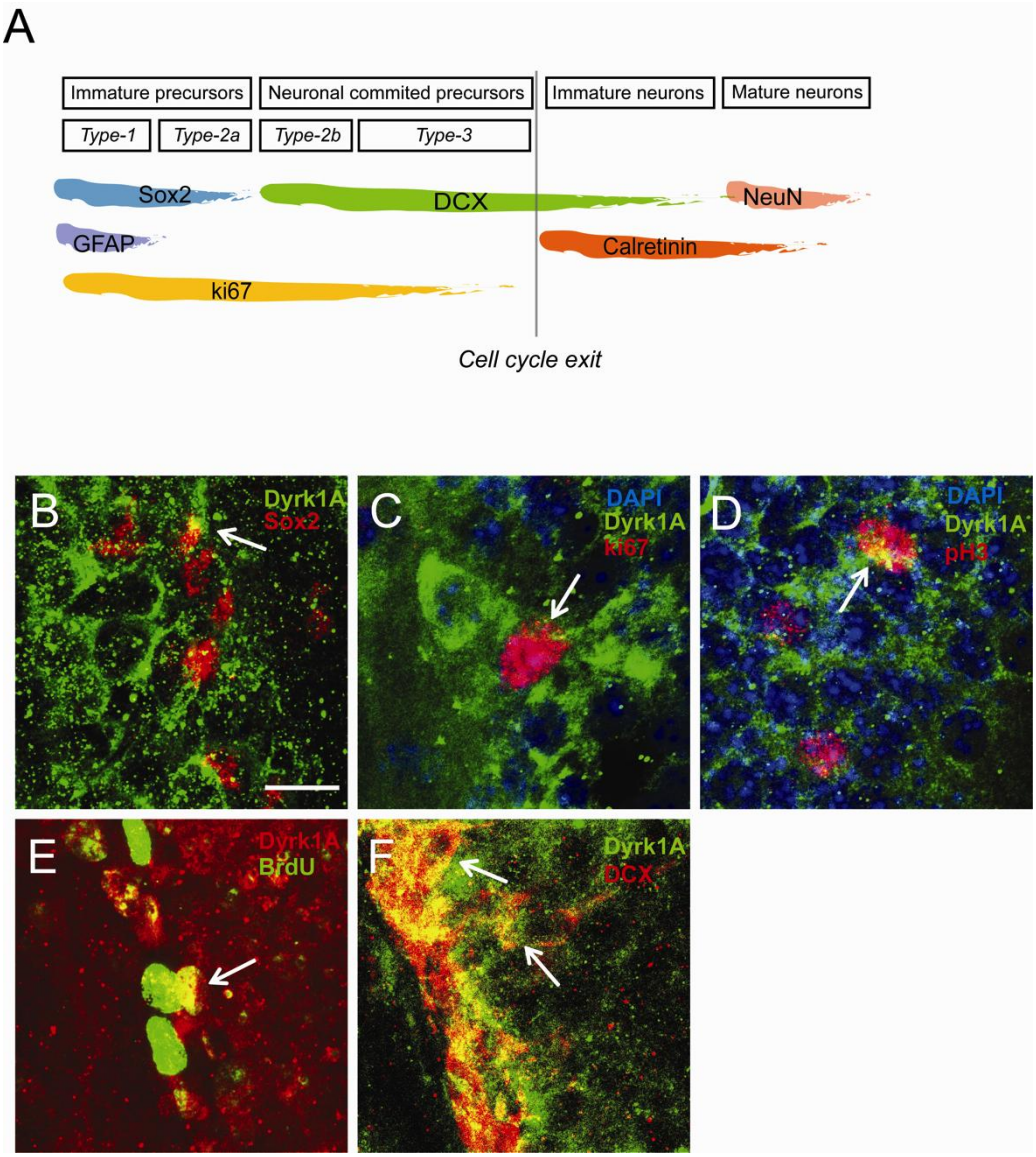

Figure 38. Dyrk1A expression in different cell types of the adult DG neurogenic region. (A) Specific cell markers used in the different stages of adult hippocampal neurogenesis (Adapted from (Beukelaers, Vandenbosch et al. 2011)). Representative confocal micrographs showing Dyrk1A expression in (B) Sox2+, (C) ki67+, (D) pH3+, (E) BrdU+ and (F) DCX+ cells. Scale bar = 10µm. Arrows show co-localization of the different markers with Dyrk1A.

4.2.2.2. Altered proliferation and cell cycle progression of newly generated cells in the adult DG in TgDyrk1A mice

Previous results have shown that newly generated cells in DS fetuses and trisomic mouse models such as Ts65Dn showed reduced proliferation rate (Contestabile, 2007). As we detected Dyrk1A expression in different proliferating cell types of the DG, we asked whether the overexpression of Dyrk1A could alter the proliferation rate.

Taking into account that the length of the cell cycle in adult mice takes around 14 hours to be completed and that the length of the S phase is around 7,6 hours (Cameron and Mc Kay, 2001), we studied the proliferation rate by quantifying the density of BrdU+ cells two hours after a single BrdU injection (Figure 39A). TgDyrk1A mice showed significantly reduced density of BrdU+ cells compared to wild types (Figures 39B and 39C, TG *vs.* WT *t* test, $t(1,9) = 2.29$, $p<0.05$).

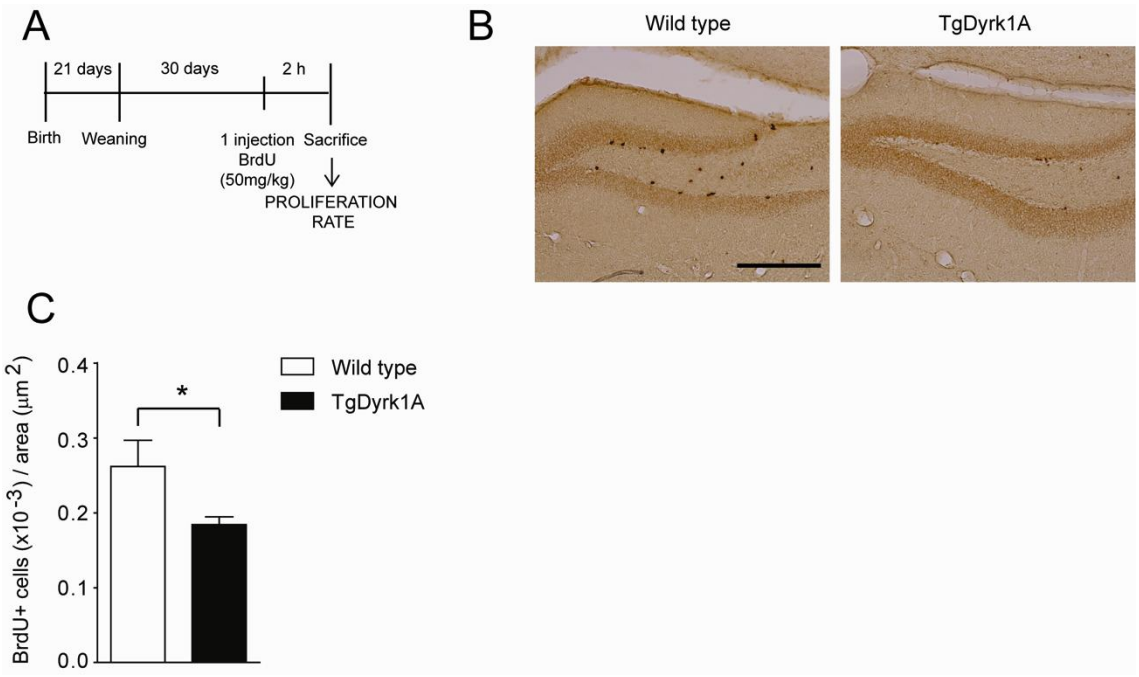

Figure 39. Proliferation rate of newly proliferating cells in the DG. (A) Experimental outline used to analyze the proliferation rate in wild type and TgDyrk1A mice. (B) Representative photomicrographs of BrdU+ cells 2 hours after the injection. Scale bar = 500µm. (C) Average density of BrdU+ cells in the DG two hours after BrdU injection. Wild type n = 5; TgDyrk1A n = 6. Data are represented as mean ± SEM. Student *t* test for independent samples * $p<0.05$.

We next examined if the reduced proliferating rate could be associated with changes in the number of progenitor cells. However, we did not detect differences between genotypes in the density of *type 1* immature precursors (GFAP+ Sox2+ cells) (Figure 40A) nor of type *2a* transient amplifying stem cells (GFAP- Sox2+ cells) (Figure 40B). In the absence of changes in the number of neuronal precursors, the differences in proliferation rate could also be due to a reduced recruitment of neural precursors to enter the cell cycle. Thus, we used ki67 (an endogenous marker of proliferating cells) to examine the actively proliferating Sox2+ cell population (ki67+ Sox2+), and showed that approximately 6% of total Sox2+ cells were proliferating in the adult DG of both genotypes (Figure 40C). Based on these results, we can also discard that the reduced

density of BrdU+ cells in TgDyrk1A mice was due to a reduced proportion of precursor cells entering in cell cycle.

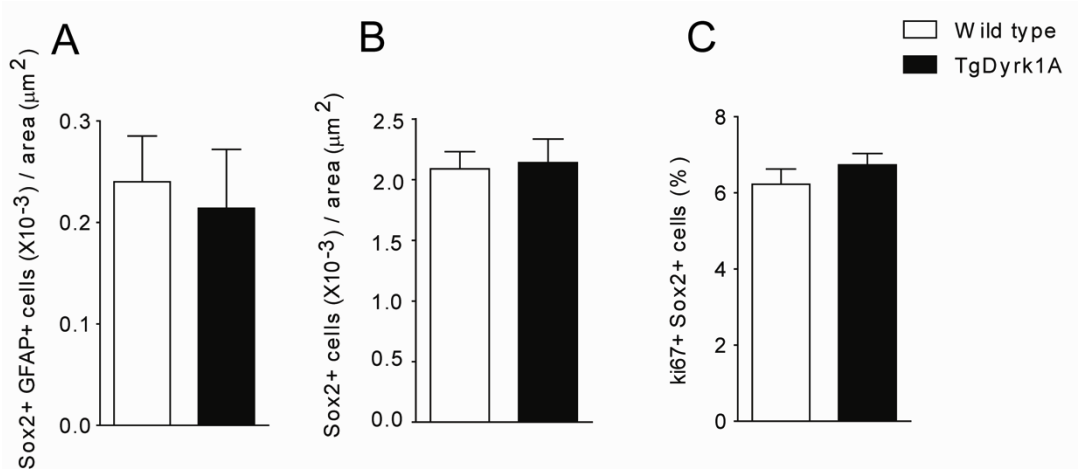

Figure 40. Proliferation of neuronal progenitors in the DG. (A) Density of Sox2+ cells co-expressing GFAP (progenitor cells *type 1*) of wild type and transgenic mice. (B) Density of Sox2+ cells (progenitor cells *type 2a*) in the DG (C) Percentage of Sox2+ cells co-expressing ki67. Wild type n = 4; TgDyrk1A n = 4. Data are represented as mean ± SEM.

Interestingly, when analyzing the total number of proliferating cells in the DG, TgDyrk1A mice showed a significant higher density of ki67+ cells compared to wild types (Figures 41A and 41B, TG *vs.* WT *t* test, $t(1,14) = -1.48$, $p<0.05$).

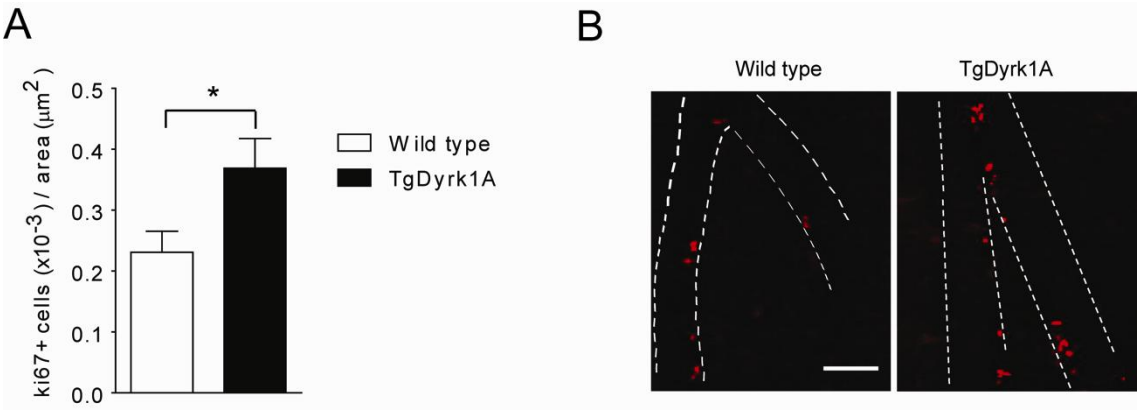

Figure 41. Proliferation in the DG. (A) Average density of ki67+ cells of wild type and transgenic mice. (B) Representative confocal micrographs showing ki67+ cells. Scale bar = 100µm. Wild type n = 8; TgDyrk1A n = 8. Data are represented as mean ± SEM. Student *t* test for independent samples * $p<0.05$.

This increased number of ki67+ cells in TgDyrk1A mice could be due to changes in the proportion of cells exiting the cell cycle. Thus, we analyzed the ratio between the proliferating cells exiting cell cycle (BrdU+ Ki67-) and total BrdU+ cells 24 hours after

one BrdU injection (Figure 42A) and found that TgDyrk1A mice had a significantly reduced percentage of cells exiting cell cycle as compared to wild types (Figures 42B and 42C, TG *vs.* WT *t* test, t (1,6) = 3.603, p<0.01).

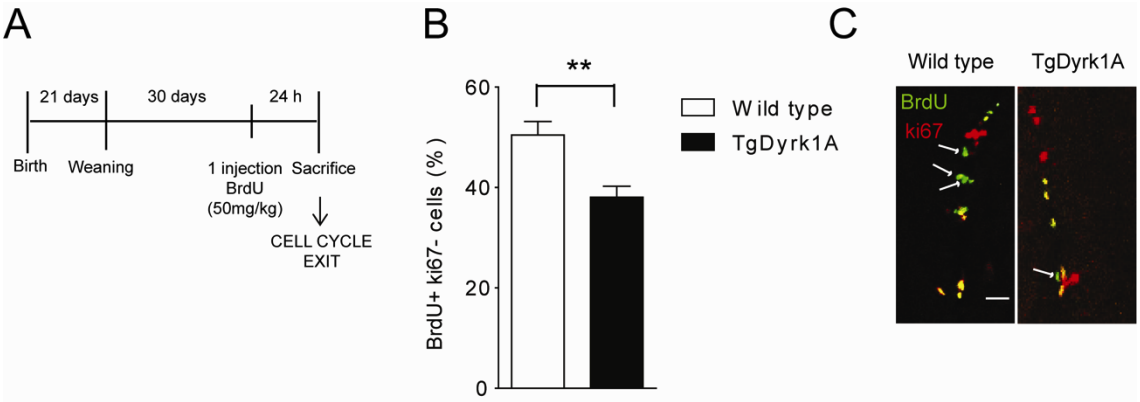

Figure 42. Cell cycle exit of newly proliferating cells in the DG. (A) Experimental outline used to analyze cell cycle exit. (B) Percentage of BrdU+ cells negative for ki67 observed twenty-four hours after one BrdU injection of wild type and transgenic mice. (C) Representative confocal micrographs showing BrdU and ki67 + cells. Scale bar = 20µm. Arrows indicate BrdU+ ki67- cells (cells exiting cell cycle). Wild type n = 4; TgDyrk1A n = 4. Data are represented as mean ± SEM. Student *t* test for independent samples ** p<0.01.

This result along with the increase of ki67+ cells suggested changes in cell cycle progression in transgenic mice. In fact, previous studies reported a G2 phase elongation in DS fetuses and trisomic mouse models (Contestabile et al., 2007). To elucidate this aspect we performed an immunohistochemistry to detect phosphorylated-(Ser10)-histone-H3 (pH3) that allowed discriminating G2 and M phases of cell cycle, based on its nuclear distribution pattern (Hendzel et al., 1997); Figure 43C). TgDyrk1A mice showed a significant increase in the density of pH3+ cells as compared to wild types (Figures 43A and 43B, TG *vs.* WT *t* test, t (1,15) = -2.44, p<0.05). Also, a significantly higher proportion of pH3+ cells with a G2 phase staining pattern were found in TgDyrk1A DG (Figure 43D, TG *vs.* WT *t* test, t (1,6) = -3.99, p<0.01), indicating that proliferating cells in TgDyrk1A remain in the G2 phase.

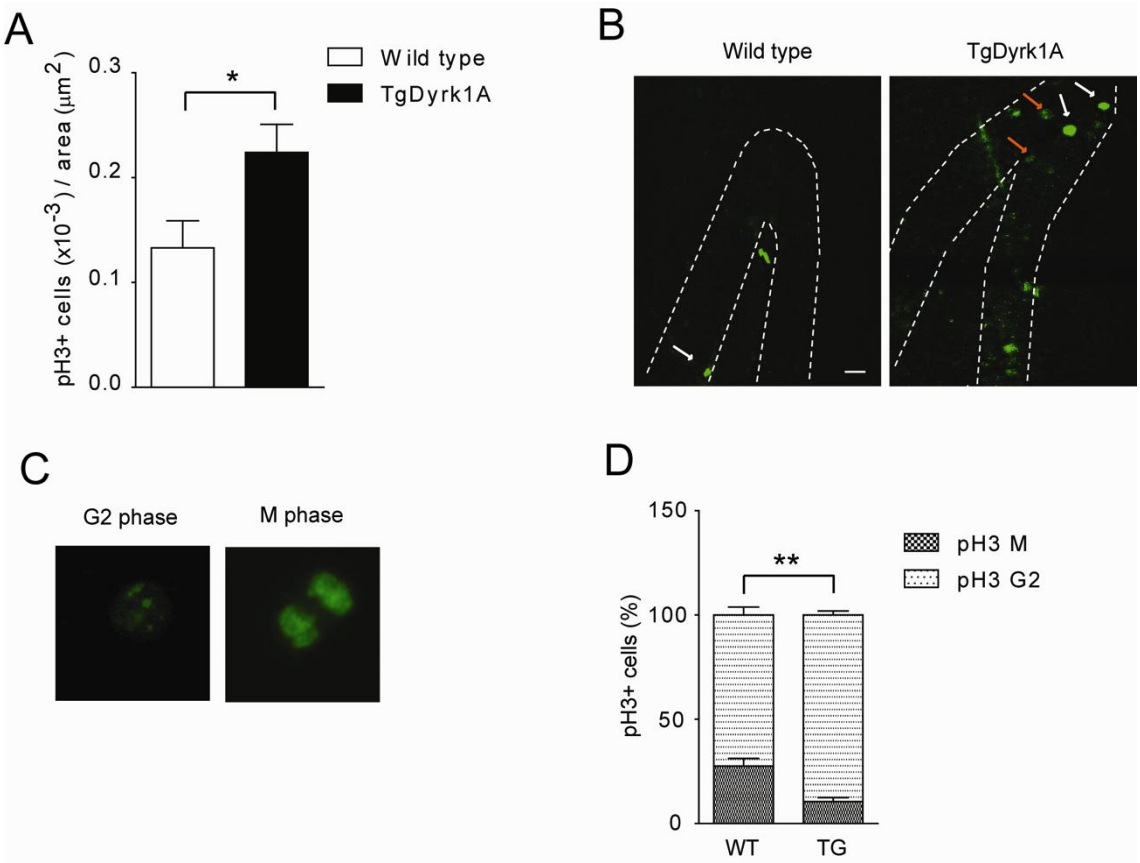

Figure 43. Characterization of pH3+cells in the DG. (A) Density of pH3+ cells in wild type and transgenic mice. (B) Representative confocal micrographs showing pH3+ cells. Scale bar = 20µm. White arrows represent pH3+ cells suggestive of M phase. Orange arrows show pH3+ cells suggestive of G2 phase. (C) Representative confocal micrographs showing pH3+ cells in the G2 and M phase of the cell cycle. (D) Percentage of pH3+ cells with a staining pattern suggestive of G2 or M phase. Wild type n = 4-8; TgDyrk1A n = 4-8. Data are represented as mean ± SEM. Student *t* test for independent samples * $p<0.05$; ** $p<0.01$.

While most of the pH3+ proliferating cells in wild type mice were placed in the subgranular zone (SGZ) (Figure 44A), where the proliferative niches are normally located, a significant higher proportion of pH3+ cells were detected in granular cell layer (GCL) of TgDyrk1A (Figure 44A, TG *vs.* WT *t* test, $t(1,6) = 5.81$, $p<0.001$). This altered pH3+ cell positioning mainly involved cells with a G2 phase staining pattern (Figure 44B, TG *vs.* WT *t* test, $t(1,6) = -6.39$, $p<0.001$).

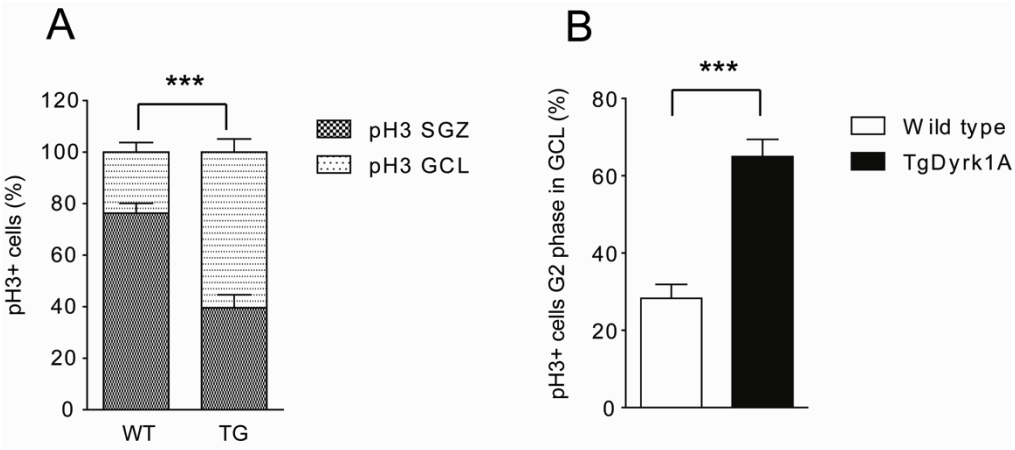

Figure 44. Localization of pH3+ cells within the DG. (A) Percentage of pH3+ cells in the SGZ and GCL in wild type and transgenic mice. Percentage of pH3+ cells with a nuclear pattern suggestive of G2 phase detected in the GCL. Wild type n = 4; TgDyrk1A n = 4. Data are represented as mean ± SEM. Student *t* test for independent samples *** $p<0.001$.

4.2.2.3. Premature migration of newly formed cells into the granular cell layer in TgDyrk1A mice

The alterations in the localization of pH3+ proliferating cells detected in TgDyrk1A mice, suggested that Dyrk1A overexpression could affect the migrating behavior of newly generated hippocampal cells. Newly formed cells in the SGZ of the DG migrate into the GCL to become granule neurons. In this experiment, mice received 4 BrdU injections and were sacrificed 24 hours, 1 week or 1 month after the last injection (Figure 45A). The percentage of BrdU+ cells in the GCL *vs.* total BrdU+ cells in the DG was analyzed.

In wild type mice the proportion of BrdU+ cells present in the GCL progressively increased starting from the first week of cell life, when immature neurons initiated the migration to their final location (Kempermann, Gast et al. 2003). Conversely, TgDyrk1A mice presented a significantly higher proportion of BrdU+ cells located in the GCL already 24 hours after the last BrdU injection (Figures 45B and 45C, TG *vs.* WT *t* test, $t\ (1,10) = -3.49$, $p<0.01$) that was also detected one week after BrdU injections as compared to wild types (Figure 45B, TG *vs.* WT *t* test, $t\ (1,10) = -2.67$, $p<0.05$). These results suggested that TgDyrk1A newborn cells undergo premature migration to the GCL. TgDyrk1A mice showed a significantly reduced percentage of surviving BrdU+ cells in the GCL one month after the last BrdU injection, as compared to wild type mice (Figures 45B and 45D, TG *vs.* WT *t* test, $t\ (1,12) = 4.505$, $p<0.001$).

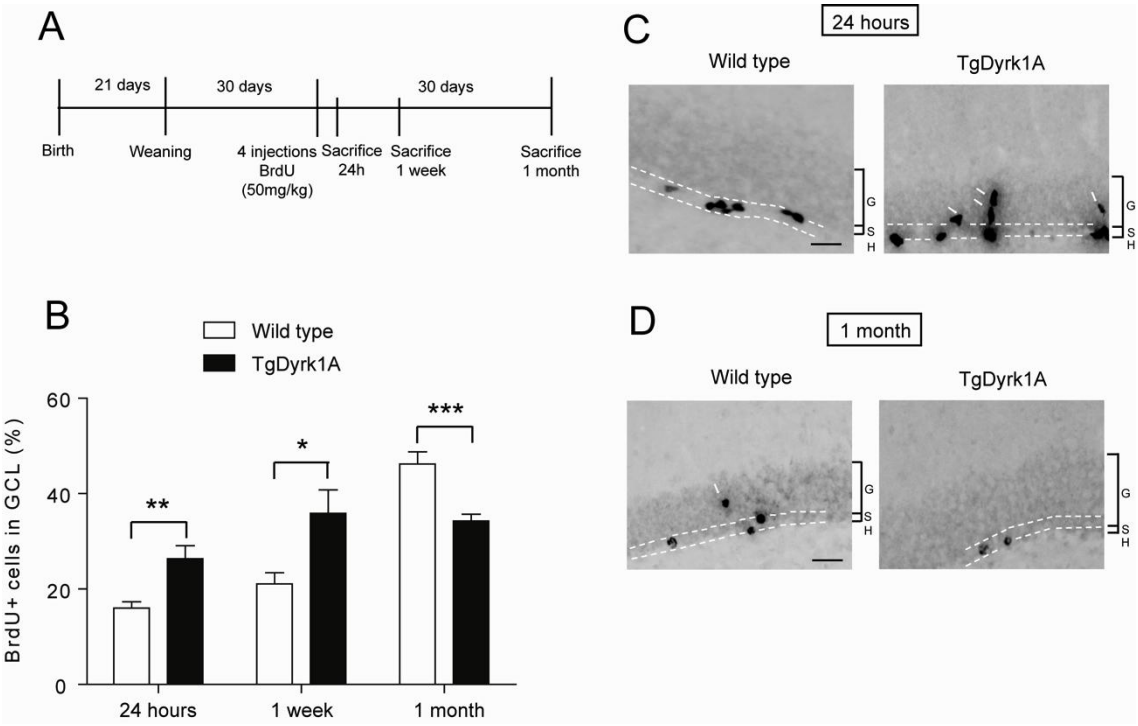

Figure 45. Migration of newly formed cells in the DG. (A) Experimental schedule used to analyze cellular migration in the DG. (B) Proportion of BrdU+ cells localized in the GCL of the DG in wild type and transgenic mice. (C-D) Representative photomicrographs of BrdU+ cells localization at (C) 24 hours and (D) 1 month after BrdU injections. Arrows represent BrdU+ cells localized in the GCL. Scale bar = 20μm. Wild type n = 6; TgDyrk1A n = 6 in every time point of sacrifice. Data are represented as mean ± SEM. Student *t* test for independent samples * $p<0.05$; ** $p<0.01$; *** $p<0.001$. G: Granular cell layer; S: Subgranular zone; H: Hilus.

The abnormal localization of proliferating cells did not arise from an atypical disposition of the precursor cells, since no differences in the localization of Sox2+ cells were detected between genotypes (Figure 46). Thus, it is plausible that the alterations in cell cycle progression observed in TgDyrk1A lead to premature migration of newborn cells through the GCL.

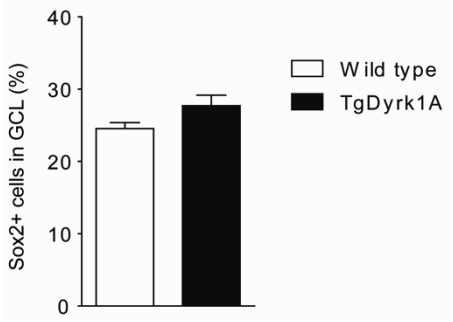

Figure 46. Localization of progenitor cells in the DG. (A) Percentage of Sox2+ cells in the GCL of wild type and transgenic mice. Wild type n = 4; TgDyrk1A n = 4. Data are represented as mean ± SEM.

4.2.2.4. Reduced survival of newly born cells in the adult DG in TgDyrk1A mice

One critical aspect of adult neurogenesis is that, while large numbers of new neurons are born in the DG, only a fraction of those survive. In fact, some studies postulate that only those cells surviving after two weeks will mature and integrate in the circuits (Kempermann et al., 2003).

As concluded from the previous experiment, Dyrk1A overexpression could be affecting cell survival. To test this hypothesis, we counted the number of BrdU+ cells throughout the DG upon repeated BrdU injections at different time points (24 h, 1 week, and 1 month) (Figure 47A). As described elsewhere, we found a progressive reduction of the density of newly generated cells in the DG in both genotypes. However, while no significant differences between genotypes were observed in the density of BrdU+ cells after 24 hours of BrdU injections (Figures 47B and 47C), at one week TgDyrk1A mice showed a marked reduction in the density of BrdU+ cells compared to wild types (Figures 47B and 47C, TG *vs.* WT *t* test, $t\ (1,10) = 1.98$, $p<0.05$) that was also detected one month after the last BrdU injection (Figures 47B and 47C, TG *vs.* WT *t* test, $t\ (1,12) = 6.97$, $p<0.001$). Accordingly, while 80% of newly formed cells survived one week in wild type mice, this percentage was only 40% in TgDyrk1A mice. However, the percentage of these cells that survive at 1 month was the same in wild type and TgDyrk1A mice, suggesting that Dyrk1A dependent effects on survival are mainly operative during the first week of life of newborn cells.

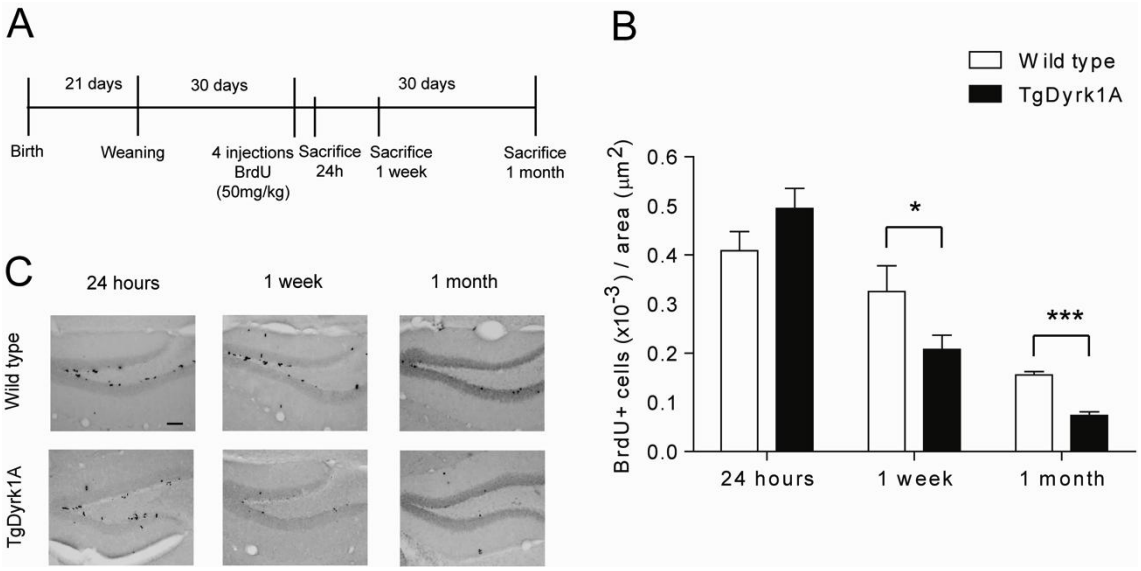

Figure 47. Survival of newly born granule cells in the DG. (A) Experimental schedule used to analyze survival in the DG. (B) Density of BrdU+ cells in the DG at 24 hours, 1 week and 1 month after BrdU injection. (C) Representative photomicrographs of BrdU+ cells at 24 hours, 1 week and 1 month after BrdU injection in wild type and transgenic mice. Scale bar = 100µm. Wild type n = 6; TgDyrk1A n = 6 in every time point of sacrifice. Data are represented as mean ± SEM. Student *t* test for independent samples * p<0.05; *** p<0.001.

As changes in survival could be related to altered apoptosis, we also analyzed caspase3+ cells (an effector of caspase 9 that has been previously related with Dyrk1A (Laguna et al., 2008). We observed a significant increase in the density of caspase3+ cells in TgDyrk1A mice as compared to wild types (Figure 48A and 48B, TG *vs.* WT *t* test, t (1, 6) = -2.43, p<0.05).

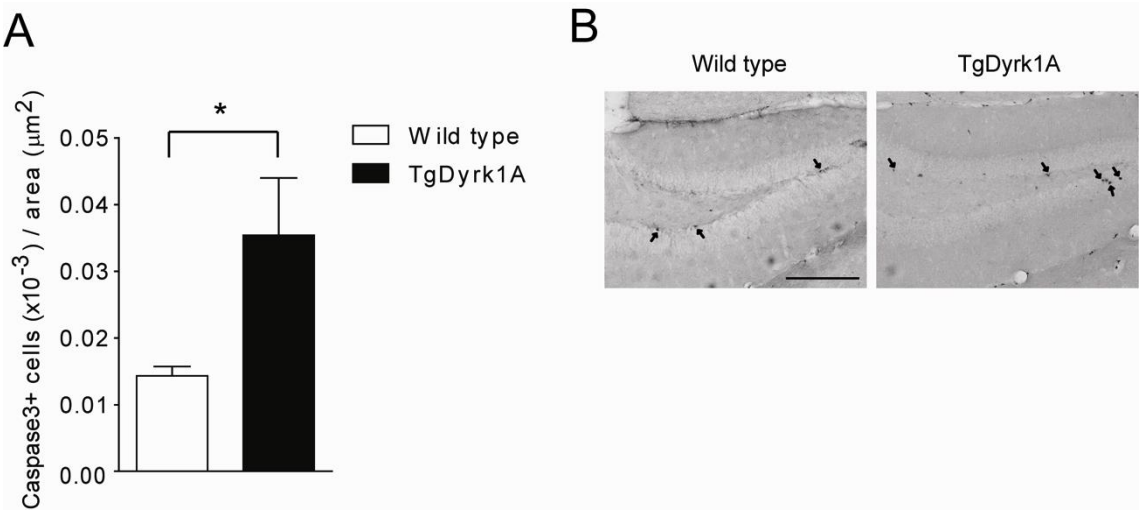

Figure 48. Apoptosis in the DG. (A) Density of caspase3+ cells in the DG of wild type and transgenic mice. (B) Representative photomicrographs of caspase3+ cells. Scale bar = 500µm. Arrows represent caspase3+ cells. Wild type n = 4; TgDyrk1A n = 4. Data are represented as mean ± SEM. Student *t* test for independent samples * p<0.05.

However, the final number of granule cells in the DG of transgenic mice was not affected (Figure 49).

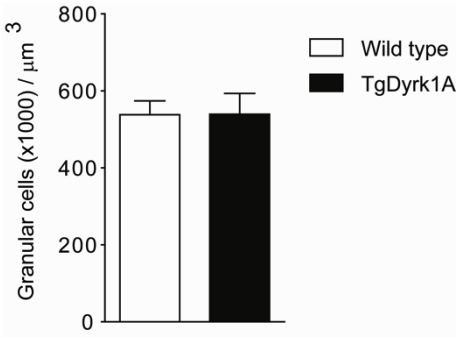

Figure 49. Granular cell density in the DG. (A) Granule cell density in the DG was represented in wild type and transgenic mice. Wild type n = 4; TgDyrk1A n = 4. Data were represented as mean ± SEM.

We next evaluated whether the alterations in survival could affect neuronal differentiation by analyzing the percentage of BrdU+ cells expressing doublecortin (DCX) or calretinin, two markers of inmature and postmitotic neurons, one week after the last BrdU injection (Figure 50A). At this stage, TgDyrk1A mice showed a reduction in the percentage of newly generated cells expressing DCX (Figures 50A and 50B) and calretinin although it only reached a tendency in the case of calretinin+ cells (Figure 50C, TG *vs.* WT *t* test, t (1, 6) = 2.05, p=0.08). Taken together, these results suggested that neuronal differentiation might be slightly impaired or delayed in TgDyrk1A newly generated cells.

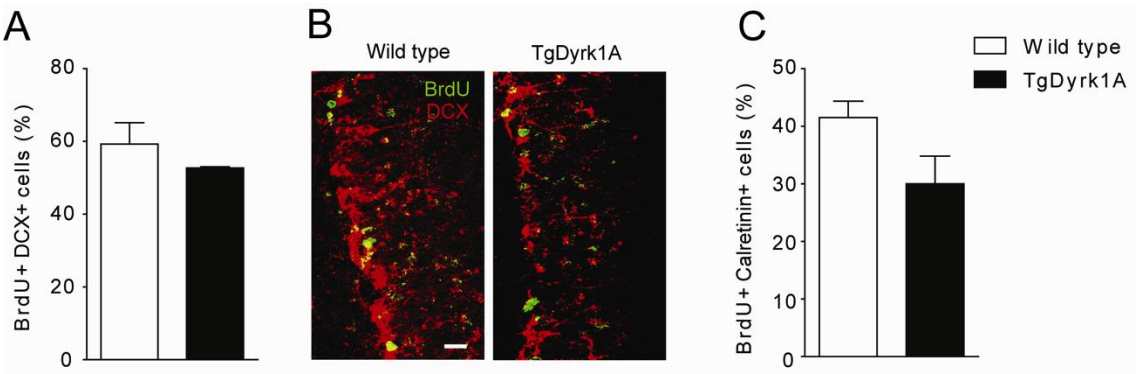

Figure 50. Differentiation of newly formed cells to immature neurons in the DG. (A-C) Percentage of BrdU+ cells coexpressing DCX (A) or calretinin (C) one week after BrdU injections in wild type and

transgenic mice. (B) Representative confocal micrographs showing BrdU+ DCX+ cells. Scale bar = 20μm. Wild type n = 4; TgDyrk1A n = 4. Data are represented as mean ± SEM.

We also quantified the number of BrdU+ cells expressing NeuN one month after BrdU injections, age in which newly formed cells acquire their final cell fate. TgDyrk1A mice showed significant reduced density of BrdU+ NeuN+ cells compared to wild type mice (Figure 51A, TG *vs.* WT *t* test, t (1, 7) = 2.95, $p<0.05$), but we did not found differences between genotypes in the percentage of BrdU+ NeuN+ cells respect to the total surviving cells (Figure 51B), indicating that neuronal fate was not influenced by Dyrk1A overexpression. Interestingly, TgDyrk1A mice showed a significant higher percentage of BrdU+ GFAP+ cells (Figure 51C, TG *vs.* WT *t* test, t (1, 8) = -3.77, $p<0.01$), suggesting that Dyrk1A overexpression shifted newly generated cells towards a glial fate.

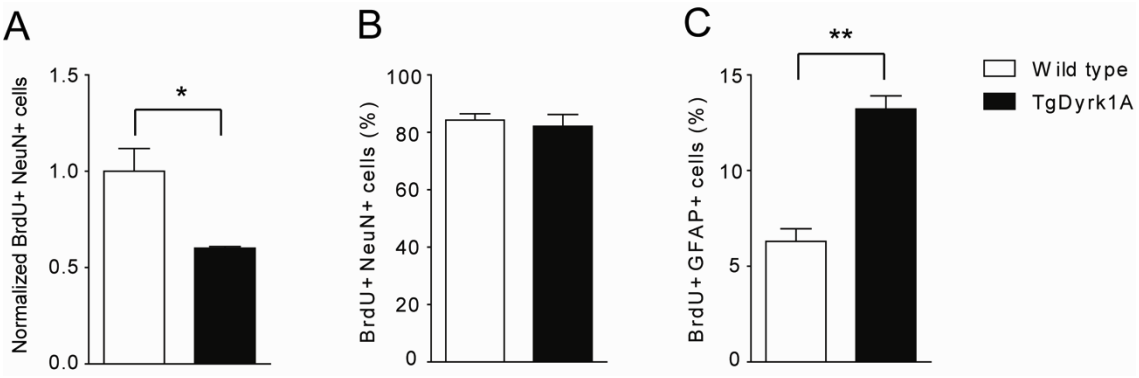

Figure 51. Differentiation of newly born surviving granule cells. (A) Average density of BrdU+ NeuN+ cells in the DG one month after BrdU injections in wild type and transgenic mice normalized respect to wild type mice. (B) Percentage of BrdU+ cells coexpressing NeuN and (C) coexpressing GFAP after 1 month from the last BrdU injection Wild type n = 5; TgDyrk1A n = 4. Data are represented as mean ± SEM. Student *t* test for independent samples * $p<0.05$; ** $p<0.01$.

4.2.2.5. Reduced dendritic arborization and functional integration of new neurons in the adult DG in TgDyrk1A mice

Once the newly formed cells migrate to their final location in the granular layer of the DG, they rapidly start to develop dendritic projections through the molecular layer. Since we observed that Dyrk1A was expressed in immature neurons of the adult DG, we investigated if Dyrk1A overexpression would affect dendritic arborization in immature neurons immunostained with DCX using digital reconstruction techniques (Figure 52A).

No differences were observed in the area or perimeter of the soma of the DCX+ cells between genotypes (data not shown). However, TgDyrk1A DCX+ neurons showed a significant reduction of dendritic length compared to wild types (Figures 52A and 52B, TG *vs.* WT *t* test, t (1, 38) = 5.20, $p<0.001$). Dendritic branching complexity was also affected in TgDyrk1A immature neurons that presented less nodes than wild type ones (Figures 52A and 52C, TG *vs.* WT *t* test, t (1, 38) = 7.035, $p<0.001$), indicating poor arborization.

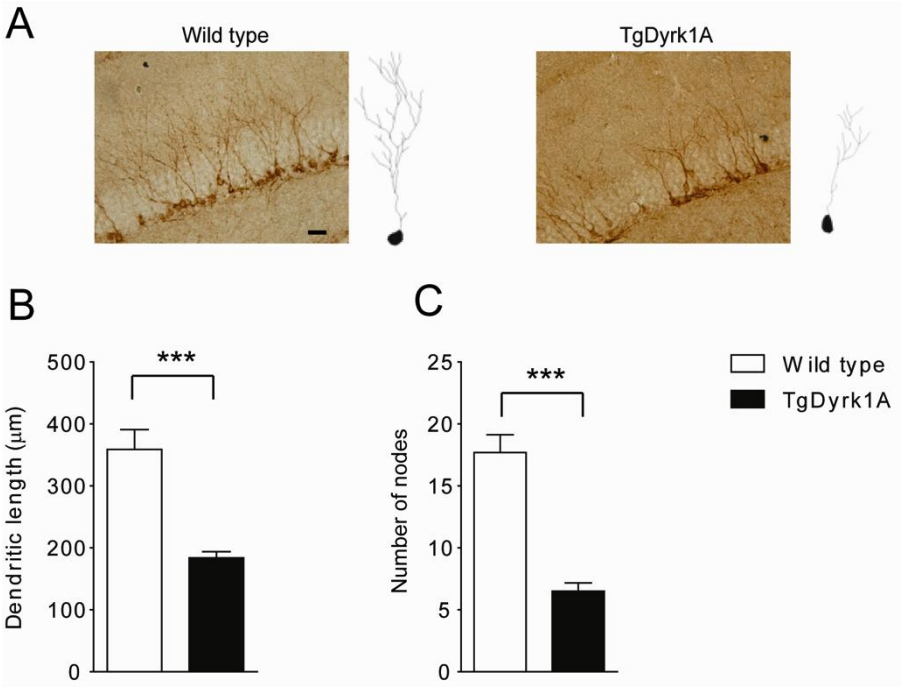

Figure 52. Dendritic morphology of newborn neurons in the DG. (A) Representative photomicrographs of DCX+ cells in the DG and digital reconstruction using the Neurolucida software in wild type and transgenic mice. Scale bar = 20µm. (B) Total dendritic length and (C) number of nodes were quantified in DCX+ cells of the DG. Wild type n = 20; TgDyrk1A n = 20; n = number of neurons. Data are represented as mean ± SEM. Student *t* test for independent samples *** $p<0.001$.

Surviving newborn neurons develop dendritic branching to interact with neurons of the preexisting network and become functional. Thus, altered microarchitecture of these structures could affect their final integration in the circuits. We analyzed the functional integration of surviving newborn cells co-immunostaining BrdU and cFos, an immediate early gene whose expression has been correlated with neuronal firing. In basal conditions, the percentage of double BrdU+ cFos+ cells respect to total BrdU+ cells was low in wild type DG (≈1.5%), and absent in TgDyrk1A DG (Figure 53B, TG *vs.* WT *t* test, t (1, 6) = 6.58, $p<0.001$).

Since the adult-generated granule cells are mainly active during spatial memory tasks, we examined whether Dyrk1A overexpression specifically altered the integration of newly born neurons (NeuN+) in those functional memory circuits. To this end, mice received four injections of BrdU and were tested one month later in a learning paradigm (MWM) (Figure 53A, and see Material and Methods section 3.2.4.3.). They were sacrificed one hour after the last session (probe trial) and the number of BrdU+ cFos+ NeuN+ cells was analyzed. TgDyrk1A mice showed a reduced percentage of newly formed neurons that were activated upon spatial learning, compared to wild type although it did not reach statistical significance (Figures 53C and 53D), suggesting a slightly impaired integration of newborn cells in the preexisting spatial learning functional circuits.

Importantly, after the spatial learning task the proportion of BrdU+ neurons expressing cFos in both genotypes was increased with respect to basal non-learning conditions, supporting the idea that newly generated cells are involved in activity-dependent learning processes (Koehl and Abrous 2011; Mongiat and Schinder 2011).

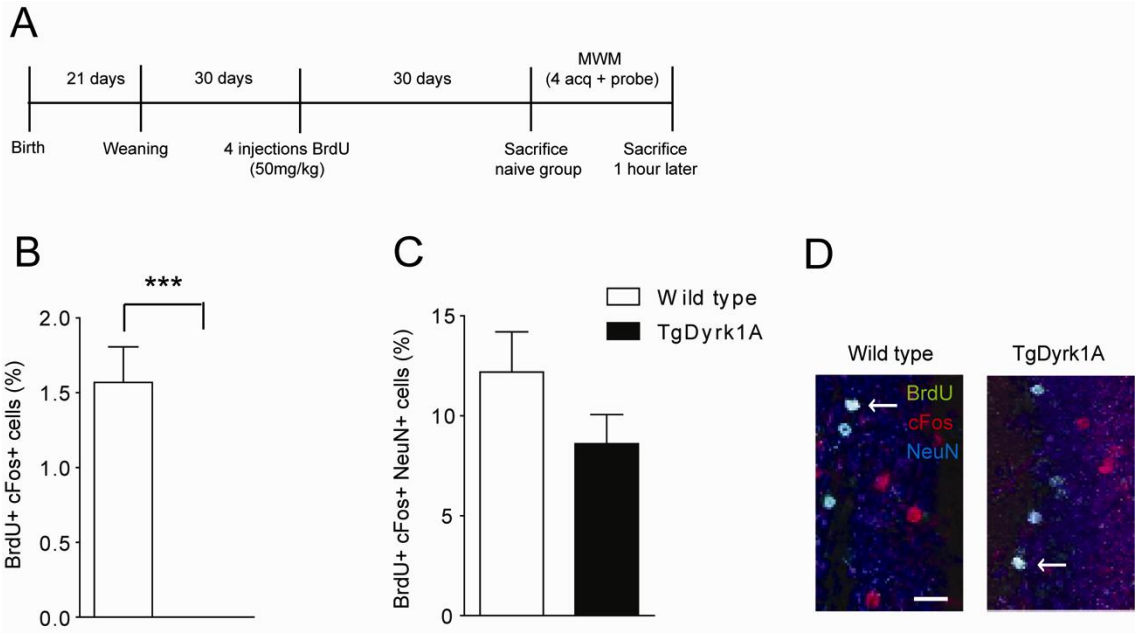

Figure 53. Activation of newborn neurons in the DG. (A) Experimental schedule used to analyze activation of newly formed neurons. (B) Percentage of BrdU+ cells coexpressing cFos in the naïve group that was not trained in the spatial learning task. (C) Percentage of BrdU+ cells coexpressing cFos and NeuN one hour after a probe session in the MWM. (D) Representative confocal photomicrographs showing BrdU+ NeuN+ cFos+ cells. Scale bar = 20μm. Arrows represent BrdU+ cFos+ NeuN+ cells. Wild type n = 6-8; TgDyrk1A n = 6-8. Data are represented as mean ± SEM. Student *t* test for independent samples *** $p<0.001$.

4.2.2.6. EGCG rescues cell proliferation alterations in the adult DG of TgDyrk1A mice

We next investigated if inhibition of DYRK1A kinase activity using EGCG would reverse the altered proliferation and cell cycle phase progression of neuronal progenitor cells in the TgDyrk1A.

Interestingly, EGCG significantly reduced the density of ki67+ cells (Figures 54A and 54B, EGCG treated TG *vs*. non treated TG one way ANOVA $F(1,17) = 4.86$, $p<0.05$; Bonferroni as *post-hoc:* $p<0.05$) and normalized the proportion of proliferating cells exiting cell cycle in TgDyrk1A mice (Figures 54C and 54D, EGCG treated TG *vs.* non treated TG one way ANOVA $F(1,11) = 28.74$, $p<0.001$; Bonferroni as *post-hoc:* $p<0.001$). Since EGCG restored cell proliferation and cell cycle exit alterations, we next evaluated whether EGCG was able to correct the differences in cell cycle phases. EGCG also normalized the density of pH3+ cells (Figures 54E and 54F, EGCG treated TG *vs.* non treated TG one way ANOVA $F(1,20) = 6.62$, $p<0.01$; Bonferroni as *post-hoc:* $p<0.05$) in the DG of TgDyrk1A mice and significantly restored the percentage of cells in G2 phase (Figures 54F and 54G, EGCG treated TG *vs.* non treated TG one way ANOVA $F(1,12) = 7.83$, $p<0.01$; Bonferroni as *post-hoc:* $p<0.05$). In addition, EGCG restored the proportion of pH3+ cells localized in the GCL of TgDyrk1A mice (Figure 54H, EGCG treated TG *vs.* non treated TG one way ANOVA $F(1,12) = 21.98$, $p<0.001$; Bonferroni as *post-hoc:* $p<0.05$) with a tendency for a specific effect in the localization of G2 phase pH3+ cells (Figure 54I, EGCG treated TG *vs.* non treated TG one way ANOVA $F(1,12) = 37.01$, $p<0.001$; Bonferroni as *post-hoc:* $p=0.08$). Taken together, these results indicate that cell proliferation and cell cycle alterations of TgDyrk1A might be DYRK1A kinase activity-dependent.

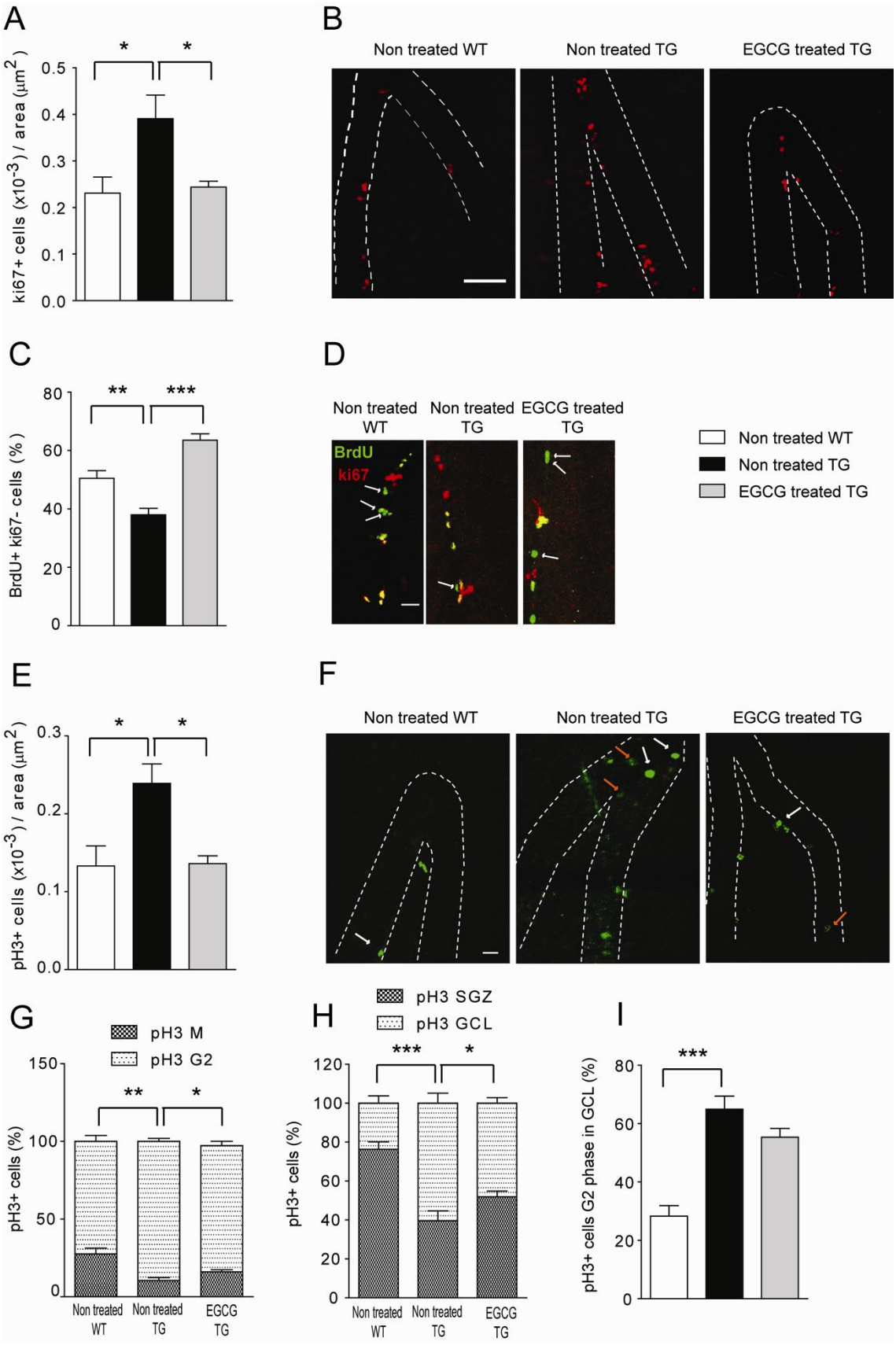

Figure 54. Specific involvement of DYRK1A kinase activity on proliferating neuronal progenitors in the DG. (A) Average density of ki67+ cells of wild type (WT) and transgenic (TG) mice untreated or EGCG treated TG mice. (B) Representative confocal micrographs showing ki67+ cells. Scale bar =

100μm. Non-treated WT n = 8; non-treated TG n = 8; EGCG treated TG n = 4. (C) Percentage of BrdU+ cells negative for ki67 observed twenty-four hours after one BrdU injection. (D) Representative confocal micrographs showing BrdU and ki67 + cells. Scale bar = 20μm. Arrows show BrdU+ ki67- cells (cells exiting cell cycle). Non treated WT n = 4; Non treated TG n = 4; EGCG treated TG n = 4. (E) Density of pH3+ cells. (F) Representative confocal micrographs showing pH3+ cells. Scale bar = 20μm. White arrows represent pH3+ cells suggestive of M phase. Orange arrows show pH3+ cells suggestive of G2 phase. (G) Percentage of pH3+ cells with a staining pattern suggestive of G2 or M phase. Non-treated WT n = 4-8; non-treated TG n = 4-8. EGCG treated TG n = 6. H) Percentage of pH3+ cells in the SGZ and GCL. (I) Percentage of pH3+ cells with a nuclear pattern suggestive of G2 phase detected in the GCL. Non-treated WT n = 4; non-treated TG n = 4. EGCG treated TG n = 6. Data are represented as mean ± SEM. Two way ANOVA Bonferroni as *post-hoc* * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

4.2.2.7. Environmental enrichment reverses adult neurogenesis impairments in the DG of TgDyrk1A mice

EE has a stimulating effect on adult hippocampal neurogenesis in rodents (Kempermann, Kuhn et al. 1997; van Praag, Christie et al. 1999; van Praag, Kempermann et al. 1999). We analyzed if one month of EE was able to rescue some of the alterations of adult neurogenesis observed in TgDyrk1A. In wild type mice, EE did not modify the cell proliferating rate (number of BrdU+ cells 2 hours post injection, Figures 55B and 55C) but it increased the density of ki67+ proliferating cells (Figures 55G and 55H, WTEE *vs.* WTNE two-way ANOVA genotype-treatment interaction $F(1,30)=6.76$, $p<0.05$; Bonferroni as *post-hoc:* $p<0.01$) without modifying the density of progenitor *type 1* or *type 2a* (Figures 55D and 55E). However, a slight increase in the proportion of double ki67+ Sox2+ actively proliferating progenitor cells upon EE (Figure 55F, two way ANOVA genotype-treatment interaction $F(1,15)=1.12$, $p=0.312$; two way ANOVA treatment effect $F(1,15)=3.68$, $p=0.08$), may suggest a tendency to an increased recruitment of precursor cells to enter the cell cycle in enriched wild types. In transgenic mice, EE induced a slight but non-significant increase in the density of BrdU+ cells two hours after a single BrdU injection (Figures 55B and 55C), but did not modify the levels of ki67 labeled cells (Figures 55G and 55H).

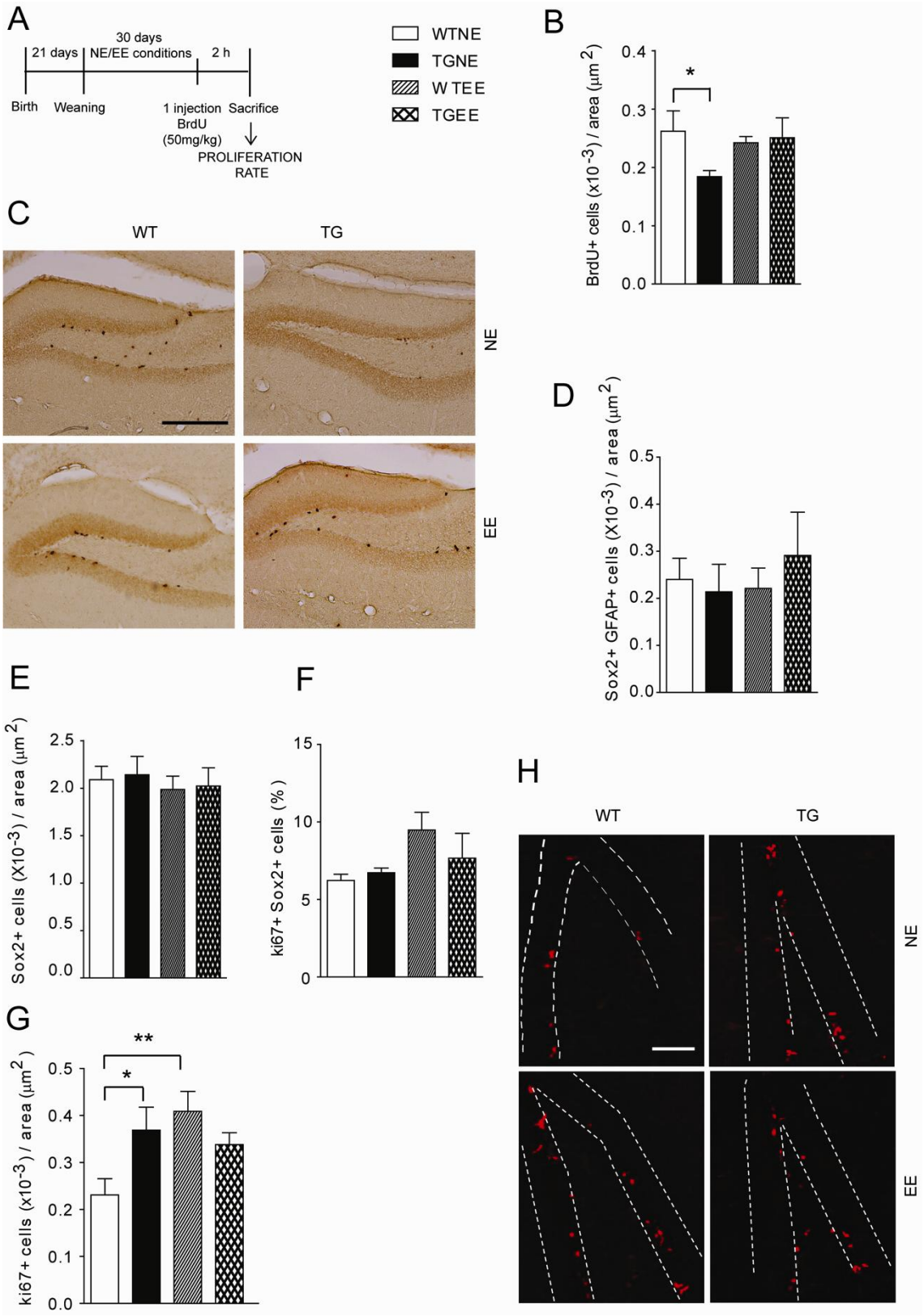

Figure 55. Effect of EE on proliferation of neuronal progenitors in the DG. (A) Experimental schedule used to analyze the proliferation rate under non-enriched (NE) or enriched (EE) conditions. (B) Average density of BrdU+ cells in the DG two hours after BrdU injection in wild type (WT) and transgenic (TG) mice reared under NE or EE conditions. (C) Representative photomicrographs of BrdU+

cells 2 hours after BrdU injection. Scale bar = 500μm. (D) Density of Sox2+ cells co-expressing GFAP (progenitor cells *type* 1). (E) Density of Sox2+ cells (progenitor cells *type 2a*). (F**)** Percentage of Sox2+ cells co-expressing ki67. (G) Average density of ki67+ cells. (H) Representative confocal micrographs showing ki67+ cells. Scale bar = 100μm. WTNE n = 4-8; TGNE n = 4-8; WTEE n = 4-8; TGEE n = 4-8. Data are represented as mean ± SEM. Two-way ANOVA Bonferroni as *post-hoc* * $p<0.05$; ** $p<0.01$.

An increased proportion of cells exiting cell cycle were observed upon EE in TgDyrk1A reaching the levels of wild type mice (Figures 56B and 56C, two-way ANOVA genotype-treatment interaction $F(1,16) = 1.078$, $p=0.32$; two-way ANOVA treatment effect $F(1,16) = 6.70$, $p<0.05$). Moreover, although EE did not modify cell cycle progression in wild type mice, EE produced an increase in the proportion of pH3+ cells with a staining pattern suggestive of M phase at expenses of a reduction of cells in G2 phase in TgDyrk1A mice (Figures 56E and 56H, TGEE *vs.* TGNE two-way ANOVA genotype-treatment interaction $F(1,16) = 4.84$, $p<0.05$; Bonferroni as *post-hoc*: $p<0.01$), thus normalizing the G2 and M proportion with respect to wild types. Also, EE reduced the proportion of pH3+ cells abnormally located in the GCL of DG in TgDyrk1A mice (Figure 56F, TGEE *vs.* TGNE two-way ANOVA genotype-treatment interaction $F(1,33) = 16.46$, $p<0.001$; Bonferroni as *post-hoc*: $p<0.001$) and the specific alteration of G2 phase pH3+ cells localization (Figure 56G, TGEE *vs.* TGNE two-way ANOVA genotype-treatment interaction $F(1,16) = 19.89$, $p<0.001$; Bonferroni as *post-hoc:* $p<0.001$).

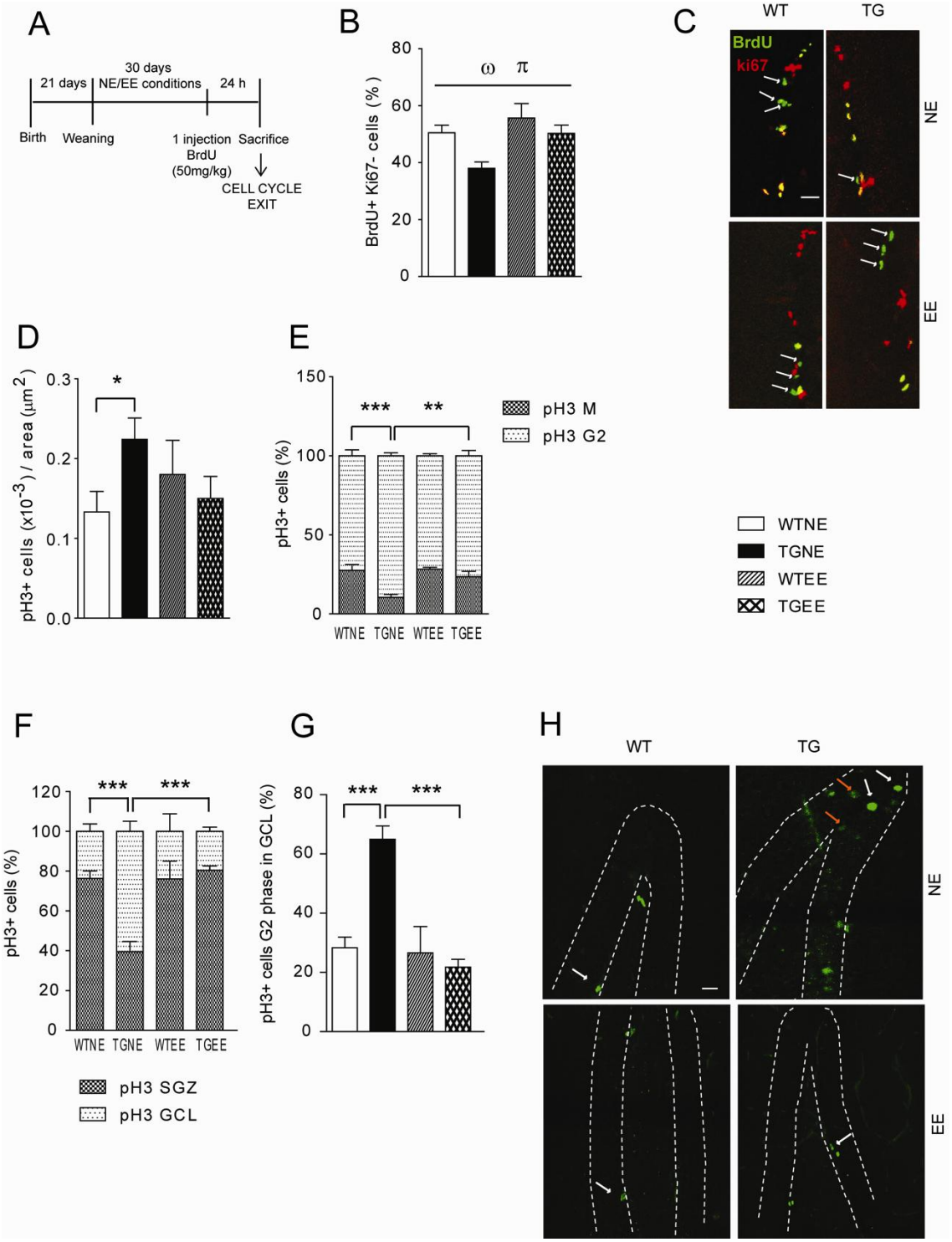

Figure 56. Effect of EE on cell cycle progression of newly proliferating cells in the DG. (A) Experimental outline used to analyze the cell cycle exit under NE or EE conditions. (B) Percentage of BrdU+ cells negative for ki67 observed twenty-four hours after one BrdU injection of wild type (WT) and transgenic (TG) mice reared under NE or EE conditions. (C) Representative confocal micrographs showing BrdU+ and ki67+ cells. Scale bar = 20μm. Arrows show cells exiting cell cycle (BrdU+ ki67- cells). WTNE n = 4; TGNE n = 4; WTEE n = 4; TGEE n = 4. (D) Density of pH3+ cells. (E) Percentage of pH3+ cells with a staining pattern suggestive of G2 or M phase. (F) Percentage of pH3+ cells in the

SGZ and GCL. (G) Percentage of pH3+ cells with a nuclear pattern suggestive of G2 phase detected in the GCL. (H) Representative confocal micrographs showing pH3+ cells. Scale bar = 20μm. White arrows represent pH3+ cells suggestive of M phase. Orange arrows show pH3+ cells suggestive of G2 phase. WTNE n = 4-8; TGNE n = 4-8; WTEE n = 4-8; TGEE n = 4-8. Data are represented as mean ± SEM. Two-way ANOVA genotype effect ω $p<0.05$; treatment effect π $p<0.05$. Two-way ANOVA Bonferroni as *post-hoc* * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

In the same line, EE prevented the premature migration to the GCL of newly born cells observed in TgDyrk1A, being the differences statistically significant 24 hours (Figures 57B and 57C, TGEE *vs.* TGNE two-way ANOVA genotype-treatment interaction $F(1,20) = 3.83$, $p=0.06$; Bonferroni as *post-hoc:* $p<0.05$) and 1 month (Figures 57B and 57D, TGEE *vs.* TGNE two-way ANOVA genotype-treatment interaction $F(1,21) = 3.203$, $p=0.08$; Bonferroni as *post-hoc:* $p<0.001$) after the last BrdU injection, reaching non enriched wild type levels.

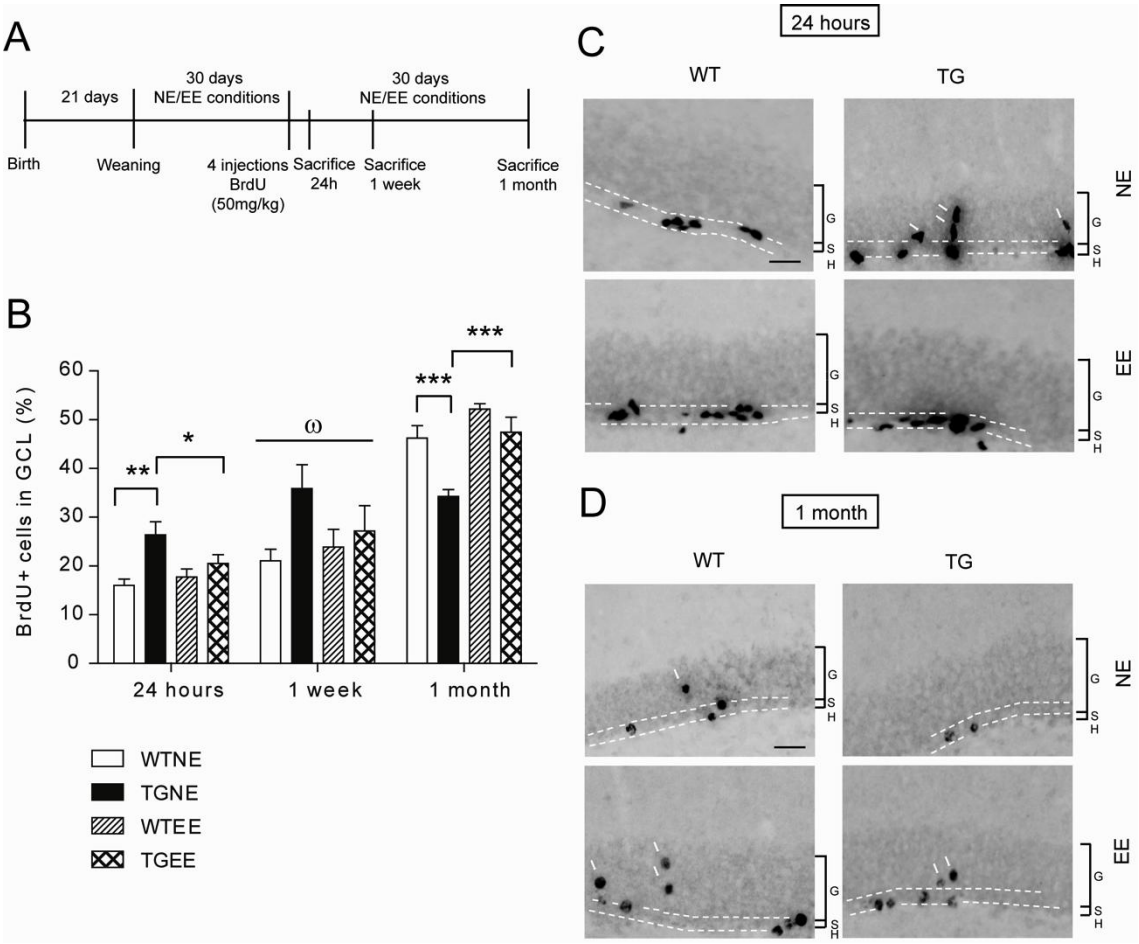

Figure 57. Effect of EE on migration of newly formed cells in the DG. (A) Experimental schedule used to analyze cellular migration in the DG under non-enriched (NE) or enriched (EE) conditions. (B) Proportion of BrdU+ cells localized in the GCL of the DG in wild type (WT) and transgenic (TG) mice reared under NE or EE conditions. (C-D) Representative photomicrographs of BrdU+ cells localization at

(C) 24 hours and (D) 1 month after BrdU injections. Arrows represent BrdU+ cells localized in the GCL. Scale bar = 20μm. WTNE n = 6; TGNE n = 6; WTEE n = 6; TGEE n = 6 in every time point of sacrifice. Data are represented as mean ± SEM. Two-way ANOVA genotype effect ω $p<0.05$. Bonferroni as *post-hoc* * $p<0.05$; ** $p<0.01$; *** $p<0.001$. G: Granular cell layer; S: Subgranular zone; H: Hilus.

One of the well-established processes that are regulated by EE is the survival of the newly formed neurons (Kempermann, Gast et al. 2003). EE significantly increased the density of BrdU+ cells in wild types and also in TgDyrk1A mice at all time points studied after BrdU injections (Figures 58A and 58B, EE effect at 24 hours: two-way ANOVA genotype-treatment interaction $F(1,20) = 1.59$, $p=0.222$; two-way ANOVA treatment effect $F(1,20)$, $p<0.01$; 1 week: two-way ANOVA genotype-treatment interaction $F(1,23) = 1.46$, $p=0.24$; two-way ANOVA treatment effect $F(1,23) = 21.63$, $p<0.001$; 1 month: two-way ANOVA genotype-treatment interaction $F(1,25) = 0$, $p=1$; two-way ANOVA treatment effect $F(1,25) = 49.01$, $p<0.001$). EE animals had similar ratios of increased surviving cells in both genotypes (1 week: ~20% and 1 month: ~10%), so that TgDyrk1A mice survival of newborn cells reached the levels of non-enriched wild type mice. In concordance with the increased density of surviving newly formed cells, we detected a reduced density of caspase3+ cells in EE mice as compared to NE ones (Figures 58C and 58D, two-way ANOVA genotype-treatment interaction $F(1,12) = 2.85$, $p=0.12$; two-way ANOVA treatment effect $F(1,12) = 11.2$, $p<0.01$). However, the final number of granule cells in the DG was not affected (Figure 58E).

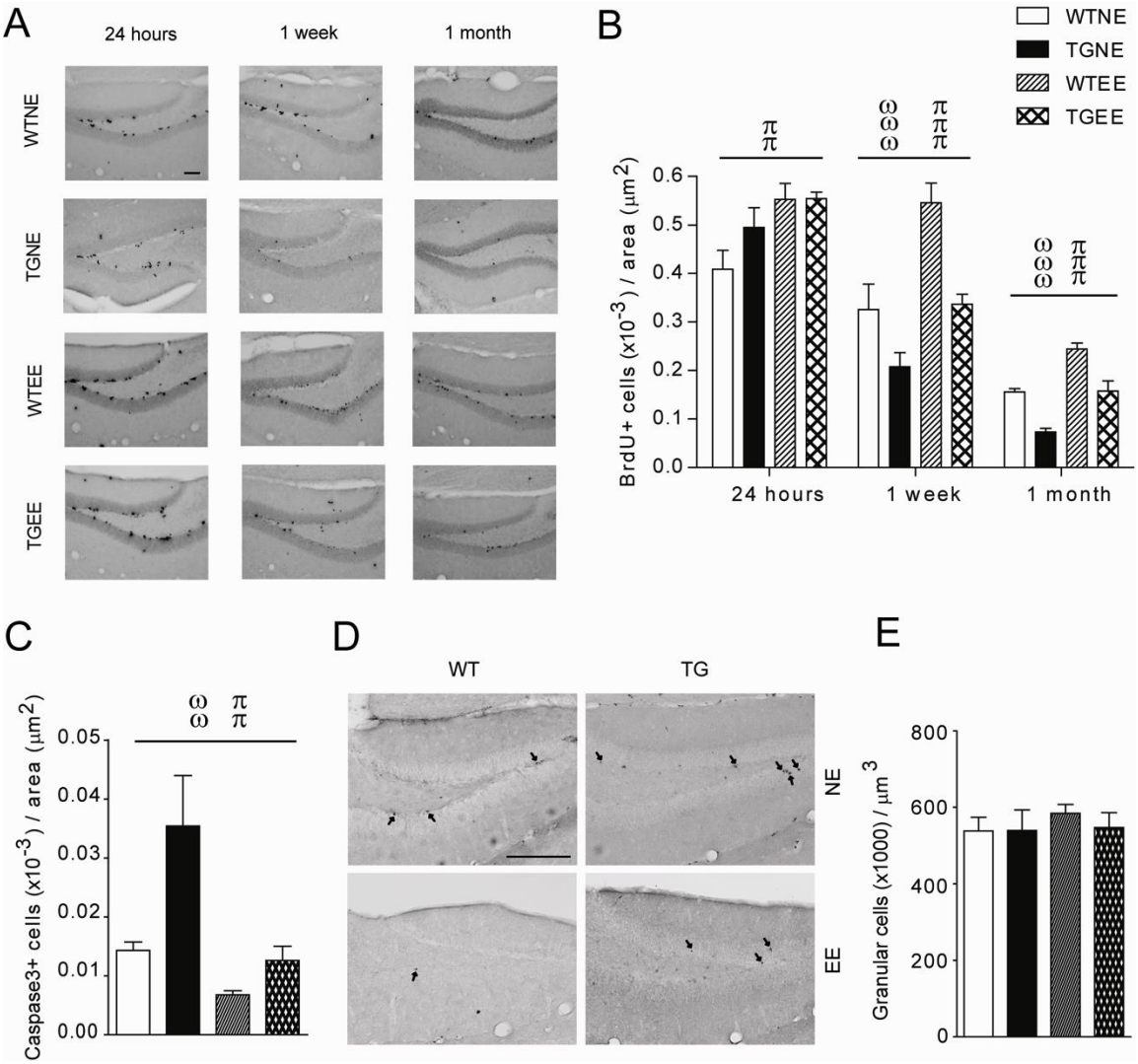

Figure 58. Effect of EE on survival and cell death of newly born granule cells. (A) Representative photomicrographs of BrdU+ cells at 24 hours, 1 week and 1 month after BrdU injection in wild type (WT) and transgenic (TG) mice reared under non-enriched (NE) or enriched (EE) conditions. Scale bar = 100μm. (B) Average density of BrdU+ cells in the DG at 24 hours, 1 week and 1 month after BrdU injection. (C) Average density of caspase 3+ cells. (D) Representative photomicrographs of caspase3+ cells. Scale bar = 500μm. Arrows represent caspase3+ cells. (E) Granule cell density in the DG. WTNE n = 4-6; TGNE n = 4-6; WTEE n = 4-6; TGEE n = 4-6. Data are represented as mean SEM. Two-way ANOVA genotype effect ωω p<0.01; ωωω p<0.001; treatment effect ππ p<0.01; πππ p< 0.001.

Importantly, the recovering effects of EE in TgDyrk1A mice on cell proliferation, cell cycle progression, migration and survival had an impact on the proportion of newly generated cells differentiating towards a neuronal phenotype. Indeed, EE increased the percentage of one week BrdU+ DCX+ (Figures 59A and 59C, two-way ANOVA genotype-treatment interaction $F(1,16) = 0.47$, $p=0.509$; two-way ANOVA treatment effect $F(1,16) = 5.91$, $p<0.05$) and BrdU+ calretinin+ surviving cells (Figure 59B, two-

way ANOVA genotype-treatment interaction F(1,16) = 0.56, p=0.46; two-way ANOVA treatment effect F(1,16) = 12.92, p<0.01) in both genotypes being the proportion completely rescued in transgenic mice. In the same line, EE significantly increased the density of surviving newborn neurons (BrdU+ NeuN+ cells) one month after BrdU injections in both genotypes (Figure 59D, two-way ANOVA genotype-treatment interaction F (1,21) = 0.125, p=1; two-way ANOVA treatment effect F (1,21), p<0.01).

As described above, although the percentage of cells differentiating into neurons was not affected by genotype or rearing conditions (Figure 59E), EE was able to restore the proportion of cells differentiating into glia in TgDyrk1A mice (Fig. 59F, TGEE *vs.* TGNE two-way ANOVA genotype-treatment interaction F (1,18) = 27.103, p<0.001; Bonferroni as *post-hoc:* p<0.001) without affecting wild types.

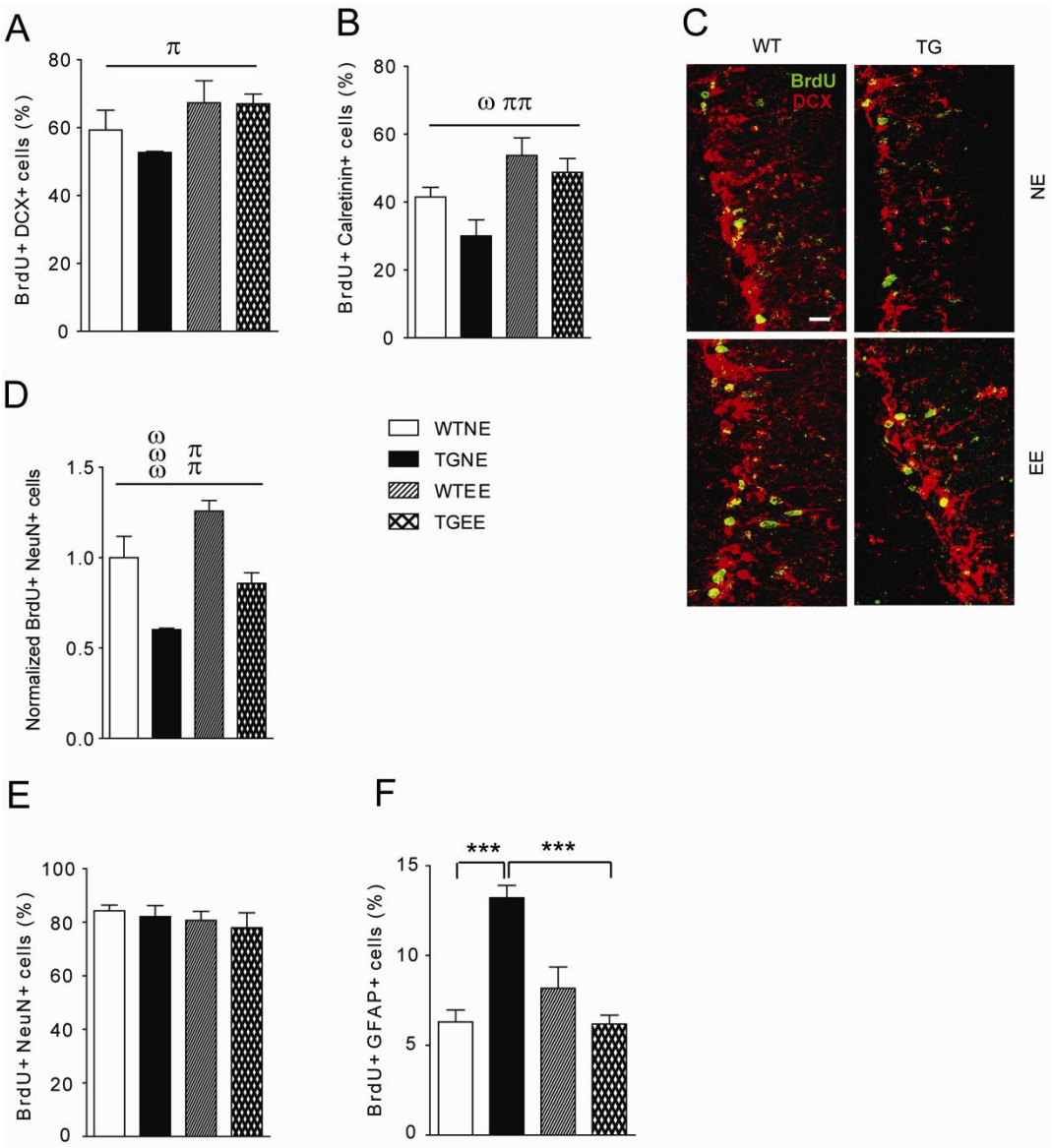

Figure 59. Effect of EE on cell fate of newly born granule cells. (A-B) Percentage of BrdU+ cells expressing (A) DCX or (B) calretinin one week after BrdU injections. (C) Representative confocal micrographs showing BrdU and DCX cells. Scale bar = 20m. (D) Average density of BrdU+ NeuN+ cells in the DG one month after BrdU injections normalized respect to WTNE mice. (E-F) Percentage of BrdU+ cells expressing (E) NeuN and (F) GFAP after 1 month of the last BrdU injection. WTNE n = 4-6; TGNE n = 4-6; WTEE n = 4-6; TGEE n = 4-6. Data are represented as mean SEM. Two-way ANOVA genotype effect ω $p<0.05$; ωωω $p<0.001$; treatment effect π $p<0.05$; ππ $p< 0.01$. Bonferroni as *post-hoc* *** $p<0.001$.

Interestingly, when we analyzed the dendritic morphology of newly formed immature neurons (DCX+ cells), EE significantly increased the total dendritic length (Figures 60A and 60B, two-way ANOVA genotype-treatment interaction $F(1,80) =7.098$, $p<0.01$; WTEE *vs.* WTNE Bonferroni as *post-hoc:* $p<0.001$; TGEE *vs.* TGNE Bonferroni as *post-hoc:* $p<0.001$) and number of nodes (Figures 60A and 60C, two-way ANOVA genotype-treatment interaction $F(1,80) =2.16$, $p<0.09$; WTEE *vs.* WTNE Bonferroni as *post-hoc:* $p<0.001$; TGEE *vs.* TGNE Bonferroni as *post-hoc:* $p<0.001$) in both genotypes, indicating that newly formed neurons were sensitive to environmental stimuli as was previously described (van Praag et al., 2000). In TgDyrk1A EE achieved a complete normalization of the abnormal dendritic microarchitecture.

Finally, when we analyzed the functional integration of surviving cells by co-immunostaining BrdU and cFos, we observed that EE was able to increase the percentage of BrdU+ cFos+ cells in the TgDyrk1A mice restoring basal activation to wild type levels (Figure 60E, TGEE *vs.* TGNE two-way ANOVA genotype-treatment interaction $F(1,16) =7.41$, $p<0.05$; Bonferroni as *post-hoc:* $p<0.001$), but had no effect on the number of basal BrdU+ cFos+ cells in wild types.

However, upon hippocampal-dependent spatial stimulation, EE significantly increased the proportion of activated newly generated neurons (BrdU+ NeuN+ cFos+ cells) in both genotypes (Figure 60F, two-way ANOVA genotype-treatment interaction $F(1,28) =0.754$, $p=0.394$; two-way ANOVA treatment effect $F(1,28) = 25.86$, $p<0.001$), rescuing the impairment observed in transgenic mice. Therefore, EE could lead to an improvement in the circuitry functionality favoring the performance in hippocampal-dependent spatial learning tasks.

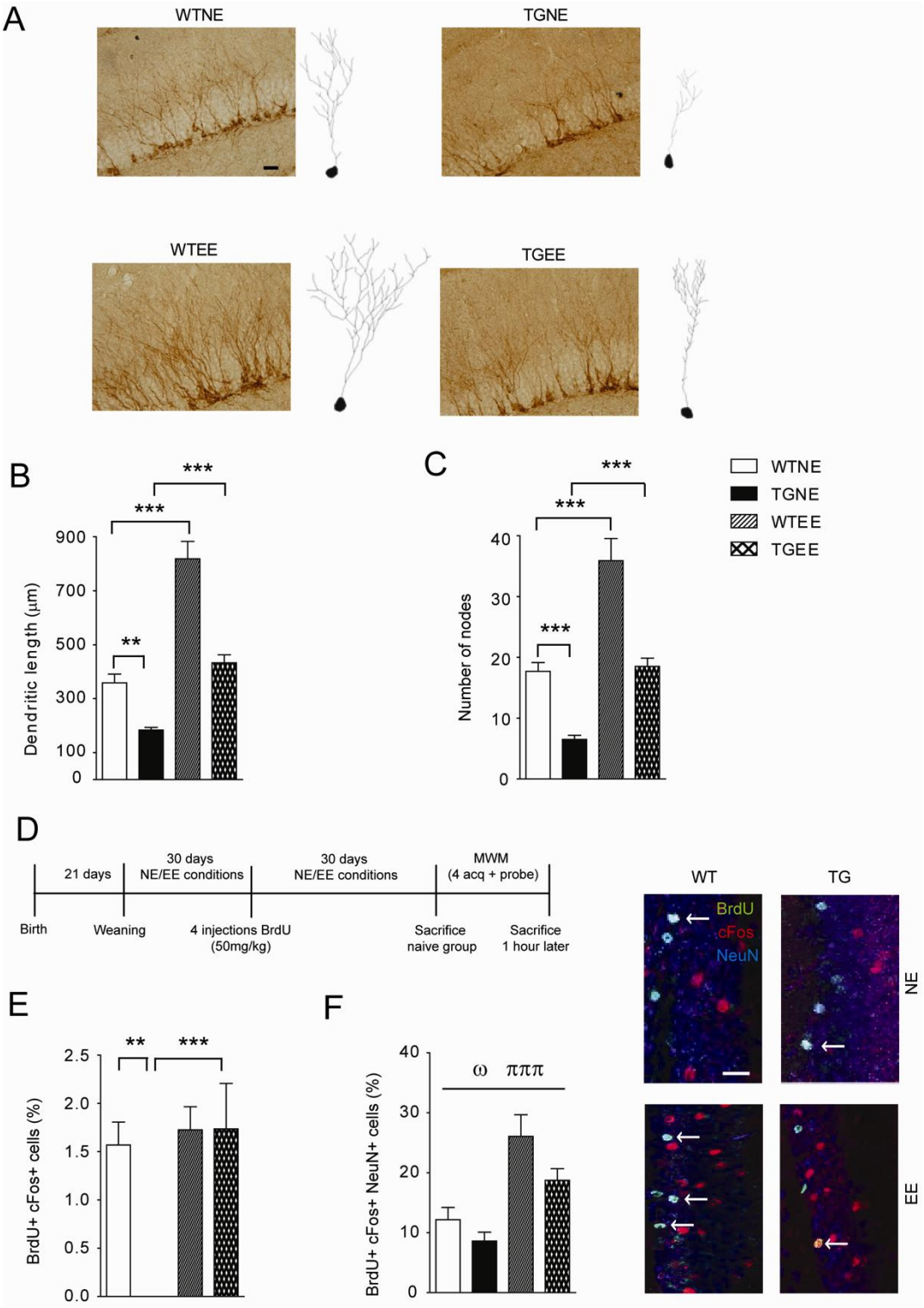

Figure 60. Effect of EE on dendritic morphology and activation upon learning of newborn hippocampal neurons. We analyzed the morphology (A,B,C) and activation pattern (D,E,F) of hippocampal newborn cells to determine their functional integration in the circuits. (A) Representative photomicrographs of DCX+ cells in the DG and digital reconstruction using the Neurolucida software in wild type (WT) and transgenic (TG) mice reared under non-enriched (NE) or enriched (EE) conditions. Scale bar = 20µm. (B) Total dendritic length and (C) number of nodes per neuron were quantified in

DCX+ cells of the DG. The number of neurons analyzed was 20 per genotype and condition. (D) Experimental schedule used to analyze activation of newly formed neurons. (E) Percentage of BrdU+ cells expressing cFos in the naïve group that was not trained in the spatial learning task. (F) Percentage of BrdU+ cells co-expressing cFos and NeuN one hour after the probe session in the MWM (left panel) and representative confocal photomicrographs (right panel). Scale bar = 20μm. Arrows represent BrdU+ cFos+ NeuN+ cells. WTNE n = 6-8; TGNE n = 6-8; WTEE n = 6-8; TGEE n = 6-8. Data are represented as mean SEM. Two-way ANOVA genotype effect ω $p<0.05$; treatment effect πππ $p< 0.001$. Bonferroni as *post-hoc* ** $p<0.01$; *** $p<0.001$.

4.2.2.7.1. Environmental enrichment normalizes Dyrk1A expression in TgDyrk1A DG

Given that one month of EE normalized DYRK1A kinase activity in the hippocampus of TgDyrk1A mice, we also evaluated whether EE could affect the levels of Dyrk1A expression in the DG by immunofluorescence. Importantly, one month of EE was also able to normalize Dyrk1A expression in the DG of TgDyrk1A mice (Figure 61).

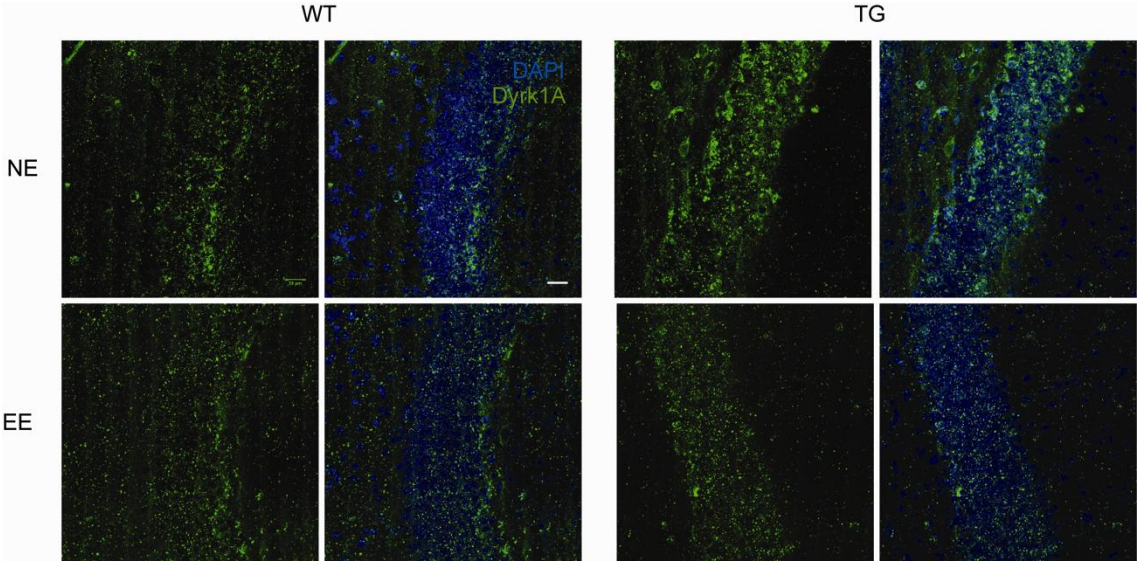

Figure 61. Dyrk1A expression in the DG. (A) Representative confocal micrographs showing DYRK1A protein expression in TgDyrk1A mice under normal and enriched rearing conditions in the DG. Scale bar = 20μm.

These data suggested that the rescuing effects of EE on adult neurogenesis, hippocampal structural plasticity and cognition in TgDyrk1A mice could be due, at least in part, through DYRK1A kinase activity and expression normalization in the hippocampus.

4.3. IMPACT OF *DYRK1A* OVEREXPRESSION ON THE STABILITY OF ACTIVITY-DEPENDENT HIPPOCAMPAL COGNITION AND NEURONAL PLASTICITY EFFECTS INDUCED BY ENVIRONMENTAL ENRICHMENT

Some studies have reported that the cognitive improvements upon early intervention programs in DS individuals are limited and temporary. Here, we evaluated whether the effects of EE on cognition and neuronal plasticity were maintained after four months. To this end, after one month of EE rearing conditions, we reared mice under NE conditions during 4 months (Figure 62).

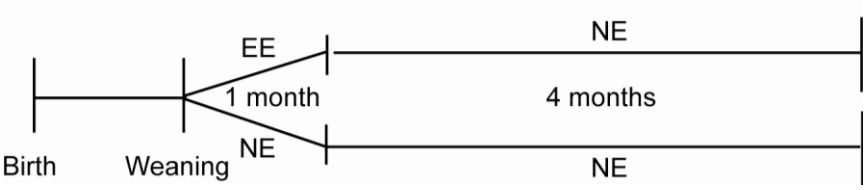

Figure 62. Experimental schedule. Representation of the experimental schedule used to analyze long-term effects of EE in wild type and TgDyrk1A mice reared during five months of non enriched conditions (NE-NE) or one month of environmental enriched followed by four months of NE conditions (EE-NE).

4.3.1. Environmental enrichment produces stable cognitive improvements in visuospatial learning and memory but failed to maintained recognition memory in TgDyrk1A mice

First, we examined whether EE hippocampal-dependent learning and memory improvements were stable along time in TgDyrk1A mice.

4.3.1.1. Long-term effects of EE on visuo-spatial learning and memory: Morris Water Maze (MWM)

Similar to the observation in young transgenic mice, at six month of age TgDyrk1A showed learning impairment, though no significant differences were reached among groups when the time to reach the hidden platform during acquisition session was analyzed (Figure 63). Both enriched wild type and transgenic mice showed better performance in the hippocampal-dependent learning (Figure 63) thus indicating that the

cognitive improvements attained by EE where stable four months after the EE treatment was discontinued.

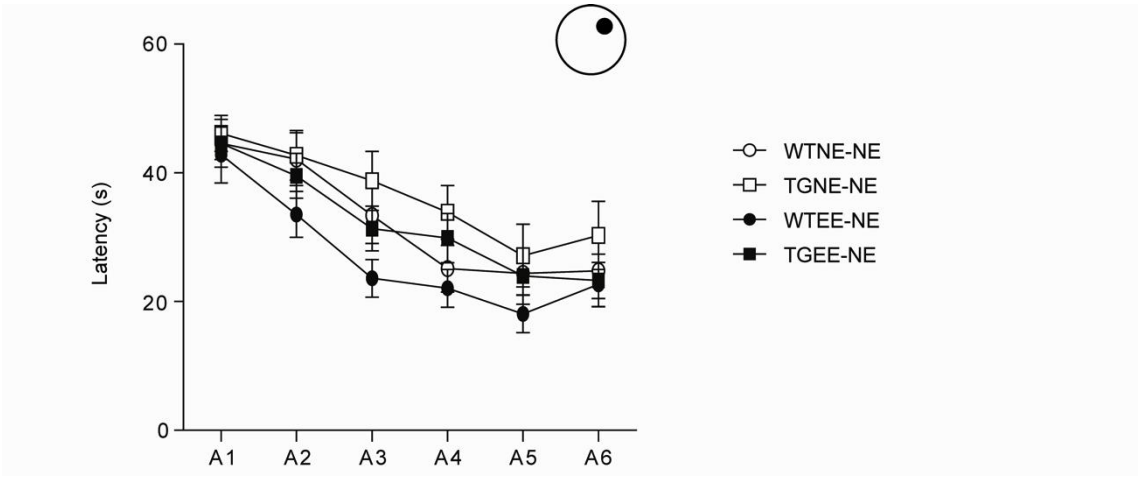

Figure 63. Long-term effects of EE on visuo-spatial learning in TgDyrk1A mice. (A) Latency to reach the platform along the learning sessions (A1-A6) in wild type (WT) and TgDyrk1A (TG) mice reared during five months in non enriched conditions (NE-NE) or one month of environmental enriched followed by four months of NE conditions (EE-NE)**.** WTNE-NE = 17; TGNE-NE n = 12; WTEE-NE n = 15; TGEE-NE n= 16. Data were represented as mean ± SEM. A: Acquisition.

We next evaluated the long-term effects of EE on reference memory in the probe test. Again, six-month old NE-TgDyrk1A mice showed a significant impairment in the reference memory compared to wild types (Figure 64, WTNE-NE paired *t* test, t (1,17) = 2.01, $p<0.05$; TGNE-NE paired *t* test, t (1,12) = 1.26, $p=0.23$), that was more pronounced as compared to young mice, suggesting the initiation of neurodegeneration processes. EE mice improved reference memory in both genotypes (Figure 64, WTEE-NE paired *t* test, t (1,15) = 3.94, $p<0.001$; TGEE-NE paired *t* test, t (1,16) = 4.60, $p<0.001$), indicating stable effects in reference memory after four months.

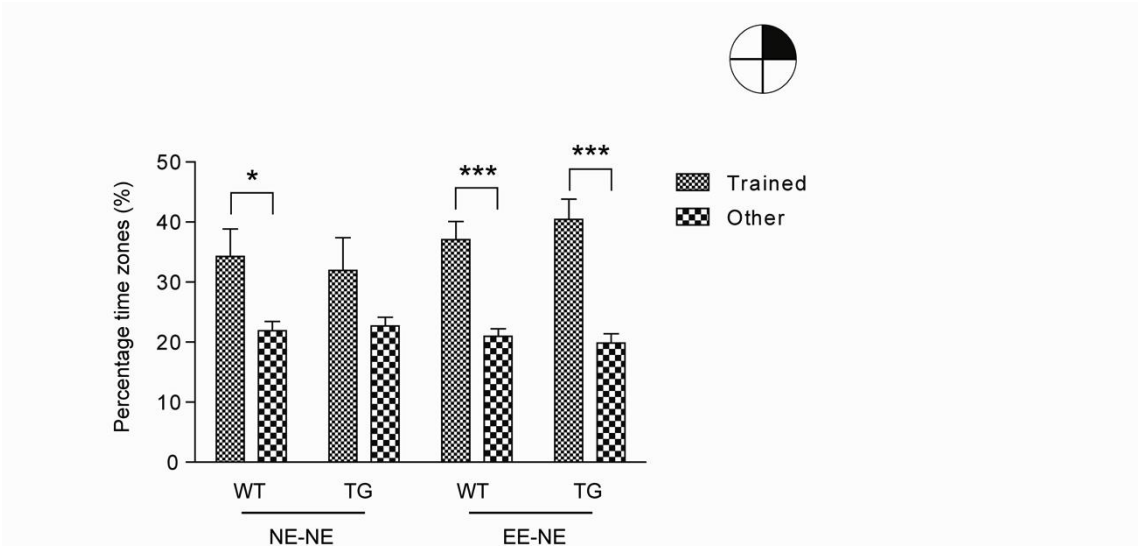

Figure 64. Long-term effects of EE on reference memory in TgDyrk1A mice. (A) Percentage of time spent in the trained zone compared to the non-trained quadrants during the probe test in non-enriched or previously enriched conditions. WTNE-NE = 17; TGNE-NE n = 12; WTEE-NE n = 15; TGEE-NE n= 16. Data were represented as mean ± SEM. Student *t* test for paired samples * $p<0.05$; *** $p<0.001$.

During the reversal sessions we evaluated whether EE produce long-term effects on cognitive flexibility. Opposite to young animals, at six months of age, only a slight impairment was detected in TgDyrk1A compared to wild types (Figure 65A). The effects of EE where maintained in time, and enriched mice of both genotypes showed a trend to reduce the time to reach the platform. Moreover, during the last reversal session, even though all groups were able to significantly discriminate where the new platform was (Figure 65B, WTNE-NE paired *t* test, $t\ (1,17) = 3.39$, $p<0.01$; TGNE-NE paired *t* test, $t\ (1,12) = 3.89$, $p=0.01$) EE mice spent significantly more time in the new position as compared to mice that never received EE (Figure 65B, WTEE-NE paired *t* test, $t\ (1,15) = 4.09$, $p<0.001$; TGEE-NE paired *t* test, $t\ (1,16) = 4.96$, $p<0.001$). These results suggest that one month of EE produces stable effects on hippocampal dependent visuo-spatial learning and memory and on cognitive flexibility after four months of discontinuation of the treatment in both genotypes.

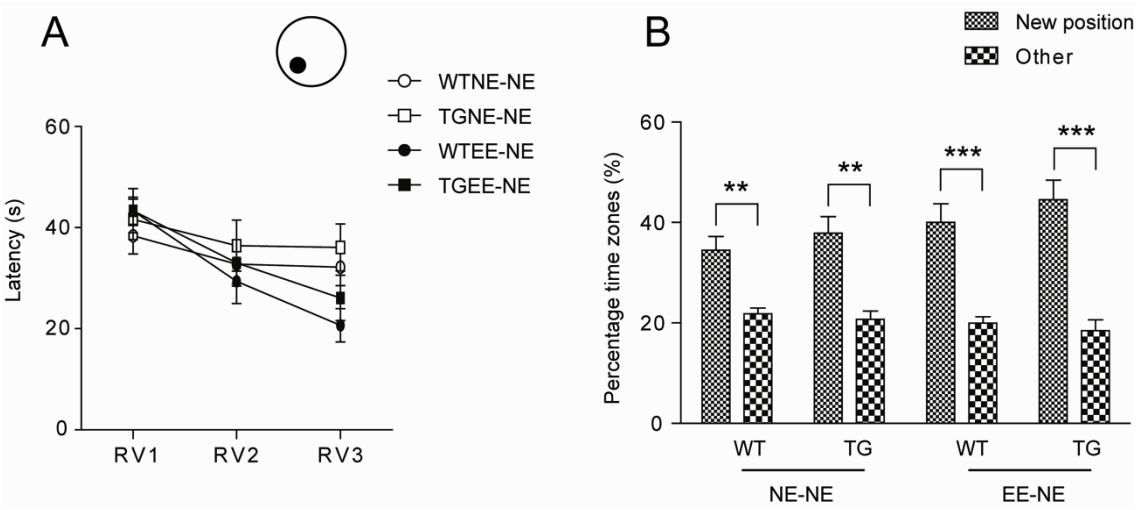

Figure 65. Long-term effects on cognitive flexibility in TgDyrk1A mice. (A) Latency to reach the platform in the new position along the reversal sessions (RV1-3) in wild type (WT) and transgenic (TG) mice reared during five months of non enriched conditions (NE-NE) or one month of environmental enriched followed by four months of NE conditions (EE-NE)**.** (B) Percentage of time spent in the new quadrant compared to the other quadrants during the last reversal session (RV3) in enriched and non-enriched conditions WTNE-NE = 17; TGNE-NE n = 12; WTEE-NE n = 15; TGEE-NE n= 16. Data were represented as mean ± SEM. Student *t* test for paired samples ** $p<0.01$; *** $p<0.001$. RV: Reversal.

4.3.1.2. Long-term effects of EE on short-term recognition memory: Novel Object Recognition (NOR)

We next evaluated the long-term effects of EE in a cortico-hippocampal dependent task such as the NOR. In this experiment no differences were observed among groups when the total time of exploration was analyzed neither in the familiarization nor in the test phase (Figures 66A and 66B). Six-month-old TgDyrk1A mice that never received EE presented an impairment in object discrimination compared to control mice (Figure 66C, TGNE-NE *vs.* WTNE-NE two-way ANOVA genotype-treatment interaction $F(1,60) = 1.26$, $p=0.266$; two-way ANOVA genotype effect $F(1,60) = 17.34$, $p<0.0001$). However, opposite to what we found in the MWM, the effects of EE in previously enriched TgDyrk1A mice were not maintained after 4 months of enrichment discontinuation (Figure 66C).

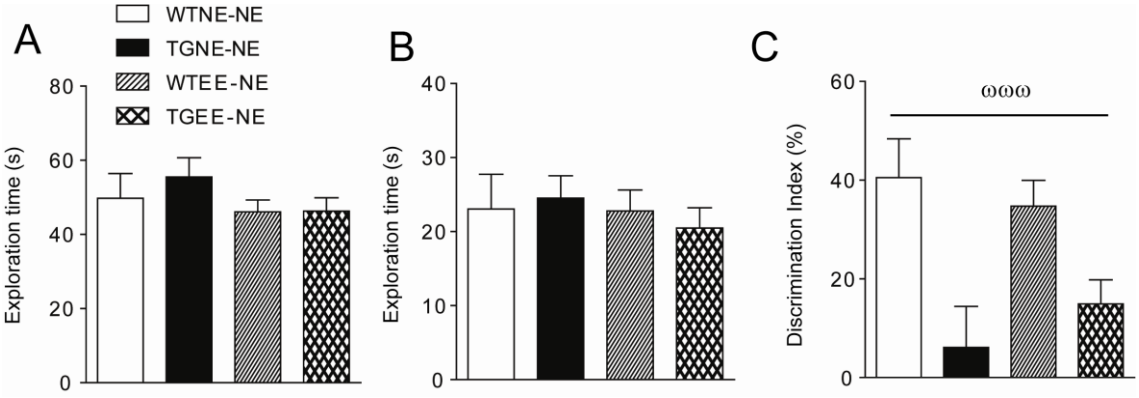

Figure 66. Long-term effects of EE on Novel Object Recognition in TgDyrk1A. (A) Average time of exploration during the familiarization session in wild type (WT) and TgDyrk1A (TG) mice reared during five months of non enriched conditions (NE-NE) or one month of environmental enriched followed by four months of NE conditions (EE-NE). (B) Average time of exploration during the test session. (C) Discrimination index during the test session in the NOR. Data are expressed as ± SEM. WTNE-NE n = 11; TGNE-NE n = 14; WTEE-NE n = 18; TGEE-NE n = 17. Two-way ANOVA Genotype effect ωωω p<0.001.

4.3.2. The effects of environmental enrichment on hippocampal structural and synaptic plasticity are not maintained after discontinuation of the treatment

As for the structural plasticity experiments described above, we used Thy1-YFP/TgDyrk1A mice and their control littermates reared under EE conditions for one month and under NE conditions during four months. The structural morphology of the different hippocampal subregions was again analyzed (Figure 66A). After discontinuation, the rescuing effects of EE were lost in TgDyrk1A mice. Thus, both enriched and non-enriched Thy-YFP/TgDyrk1A mice showed a significant reduction of CA1 *stratum oriens* (Figures 67A and 67B, two-way ANOVA genotype-treatment interaction F (1,26) =0.511, p=0.482; two-way ANOVA genotype effect F(1,26) =5.48, p<0.05) and *stratum radiatum* width (Figures 67A and 67C, two-way ANOVA genotype-treatment interaction F (1,26) =0.02, p=0.885; two-way ANOVA genotype effect F(1,26) =35.75, p<0.001) compared to wild types. Similarly, both groups of Thy-YFP/TgDyrk1A mice showed increased pyramidal cell area compared to their control littermates (Figures 67A and 67E, two-way ANOVA genotype-treatment interaction F (1,26) =0.217, p=0.646; two-way ANOVA genotype effect F(1,26) =13.16, p<0.001). Thus, the recovering structural effects observed in CA1 subregion produced by one month EE in Thy-YFP/TgDyrk1A mice were lost after four months without treatment.

As in two-months-old transgenic mice, no differences between groups were observed when we analyzed the area of CA3 region pyramidal layer (Figures 67C and 67E), but Thy-YFP/TgDyrk1A mice showed a significant reduced numbers of YFP+ cells in CA3 pyramidal layer. This alteration, was not modified by rearing conditions (Figures 67C and 67D, two-way ANOVA genotype-treatment interaction $F(1,26) = 2.25$, $p = 0.147$; two-way ANOVA genotype effect $F(1,26) = 100.183$, $p < 0.001$).

Regarding the DG, EE Thy-YFP/TgDyrk1A mice showed a reduced dendritic width in the molecular layer as compared to never enriched Thy-YFP/TgDyrk1A (Figure 67F, TGEE-NE vs. TGNE-NE two-way ANOVA genotype-treatment interaction $F(1,26) = 7.03$, $p < 0.01$; Bonferroni as post-hoc $p < 0.05$) an effect that was not observed in young mice (see fig 35F), thus suggesting that it is not related to EE. Moreover, we observed a significant increase in the granular layer width in never enriched Thy-YFP/TgDyrk1A mice as compared to their littermates (Figure 67F, TGNE-NE vs. WTNE-NE two-way ANOVA genotype-treatment interaction $F(1,26) = 4.86$, $p < 0.05$; Bonferroni as post-hoc $p < 0.05$) that was probably associated to age, and recovered in enriched Thy-YFP/TgDyrk1A mice (Figure 67F, TGEE-NE vs. TGNE-NE Bonferroni as post-hoc $p < 0.05$). This may suggest that EE could have protective effects against age-related phenotypes.

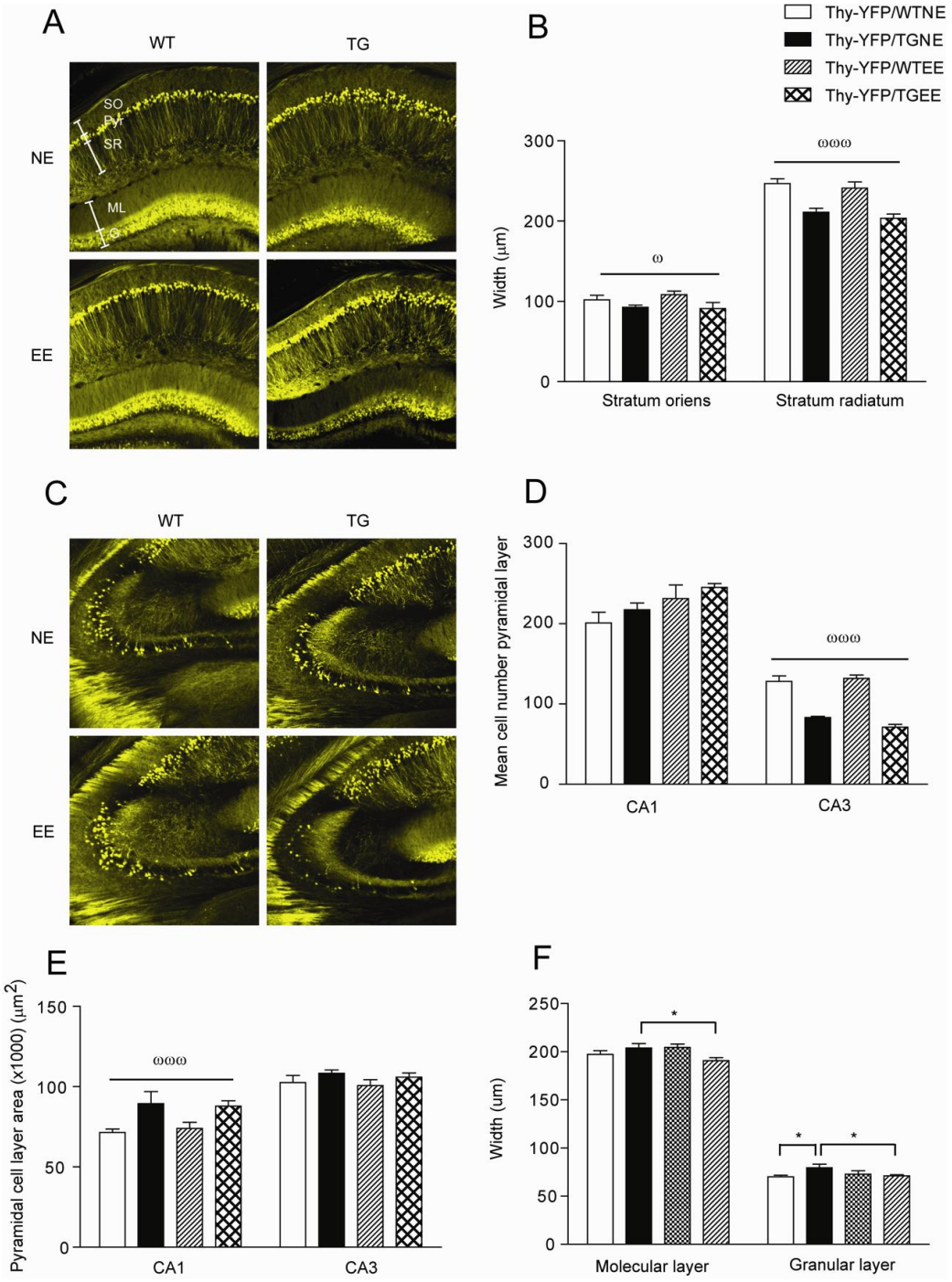

Figure 67. Long-term effects of EE on hippocampal structural plasticity in TgDyrk1A mice. (A) Representative confocal micrographs showing Thy1-YFP+ cells in the dentate gyrus and CA1 region in wild type (WT) and TgDyrk1A (TG) mice reared during five months of non enriched conditions (NE) or one month of environmental enriched followed by four months of NE conditions (EE). (B) Average width of *stratum oriens* and *stratum radiatum* in CA1 region. (C) Representative confocal micrographs of Thy1-YFP+ cells in CA3 region. (D) Mean number of Thy1-YFP+ pyramidal cells in the CA1 and CA3 regions. (E) Area of Thy1-YFP+ pyramidal layer in CA1 and CA3 regions. (F) Dendritic molecular layer and granular layer width in the DG. Data are expressed as ± SEM. Thy-YFP/WTNE n = 8; Thy-YFP/TGNE n = 6; Thy-YFP/WTEE n = 6; Thy-YFP/TGEE n = 6. Two-way ANOVA Genotype effect ω $p<0.05$; ωωω $p<0.001$. Bonferroni as *posthoc* * $p<0.05$.

We also found that the effects of EE on LTP in TgDyrk1A mice were lost after four months of treatment discontinuation (see results in Annex III). We next tested whether the EE effects on excitatory-inhibitory balance were maintained. Interestingly, four months after EE treatment, the VGLUT1/VGAT ratio was reduced in both genotypes as compared to two-months-old EE mice. However, while enriched wild type mice maintain the balance between excitatory and inhibitory vesicles (Figures 68A and 68B), in enriched TgDyrk1A the rescuing effects of EE on the excitatory-inhibitory balance are lost (Figures 68A and 68B, two-way ANOVA genotype-treatment interaction $F(1,20) = 0.001$, $p=0.96$; two-way ANOVA genotype effect $F(1,20) = 8.007$, $p<0.01$).

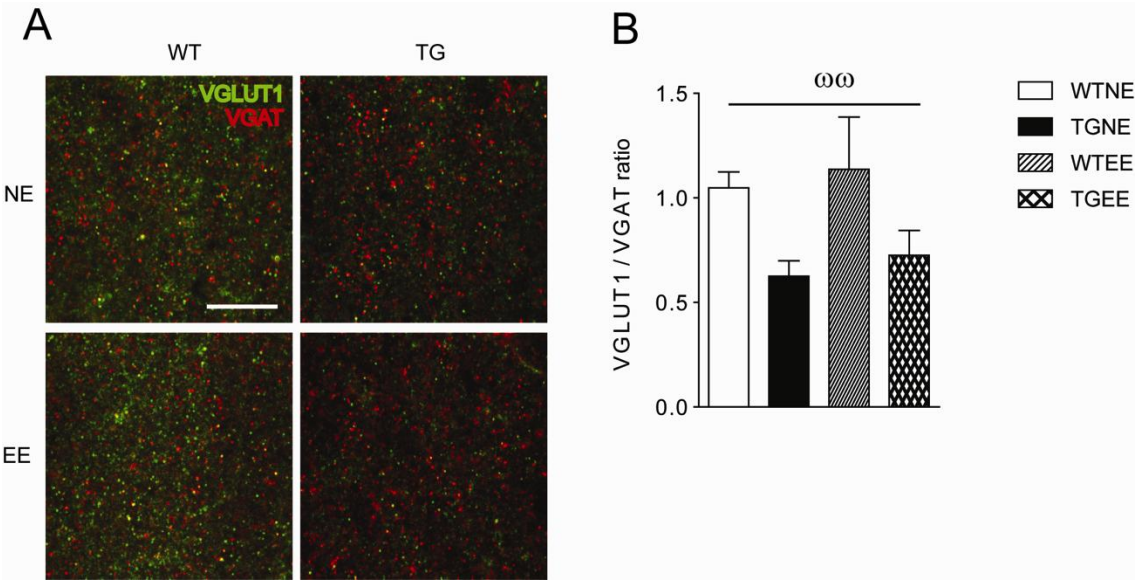

Figure 68. Long-term effects of EE on excitatory-inhibitory balance in CA1 stratum radiatum. (A) Representative confocal images showing VGLUT1 and VGAT puncta in CA1 stratum radiatum hippocampal subfield in wild type (WT) and TgDyrk1A (TG) mice reared during one month under NE or EE conditions followed by four months under NE conditions. Scale bar = 10 μm. (B) Histograms showing the ratio between VGLUT1 and VGAT puncta. Data are expressed as ± SEM. WTNE = 5; TGNE = 6; WTEE = 5; TGEE = 4. Two-way ANOVA genotype effect ωω $p<0.01$.

4.3.3. Increased DYRK1A kinase activity in previously enriched TgDyrk1A hippocampus

Our results indicated that the normalization of DYRK1A kinase activity in TgDyrk1A mice exposed to EE was lost after discontinuation of the treatment, and thus a significant increase was detected in transgenic mice regardless the rearing conditions (Figure 69, two-way ANOVA genotype-treatment interaction $F(1,45) = 0.468$, $p=0.498$; two-way ANOVA genotype effect $F(1,45) = 8.85$, $p<0.01$).

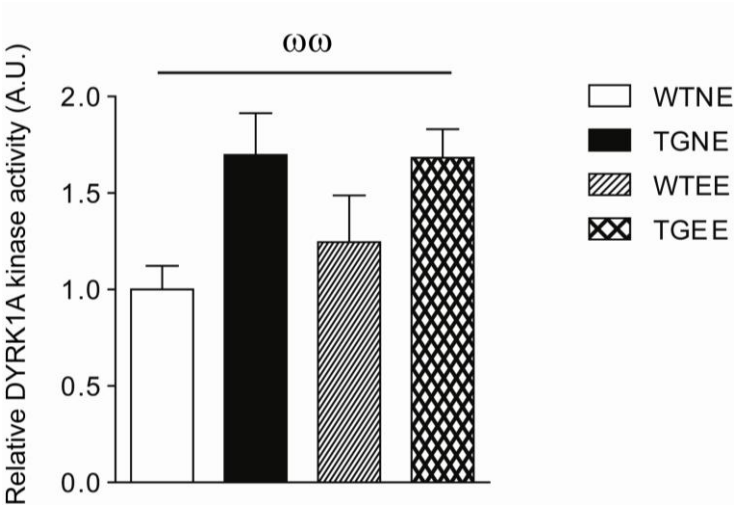

Figure 69. Long-term effects of EE on relative DYRK1A kinase activity in TgDyrk1A mice. (A) Relative DYRK1A kinase activity in the hippocampus in wild type (WT) and TgDyrk1A (TG) mice reared during five months of non enriched conditions (NE-NE) or one month of environmental enriched followed by four months of NE conditions (EE-NE). (WTNE-NE = 12; TGNE-NE n = 12; WTEE-NE n = 12; TGEE-NE n = 12). Data are represented as mean ± SEM. Two-way ANOVA Genotype effect ωω $p<0.01$.

DISCUSSION

5. DISCUSSION

The morphological and cognitive deficits of Down syndrome (DS) have been consistently associated with aberrant neural plasticity. Examination of postmortem brain tissue from DS individuals reveals profound alterations in dendritic and neuronal densities and morphology across many regions that would be expected to adversely affect the information storage capacity of neural networks. Here we aimed at understanding the genetic dependence and underlying mechanisms of the impairments in activity-dependent neuronal plasticity in DS. This is still an open issue in DS, since there is a global upregulation of gene expression due to trisomy 21. However, evidence from mouse models and human studies points towards specific dosage-sensitive molecular pathways potentially associated with the cognitive and neuronal plasticity defects observed in DS. *DYRK1A* is a strong candidate gene which overexpression is sufficient to recapitulate DS hippocampal learning and memory deficits (Altafaj, Dierssen et al. 2001; Ahn, Jeong et al. 2006), aberrant neuronal architecture (Martinez de Lagran, Benavides-Piccione et al. 2012) and embryonic neurogenesis alterations (Tejedor and Hammerle 2011).

The link between synaptic plasticity and cognitive processes such as learning and memory is frequently studied within the hippocampus, a structure involved in diverse cognitive processes such as those related to acquisition, coding, storing, and recalling information in spatial environments. Here, we used TgDyrk1A mice to proof the involvement of DYRK1A in the reduced adult hippocampal neuronal plasticity that may explain some of the intellectual deficits of DS individuals.

We report for the first time that overexpression of DYRK1A in mice is sufficient to produce structural changes in the hippocampus, recapitulate the adult neurogenesis defects detected in trisomic mice and human cell lines, and alter hippocampal-dependent cognitive processes. Most of these alterations could be completely rescued by pharmacological inhibition of DYRK1A kinase activity. Moreover, we found that environmental enrichment, a strategy that improves cognitive functions and rescues the neural plasticity deficits of transgenic and trisomic mice, normalizes DYRK1A expression levels and kinase activity in TgDyrk1A. Our results highlight the key role of DYRK1A in neural plasticity in the hippocampus, and indicate that DYRK1A kinase

inhibitors could boost physiological plasticity *in vivo*, thus providing a new therapeutic strategy for DS.

5.1. DYRK1A OVEREXPRESSION PRODUCES DEFICITS IN HIPPOCAMPAL NEURONAL PLASTICITY IN ADULT TgDyrk1A MICE

The first question addressed in this Thesis was to what extend the overexpression of a single gene, *Dyrk1A*, was sufficient to produce the hippocampal neuronal plasticity deficits observed in DS individuals and trisomic mouse models (Dierssen, Benavides-Piccione et al. 2003; Nadel 2003; Creau 2012). However, first we had to understand if hippocampal functioning was preserved in TgDyrk1A mice.

5.1.1. Impact of Dyrk1A overexpression on hippocampal-dependent learning and memory in adult TgDyrk1A mice

DS individuals show a variety of cognitive deficits that have been associated with alterations in brain structures involved in learning and memory, such as the cortex and the hippocampus (for review see (Cramer and Galdzicki 2012). The insights provided by mouse models of this neurodevelopmental disorder into the potential molecular mechanisms contributing to these deficits have revealed the importance of some candidate genes (Dierssen 2012). Among them, Dyrk1A overexpression was previously shown to produce hippocampal-dependent cognitive defects in transgenic mouse models (Smith, Stevens et al. 1997; Altafaj, Dierssen et al. 2001; Ahn, Jeong et al. 2006) and trisomic mouse models showing learning and memory deficits present DYRK1A overdosage (Reeves, Irving et al. 1995; Sago, Carlson et al. 1998; Belichenko, Belichenko et al. 2009). In our experiments, we used females to perform the environmental enrichment (EE) experiments because EE is known to produce a shift towards a more territorial organization in males, which produces an increase in the aggressiveness level among mice reared in the same cage (Haemisch and Gartner 1997). Thus, since previous studies only evaluated the performance of male mice, and in DS there are gender-related cognitive differences (Martinez-Cue, Baamonde et al. 2002), we evaluated whether DYRK1A overexpression recapitulate hippocampal-mediated

visuo-spatial learning and memory performance in the Morris water maze (MWM) paradigm in female TgDyrk1A mice. In this test, spatial learning is assessed across repeated trials and reference memory is determined by preference for the platform area when the platform is absent. Our results show that while in the initial acquisition sessions, where mice have to learn the spatial relationships between extramaze cues and the position of the platform to escape from the water, both genotypes equally perform the task, female TgDyrk1A mice were unable to reach similar execution levels as wild type littermates. As mice learn the location of the escape platform in the MWM they typically display a progression of search strategies with an increasing use of visuo-spatial cues. Thus, one explanation of the impaired performance of transgenic mice could be that they were using inefficient (non-searching or non-spatial) learning strategies as has also been proposed in other trisomic mouse models (Stasko and Costa 2004). A marked thigmotactic behavior was detected in TgDyrk1A that increased during the last acquisition sessions (Figure 1B) indicating that as training continues, transgenic mice progress to non-spatial procedural strategies, such as swimming in a circle around the wall in a fixed distance from the edge of the pool. To investigate further if impaired learning strategies could account for the observed deficits we performed a careful analysis of the swimming trajectories. The J-track analysis revealed more erratic swimming trajectories in TgDyrk1A mice as compared to a more persistent activity for the target platform in wild types, indicating that spatial preference for the platform quadrant was only developed by wild type mice, whereas transgenic mice distributed their activity similarly across all quadrants with the consequent reduced spatial learning. DYRK1A overdose may thus contribute to impairment in the development of adequate spatial learning strategies, although it does not completely abolish the learning capacities of these mice. As in Ts65Dn mice, thigmotaxis is an index to reflect that TgDyrk1A is not problem-solving and not focusing on the task appropriately. Previous studies in male TgDyrk1A mice have also shown impairments in spatial learning although the main differences appeared during the first acquisition sessions (Altafaj, Dierssen et al. 2001). Moreover, Ts65Dn female mice showed a more pronounced impairment in visuo-spatial learning with increased thigmotatic behavior compared to Ts65Dn males (Martinez-Cue, Baamonde et al. 2002), indicating that visuo-spatial learning performance has a clear gender-dependent effect.

After the learning sessions, mice were tested in a probe trial, where the platform is removed from the pool and the mouse is allowed to search for it, to investigate whether female TgDyrk1A mice show defects in reference memory similar to their male counterparts. Mice that have learned the spatial localization of the platform during learning sessions will selectively search the place where the hidden platform had been located. This session also helps to discriminate spatial and non-spatial strategies, since mice having adopted a spatial strategy will search focally near the former location of the platform. In our experiments, although both genotypes preferentially searched the trained quadrant, TgDyrk1A mice spent less time and showed swimming trajectories less focalized in the target quadrant than wild types. These results indicated that DYRK1A overexpression does not abolish but significantly interferes with reference memory. Our results confirm and extend previous results obtained in trisomic mice (Ts65Dn and Ts1Cje) and in other mouse models overexpressing Dyrk1A such as the YAC152F7 or TgBACDyrk1A that present severe defects in spatial learning and memory (Reeves, Irving et al. 1995; Smith, Stevens et al. 1997; Sago, Carlson et al. 1998).

A control condition that is frequently used in the MWM is to test the animals for their ability to learn to swim to a cued goal. In this procedure, curtains are closed around the maze to interfere with the animal's access to distal cues that could be used to spatially navigate. During this cued session (where the platform was visible) no differences between genotypes were detected. Since the mouse' performance is only constrained by its motor capabilities or its motivation to escape from the water, our results suggest that Dyrk1A overexpression specifically impairs hippocampal-dependent learning without affecting other functional domains such as sensorimotor abilities or motivation.

We also tested the possible deleterious effect of DYRK1A overexpression in a visuo-spatial reversal-learning task, in which mice are required to learn a new position of the platform. Reversal learning in the MWM reveals whether animals can extinguish their initial learning of the platform's position and can acquire a direct path to the new goal position. Tracking patterns typically reveal that mice swim to the previous location first, and then begin to search in an arching pattern to reach the new goal. In the reversal test, TgDyrk1A mice spent significantly more time to reach the hidden platform in the new position as compared to wild types. Moreover, the analysis of the swimming trajectory revealed that while wild type mice presented a spatial searching strategy, with a

preference for the trained quadrant during the first sessions that changes towards the new target quadrant in the last session. Even after multiple trials, transgenic mice do not entirely abandon their initial learning strategy and begin trials by starting to move towards the original platform position not showing any preference for the new platform position even in the last session. These results highlight enhanced perseveration on the platform position trained during the initial learning in TgDyrk1A and demonstrate that besides spatial learning and memory, DYRK1A overexpression also affects cognitive flexibility. Reversal learning is considered a measure of executive function because it involves inhibiting a previously learned response and shifting to a new one. The ability to consolidate memories and relearn a new task depends on the functionality of the hippocampus (Dong, Bai et al. 2013), but also on other brain regions such as prefrontal cortex and striatum (Squire 2004; Castane, Theobald et al. 2010), suggesting an impact of DYRK1A imbalance on other forebrain regions. In this line, our previous studies using male TgDyrk1A mice have also shown a significant cognitive impairment in a more cognitively demanding version of the MWM (including repeated reversal learning platform position during 8 days) that depends on the hippocampus, prefrontal cortex and striatum (Altafaj, Dierssen et al. 2001).

There is wide agreement that spatial memory is highly dependent on the hippocampus (O'Keefe and Conway 1978; Morris, Garrud et al. 1982; Maren and Holt 2000), however, its integrity is also important for non-spatial tasks including recognition memory (Clark, Zola et al. 2000; Broadbent, Squire et al. 2004; Broadbent, Gaskin et al. 2009), that is affected in trisomic mouse models (Fernandez and Garner 2008) and DS individuals (Nadel 2003). One hour after the training session in a novel object recognition (NOR) paradigm, TgDyrk1A mice were not able to recognize a novel object, and spent the same amount of time in the novel and familiar object indicating a failure in discrimination. This impairment was not due to reduced exploration since no differences were detected between genotypes. Importantly, similar cognitive impairments occurred in Dyrk1A heterozygous mice (Dyrk1A +/-) (Arque, Fotaki et al. 2008), indicating that correct dose of DYRK1A is necessary to efficiently perform this cortico-hippocampal dependent task. Previous work showed that Ts65Dn and Ts1Cje mice react normally to object novelty over short intervals of a few minutes, but cannot detect object novelty over 24 hours (a typical time frame used to evaluate rodent long-

term memory). Our results suggest that overexpression of DYRK1A is sufficient to produce this phenotype.

Interestingly, human studies have demonstrated altered performance of DS adults on tasks of object discrimination learning, reversal learning, and object and spatial memory (Nelson, Johnson et al. 2005). Our results indicate that DYRK1A overexpression is sufficient to induce learning defects in hippocampal dependent tasks, and may contribute to the hippocampal-dependent learning and memory impairments observed in DS individuals and trisomic mouse models.

5.1.2. Impact of Dyrk1A overexpression on hippocampal structural and synaptic plasticity in adult TgDyrk1A mice

One of best established feature in intellectual disabilities refers to neuronal dendritic abnormalities and synaptic plasticity alterations in brain regions involved in learning and memory (Kaufmann and Moser 2000). In DS postmortem brains there is reduced dendritic complexity in pyramidal neurons of the cortex (Becker, Mito et al. 1991) that present fewer and abnormally enlarged spines (Marin-Padilla 1976). In the hippocampus of adult DS individuals, in addition to altered neuronal numbers, the number of dendritic spines in CA1 and CA3 pyramidal neurons is also reduced (Ferrer and Gullotta 1990). The anatomo-functional alterations detected in previous studies in the Ts65Dn mouse suggest that altered signal processing by the hippocampal trisynaptic circuit underlies memory impairment in DS (Dierssen 2012). However, the genetic dependence of these phenotypes has not been completely elucidated. In the context of this Thesis it is important to highlight that previous results demonstrated that both heterozygous and transgenic Dyrk1A mice showed dendritic defects in cortical pyramidal neurons (Benavides-Piccione, Dierssen et al. 2005; Martinez de Lagran, Benavides-Piccione et al. 2012). However, no one has ever analyzed whether similar alterations are present in the hippocampus of TgDyrk1A mice.

We studied the morphology of the hippocampus in TgDyrk1A mice. To this end, we used double transgenic mice Thy-YFP/TgDyrk1A mice in which pyramidal neurons are easily visualized. We analyzed the width of different hippocampal strata and the number of neurons of CA1, CA3 and DG subfields. The CA1 hippocampus receives two main monosynaptic glutamatergic excitatory inputs (Ramon y Cajal, 1893). The first

originates from the CA3 hippocampal subfield and reaches the dendrites of pyramidal cells in *stratum oriens* and *stratum radiatum* via the Schaffer collateral. The second is generated by neurons in layer III of the entorhinal cortex and targets the distal dendrites of pyramidal cells in *stratum lacunosum-moleculare* (temporoammonic pathway). The Schaffer collaterals make synapses in the CA1 *stratum radiatum* on proximal apical dendrites (Steward and Scoville 1976). The *stratum oriens*, contains the basal dendrites of CA1 neurons, and receives inputs from other pyramidal cells, septal fibers and commissural fibers from the contralateral hippocampus, usually from CA3. Finally, the *stratum pyramidale* includes the cell bodies of the excitatory pyramidal neurons. Adult TgDyrk1A mice showed significantly increased area of the *stratum pyramidale* in CA1 region without changes in the cell number. We also found that the number of TgDyrk1A YFP+ pyramidal cells in the CA3 region was reduced when compared to wild types, while no differences in DG were observed. Studies performed in Ts65Dn mice have revealed hippocampal structural abnormalities as compared to euploid mice mainly involving neuronal numbers (Insausti, Megias et al. 1998; Kurt, Kafa et al. 2004; Lorenzi and Reeves 2006). Concretely, Insausti and colleagues found that Ts65Dn mice contain 44% more pyramidal cells in the CA3 subregion and 30% fewer granule cells in the DG at 5-7 months of age (Insausti, Megias et al. 1998). In contrast, Kurt and colleagues showed a significantly lower (15%) pyramidal neuronal density in CA1 of Ts65Dn compared to diploid mice, with no differences in granule cell density in the DG or pyramidal neuron density in CA3 in 16-months-old mice (Kurt, Kafa et al. 2004). Another study demonstrated that already at PD6 Ts65Dn mice had 20% fewer granule cells in the DG (Lorenzi and Reeves 2006). The reason for these discrepancies is not clear, but may be due to a variety of factors such as differences in the ages of the animals used, tissue preparation techniques and methods of quantification. Although our counting results in the hippocampus of TgDyrk1A mice are not in concordance with previously described in Ts65Dn mice, we have to take into account that, we selectively only counted excitatory (YFP+) neurons from the different hippocampal subfields. Moreover, Ts65Dn mice overexpress Dyrk1A among other genes, which could also contribute to the misbalance in the number of cells. In this line, transgenic mice overexpressing *Rcan1*, located in HSA21 and overexpressed in DS brains, showed reduced number of neurons in CA1 and DG subfields with no changes in the CA3 region (Martin, Corlett et al. 2012). However, the main important idea of these results relies on the observation that although Ts65Dn mice present significant visuo-spatial

learning and memory impairments, the density of neurons in different hippocampal subfields is highly variable, suggesting that neuronal numbers are not key determinants in cognitive defects.

Our results of a selective around 40% decrease in CA3 pyramidal cell numbers may well lead to a concomitant decrease in the number of synaptic sites available in CA1 for receiving information through the Schaffer collaterals. Since CA1-CA3 synapses are involved in the formation of long-term potentiation (LTP), the observed reduction may have an impact on synaptic plasticity. It is difficult to predict to what extent the observed phenotype may be contributing to the learning and memory alterations observed in transgenic mice. CA3 pyramidal cells are an essential link in the trisynaptic circuit. Several studies have suggested that the CA3 region of dorsal hippocampus mediates the acquisition and encoding of spatial information, or of information requiring multiple trials to construct relational representations (Kesner 2007).

The hippocampus is laminated into cytoarchitectonic layers (Forster, Zhao et al. 2006). While the pyramidal and granular layers are densely packed with cell bodies, the majority of dendrites, axons and synapses are distributed in the other layers. Therefore, quantifying the width of the different layers across the hippocampus may shed a light on circuitry organization. Regarding CA1 region, adult TgDyrk1A mice showed significantly reduced width of *stratum oriens* and *radiatum* together with increased *stratum pyramidale* area, without changes in the cell number, suggesting a more scattered distribution of cell bodies in CA1 region. Recently, some authors have proposed that the structural organization in the space of neurons would have implications in the neuronal circuit computation (Cuntz, Mathy et al. 2012). In this line, a previous work performed by Chklovskii and colleagues revealed that the volume of axons and dendrites combine to occupy 3/5 of the neuropil volume of CA1 *stratum radiatum* in order to efficiently perform connections (Chklovskii, Schikorski et al. 2002). Therefore, our observation of changes in the size of the CA1 dendritic and cellular layers of TgDyrk1A suggests an altered neurite distribution compared to wild type mice. As mentioned before, DS postmortem brains and trisomic mouse models showed reduced hippocampal volumes, however, it is important to mention that nowadays there are no studies describing a subregional hippocampal strata analysis in intellectual disabilities. Hence, our results shed a light on a new level of morphological analysis, meaning concrete stratum alterations while preserving intact other

hippocampal subfields, probably contributing to cognitive phenotypes

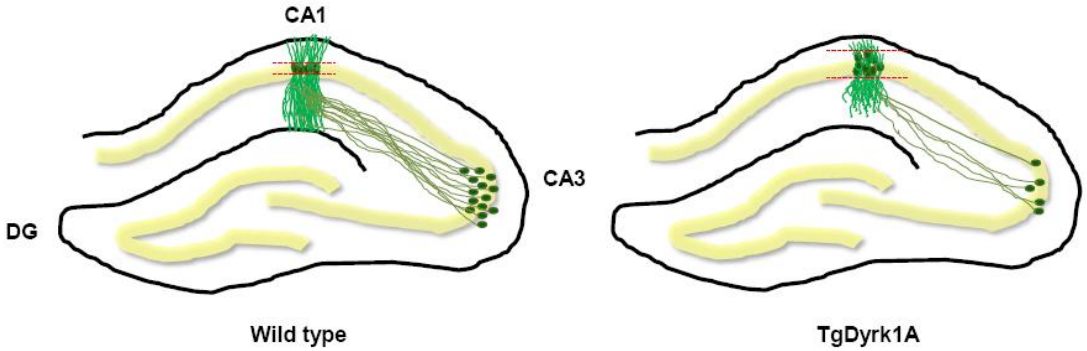

Figure 1. Hippocampal morphology in TgDyrk1A mice. Schematic representation summarizing the main results obtained in this study. TgDyrk1A mice showed a significant reduced dendritic width in *stratum oriens* and *stratum radiatum* accompanied with increased *stratum pyramidale* area of CA1 subregion together with reduced number of YFP+ cells in CA3 subfield as compared to wild type mice.

Taken together with previous data on the hippocampal structure of Ts65Dn mice, our results strongly suggest that discrete hippocampal dysplasia gives rise to functional abnormalities, which are sufficient to render transgenic animals less efficient in cognitive tasks. Interestingly, mouse models associating a susceptibility to epilepsy and cognitive disturbances, such as p35, Lis1 and reeler mutant mice, show hippocampal pyramidal cell defects affecting both the CA1 and CA3 regions (Deller, Drakew et al. 1999; Fleck, Hirotsune et al. 2000; Wenzel, Robbins et al. 2001) and hippocampal lesions restricted to CA1 and/or CA3 subfields produce memory impairments.

As suggested above, the spread distribution of the neurons affects also the occupancy of their dendrites and axons into the different subfields, and thus their neuroarchitecture might be altered to compensate a suboptimal connectivity. Thus, we evaluated the dendritic arborization of the apical dendrite in the *stratum radiatum* of CA1 using Lucifer yellow injections (collaboration with Dr Imma Ballesteros, UCLM). We focused on this area because the apical dendrite of CA1 neurons is the main target of CA3 inputs. In these experiments, we did not detect differences between genotypes in the complexity of the dendritic trees located in CA1 *stratum radiatum*. However, the longest axis of *stratum radiatum* as defined by the longest apical dendrite of the arbor, was significantly shorter in pyramidal neurons from TgDyrk1A mice. This is relevant

since distal apical dendrites form non-local synapses while shorter proximal apical dendrites project radially to local pyramidal cells and interneurons.

Previous studies have demonstrated reduced branching and length of the basal dendritic arbor of pyramidal neurons in layer III of the secondary motor cortex in adult TgDyrk1A mice suggesting that Dyrk1A contributes to the altered cortical dendritic morphology in DS (Kaufmann and Moser 2000; Benavides-Piccione, Dierssen et al. 2005; Martinez de Lagran, Benavides-Piccione et al. 2012). However, the results obtained in this Thesis suggest that the impact of DYRK1A overexpression may be different on different dendritic territories probably due to the fact that pyramidal neurons from the hippocampus and motor cortex are involved in a completely different computational circuit. Taken together, our results suggest that the reduced numbers of CA3 neurons targeting CA1 *stratum radiatum* dendrites in TgDyrk1A mice, leads to subtle, possibly compensatory changes in hippocampal lamination.

DYRK1A has been shown to phosphorylate several MAPs proteins such as MAP1B compromising neuritogenesis (Scales, Lin et al. 2009), and TAU proteins at several phosphorylation sites (Woods, Rena et al. 2001; Ryoo, Jeong et al. 2007). Deregulation of these proteins leads to significant modifications in arbor size (Teng, Takei et al. 2001; Harada, Teng et al. 2002). Although we have not explored the molecular pathways involved in the altered hippocampal morphology present in our transgenic mice, it can be speculated a putative disruption in the regulation of cytoskeleton rearrangements. Hence, further experiments would be necessary to elucidate the impact of *in vivo* Dyrk1A overexpression on microtubule organization and its relevance for the alterations observed in the hippocampal morphology.

Given the deficient connectivity between CA3 and CA1 layers, we also examined dendritic spines of CA1 *stratum radiatum* proximal dendrites in Lucifer yellow injected neurons. No differences in spine density were detected between genotypes; however, increased number of immature spines (filopodia-like) and reduced mature (mushroom-like stable) ones were observed in TgDyrk1A mice, suggesting altered spine maturation in the hippocampus. Concomitant with the formation of novel spines in response to memory-inducing stimuli, neurons had to stabilize immature spines that are necessary and retract the ones that are non-functional in order to maintain an efficient neuronal circuitry (Trachtenberg, Chen et al. 2002; Holtmaat, Wilbrecht et al. 2006). Our results

suggested alterations in the stabilization of immature spines to mature ones in TgDyrk1A mice, similar to previously described phenotypes in primary cortical neuronal cultures of TgDyrk1A mice (Martinez de Lagran, Benavides-Piccione et al. 2012). The morphology of dendritic spines is determined by cytoskeleton rearrangements mainly depending on actin protein (Hotulainen and Hoogenraad 2010) and changes in the amount and structure of F-actin have been shown to mediate the spine size and synaptic efficacy (Matus 2000). Interestingly, in TgDyrk1A primary cultures the morphological alterations were accompanied with a significant reduction of the percentage of actin mobile fraction in TgDyrk1A spines, suggesting a lower rate of treadmilling of actin filaments (Martinez de Lagran, Benavides-Piccione et al. 2012). This behavior may partly be related to altered actin polymerization, since a similar phenotype was previously described in wild type primary cultures treated with cytochalasin D, which prevents actin polymerization (Star, Kwiatkowski et al. 2002). In addition, DYRK1A has been shown to interact with several proteins involved in actin cytoskeleton organization both *in vitro* and *in vivo* (Adayev, Chen-Hwang et al. 2006; Murakami, Xie et al. 2006; Kimura, Kamino et al. 2007; Ryoo, Jeong et al. 2007; Murakami, Bolton et al. 2009; Park, Sung et al. 2012) and its overexpression in DS brains reduces its association with actin (Dowjat, Adayev et al. 2012). Therefore, our results reinforce the idea that DYRK1A could be responsible for the altered spine shape in DS (Ferrer and Gullotta 1990; Dierssen, Benavides-Piccione et al. 2003; Belichenko, Masliah et al. 2004).

The observed morphological changes and the deficits in hippocampus-dependent learning tasks detected in TgDyrk1A mice could be associated with abnormalities on synaptic plasticity (Gruart, Munoz et al. 2006; Whitlock, Heynen et al. 2006). Interestingly, DS trisomic mouse models have been shown to present significantly lower synapse densities in hippocampal neurons (Kurt, Kafa et al. 2004) along with impaired LTP postsynaptic response in the CA1 *stratum radiatum* when CA3 pyramidal neurons were stimulated (Siarey, Stoll et al. 1997; Siarey, Carlson et al. 1999; Costa and Grybko 2005). Our results suggested that defects in hippocampal architecture and spine morphology could affect the functional network connectivity in the CA3 - CA1 synapse, and thus we analyzed *in vitro* synaptic transmission and activity-dependent synaptic plasticity using extracellular field recordings (fEPSPs) in brain slices containing the CA1 region (collaboration with Dr Thomas Gener, CRG). Two different protocols were

applied: paired pulse facilitation (PPF), and theta burst stimulation (TBS) in the Schaffer collateral pathway. We found reduced PPF in TgDyrk1A mice as compared to wild types. PPF has been reported to represent a form of short-term synaptic plasticity that results mostly from presynaptic mechanisms. Three main processes are part of this phenomenon: presynaptic residual calcium ([Ca2 +] res), number of released vesicles and sensitivity of vesicle release mechanisms to calcium (Zucker and Regehr 2002). Therefore, the reduced PPF in TgDyrk1A mice could reflect altered presynaptic calcium behaviour or altered sensitivity of the neurotransmitter release machinery. In support to this idea, Altafaj and colleagues demonstrated a more prolonged calcium response after NMDA stimulation in TgDyrk1A mice, indicating altered calcium homeostasis due to the inability of synaptosomes to re-establish calcium levels (Altafaj, Ortiz-Abalia et al. 2008). These results could be in concordance with previous observations showing that reduced PPF is an indicative of reduced evoked glutamate release probability (He, Mahnke et al. 2012). Computational analysis of short-term potentiation using a model of synaptic dynamics at CA1 hippocampal synapses have shown that facilitation is necessary for the rapid increase in synaptic strength in response to discharges of place cells (Kandaswamy, Deng et al. 2010), pointing to PPF as a crucial phenomenon to understand how synaptic properties fast changes are involved in memory processes. Therefore, our results could indicate that Dyrk1A overexpression would be responsible for the reduced short-term presynaptic plasticity.

The major form of synaptic plasticity in the hippocampus is long-term potentiation (LTP) (Bliss, Lancaster et al. 1983; Bliss and Collingridge 1993), which has been correlated with morphological and functional changes of the hippocampus (Matsuzaki, Honkura et al. 2004; Nagerl, Eberhorn et al. 2004). We detected a significant reduced TBS-induced LTP in TgDyrk1A mice as compared to wild types. Previous results using the partial trisomic Ts1Cje found similar LTP reductions in the CA1 region accompanied with enhanced LTD (Siarey, Villar et al. 2005). Studies performed in Ts65Dn mice have also reported reduced LTP in the CA1 regions in both young (2 months) and older mice (9 months) when a single tetanizing pulse train (1 sec in duration) at 100 Hz was applied, also known as high-frequency-stimulation (HFS) (Siarey, Stoll et al. 1997). In contrast to the previous reports, however, Costa and colleagues demonstrated that the type of LTP inducing stimuli clearly had an impact on the postsynaptic response in Ts65Dn mice in the CA1 region. Indeed, they showed no

significant difference in HFS-induced LTP response in CA1 region between Ts65Dn and euploid littermate mice but a significant reduction in the amount of LTP induced by TBS (5 trains of 4 pulses; at 100 Hz and 200 ms apart; 100 μs individual pulse-duration) (Costa and Grybko 2005). The main difference between those studies relies on the absolute value of stimuli intensity applied. Here, using the same protocol applied by Costa and colleagues we were able to recapitulate LTP disruptions in TgDyrk1A CA1 region supporting the importance of Dyrk1A overexpression on the reduced synaptic plasticity in DS.

Previous results in the literature have related changes in spine morphology and synaptogenesis with LTP induction (Peebles, Yoo et al. 2010; Oe, Tominaga-Yoshino et al. 2013), being the capacity of neurons to convert silent and instable spines into functional ones a key determinant in LTP induction and learning (Liao, Hessler et al. 1995; Geinisman, Disterhoft et al. 2000). Our observation of predominant filopodia-like immature spines in TgDyrk1A *stratum radiatum* suggested the idea of disability for spine maturation with consequences in the disruption of synaptic functional contacts in Dyrk1A overexpressing mice. Besides, excessive GABA-mediated neurotransmission in DS (Siarey, Carlson et al. 1999; Kleschevnikov, Belichenko et al. 2004; Fernandez, Morishita et al. 2007) is also a plausible mechanism underlying cognitive deficits and reduced LTP. In TgDyrk1A mice we detected an imbalance between excitatory and inhibitory neurotransmission in CA1 *stratum radiatum* towards inhibition similarly as the imbalance observed in the hippocampus of adult DS individuals (Reynolds and Warner 1988; Risser, Lubec et al. 1997) and in trisomic mice which had fewer excitatory synapses in CA1 region (Kurt, Kafa et al. 2004). Recently, it has been demonstrated that treating Ts65Dn mice with a selective GABA-A α5 inverse agonist or GABA-B receptor antagonist significantly reverse LTP in CA1 pyramidal neurons, neuromorphological and cognitive deficits (Kleschevnikov, Belichenko et al. 2012; Martinez-Cue, Martinez et al. 2013). Similar to the findings in Ts65Dn mice (Kurt, Kafa et al. 2004), the imbalance between excitatory and inhibitory neurotransmission that we detected in transgenic mice was mainly due to reduced VGLUT1 vesicles (excitatory) rather than alterations in VGAT puncta (inhibitory), the overall functional consequence would be increased inhibition in TgDyrk1A CA1 *stratum radiatum*. The reduced presynaptic excitatory input due to the lower number of pyramidal excitatory neurons in the CA3 region along with the reduced number of mature dendritic spines

receiving excitatory contacts may well account for the reduction in VGLUT puncta and the reduced hippocampal synaptic plasticity shown by the LTP response in CA1.

5.1.3. Impact of Dyrk1A overexpression on adult neurogenesis in the dentate gyrus of TgDyrk1A mice

Although dendritic abnormalities are one of the main neuronal plasticity mechanisms underlying cognitive impairments, adult neurogenesis in the dentate gyrus (DG) has become another cellular process highly related to hippocampal learning and memory. Importantly, before discussing the main results is important to take into account that no information is available in adult humans with DS, and thus, our knowledge only derives from experiments performed in animal models of this disease. Moreover, since classically DS is considered a developmental disease, the majority of the data in the literature is limited to embryogenesis stages and very few information involves adult neurogenesis.

DYRK1A has been involved in neuronal proliferation and differentiation during embryogenesis (Aranda, Laguna et al. 2011; Tejedor and Hammerle 2011); however, its role in adult hippocampal neurogenesis has not been established. In this Thesis, we provide for the first time evidence that DYRK1A overexpression impairs adult neurogenesis in the DG. Specifically, DYRK1A overdosage induces alterations in cell proliferation, cell cycle progression and cell cycle exit of DG proliferating cells with consequences in neuronal survival and migration. Since adult neurogenesis is widely related to hippocampal learning and memory (see Introduction section), we could speculate that the adult neurogenesis impairment observed in TgDyrk1A would also participate in hippocampal learning deficits that we observed. Importantly, in support of this assumption, the functional integration of newly born cells in learning and memory networks was altered in transgenic mice.

Previous reports already had suggested that DYRK1A overexpression might disturb cell proliferation (reviewed in (Tejedor and Hammerle 2011)) during the embryonic period. Concretely, gain of function of DYRK1A leads to neuronal proliferation arrest, while its loss of function causes over proliferation and cell death (Hammerle, Ulin et al. 2011). Consistent with these previous observations, we observed reduced number of BrdU+ cells 2 hours post injection in the adult DG of TgDyrk1A mice, indicating reduced proliferation rate. This was not due to a reduction of the number of progenitor cells,

since no differences were observed in the numbers of *type 1* (GFAP+ Sox2+ cells) or *type 2* (GFAP- Sox2+) cells, nor to reduced recruitment of either neural precursor to enter the cell cycle (ki67+ Sox2+ cells). Although previous work had shown reduced density of *type 1* (GFAP+ Sox2+) cells in the subependimal zone of adult *Dryk1A* +/- mice (Ferron, Pozo et al. 2010), our observation does not conflict with these experiments since *Dyrk1A* is a dosage-sensitive gene, which gain- or loss-of-function may produce different consequences in different neurogenic niches, and thus a strict comparison is not possible.

Despite the number of TgDyrk1A BrdU+ cells at 2 hours post injection (in S-phase of the cell cycle) was reduced in transgenic mice, the total number of proliferating ki67+ cells (endogenous marker expressed in all phases of the cell cycle except the resting phase) was increased, and the proportion of cells exiting cell cycle (BrdU+ ki67- cells 24 hours post injection) was reduced. Taken together, these results suggested an impairment of cell cycle progression, as supported by the increased proportion of pH3+ cells in the G2 phase. Thus, we can conclude that in the adult TgDyrk1A hippocampus, progenitor cells arrest in G2 phase. This is in accordance with the high levels of cyclin B1, involved in G2/M cell cycle phase transition, detected in a YAC transgenic mouse model overexpressing *Dyrk1A* (YAC152F7; (Branchi, Bichler et al. 2004). Moreover, DS fetuses and newborn Ts65Dn mice, which also overexpress DYRK1A, present a higher number of proliferating cells in G2 phase and a reduced number of cells in M phase in the DG (Contestabile, Fila et al. 2007). In the other hand, Dyrk1A has also been related to G1-S phase transition during embryogenesis in different organisms (Park, Oh et al. 2010; Yabut, Domogauer et al. 2010; Hammerle, Ulin et al. 2011) and Ts65Dn show impairment in this transition due to decreased expression of Skp2 leading to cell cycle elongation (Contestabile, Fila et al. 2009). Thus, the possibility of a reduction of the S phase length or an elongation of the G1 phase leading to less progenitor cells entering the S phase (BrdU + cells two hours after injection) should also be considered. The rescue of proliferation and cell cycle alterations in TgDyrk1A mice adult neurogenesis using a DYRK1A kinase inhibitor (EGCG) confirmed their dependence on DYRK1A kinase activity. All together our experiments further support the involvement of DYRK1A in the regulation of the cell cycle and indicate for the first time that DYRK1A overexpression could affect cell cycle progression in the adult DS dentate gyrus.

In our experiment newly generated TgDyrk1A cells prematurely migrate to the granular cell layer (GCL). Remarkably, most of BrdU+ cells found at 24 hours in the GCL of transgenic mice were in G2 cell cycle phase. However, one week after the last BrdU injection in TgDyrk1A mice we detected a marked reduction of the number of BrdU+ cells in the DG: while 80% of BrdU+ cells survived in wild type mice, only 40% did so in transgenic mice. However, late survival (between 1 week and 1 month) was similar in both genotypes indicating that DYRK1A overexpression affects survival only during the first week of life of newborn cells when premature migration is taking place in TgDyrk1A mice.

In adult organisms, neurons born in the SGZ migrate into the GCL to become granule neurons and to functionally integrate in the preexisting network (van Praag, Schinder et al. 2002). Once the newborn cells have migrated into the GCL, those that survive after two weeks would mature, developing dendrites that synapse with preexisting hippocampal granule cells (Kempermann, Gast et al. 2003). Interestingly, we detected reduced percentage of BrdU+ cells expressing immature neuronal markers (DCX+ and calretinin+ cells), suggesting that DYRK1A overexpression is impairing or delaying neuronal differentiation. Accordingly, the surviving TgDyrk1A immature cells had reduced dendritic complexity. These newborn neurons were less capable of being activated in basal conditions, thus suggesting that the reduced complexity of the dendritic tree is probably affecting the functional integration in the preexisting network. Moreover, the number of newborn neurons that are specifically activated upon hippocampal-dependent spatial learning was also reduced in transgenic mice.

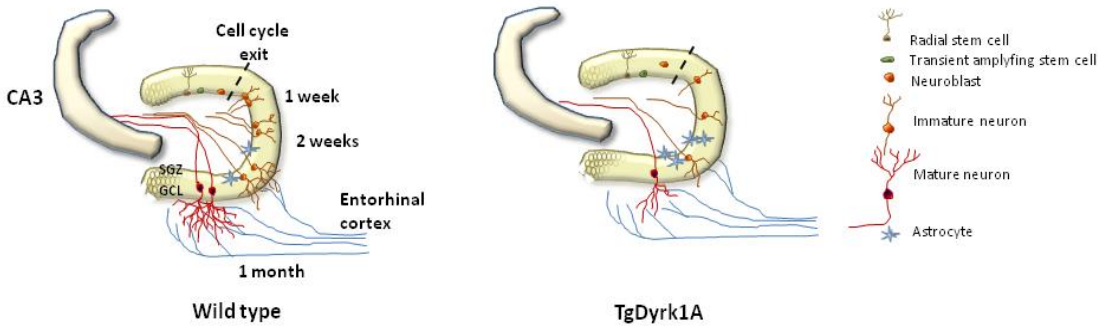

Figure 2. Hippocampal adult neurogenesis in TgDyrk1A mice. Schematic representation summarizing the main results obtained in this study. Briefly, DYRK1A overdose induces impaired cell cycle progression of proliferating cells with consequences on cell cycle exit, premature migration, and survival

of newly generated cells that could in part underlie the hippocampal-dependent learning impairment in TgDyrk1A mice.

Mounting evidence supports contributions of adult-born neurons to hippocampal function (Ramirez-Amaya, Marrone et al. 2006; Deng, Aimone et al. 2010) and thus, our results suggest that the decrease activation of newborn granule transgenic neurons could contribute to the impairment observed in hippocampal visual-spatial tasks in DS. However, we have to take into account that TgDyrk1A mice constitutively express DYRK1A from the development and therefore the impaired neurogenesis could be contributed both by developmentally impaired network connectivity and also by a neural plasticity defect during the adulthood.

5.2. SPECIFIC INVOLVEMENT OF DYRK1A KINASE ACTIVITY ON HIPPOCAMPAL PHENOTYPES

Our results suggested that overexpression of DYRK1A is sufficient to alter hippocampal structural morphology and adult neurogenesis in TgDyrk1A mice. The next question we had to address was to what extent DYRK1A kinase activity is responsible of these neuronal phenotypes. To elucidate this question we have treated mice with epigallocatechin-3-gallate (EGCG), a potent and safe DYRK1A kinase inhibitor.

EGCG**,** the major catechin in green tea leaves, was identified as one of the most specific inhibitors of DYRK1A, with an apparent IC50 of 0.33 µM (Bain, McLauchlan et al. 2003). Here, we demonstrated that one month of oral administration of EGCG normalizes DYRK1A kinase activity *in vivo* in the hippocampus of TgDyrk1A mice without affecting wild types. Importantly, EGCG was able to rescue the hippocampal-dependent phenotypes in adult TgDyrk1A, reinforcing the idea that DYRK1A overdosage is a major contributor of the reduced neuronal plasticity. In our experiments, one month of EGCG treatment increased the distance travelled both in the open field and in the EPM indicating enhanced locomotor activity. Although some reports in the literature suggested that EGCG reduces locomotor activity in hypertension rats (Wang, Chang et al. 2012), others have reported enhanced hyperactivity effect (Nakae, Dorchies et al. 2012). TgDyrk1A mice showed increased anxiety-related behavior in the EPM

(Altafaj, Dierssen et al. 2001) as shown by the reduced percentage of time in the open arms. However, even though in the literature it has been reported that EGCG had anxiolytic properties in wild type mice by increasing the amount of time spent in open arms in a dose-dependent manner (Vignes, Maurice et al. 2006; Park, Oh et al. 2010), one-month treatment with EGCG did not rescue the anxious phenotype in transgenic mice.

Interestingly, EGCG rescued the hippocampal-dependent learning and reference memory deficits in the MWM paradigm both during acquisition and probe sessions in TgDyrk1A mice without affecting the performance of wild types. The improved learning strategy that EGCG-treated TgDyrk1A mice used to find the platform clearly indicates a specific learning and memory improvement. These results are similar to those obtained in Ts65Dn mice treated with EGCG (De la Torre, 2013, *in press*), and support the idea that normalizing DYRK1A activity can reverse the cognitive impairment not only in conditions of single DYRK1A overdose, but also in a trisomic context. In the reversal sessions of the MWM and in the NOR paradigm, while EGCG rescued cognitive flexibility and recognition memory deficits in TgDyrk1A, it impaired the performance of wild types. Given that EGCG disrupts wild type performance of cognitive tasks that depend on cortico-hippocampal pathways without affecting more hippocampal-dependent tasks, it could suggest that, in the young adults, cortical-dependent performances would be more sensitive to reduction of DYRK1A kinase activity. In this regard, Dyrk1A haploinsufficient mice (Dyrk1A +/-) showed similar cognitive impairments in the NOR task (Arque, Fotaki et al. 2008) compared to wild types, suggesting that the reduced dosage of DYRK1A could impair certain cortico-hippocampal-dependent tasks. Thus, even though EGCG did not affect DYRK1A kinase activity in the hippocampus, we cannot discard an effect of the polyphenol in the cortex of wild type mice. However, other mechanisms not specifically related to DYRK1A kinase activity should also be considered.

Surprisingly, while EGCG rescued CA1 lamination alterations in Thy-YFP/TgDyrk1A mice, increasing the width of *stratum oriens* and *radiatum*, it reduced the size of CA1 dendritic layers in wild type mice. Previous results have demonstrated that lesions in the prefrontal cortex reduce the myelin sheaths of hippocampal neurons affecting the functional activated CA1 hippocampal neurons (Muir and Bilkey 2003; Klein, Koch et al. 2008). Therefore, the changes observed in CA1 dendritic layers after EGCG

treatment in wild types could be a consequence of changes in other brain regions. In our experiments, we are feeding mice with a green tea drink (usually drinking 3–5 ml/day) which is equivalent to administering 0.6 mg/day pure EGCG. It has been previously reported that at this dose EGCG effectively rescued some cognitive deficits and brain morphological alterations in transgenic TgYAC152F7 pups overexpressing *DYRK1A* along with other four genes *PIGP*, *TTC3*, *DSCR9* and *DSCR3* (Guedj, Sebrie et al. 2009). Among the molecular mechanisms of these improvements, EGCG normalized the levels of some synaptic plasticity related proteins in the hippocampus such as BDNF and CREB in TgYAC152F7 mice (Guedj, Sebrie et al. 2009) and promotes neuronal plasticity in wild type and trisomic mice (Xie, Ramakrishna et al. 2008). Moreover, EGCG has both antioxidant and pro-oxidative activities (Sang, Hou et al. 2005) and is able to reduce oxidative stress (Yamamoto, Hsu et al. 2003), indicating that other EGCG molecular targets may be involved. Although other proteins apart from DYRK1A could also be affected by EGCG treatment, the normalization of DYRK1A kinase activity in the hippocampus of transgenic mice might be at least in part involved in the rescuing hippocampal neuronal plasticity effects. In support of this argument, previous studies demonstrated that inhibiting *Dyrk1A* expression using adenoviral vectors (AAVshDyrk1A) corrected functional cortico-striatal defects in TgDyrk1A mice (Ortiz-Abalia, Sahun et al. 2008) and attenuated hippocampal-dependent defects in Ts65Dn mice when injected directly in the hippocampus (Altafaj, Martin et al. 2013). However, although the use of short hairpin RNAs would target more specifically *Dyrk1A*, EGCG has been demonstrated to be a safe substance after repeated administration in humans in a range of doses (Chow, Cai et al. 2003) with no significant toxic effects (Isbrucker, Edwards et al. 2006; Lambert, Sang et al. 2007). Thus, our results on hippocampal morphology and cognition *in vivo* support the clinical use of EGCG as a potential therapeutic agent for improving cognitive deficits in DS individuals.

5.3. ENVIRONMENTAL ENRICHMENT STIMULI IN ADULT TgDyrk1A MICE

In human, early intervention programs are the only effective non-pharmacological therapy to improve cognitive impairments in intellectual disability disorders, and specifically in DS individuals (Mahoney, Perales et al. 2006; Bonnier 2008). In trisomic DS mouse models, a number of studies have reported that postnatal environmental enrichment (EE) was able to completely recover hippocampal-dependent learning and memory deficits (Martinez-Cue, Baamonde et al. 2002; Dierssen, Benavides-Piccione et al. 2003; Chakrabarti, Scafidi et al. 2011; Kida, Rabe et al. 2013). A myriad of reports have provided evidence that EE improves hippocampal-dependent learning and memory through the modulation of neuronal structural plasticity mechanisms involving dendritic structure, synaptic plasticity and adult neurogenesis (Kempermann, Kuhn et al. 1997; Rampon, Tang et al. 2000; Nithianantharajah and Hannan 2006). Therefore, since DYRK1A overexpression affected these cellular mechanisms we evaluated whether EE was able to modulate the alterations in cognition due to DYRK1A overdosage.

As stated above, we used female mice instead of males to perform the experiments. This is due to the well-established fact that EE can increase levels of aggression among male mice, and concretely, EE produced negative effects in Ts65Dn male mice and positive effects in females when hippocampal-dependent tasks were analyzed (Martinez-Cue, Baamonde et al. 2002). Although the underlying mechanisms are not well understood, EE can substantially modify the males hierarchy causing a shift towards a more territorial organization, which produces an increase in the aggressiveness level among mice reared in the same cage (Haemisch and Gartner 1997). However, it should be taken into account that EE produces gender-dependent effects in cortical and hippocampal dendritic branching (Juraska 1984; Juraska, Fitch et al. 1985) and exploratory behavior (Joseph, 1979), and thus our conclusions cannot be easily extrapolated to both genders. In fact Ts65Dn males showed increased levels of stress hormones that have a deleterious effect on hippocampal structure and function (Martinez-Cue, Rueda et al. 2005).

In our experiments, EE did not alter the locomotor activity in young adult mice. While several authors found that animals from enriched environments had higher activity levels than animals from control environments (Manosevitz and Joel 1973; Holson 1986; Sachser, Lick et al. 1994), others, however, found the opposite: mice and rats from enriched housing conditions were less active in the open field test or their activity did not differ from standard housed animals (van de Weerd, Baumans et al. 1994;

Amaral, Vargas et al. 2008). The main differences between studies with opposite results are related to test duration. Indeed, it appeared that in short open field tests (2-3 min trials on consecutive days) mice reared under EE conditions were more active than NE-mice whereas in longer open field tests (1 trial for 8-15 min) it was found that NE-mice were overall more active than EE-mice. Other authors have observed that while no overall differences in locomotor activity appeared in 10 min of open field, during the first 2.5 min of the test, rats from the enriched housing conditions showed significantly more locomotion than rats from standard conditions (Van de Weerd, Aarsen et al. 2002). We used a one trial-5 min open field in which EE-mice behave similar to NE mice of both genotypes.

Similarly, rearing conditions did not modify the total distance travelled nor the percentages of time spend in the open and closed arms of the EPM. Previous results demonstrated that EE decreased anxiety-like behavior as revealed by a greater percentage of time spent in the open arms of the EPM (Benaroya-Milshtein, Hollander et al. 2004). However, others did not find any differences. This contradiction may be accounted for the variable impact that EE has on different strains (van de Weerd, Baumans et al. 1994; Chapillon, Manneche et al. 1999; Tsai, Pachowsky et al. 2002). In this line, Chapillon and colleagues demonstrated that two months of EE reduced the anxiety-like behavior in BALB/c mice without modifying the levels of anxiety in C57BL/6(B6) mice that are more similar to the background strain of our transgenic mice.

The most important question in TgDyrk1A mice, was to what extend EE could rescue the DS-like hippocampal learning and memory impairments in the MWM paradigm. As also described previously for Ts65Dn mice, EE significantly improved spatial learning, reference memory and cognitive flexibility performances in TgDyrk1A mice and had also beneficial effects in wild types. In TgDyrk1A mice, EE restored hippocampal functionality to NE-wild type levels. Although we found increases in swimming speed in EE groups of both genotypes that may contribute to the reduced escape latencies, we clearly observed an improvement of the learning strategy as shown by more focalized path trajectories in the platform quadrant in TgDyrk1A mice. Similar rescuing effects were obtained in the NOR where the impaired recognition memory was returned to wild type levels in TgDyrk1A mice. However, EE did not enhance object discrimination in wild type mice. Previous results in the literature have shown that rearing mice under EE

improves recognition memory (Mesa-Gresa, Perez-Martinez et al. 2013), but others did not find any effect (Viola, Botton et al. 2010). The main reasons for these discrepancies are related to the mouse strains but also the duration of the environmental stimuli. While Mesa-Gresa and colleagues reared mice under EE during 3 months, the experiments performed by Viola and colleagues were done in 2 months treatment. Hence, probably we could not see the improvement in wild type mice because we reared our mice only during one month under EE conditions.

Regarding the possible mechanisms underlying the effects of EE, several studies demonstrate that environmental stimuli and learning experiences promote dendritic and axonal rearrangements in order to allow the brain develop a more efficient neuronal connectivity prepared for new complex challenges. However, to our knowledge, no one has ever evaluated hippocampal gross morphology plasticity. In our wild type mice even though we found a significant improvement in some cognitive tests upon EE, we did not find any significant change when the gross hippocampal morphology was analyzed. However, when evaluating the dendritic complexity of CA1 individual pyramidal neurons in *stratum radiatum* using Lucifer yellow injections, we detected a significant increase of the apical dendritic length and complexity in EE wild types as previously described (Johansson and Belichenko 2002; Bindu, Alladi et al. 2007; Beauquis, Roig et al. 2010). Together, these results could suggest that upon environmental stimuli wild type CA1 pyramidal neurons are able to increase the complexity of the dendritic arbor to receive more inputs from CA3 pyramidal neurons.

However, in the case of TgDyrk1A mice results are completely different. One month of EE rescued the reduced width of *stratum oriens* and *radiatum*, and increased YFP+ pyramidal area in CA1 region in adult TgDyrk1A mice. However, detailed cellular analysis of *stratum radiatum* neurons revealed that EE only normalized the length of the largest dendrite without modifying dendritic branching. These results suggested that under Dyrk1A overexpression, EE is not able to modulate the complexity of the dendritic arbor, a result similar to the previously described in Ts65Dn cortical pyramidal neurons (Dierssen, Benavides-Piccione et al. 2003). However, opposite to the preconception that individuals with intellectual disabilities are not able to respond to environmental stimuli, TgDyrk1A mice significantly reacted to the activity-dependent stimuli but instead of changing dendritic complexity they changed their gross hippocampal morphology indicating that in fact the hippocampus of Dyrk1A

overexpressing mice is highly plastic. It may be argued that this change could improve the dendritic organization to increase CA3 target space. Several studies demonstrated that EE increase the expression of cytoskeleton proteins such as ARC (Pinaud, Penner et al. 2001) and NF-70 (neurofilament protein) (Teather, Magnusson et al. 2002) in the hippocampus and recent data in the literature pointed that postnatal EE in female Ts65Dn mice normalized the levels of MAP2ab, a protein involved in cytoskeleton stabilization (Kida, Rabe et al. 2013), so probably relevant cytoskeleton modifications could be taken place in the hippocampus of our transgenic mice.

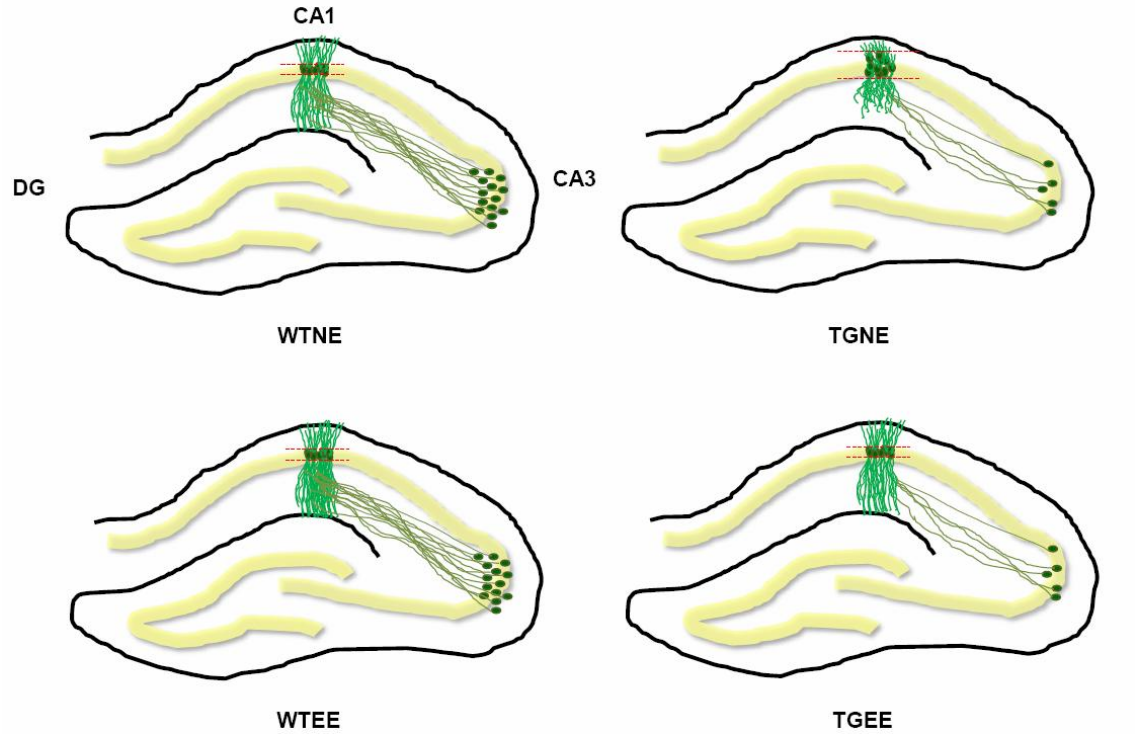

Figure 3. Activity-dependent hippocampal structural plasticity in TgDyrk1A mice. Schematic representation summarizing the main results obtained in this study. Briefly, environmental enrichment normalizes *stratum oriens* and *radiatum* dendritic widths, and *stratum pyramidale* area in CA1 region of TgDyrk1A mice.

We also examined whether EE was able to modulate spine density and morphology of CA1 apical dendrites. In wild types one month of EE stimulated spinogenesis mainly increasing the number of immature spines. In TgDyrk1A mice, even though the density of spines was not modified upon EE, similar as occurs in Ts65Dn cortical neurons (Dierssen, Benavides-Piccione et al. 2003), EE stimulated the stabilization of preexisting spines by reducing the amount of immature spines at expense of increasing the density of mature ones to NE-wild type levels. Given that EE improves hippocampal-dependent learning and memory in both genotypes, these results suggested

that both wild type and TgDyrk1A hippocampal neurons respond very different upon environmental stimuli to ultimately enhance the functional connections in neuronal circuits involved in cognition.

EE has been shown to increase spine density and to stabilize new synapses through a cytoskeleton protein known as β-ADDUCIN that cap actin filaments in the cytosol and link them to the cell membrane (Bednarek and Caroni 2011). Given that previous results in our laboratory demonstrated that morphological alterations in TgDyrk1A primary cultures were accompanied with a significant reduction of the percentage of actin mobile fraction in TgDyrk1A spines (Martinez de Lagran, Benavides-Piccione et al. 2012), we could speculate that the normalization of DYRK1A kinase activity by EE could affect the polymerization of actin filaments with consequences in spine maturation.

Regarding synaptic transmission or plasticity in CA3-CA1 regions, electrophysiological recordings in CA1 showed that reduced PPF in TgDyrk1A mice was completely rescued by EE. EE also significantly increased TBS-induced LTP in hippocampal slices as previously described (Artola, von Frijtag et al. 2006; Kumar, Rani et al. 2012) in wild type and in TgDyrk1A mice, in which EE completely reversed LTP impairments to NE-wild type levels. These results suggest that the dendritic and spine modifications upon EE had an impact on the functional neuronal connections, probably explaining the improving effects on LTP and cognition in EE-wild type mice and the rescuing hippocampal alterations in TgDyrk1A. Our results demonstrated that EE increased the excitatory-inhibitory balance towards excitation in the CA1 *stratum radiatum* in wild types. Although we have not evaluated whether EE changes the receptor abundance in the postsynaptic terminals of CA1 pyramidal dendrites (synaptic scaling) in our mice, previous reports found higher AMPA receptor binding and density of AMPA receptor subunits in the hippocampus of EE mice (Gagne, Gelinas et al. 1998; Naka, Narita et al. 2005). Hence, similar mechanisms could underlie the increased CA1 LTP response in EE-wild type mice. In the case of TgDyrk1A, EE restored the excitatory-inhibitory balance in the CA1 *stratum radiatum*, which correlates with a significant recover of the LTP. Importantly, our results are in accordance with previous work in Ts65Dn mice demonstrating that EE is able to rescue hippocampal cognition and synaptic plasticity

alterations through modulating the excitatory-inhibitory balance to wild type levels (Begenisic, Spolidoro et al. 2011).

Taken together, the results suggested that in wild type mice EE increased dendritic complexity, length and number of spines in CA1 *stratum radiatum* to significantly enhance the functional neuronal circuitry with consequences on visuo-spatial learning and memory. Interestingly, TgDyrk1A mice also react to environmental stimuli but through other mechanisms. In fact, although EE did not significantly affect dendritic complexity, it increases the largest apical dendrite in the *stratum radiatum*, stimulates the maturation of instable spines into stable ones and more importantly, it normalizes the gross *stratum radiatum* and *oriens* width to increase the synaptic plasticity in the hippocampus and rescues visuo-spatial learning and memory defects.

Another plausible cellular mechanism to explain the cognitive recovery after EE refers to adult neurogenesis (see Introduction section). As explained above, TgDyrk1A mice showed disrupted adult neurogenesis in the DG similar to trisomic DS mouse models (Contestabile, 2007; Chakrabarti, 2011). In our experiments, EE increased neuronal survival of newborn cells, dendritic complexity and the number of DG cells activated after spatial learning in both wild type and transgenic mice. Moreover, in TgDyrk1A mice EE was also able to rescue cell cycle alterations, normalizing cell cycle exit probably because EE cells adequately progressed from G2 to M phase.

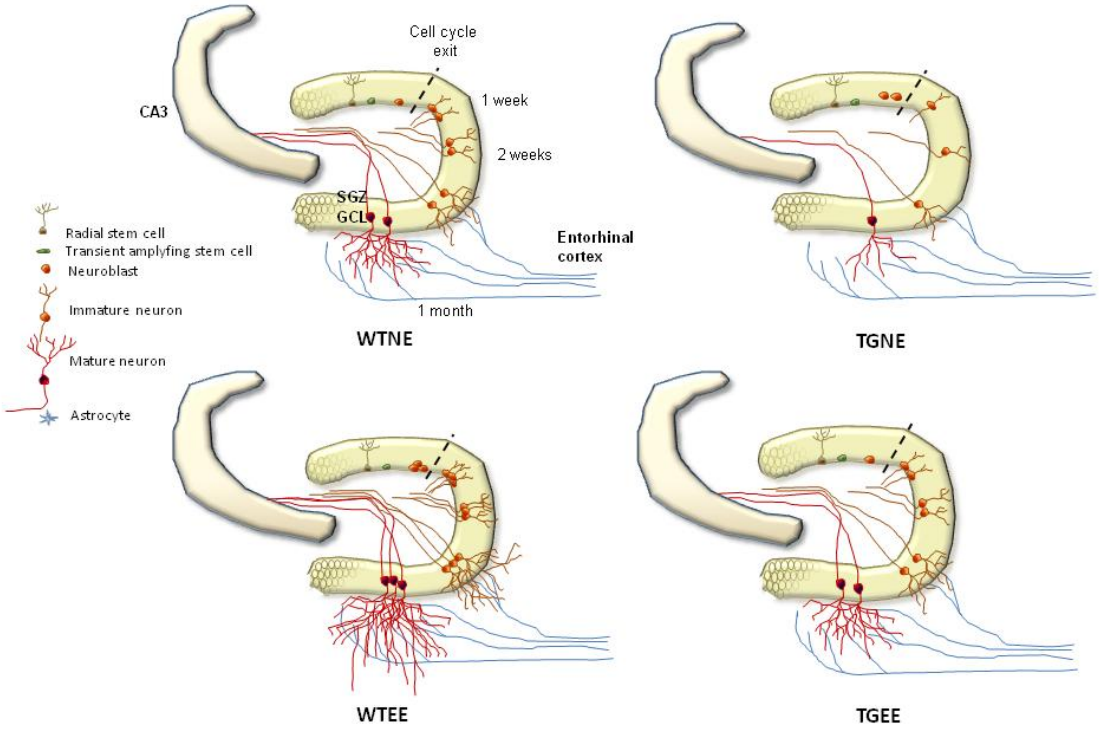

Figure 4. Hippocampal adult neurogenesis in TgDyrk1A mice under normal and enriched rearing conditions. Schematic representation summarizing the main results obtained in this study. Briefly, environmental enrichment increases hippocampal adult neurogenesis in both genotypes and in the case of TgDyrk1A mice it rescues completely adult neurogenesis defects probably contributing to learning and memory improvements.

Previous results analyzing the effects of EE on adult neurogenesis in Ts65Dn mice have found contradictory results. While some authors described that postweaning EE rescued specifically adult neurogenesis impairments in the DG with a complete recover on hippocampal-dependent cognitive tasks (Chakrabarti, Scafidi et al. 2011), others demonstrated that even though physical exercise rescued hippocampal-dependent learning deficits in 10-12 months old Ts65Dn mice, it did not recover adult neurogenesis defects (Llorens-Martin, Rueda et al. 2010). The age at which trisomic mice receive EE (PD21 *vs.* 10-months-old) could be a key determinant in the different effects observed. In this line, some studies showed that in contrast to postweaning EE, preweaning enrichment had no lasting measurable effect on adult hippocampal neurogenesis in four-months-old mice (Kohl, Kuhn et al. 2002). Therefore, these results pointed that EE effectively modifies adult neurogenesis only in a specific critical period of time.

Many studies have shown that EE enhances learning and memory (Nithianantharajah and Hannan 2006) through changes in the expression of genes involved in neuronal structure, synaptic signaling and plasticity (Rampon, Jiang et al. 2000). Our results using a DYRK1A kinase inhibitor demonstrated that DYRK1A kinase activity is involved in modulating hippocampal structural plasticity and adult neurogenesis in the DG. Importantly, EE normalized DYRK1A kinase activity selectively in TgDyrk1A hippocampus thus suggesting that the rescuing effects of EE on cognition and neuronal plasticity are at least in part through DYRK1A kinase activity normalization.

5.3.1. Long-term stability of the neural plasticity effects of environmental enrichment in TgDyrk1A mice

One concern in DS is that early intervention programs improve only transiently certain cognitive capacities. To analyze the long-term stability of EE effects on cognition and neuronal plasticity in our model, we reared wild type and TgDyrk1A mice during one month under EE conditions followed by four months under NE conditions. Four months after discontinuing EE rearing conditions, wild type and Dyrk1A overexpressing mice still showed an improved learning, reference memory and cognitive flexibility capacities in the MWM as compared to NE- mice being the improvement similar as that detected immediately after EE. Conversely, the improvement detected upon EE in the object recognition memory performance, was not maintained after four months in TgDyrk1A mice after EE discontinuation. These results indicated that the stability of EE effects on hippocampal-dependent cognition in TgDyrk1A mice are not homogeneous, again suggesting that the neuronal mechanisms involved in spatial learning tasks are more stable whereas recognition memory would be more influenced by environmental changes.

We also analyzed whether the discontinuation of EE would also affect the effects of the treatment on hippocampal structural and synaptic plasticity. In fact, upon EE discontinuation, TgDyrk1A mice do not maintain the rescuing effects of EE on structural plasticity in CA1 region and on LTP. Taken together these results suggest that DYRK1A is key in the maintenance of activity-dependent neuronal plasticity and cognitive effects of EE.

5.3.2. Synergistic effects of environmental enrichment and EGCG on hippocampal-dependent cognition in TgDyrk1A mice

As described above, both EGCG and EE alone were able to rescue hippocampal-dependent learning and memory and neural plasticity deficits in TgDyrk1A mice and EE normalized DYRK1A kinase activity in transgenic hippocampus. Thus, the combination of both treatments might enhance the cognitive improvements in TgDyrk1A mice. We treated transgenic mice during one month with EGCG and EE and detected a synergistic positive effect in TgDyrk1A mice that significantly improved spatial learning, reference memory and cognitive flexibility in the MWM as compared to EE or EGCG effects alone. It could be argued that EE and EGCG modulate similar synaptic plasticity related proteins in the hippocampus that have been shown to underlie cognition. Among the huge amount of possible candidate proteins, it has been reported that EE increases BDNF levels in the hippocampus (Sun, Zhang et al. 2010; Kuzumaki, Ikegami et al. 2011) and similarly, EGCG increases the expression of BDNF in hippocampal neurons (Nath, Bachani et al. 2012). For interest in this Thesis, studies performed in another Dyrk1A overexpressing mouse model have shown that EGCG normalized the levels of BDNF (Guedj, Sebrie et al. 2009). In the same line, both treatments have been shown to increase the levels of hippocampal PKC, a well-known signaling protein involved in learning and memory (Paylor, Morrison et al. 1992; Levites, Amit et al. 2003). Therefore, it could be speculate that probably both treatments are enhancing the levels of synaptic proteins in a synergic manner. However, our results showed an impaired performance in the NOR in TgDyrk1A mice treated with EE and EGCG. Again, these results suggested that both hippocampal-dependent tasks depend on different neuronal mechanisms. We have not analyzed DYRK1A kinase activity in mice treated with both EE and EGCG, but it could be speculated that whereas in some brain regions EE and EGCG could produce a synergic inhibition of DYRK1A kinase levels reaching the same amount as Dyrk1A+/- mice and thus leading to a deleterious effect, in other regions there could be some compensatory mechanisms to keep DYRK1A kinase activity levels in a narrow range of normalization leading to positive effects. Nevertheless, the synergic positive effects observed in the MWM task, support the idea of using a new therapeutical strategy by combining both therapies to improve certain types of hippocampal-dependent tasks in DS individuals.

CONCLUSIONS

6. CONCLUSIONS

1. DYRK1A overexpression impairs hippocampal-dependent spatial learning, reference memory, cognitive flexibility and recognition memory in TgDyrk1A mice recapitulating the cognitive impairments observed in trisomic mouse models that are relevant to Down syndrome individuals.
2. TgDyrk1A mice present a discrete hippocampal dysplasia, affecting specific hippocampal strata of the CA1 subfield, and reduced numbers of pyramidal cells in CA3. Although dendritic complexity is not altered in transgenic mice, increased proportion of immature-like dendritic spines are found in CA1 *stratum radiatum* receiving CA3 projections. These alterations along with an excitatory/inhibitory imbalance are possibly contributing to the decreased synaptic function and TBS-induced LTP detected in these mice
3. DYRK1A overexpression disrupts hippocampal adult neurogenesis in the DG. Transgenic mice present reduced cell proliferation rate, altered cell cycle progression and reduced cell cycle exit, possibly leading to premature migration, impoverished differentiation and reduced survival of newly born cells. In addition less proportion of newborn TgDyrk1A neurons are activated upon learning, suggesting reduced integration in learning circuits.
4. Hippocampal-dependent learning and memory impairments, as well as some hippocampal morphology and adult neurogenesis alterations in TgDyrk1A mice are reversed by treatment with epigallocatechin-3-gallate (EGCG), a DYRK1A kinase activity inhibitor.
5. Environmental enrichment (EE) reduces DYRK1A kinase activity in the hippocampus and normalizes the defects in cognition, hippocampal structure, spine morphology, activity-dependent synaptic plasticity and adult neurogenesis in TgDyrk1A mice. This indicates that, when overexpressed, DYRK1A is susceptible to an activity-dependent regulation and suggests that behavioral improvements of EE and neuronal plasticity changes in transgenic mice are in part due to DYRK1A kinase modulation.
6. The effects of EE in TgDyrk1A mice are only partially maintained after four months of treatment discontinuation, on cognition and completely lost on hippocampal structural and synaptic plasticity.

7. Concomitant treatment with EE and EGCG produces a synergistic positive effect on cognition, suggesting that pharmacological targeting of DYRK1A kinase activity excess can boost the therapeutic effects of environmental treatments.

In conclusion, our results demonstrate that *in vivo* Dyrk1A overexpression is relevant for the reduced activity-dependent hippocampal neuronal plasticity underling learning and memory impairments. We demonstrate that DYRK1A kinase activity is sensitive to environmental stimulation. This opens a new view in which more effective therapies should be directed to pharmacological boosting of environmental therapies for DS.

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BIBLIOGRAPHY

ANNEX

ANNEX

ANNEX I: ABREVIATIONS

APP	Amyloid Precursor Protein
BrdU	5'-Bromo-2'-deoxyuridine
CA1	Cornu Ammonis 1
CA3	Cornu Ammonis 3
cm	centimeter
CNS	Central Nervous System
DCX	Doublecortin
DG	Dentate Gyrus
DS	Down Syndrome
DYRK1A	Dual specificity Yak1-Related Kinase
EE	Environmental Enrichment
EGCG	Epigallocatechin-3-gallate
EPM	Elevated Plus Maze
fEPSP	field Evoked Post-Synaptic Potential
FBS	Fetal Bovine Serum
GABA	Gamma Aminobutyric Acid
GCL	Granular Cell Layer
GFAP	Glial Fibrillary Acidic Protein
h	hour
HCL	Hydrochloric acid
HSA21	Homo sapiens autosome 21
LTD	Long-Term Depression
LTP	Long-Term Potentiation
LY	Lucifer Yellow
M	Molar
min	minute
ml	milliliter
mM	millimolar
mm	millimeter
MMU10	*Mus musculus* chromosome 10

MMU16	*Mus musculus* chromosome 16
MWM	Morris Water Maze
n	number
NE	Non Enriched
NeuN	Neuronal nuclear antigen
NOR	Novel Object Recognition
NSC	Neural Stem Cell
PBS	Phosphate Buffered Saline
PBS-T	Saline Phosphate Buffer with Triton X-100
PFA	Paraformaldehid
pH3	Phosphorylated-(Ser10)-histone-H3 (pH3)
PPF	Paired-Pulse Facilitation
RT	Room Temperature
s	second
SEM	Standard Error of the Mean
SVZ	Subventricular Zone
TBS	Theta Burst Stimulation
TG	Transgenic mice overexpressing Dyrk1A
TgDyrk1A	Transgenic mice overexpressing Dyrk1A
VGAT	Vesicular GABA Transporter
VGLUT	Vesicular glutamate Transporter
WT	Wild Type
YFP	Yellow Fluorescent Protein
µl	microliter
µm	micrometer

ANNEX II: SUPPLEMENTARY TABLE

Table 1. List of antibodies used for immunostaining

Antibody	Dilution	Specie	Company
BrdU	1/75	Rat	Serotec
Calretinin	1/1000	Rabbit	Sigma
Capase3	1/300	Rabbit	BD Pharmigen
cFos	1/500	Rabbit	Santacruz
DCX	1/300	Goat	Santacruz
Dyrk1A	1/100	Mouse	Abnova
GFAP	1/500	Mouse	Abnova
GFAP	1/500	Rabbit	Dako
pH3	1/100	Rabbit	Cell signaling
Ki67	1/100	Mouse	Novocastra
Ki67	1/200	Mouse	BD Pharmigen
Ki67	1/200	Rabbit	Abcam
NeuN	1/200	Mouse	Chemicon
Sox2	1/400	Rabbit	Millipore
VGLUT	1/200	Mouse	Synaptic systems
VGAT	1/200	Guinea pig	Synaptic systems

ANNEX II

ANNEX III: SUPPLEMENTARY RESULTS

1. Environmental enrichment restores dendritic structural deficits in CA1 *stratum radiatum* of TgDyrk1A mice

Since we detected gross morphological alterations in the CA1 hippocampal subfield that were normalized by one month of EE, we evaluated whether the complexity of CA1 apical dendrites in the *stratum radiatum* was changed by EE in TgDyrk1A mice using Lucifer yellow injections (collaboration with Dr. Inma Ballesteros, UCLM).

1.1. Materials and Methods

Intracellular injections of hippocampal pyramidal cells

Four two-months-old mice per genotype and treatment were used to study the dendritic morphology in the *stratum radiatum* of pyramidal CA1 cells. Intracellular injections of Lucifer yellow (LY) were performed in dorsal hippocampus CA1 pyramidal neurons. Animals were perfused with 4% PFA and 150 μm coronal sections were obtained with a vibratome, mounted and coverslipped with mowiol reagent.

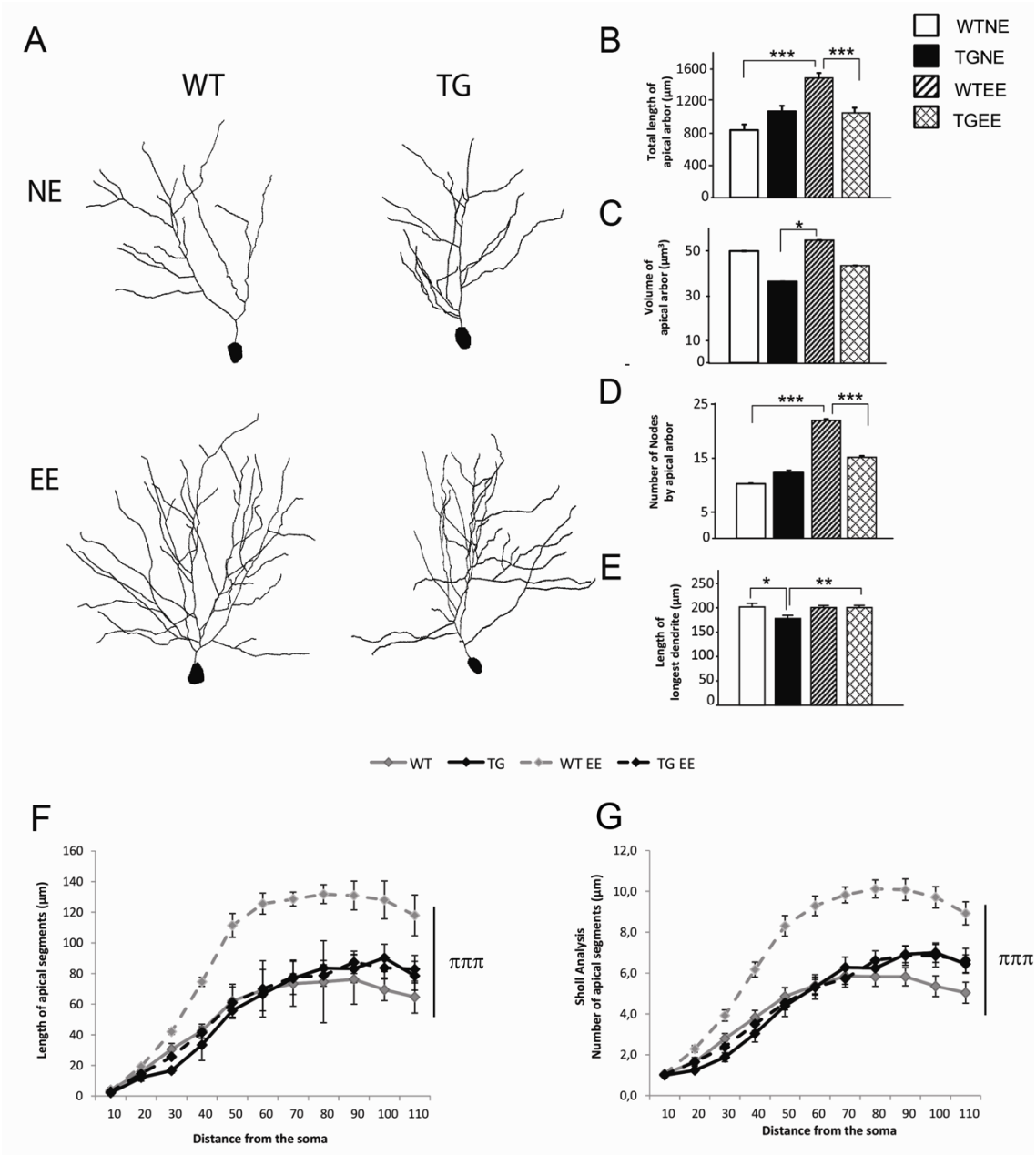
Cell injection methodology was previously described in detail (Elston and Rosa 1997; Elston, Benavides-Piccione et al. 2001). Briefly, cells were injected with fluorescent LY by continuous current. Only pyramidal neurons whose entire apical dendritic arbor was completely filled were included in the analysis. Images were acquired with a confocal microscope (SPE; Leica Microsystems). Morphometric analysis was performed using NIH image software (NIH Research Services) (Ballesteros-Yanez, Benavides-Piccione et al. 2006). The size of dendritic arbors was determined by calculating the area contained within a polygon that joined the outermost distal tips of the apical dendrites (Elston and Rosa 1997). Branching pattern was determined by counting the number of dendritic branches that intersected concentric circles (centered on the cell body) of radii that incremented by 10 μm (Sholl 1953).

1.2. Results

Under NE conditions we did not observe important differences neither in the length of the apical arbor nor in the number of nodes between genotypes (Supplementary Figures

S1A, S1B, S1C and S1D). However, the longest axis of stratum radiatum as defined by the longest apical dendrite of the arbor, was significantly shorter in pyramidal neurons from TgDyrk1A mice (Supplementary Figure S1E, TGNE *vs.* WTNE two-way ANOVA genotype-treatment interaction $F(1,130) = 3.73$, $p<0.01$; Bonferroni as *post-hoc* $p<0.05$). This is relevant since distal apical dendrites form non-local synapses while shorter proximal apical dendrites project radially to local pyramidal cells and interneurons.

EE produced different effects depending on the genotype. While in wild type mice EE significantly increased the dendritic complexity of the pyramidal CA1 *stratum radiatum* neurons as shown by the total length of dendritic segments (Supplementary Figures S1B and S1F, WTEE *vs.* WTNE two-way ANOVA genotype-treatment interaction $F(1,130) = 19.1$, $p<0.001$; Bonferroni as *post-hoc* $p<0.001$) and the number of nodes (Supplementary Figures S1D and S1G, WTEE *vs.* WTNE two-way ANOVA genotype-treatment interaction $F(1,130) = 33.80$, $p<0.001$; Bonferroni as *post-hoc* $p<0.001$), in TgDyrk1A EE only reduced the length of the longest apical dendrite (Supplementary Figure S1E, TGEE *vs.* TGNE Bonferroni as *post-hoc* $p<0.01$).



Supplementary Figure 1. Activity-dependent dendritic complexity in CA1 *stratum radiatum* pyramidal neurons. (A) Representative apical dendritic arbor in CA1 pyramidal neurons stained with Lucifer yellow of wild type and TgDyrk1A mice reared during one month under non enriched (NE) or environmental enriched (EE) conditions. (B) Total length of apical dendritic segments. (C) Volume of apical arbor. (D) Number of nodes of each apical arbor. (E) Length of the longest dendrite. (F-G) Sholl analysis (concentric circles around a cell of radii incremented by 10 µm) showing (F) the length of the apical dendritic segments or (G) the number of nodes by the distance from the soma. Data are expressed as ± SEM. n = number of CA1 pyramidal neurons. WTNE n = 28; TGNE n = 25; WTEE n = 52; TGEE n = 26. Two-way ANOVA treatment effect πππ $p<0.001$; Bonferroni as post-hoc: * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

2. Activity-dependent synaptic plasticity in CA1 hippocampal subregion of TgDyrk1A mice

We performed an *in vitro* analysis of activity-dependent synaptic plasticity by carrying out field excitatory post-synaptic potential (fEPSP) recordings in the CA1 region of the hippocampus in brain slices.

2.1. Materials and Methods

Electrophysiological recordings

Field excitatory postsynaptic potentials (fEPSPs) were recorded in the *stratum radiatum* of dorsal hippocampal CA1 region in response to stimulation of the Schaffer collateral pathway. After decapitation, the brain was quickly removed and placed on ice-cold cutting solution (in mM): KCl 2.5; MgSO4 3; NaHPO4 1.25; CaCl2 1; NaHCO3 26; sucrose 10 and gassed with 95% O2-5% CO2 to a final pH of 7.4. Coronal slices (400 µm thick; Bregma -1.06 mm to – 2.18 mm) were obtained with a vibratome (Leica); placed in an interface style recording chamber (Fine Science Tools, Foster City, CA) and bathed in artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl, 124; KCl, 2.5; MgSO4, 1; NaHPO4, 1.25; CaCl2, 2; NaHCO3, 26; Dextrose, 10; and was aerated with 95% O2-5% CO2 to a final pH of 7.4. Bath temperature was maintained at 32–34°C. Unfiltered recordings were obtained by means of glass electrodes (impedance 1-2 MΩ) through a Neurolog system amplifier (Digitimer). Electrical stimuli were delivered with concentric bipolar electrode (platinum-iridium) with the stimulus strength adjusted to a stimulation intensity that yielded a half-maximal response. For each slice, after establishing a stable baseline, paired-pulse facilitation (PPF) was induced by a double-pulse (50 ms apart) stimulation protocol. After 15 min baseline registering (pulse at 0.016 Hz), long-term potentiation (LTP) was induced by theta burst stimulation (TBS) (5 episodes at 0.1 Hz; each episode was 10 stimulus trains of 4 pulses at 100 Hz; delivered at 5 Hz) and registered for 60 min (pulse at 0.016 Hz). Recordings were digitized, acquired, and analyzed using a data acquisition interface and software from Cambridge Electronic Design (Spike2, Cambridge, UK).

2.2. Results

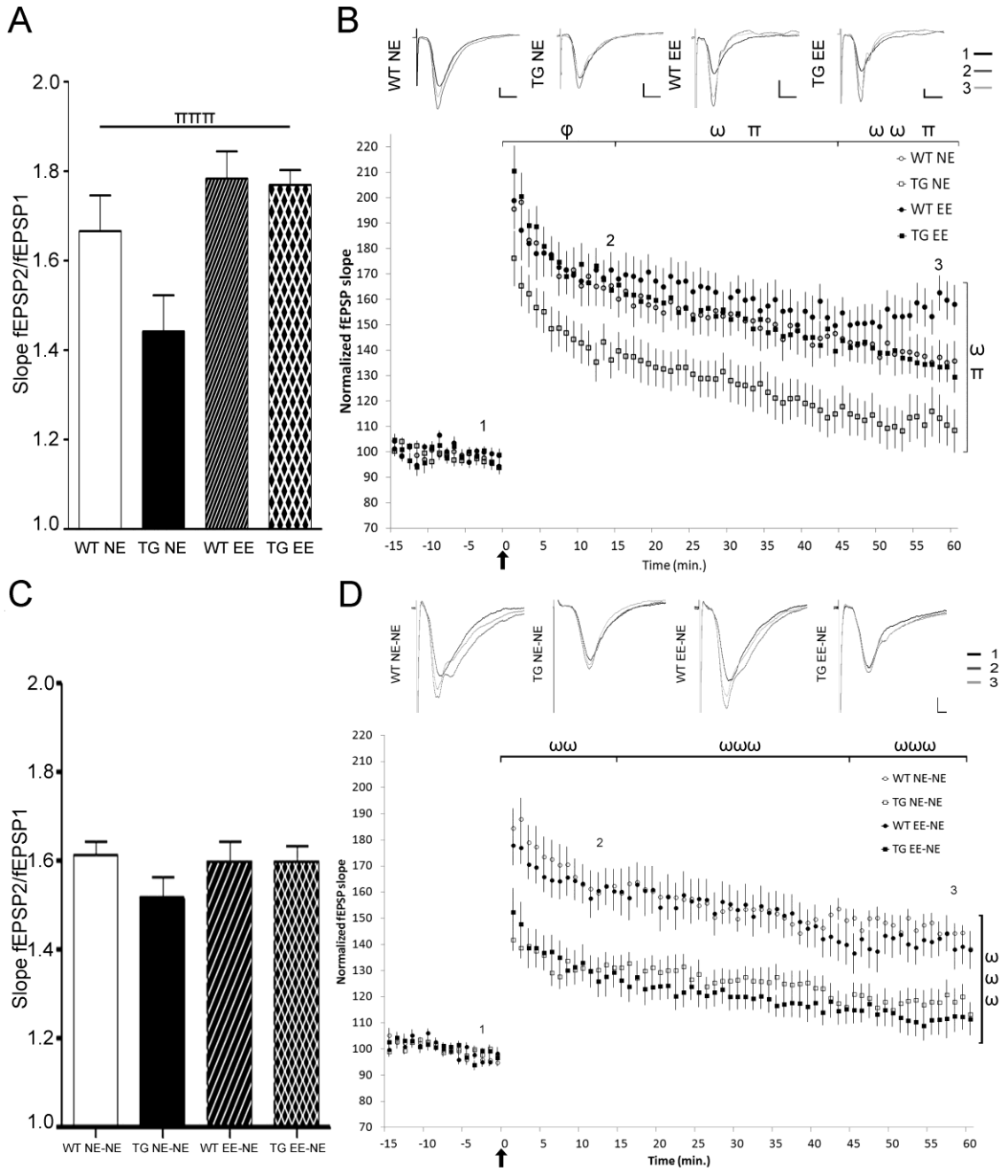
Two different protocols were applied: paired-pulse facilitation (interstimulus intervals (ISI) of 50 ms) to study the presynaptic component of signal transduction and theta burst stimulation (TBS) in order to induce long-term potentiation (LTP) in the Schaffer collateral pathway.

To examine presynaptic function, we compared paired-pulse facilitation (PPF) exhibited by TgDyrk1A mice and their control littermates under NE or EE conditions. Under NE conditions TgDyrk1A mice presented a trend to reduced PPF (ratio between both stimulations fEPSP2/fEPSP1) as compared to wild types (Supplementary Figure S2A, two way ANOVA genotype effect $F(1,45)=3.29$, $p=0.076$) that was significantly increased by one month of EE (Supplementary Figure S2A, two-way ANOVA treatment effect $F(1,45)=11.5$, $p<0.001$). These results demonstrated that although TgDyrk1A mice slightly impaired pre-synaptic plasticity, environmental enrichment is able to normalize PPF to wild type levels.

We then investigated hippocampal LTP, which is considered a major physiological mechanism of learning and memory and has been widely related with synaptic alterations (Yuste and Bonhoeffer 2001). The fEPSP of hippocampal CA1 was induced by TBS in the Schaffer collateral pathway. The baseline fEPSPs, as well as the evoked potentials after the TBS induction were recorded for each brain slice. No significant differences were found at the baseline level between groups. In contrast, the comparative analysis of the time-course after TBS stimulation revealed that NE-TgDyrk1A mice had a significant deficit in hippocampal LTP compared to wild types (Supplementary Figure S2B, two-way ANOVA repeated measures genotype effect $F(1,43)=4.83$, $p<0.05$) as shown by the reduced slope of the curve. Importantly, EE significantly increased fEPSP in both genotypes that in the TgDyrk1A mice gave rise to a completely recovery of the fEPSP slope (Supplementary Figure S2B, two-way ANOVA repeated measures treatment effect $F(1,43)=3.76$, $p<0.05$). Detailed values and statistical analysis are summarized in Tables 1 and 2. These results demonstrated that EE treatment completely rescued the synaptic plasticity shown by the LTP response in the CA1 hippocampal subfield in TgDyrk1A mice.

We also evaluated whether discontinuing environmental enrichment during four months had a consequence on synaptic plasticity by analyzing *in vitro* activity-dependent synaptic plasticity in CA1 region after treatment discontinuation. No differences were

detected in PPF between groups (Supplementary Figure S2C). The effects of EE on LTP response upon TBS stimulation were lost after discontinuation of the treatment (Supplementary Figure S2D). Thus, TgDyrk1A mice showed a significant impairment in LTP response independent of the rearing conditions (Supplementary Figure S2D, two-way ANOVA repeated measures genotype effect $F(1,50)=9.75$, $p<0.001$). Detailed values and statistical analysis are summarized in TableS 3 and 4. Taking together, our results showed that the hippocampal synaptic plasticity induced by one month of EE did not last across time.



Supplementary Figure 2. Activity-dependent synaptic plasticity in the Schaffer collateral pathway in TgDyrk1A mice. (A-C) Paired pulse facilitation (fEPSP2/fEPSP1) in the hippocampus of wild type (WT) and TgDyrk1A (TG) mice (A) reared under non enriched (NE) or environmental enriched (EE) conditions during one month (number of slice/animals: WTNE = 11/5; TGNE = 11/5; WTEE = 9/5; TGEE= 16/5) and (C) reared under NE or EE conditions during one month followed by four months under NE conditions (number of slice/animals: WTNE-NE = 16/5; TGNE-NE = 13/6; WTEE-NE = 15/7; TGEE-NE= 16/5). The inter-stimuli interval (ISI) was 50ms. (B-D) Long-term potentiation in the CA1 region induced by theta burst stimulation (TBS) in the CA3 region after (B) one month of EE (WTNE: 11/5; TGNE: 14/5; WTEE: 14/6; TGEE: 16/5) or (D) after one month under NE or EE conditions followed by four months under NE conditions (WTNE-NE: 16/5; TGNE-NE: 15/6; WTEE-NE: 15/6; TGEE-NE: 16/5). Upper panel: representation of the mean of 5 responses to a stimulation at time 1 (-5 min), 2 (15 min) and 3 (60 min). Black vertical bar represents 500µV. Black horizontal bar represents 5ms. Lower panel: normalized fEPSP slope before and after TBS. Black horizontal bar represents 2ms. Lower panel: normalized fEPSP slope before and after TBS stimulation. Data are represented as mean ± SEM. (A-C) Two-way ANOVA treatment effect πππ $p< 0.001$. (B-D) Two-way ANOVA repeated measures genotype-treatment interaction φ<0.05; genotype effect ω $p<0.05$; ωωω $p<0.01$; ωωω $p<0.001$; treatment effect π $p<0.05$.

Table 1: Mean values of paired pulse and long term potentiation (LTP) obtained in wild type (WT) and TgDyrk1A (TG) mice reared during one month under non enriched (NE) or environmental enriched (EE) conditions.

Paired Pulse		**WTNE**	**WTEE**	**TGNE**	**TGEE**
Slope $fEPSP_1/fEPSP_2$		1.66±0.07	1.78±0.07	1.44±0.62	1.77±0.05
LTP					
Slope fEPSP	Baseline	99.25±2.3	95.9±2.06	99.42±2.06	100.02±1.9
	15’	160.17±7.9	168±7	139.7±7.2	165.8±6.5
	60’	135.7±12.6	157.91±10.3	108.3±10.3	129.45±9.2

Table 2: Statistical analysis of paired pulse and LTP for mice reared during one month under NE or EE conditions.

Paired pulse		Genotype	Treatment	Genotype x Treatment
Two-way ANOVA		$F_{(1,45)}$=3.29, p=0.076	$F_{(1,45)}$=11.5, p=0.001	$F_{(1,45)}$=2.55, p=0.117
LTP		Genotype	Treatment	Genotype x Treatment
Baseline		$F_{(1,18)}$=0.795; P=0.384	$F_{(1,18)}$=0.072; P=0.792	$F_{(1,18)}$=0.006; P=0.939
Two-way ANOVA RM after TBS	all	$F_{(1,43)}$=4.83; P=0.033	$F_{(1,43)}$=3.76; P=0.05	$F_{(1,43)}$=1.71; P=1.97
	0-15	$F_{(1,50)}$=2.23; P=0.141	$F_{(1,50)}$=3.89; P=0.05	$F_{(1,50)}$=3.76; P=0.05
	15-45	$F_{(1,46)}$=6.15; P=0.017	$F_{(1,46)}$=5.24; P=0.027	$F_{(1,46)}$=2.16; P=0.148
	45-60	$F_{(1,43)}$=7.02; P=0.01	$F_{(1,43)}$=5.11; P=0.029	$F_{(1,43)}$=0.38; P=0.54

Table 3: Mean values Paired pulse and LTP obtained in wild type (WT) and TgDyrk1A (TG) mice reared during one month under non enriched (NE) or environmental enriched (EE) conditions followed by four months under NE conditions.

Paired Pulse		**WTNE-NE**	**WTEE-NE**	**TGNE-NE**	**TGEE-NE**
Slope $fEPSP_1/fEPSP_2$		1.61±0.12	1.59±0.17	1.51±0.17	1.59±0.15
LTP					
Slope fEPSP	Baseline	95.78±2.1	101.05±2.1	97.84±2.1	96.61±2.3
	15’	164.36±9.2	158.67±9.2	135.59±9.2	138.74±9.9
	60’	138.34±5.5	137.74±5.5	117.84±5.5	115.68±6

Table 4: Statistical analysis of paired pulse and LTP for mice reared during one month under NE or EE conditions followed by four months under NE conditions.

Paired pulse		Genotype	Treatment	Genotype x Treatment
Two way ANOVA		$F_{(1,60)}$=1.439, p=0.235	$F_{(1,60)}$=0.734, p=0.395	$F_{(1,60)}$=1.433, p=0.236
LTP		Genotype	Treatment	Genotype x Treatment
Baseline		$F_{(1,57)}$=0.507; P=0.479	$F_{(1,57)}$=0.005; P=0.942	$F_{(1,57)}$=3.788; P=0.057
Two-way ANOVA RM after TBS	all	$F_{(1,50)}$=9.75; P=0.003	$F_{(1,50)}$=0.04; P=0.832	$F_{(1,50)}$=0.37; P=0.543
	0-15	$F_{(1,61)}$=5.88; P=0.01	$F_{(1,61)}$=0.2; P=0.654	$F_{(1,61)}$=0.64; P=0.428
	15-45	$F_{(1,57)}$=15.3; P=0.000	$F_{(1,57)}$=0.35; P=0.557	$F_{(1,57)}$=0.13; P=0.716
	45-60	$F_{(1,50)}$=16.5; P=0.000	$F_{(1,50)}$=0.58; P=0.449	$F_{(1,50)}$=0.01; P=0.905

ANNEX III

ANNEX IV: PUBLICATIONS

Original papers

1. **Pons-Espinal M**, Martinez de Lagran M, & Dierssen M. **Environmental enrichment rescues Dyrk1A activity and hippocampal adult neurogenesis in TgDyrk1A.** Neurobiology of Disease. 2013. *In press.*

2. De la Torre R, De Sola S, **Pons-Espinal M**, Martínez de Lagran M, Farré M, Fitó M, Benejam B, Langohr K, Rodriguez J, Pujadas M, Duchon A, Bizot JC, Cuenca A, Janel N, Covas M, Blehaut H, Herault Y, Delabar JM, Dierssen M. **Epigallocatechin-3-gallate, a Dyrk1A inhibitor, rescues cognitive deficits in Down syndrome mouse models and in humans.** Molecular Nutrition and Food Research. 2013. *In press.*

3. **Pons-Espinal M**, Gener T, Ballesteros-Yanez I, Sanchez-Vives MV, Martinez de Lagran M & Dierssen M. **Dyrk1A overdosage impairs long-term hippocampal structural plasticity effects of environmental enrichment** *(In preparation)*

Reviews

1. **Pons-Espinal M**, Martinez de Lagran M, & Dierssen M. **Functional implications of hippocampal adult neurogenesis in intellectual disabilities.** Amino Acids. 2013; 45(1): 113-131. Review.

Scientific international meetings

- **Pons-Espinal M**, Martinez de Lagrán M, Dierssen M. Environmental enrichment normalizes Dyrk1A activity and hippocampal plasticity in TgDyrk1A mice. Society for Neuroscience, San Diego, USA-9th-13rd November 2013 (poster presentation).
- Martínez de Lagrán M., **Pons-Espinal M.**, Ruiz-Mejías M., Ciria-Suarez L., Mattia M., de la Torre R., Sanchez-Vives M.V., Dierssen M.. Narrowing "Down" the genes: Dyrk1A as a multi-level regulator of adult plasticity. Workshop on Cognition in Down syndrome. Washington, USA-13th-15th April 2013 (poster presentation).
- **Pons-Espinal M**, Martinez de Lagrán M, Dierssen M. The effect of environmental enrichment on adult neurogenesis in a mouse model overexpressing Dyrk1A, a

candidate gene for Down syndrome. 8th FENS Forum on European Neuroscience, Barcelona, Spain-14th-18th July 2012 (poster presentation).

- **Pons-Espinal M**, Martinez de Lagrán M, Dierssen M. EGCG improves cognitive impairments in a mouse model overexpressing Dyrk1A, a candidate gene for Down syndrome. Jounées Internationationales Jérôme Lejeune, Paris - March 24-26, 2011 (poster presentation).
- **Pons-Espinal M**, Martinez de Lagrán M, Dierssen M. The effect of environmental enrichment in a murine model that overexpresses Dyrk1A, a candidate gene for Down syndrome. Jounées Internationationales Jérôme Lejeune, Paris - March 24-26, 2011 (poster presentation).
- **Pons-Espinal M**, Martinez de Lagrán M, Dierssen M. The effect of environmental enrichment in a murine model that overexpresses Dyrk1A, a candidate gene for Down syndrome. CNRS Jacques Monod Conference "Mental retardation", Roscoff - October 7-11, 2010 (poster presentation).
- **Pons-Espinal M**, Martinez de Lagrán M, Dierssen M. The effect of environmental enrichment in a murine model that overexpresses Dyrk1A, a candidate gene for Down syndrome. 2nd AnEUploidy Workshop, Split, Croatia, September 16-19, 2010 (poster presentation).
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Environmental enrichment rescues Dyrk1A activity and hippocampal adult neurogenesis in TgDyrk1A

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ABSTRACT

Hippocampal adult neurogenesis disruptions have been suggested as one of the neuronal plasticity mechanisms underlying learning and memory impairment in Down syndrome (DS). However, it remains unknown whether specific candidate genes are implicated in these phenotypes in the multifactorial context of DS. Here we report that transgenic mice (TgDyrk1A) with overdosage of *Dyrk1A*, a DS candidate gene, show important alterations in adult neurogenesis including reduced cell proliferation rate, altered cell cycle progression and reduced cell cycle exit leading to premature migration, differentiation and reduced survival of newly born cells. In addition, less proportion of newborn hippocampal TgDyrk1A neurons are activated upon learning, suggesting reduced integration in learning circuits. Some of these alterations were DYRK1A kinase-dependent since we could rescue those using a Dyrk1A inhibitor, epigallocatechin-3-gallate. Environmental enrichment also normalized DYRK1A kinase overdosage in the hippocampus, and rescued adult neurogenesis alterations in TgDyrk1A mice. We conclude that *Dyrk1A* is a good candidate to explain neuronal plasticity deficits in DS and that normalizing the excess of DYRK1A kinase activity either pharmacologically or using environmental stimulation can correct adult neurogenesis defects in DS.

Introduction

Down syndrome (DS; trisomy 21) is the most common genetic cause of intellectual disability. Neural plasticity mechanisms underlying intellectual disability may include defects in the functional synaptic connections between preexisting neurons and/or defects in adult neurogenesis, that are believed to be important for information processing. Brains of individuals with DS are characterized by their reduced size, decreased neuronal density, dendritic atrophy and spine dysgenesis in the hippocampus and cortex (Becker et al., 1991; Guidi et al., 2008). These morphological alterations are assumed to underlie some cognitive impairments such as deficits in place learning and recall, and episodic and long-term memory deficits in DS individuals (Lott and Dierssen, 2010).

Among the triplicated genes in trisomy 21, *Dyrk1A* has been proposed as a major candidate gene due to its location in the DS critical region of human chromosome 21 and its overexpression in fetal and adult DS brains (Dierssen and de Lagran, 2006). The encoded protein is expressed in the adult hippocampus and engineered excess or defect of Dyrk1A in mice impairs hippocampal-dependent learning and memory (Ahn et al., 2006; Altafaj et al., 2001; Arque et al., 2009; Fotaki et al., 2002) thus indicating its dosage sensitivity. *Dyrk1A* encodes for a serine/threonine kinase that plays key roles in cell proliferation and survival during embryogenesis (see Dierssen, 2013; Dierssen and de Lagran, 2006; Tejedor and Hammerle, 2011). However, much less is known about its possible involvement in adult hippocampal neurogenesis.

Although the strengthening of functional synaptic connections between preexisting hippocampal neurons upon environmental enrichment (EE) contributes to learning and memory improvements, the generation of new hippocampal neurons and their integration in the dentate gyrus (DG) also add new computational units to the neuronal network enhancing hippocampal-dependent spatial learning and memory in adult mice (Kempermann et al., 2003; van Praag et al., 2002). In individuals with DS, cognitive impairments can be improved through environmental stimulation in early intervention programs (Bonnier, 2008; Mahoney et al., 2006) and EE is able to rescue behavioral performance and learning abilities of Ts65Dn mice (a DS model bearing a partial trisomy of mouse chromosome 16), improving hippocampal dependent memories (Martinez-Cue et al., 2002). Among the plausible mechanisms of this improvement, EE is able to rescue specifically adult neurogenesis impairment in the hippocampal DG of Ts65Dn mice (Chakrabarti et al., 2011), although the underlying mechanisms

Abbreviations: DS, Down syndrome; TgDyrk1A, transgenic mice overexpressing Dyrk1A; EE, environmental enrichment; DG, dentate gyrus; EGCG, epigallocatechin-3-gallate.

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are not well understood. Moreover, we reported modifications in neurotransmitter signaling pathways (Baamonde et al., 2011) and others have observed regulation of excitatory–inhibitory imbalance (Begenisic et al., 2011).

Here, we demonstrate that single overexpression of Dyrk1A *in vivo* leads to abnormalities in hippocampal adult neurogenesis similar to those described in the trisomic Ts65Dn mice, indicating the relevance of the phenotype in DS. Those cellular alterations are rescued with EE, but also using a pharmacological Dyrk1A inhibitor. Interestingly, EE reduces DYRK1A activity in the TgDyrk1A hippocampus to wild type levels, suggesting that DYRK1A kinase activity normalization could be one of the mechanisms for the improvement seen in DS individuals submitted to early intervention programs.

Materials and methods

Animals

Transgenic mice overexpressing Dyrk1A (TgDyrk1A) were obtained as previously described (Altafaj et al., 2001). The non-transgenic littermates of TgDyrk1A mice served as controls. Of interest for the present work, Dyrk1A overdosage in TgDyrk1A yielded similar levels of overexpression than those detected in both the fetal DS brains and the partial trisomic Ts65Dn model (Toiber et al., 2010). The present experiments were conducted using only females since males showed hierarchical behavior (data not shown), similar to that observed in Ts65Dn mice (Martinez-Cue et al., 2002) that may cause stress affecting adult hippocampal neurogenesis. All animal procedures were approved by the local ethical committee (Comité Ético de Experimentación Animal del PRBB (CEEA-PRBB); procedure numbers MDS-08-1060P1 and JMC-07-1001P1-MDS), and met the guidelines of the local (law 32/2007) and European regulations (EU directive no. 86/609, EU decree 2001-486) and the Standards for Use of Laboratory Animals no. A5388-01 (NIH). The CRG is authorized to work with genetically modified organisms (A/ES/05/I-13 and A/ES/05/14).

Housing and enrichment conditions

After weaning (21 days of age) female TgDyrk1A and wild type mice were randomly reared under either non-enriched (NE) or enriched (EE) conditions for 30 days. As newly generated cells take between one and two months of being completely integrated in the preexisting DG network, mice were reared for 60 days under NE or EE conditions when survival and activity-dependent integration of newly surviving cells were analyzed. In the NE conditions animals were reared in conventional cage (20 × 12 × 12 cm height, Plexiglas cage) in groups of 2–3 animals. EE was performed in a spacious (55 × 80 × 50 cm height) Plexiglas cage with toys, small houses, tunnels and platforms. Wheels were not introduced in the cages to avoid the effect of physical exercise on adult neurogenesis. The arrangement was changed every 3 days to keep novelty conditions. To stimulate social interactions, 6–8 mice were housed in each cage. All groups of animals were maintained under the same 12 hour light-dark cycle (8:00 to 20:00) in controlled environmental conditions of humidity (60%) and temperature (22 ± 1 °C) with free access to food and water.

Treatment with epigallocatechin-3-gallate (EGCG)

To reduce DYRK1A kinase activity *in vivo*, TgDyrk1A mice were treated for one month with EGCG dissolved in drinking water (EGCG concentration: 90 mg/ml for a dose of 2–3 mg per day). EGCG solution was prepared freshly every 3 days from a green tea leaf extract [Mega Green Tea Extract, Decaffeinated, Life Extension®, USA; EGCG content of 326.25 mg per capsule].

DYRK1A activity assay

Kinase activity of DYRK1A protein was determined from the hippocampus (6 mice per group) according to previously published protocol (Papadopoulos et al., 2011). Briefly, samples were extracted in a Hepes lysis buffer and immunoprecipitated with a mouse anti-Dyrk1A antibody (Abnova, 3 μg/sample) immobilized on glutathione-Sepharose beads (GE Healthcare). The recovered samples were analyzed by immunoblotting and *in vitro* kinase assays.

For immunoblotting analysis, immunocomplexes were resolved by 7.5% SDS-PAGE, transferred onto a nitrocellulose membrane and incubated with anti-Dyrk1A antibody (1/1000) overnight at 4 °C. After that, membranes were incubated for 1 h at room temperature with a secondary anti-mouse immunoglobulin HRP antibody (Dako, 1/2000). Detection was performed using ECL (GE Healthcare) and determined with LAS-3000 image analyzer (Fuji PhotoFilm). Protein quantification was performed using Image Gauge software version 4 (Fuji PhotoFilm).

To determine the catalytic activity of DYRK1A, 500 μg of the purified protein were incubated for 50 min at 30 °C in 30 μl of phosphorylation buffer containing 2 mM of specific DYRK1A peptide [DYRKtide-RRRFRPASPLRGPPK; (Himpel et al., 2000)], 1 mM ATP and [γ-^{32}P]ATP (2 μCi/sample). 5 μl of reaction aliquots were dotted onto P81 Whatman paper. After washing extensively with 5% phosphoric acid, counts were determined in a liquid scintillation counter. Relative kinase activity was obtained normalizing against the amount of DYRK1A protein present in the immunocomplexes previously quantified.

Visuo-spatial learning and memory paradigm

To test the effect of EE on hippocampal-dependent spatial cognition, mice were trained in a Morris water maze (MWM) after being reared 30 days under EE or NE conditions. 13–16 mice per experimental group were tested. The water maze consisted of a circular pool (diameter, 1.70 m; height, 0.6 m) filled with tepid water (20 ± 1 °C) opacified by addition of white paint. A white escape platform (12 cm diameter, height 24 cm) was located 1 cm below the water surface in a fixed position (22 cm away from the wall). White curtains with affixed black patterns to provide an arrangement of spatial cues surrounded the maze.

To characterize the learning efficiency, performance of the task was assessed during 5 acquisition days (4 trials per day) using a hidden platform until mice reach an asymptotic execution level in the learning curve. In each trial, mice were placed at one of the starting locations in random order and were allowed to swim until they located the platform. Mice failing to find the platform within 60 s were placed on it for 20 s. Mice were returned to their home cage at the end of every trial. To assess the reference memory a probe test 24 h after last acquisition session was performed. The platform was removed and mice were allowed to swim for 60 s.

All the trials were recorded and traced with an image tracking system (SMART, Panlab) connected to a video camera placed above the pool. Escape latencies, swimming speed, percentage of time spent in each quadrant of the pool were monitored and computed. Probe test performance was quantified comparing the amount of time mice spent in the target zone *versus* the average of the three other equivalent zones of the pool. To more accurately analyze learning strategy J-tracks analysis was used, where the frequency of stay in a group of mice was represented (Arque et al., 2009).

BrdU administration

To analyze adult neurogenesis in the DG, newly generated cells were identified using 5′-bromo-2′-deoxyuridine (50 mg/kg BrdU intraperitoneal injection). Proliferation rate was analyzed in mice receiving a single BrdU injection and sacrificed 2 h later. Cell cycle exit index was evaluated 24 h after a single BrdU injection. To examine

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the migrating behavior, survival and differentiation of newly generated cells mice received four BrdU injections every two hours and sacrificed 24 h, 1 week or 1 month after the last BrdU injection.

Immunofluorescence and immunohistochemistry

Mice were sacrificed and perfused intracardially with 15 ml phosphate buffered saline (PBS) followed by 60 ml chilled 4% paraformaldehyde. The brains were removed from the skull, postfixed in the same fixative at 4 °C overnight and cryoprotected in 30% sucrose. 40 μm coronal sections were obtained using a cryostat.

To identify proliferating cells, an antibody against ki67, an endogenous marker expressed during G1, S, G2 and M cell cycle phases, was used. Progenitor precursors were identified using an antibody against Sox2 that is expressed in type 2a cells. Type 1 population was distinguished using Sox2+ GFAP+ double staining. An antibody against pH3 was used to discriminate proliferating cells in either the G2 or M phases based on their nuclear pattern [(Hendzel et al., 1997); Fig. 5E]. To evaluate the proportion of proliferating progenitor cells, double immunostaining of Sox2+ ki67+ was used.

Doublecortin (DCX) antibody was used to study the dendritic morphology of newborn neurons. Neuronal differentiation of one week surviving cells was evaluated using double immunostaining of BrdU+ DCX+ and BrdU+ calretinin+ for immature neurons. The cell fate of the surviving cells was evaluated using BrdU+ NeuN+ and BrdU+ GFAP+ for neuronal and glial population respectively, in a group of mice sacrificed one month after the last BrdU injection. Schematic representation of the markers used to identify the different cellular types of the DG neurogenic niche is shown in Fig. 3A.

Neuronal integration of surviving cells in the preexisting network was determined using triple immunostaining of BrdU+ NeuN+ cFos+, considering cFos as an activity-dependent early immediate gene. The immunostaining procedure consisted on the incubation of free-floating sections for 1 h at room temperature with blocking solution (Phosphate Buffer Saline-Triton (PBS-T 0.5%) containing 0.2% gelatin, 0.2 M Glycine, 10% fetal bovine serum). Then, sections were incubated with primary antibodies overnight at 4 °C and subsequently incubated for 1 h with the corresponding secondary fluorescent antibody (1/500, Alexa 488, 555, 594 or 647 nm, Molecular Probes). Sections were mounted and coverslipped with Mowiol reagent.

For immunohistochemistry experiments using peroxidase detection, sections were preincubated in 3% H_2O_2, 10% methanol in PBS for 20 min to block endogenous peroxidase and visualized using the corresponding biotinylated secondary antibodies (1/150, Vector Laboratories) followed by 2 h of incubation with ABC kit system (Vectastain, Vector Laboratories). Diaminobenzidine (DAB) chromogen was used to visualize the positive cells. Finally, sections were washed, mounted on slides, dehydrated and coverslipped with DPX. Special treatments were used for the following stainings: for BrdU detection, sections were pretreated with 0.1 N HCL at 4 °C during 10 min, 2 N HCL at 37 °C for 30 min and 0.1 M borate buffer pH = 8.5 to denature DNA. To detect Dyrk1A, Ki67, Sox2, pH3 and caspase3 immunostaining citrate buffer 10 mM pH = 6 treatment during 10 min at 95 °C was used.

As primary antibodies rat anti-BrdU (1/75, Serotec), rabbit anti-cFos (1/500, Santa Cruz), rabbit anti-calretinin (1/1000, Sigma), rabbit anti-caspase3 (1/300, BD Pharmingen), goat anti-Doublecortin (1/300, Santacruz), mouse-anti-Dyrk1A (1/100, Abnova), mouse anti-GFAP (1/500, Abnova), rabbit anti-pH3 (1/100, Cell Signaling), mouse anti-ki67 (1/100, Novocastra), mouse anti-ki67 (1/200, BD Pharmigen), rabbit anti-ki67 (1/200, Abcam), mouse anti-NeuN (1/200, Chemicon) and rabbit anti-Sox2 (1/400, Millipore) were used.

Quantification of labeled cells

For the quantification of labeled cells we used stereological counting of BrdU+ and total granule cell number, using the optical fractionator. Cells were quantified with the aid of CAST-GRID software package using an OLYMPUS BX51 microscope (Olympus) in one hemi-hippocampus of the DG (Bregma, −1.3 to −3.4 mm (Paxinos and Franklin, 2001); Q4 4–8 animals per group). Given that in the rest of the experiments the density of the positive cells was relatively low, confocal stacks obtained with a Confocal TCS SP5 microscope (Leica, 40×) were merged using Image J software for counting. Final cell number was corrected after checking along z-stack that no overlapping cells were counted twice.

Dendritic morphology analysis

Morphological analysis of the dendritic tree of DCX+ cells was performed using Neuroexplorer (Neurolucida software) in an OLYMPUS BX51 microscope (Olympus). A total of 80 neurons (5 animals per experimental group) from the DG-free blade (infrapyramidal) were traced and, dendritic length and number of nodes were measured. Only DCX+ cells with clear somatic boundaries, a visible dendritic tree and absence of severed dendrites were chosen for reconstruction.

Activation of newly generated cells after spatial learning

To test the capability of newborn neurons to be activated upon learning, mice received four BrdU injections and one month later an intensive MWM protocol (four acquisitions of six trials per day and a probe test 24 h after the last acquisition session) was performed (Kee et al., 2007) in order to train more rapidly mice in the learning test and to efficiently activate newly born neurons. One hour after the probe session animals were sacrificed and brain sections were immunostained with BrdU, cFos and NeuN antibodies.

Statistical analysis

Data were expressed as mean ± SEM and analyzed using two-way ANOVA. Bonferroni was used for *post-hoc* analysis when a significant, or a trend to, genotype × treatment interaction was found ($p < 0.09$). Performances on the MWM were compared using two-way ANOVA repeated measures. Paired sample *t*-test was used to analyze the probe session of MWM. Results were considered significant when $p < 0.05$. All the analyses were performed using the statistical package SPSS for Windows, version 12.0.

Results

Increased DYRK1A kinase activity in the TgDyrk1A hippocampus is normalized by EE

It is well established that EE enhances cognition through the change in the expression and/or activity of several proteins involved in learning and memory, but no data regarding its effect on DYRK1A have been previously studied. Since *Dyrk1A* is a dosage-sensitive gene, involved in neuronal plasticity mechanisms underlying cognition, we evaluated whether EE could modulate DYRK1A kinase activity or expression *in vivo*. DYRK1A kinase activity was measured from hippocampal extracts (six animals per group) analyzing the incorporation of radioactive phosphate in a specific DYRK1A peptide. Relative DYRK1A kinase activity was 1.6 fold higher in non-enriched (NE) TgDyrk1A hippocampus (Fig. 1A, TGNE *vs.* WTNE genotype-treatment interaction $F(1,28) = 5.39$, $p < 0.05$; Bonferroni as *post-hoc*: $p < 0.01$). One month of EE was able to normalize the levels of DYRK1A kinase activity in TgDyrk1A (Fig. 1A, TGEE *vs.* TGNE Bonferroni as *post-hoc*: $p < 0.05$). Moreover, EE reduced Dyrk1A expression in the TgDyrk1A DG without affecting wild types (Fig. 1B). These data indicated that EE normalizes DYRK1A kinase activity/expression in the hippocampus of TgDyrk1A mice.

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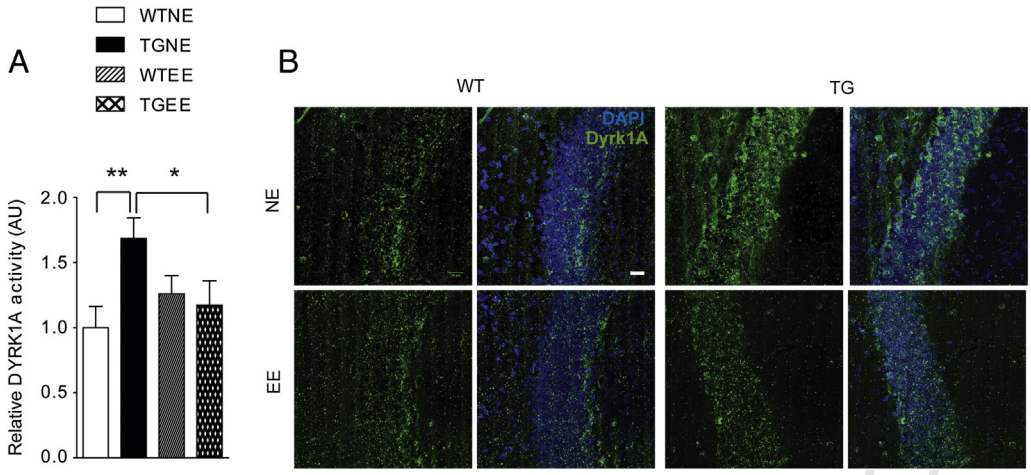

Fig. 1. Relative DYRK1A kinase activity and expression in TgDyrk1A mice under normal and enriched rearing conditions. (A) Relative DYRK1A kinase activity in the hippocampus (WTNE = 12; TGNE n = 12; WTEE n = 12; TGEE n = 12). (B) Representative confocal micrographs of Dyrk1A expression in the DG. Scale bar = 20 μm. Data are represented as mean ± SEM. Two-way ANOVA Bonferroni as *post-hoc* * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

EE improved hippocampal-dependent learning and memory impairments in TgDyrk1A mice

Since one month of EE was able to normalize DYRK1A overdosage in TgDyrk1A hippocampus, we next investigated if EE was able to rescue the cognitive phenotype in TgDyrk1A mice (Altafaj et al., 2001). TgDyrk1A mice and their wild type littermates were trained on a standard MWM paradigm, widely used to test hippocampal cognitive visuo-spatial deficits in mice. During the acquisition sessions, NE TgDyrk1A mice presented a significant learning impairment as shown by the shift to the right of the learning curve (Fig. 2A, genotype-treatment interaction $F(1,55) = 0.015$, $p = 0.902$; genotype effect $F(1,55) = 5.022$, $p < 0.05$), similar to the impaired performance of Ts65Dn mice (Martinez-Cue et al., 2002). One month of EE significantly improved acquisition as shown by the reduction of the escape latency (time to reach the hidden platform) in both genotypes (Fig. 2A, treatment effect $F(1,55) = 10.977$, $p < 0.01$). EE increased the preference for the target zone in enriched wild type and TgDyrk1A mice as shown in the depicted

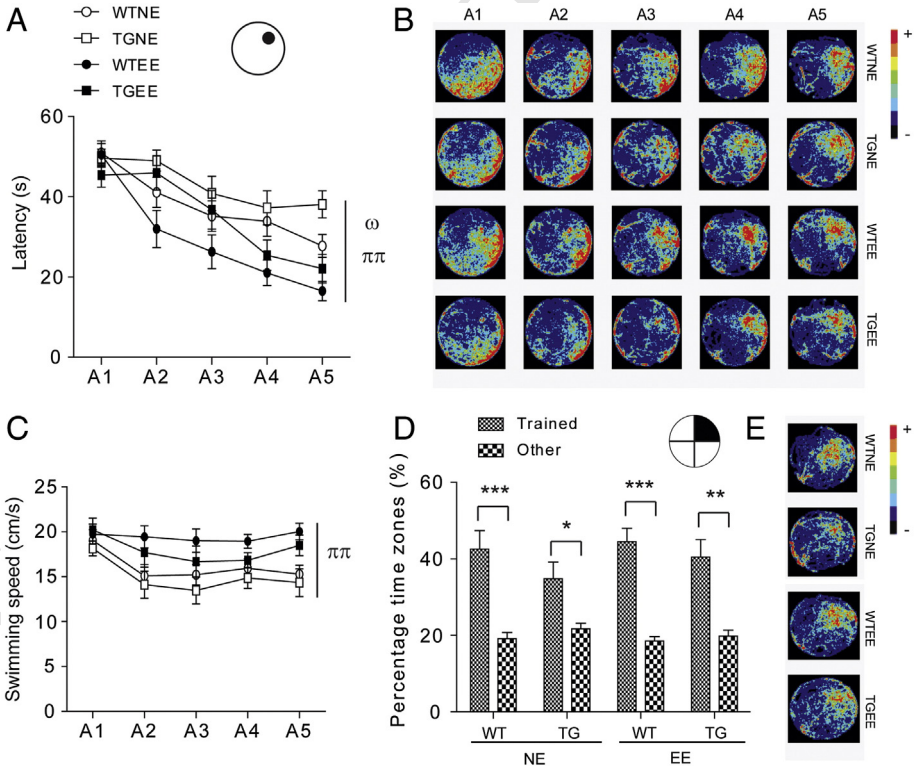

Fig. 2. Visuo-spatial learning and memory in TgDyrk1A mice under normal and enriched rearing conditions. (A) Latency to reach the escape platform along the learning sessions (A1–A5; two-way ANOVA repeated measures) in wild type (WT) and transgenic (TG) mice reared under non-enriched (NE) or enriched (EE) conditions. (B) Color-coded histograms representing the spatial distribution of activity of wild type and transgenic mice in the maze. Color scale is given on the right of the histogram, where red color corresponds to the most visited zones and black color to the less or non-visited zones (J-tracks' plots) during learning sessions (A1–A5). (C) Swimming speed during the acquisition sessions (A1–A5). WTNE = 12; TGNE n = 16; WTEE n = 16; TGEE n = 12. Data are represented as mean ± SEM. Two-way ANOVA repeated measures genotype effect ω $p < 0.05$; treatment effect ππ $p < 0.01$. (D) Percentage of time spent in the trained compared to the non-trained quadrants during the probe test in enriched and non-enriched conditions (Paired *t*-test). (E) Color-coded histograms representing the spatial distribution of activity of wild type and transgenic mice during probe session. A: Acquisition. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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heat maps representing the spatial distribution of activity of wild type and transgenic mice (Fig. 2B). Importantly, enriched TgDyrk1A mice showed a more focused swimming pattern similar to NE wild type mice, suggesting that EE was able to rescue visuo-spatial learning deficits in adult TgDyrk1A mice (Fig. 2B). EE mice of both genotypes swam faster than the NE counterparts (Fig. 2C, genotype-treatment interaction $F(1,55) = 0.023$, $p = 0.880$; treatment effect $F(1,55) = 8.99$, $p < 0.01$) as was previously described (Wood et al., 2010).

In the probe trial, all groups spent a significant higher percentage of time in the trained zone (Fig. 2D) that is a measure of reference memory. Under NE conditions, however, TgDyrk1A showed less preference for the target zone than wild type mice (Fig. 2D, WTNE $t(1,12) = 3.60$, $p < 0.001$; TGNE $t(1,16) = 2.26$, $p < 0.05$). Importantly, EE improved reference memory in both genotypes (Fig. 2D, WTEE $t(1,16) = 5.55$, $p < 0.0001$; TGEE $t(1,12) = 3.37$, $p < 0.01$). Enriched TgDyrk1A mice were as efficient as NE wild types in the probe test (Fig. 2E). Taken together, those results suggest that TgDyrk1A mice presented altered spatial learning that could be improved with EE treatment.

EE rescued adult neurogenesis disruptions in the DG in TgDyrk1A mice

Although the strengthening of the synaptic connections between preexisting hippocampal neurons upon EE contributes to learning and memory improvements, EE also stimulates adult hippocampal neurogenesis by adding new computational units to the neuronal network and contributing to cognition (Kempermann et al., 1997; van Praag et al., 1999a, 1999b). Several studies have demonstrated that adult neurogenesis is regulated by many physiological and pathological stimuli at almost every stage, from proliferation of neuronal precursors until integration of newly formed neurons in the preexisting network (Zhao et al., 2008). Here, we examined the activity-dependent regulation of adult neurogenesis in the DG of Dyrk1A overexpressing mice.

Dyrk1A is expressed in the adult DG neurogenic zone

Although Dyrk1A expression in adult brain of mice was described several years ago (Marti et al., 2003), its expression in adult neurogenic regions had only been studied in the subventricular zone (Ferron et al., 2010). We here demonstrate that in the DG neurogenic region, Dyrk1A was expressed in Sox2 neuronal precursors (Fig. 3B) and in proliferating cells positive for ki67 and pH3 (Figs. 3C and D). Moreover, we detected Dyrk1A expression in cells that expressed BrdU (Fig. 3E) and in neuroblasts and immature neurons expressing DCX (Fig. 3F). These results suggested that Dyrk1A is expressed during all phases of adult neurogenesis in the DG.

EE rescued the altered proliferation of newly generated cells in the adult DG in TgDyrk1A mice

First, we studied the proliferation rate by quantifying the density of BrdU+ cells two hours after a single BrdU injection (Fig. 4A). NE-TgDyrk1A mice showed significantly reduced density of BrdU+ cells compared to wild types (Figs. 4B and C, TGNE *vs.* WTNE genotype-treatment interaction $F(1,20) = 3.32$, $p = 0.08$; Bonferroni as *post-hoc*: $p < 0.05$). Interestingly, although one month of EE did not modify

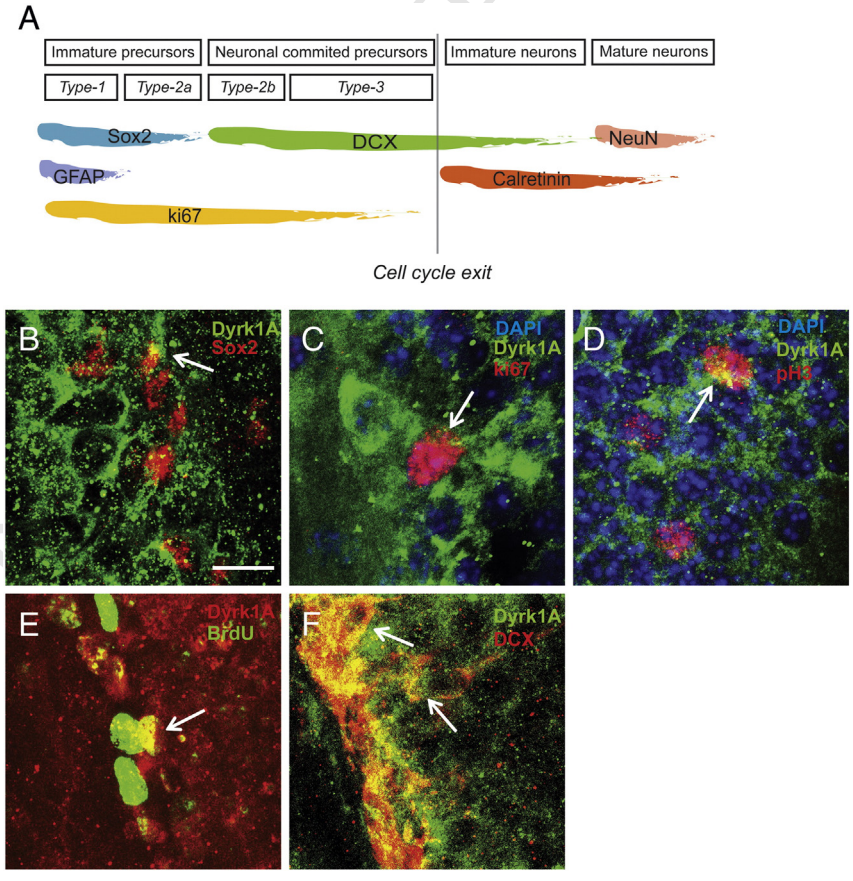

Fig. 3. Dyrk1A expression in different cell types of the adult DG neurogenic region. (A) Specific cell markers used in the different stages of adult hippocampal neurogenesis. Representative confocal micrographs showing Dyrk1A expression in (B) Sox2+, (C) ki67+, (D) pH3+, (E) BrdU and (F) DCX+ cells. Scale bar = 10 μm. Arrows show co-localization of the different markers with Dyrk1A.
Adapted from Beukelaers et al. (2011).

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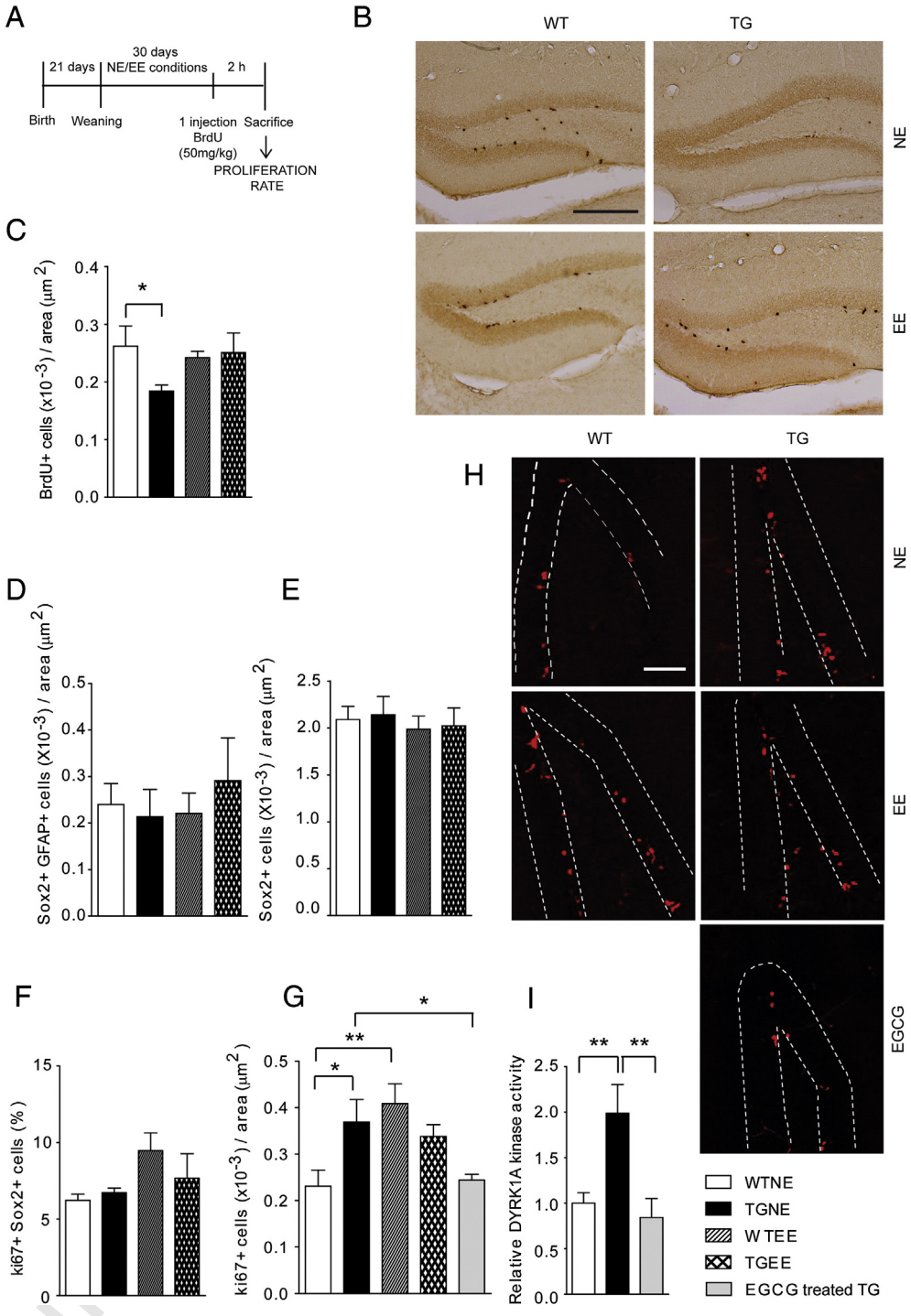

Fig. 4. Proliferation of neuronal progenitors in the DG. (A) Experimental schedule used to analyze the proliferation rate under non-enriched (NE) or enriched (EE) conditions. (B) Representative photomicrographs of BrdU+ cells 2 h after BrdU injection in wild type (WT) and transgenic (TG) mice reared under NE or EE conditions. Scale bar = 500 µm. (C) Average density of BrdU+ cells in the DG two hours after BrdU injection in WT and TG mice reared under NE or EE conditions. (D) Density of Sox2+ cells co-expressing GFAP (progenitor cells type 1). (E) Density of Sox2+ cells (progenitor cells type 2a). (F) Percentage of Sox2+ cells co-expressing ki67. (G) Average density of ki67+ cells in WT and TG mice reared under NE or EE conditions and EGCG treated TG mice. (H) Representative confocal micrographs showing ki67+ cells in WT and TG mice reared under NE or EE conditions and TG mice treated with EGCG. Scale bar = 100 µm. WTNE n = 4–8; TGNE n = 4–8; WTEE n = 4–8; TGEE n = 4–8; TG EGCG n = 4. (I) Relative DYRK1A kinase activity in the hippocampus. WTNE n = 8; TGNE n = 8; TG EGCG n = 8. Data are represented as mean ± SEM. Two-way ANOVA Bonferroni as *post-hoc* * $p < 0.05$; ** $p < 0.01$.

the cell proliferation rate in wild type mice, it induced a slight but non-significant increase in transgenic mice (Figs. 4B and C).

We next examined the number of progenitor cells to explore if this could be associated with changes in proliferating rate. We did not detect differences between genotypes or rearing conditions in the density of type 1 immature precursors (GFAP+ Sox2+ cells, Fig. 4D) nor of type 2a transient amplifying stem cells (GFAP− Sox2+ cells, Fig. 4E). In the absence of modifications in the number of neuronal precursors, the differences in cell proliferation could also be due to a difference in the recruitment of neural precursors to enter the cell cycle. Thus, we used ki67 (an endogenous marker of proliferating cells) to examine the actively proliferating Sox2+ cell population (ki67+ Sox2+ cells),

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and showed that approximately 6% of total Sox2 + cells were proliferating in the adult DG of both genotypes under NE conditions (Fig. 4F). Based on these results, we can also discard that the reduced number of BrdU + cells in NE-TgDyrk1A mice was due to a reduced proportion of precursor cells entering in cell cycle. However, a slight increase in the proportion of double ki67 + Sox2 + actively proliferating progenitor cells upon EE (Fig. 4F, genotype-treatment interaction $F(1,15) = 1.12$, $p = 0.312$; treatment effect $F(1,15) = 3.68$, $p = 0.08$), may suggest a tendency to an increased recruitment of precursor cells to enter the cell cycle in EE wild types.

The reduced proliferation rate observed in NE-transgenic mice, may also affect the total number of proliferating cells. To this end we analyzed the density of ki67 + cells in the DG. NE-TgDyrk1A mice showed a significantly higher density of ki67 + cells compared to wild types (Figs. 4G and H, TGNE *vs.* WTNE genotype-treatment interaction $F(1,30) = 6.76$, $p < 0.05$; Bonferroni as *post-hoc*: $p < 0.05$). Interestingly, although EE did not modify the density of ki67 + cells in TgDyrk1A mice it increased the density of ki67 + proliferating cells in wild type mice (Figs. 4G and H, WTEE *vs.* WTNE Bonferroni as *post-hoc*: $p < 0.01$).

This increased number of ki67 + cells in NE-TgDyrk1A mice pointed to changes in the proportion of cells exiting the cell cycle. To this end, we next analyzed the ratio between the proliferating cells exiting cell cycle (BrdU + Ki67 −) and total BrdU + cells 24 h after one BrdU injection (Fig. 5A). NE-TgDyrk1A mice showed a significantly reduced percentage of cells exiting cell cycle as compared to wild types (Figs. 5B and C, genotype-treatment interaction $F(1,16) = 1.078$, $p = 0.32$; genotype effect $F(1,16) = 6.99$, $p < 0.05$) that was rescued by EE reaching the levels of wild type mice (Figs. 5B and C, treatment effect $F(1,16) = 6.70$, $p < 0.05$). The reduced cell cycle exit along with an increase in total density of ki67 + cells suggested changes in cell cycle progression in transgenic mice. Since previous studies reported a G2 phase elongation in DS fetuses and trisomic mouse models (Contestabile et al., 2007), we performed an immunohistochemistry for phosphorylated-(Ser10)-histone-H3 (pH3) to analyze G2 and M phases of cell cycle. As this marker also allows to discriminate cells in either the G2 or M phases based on its nuclear distribution pattern (Hendzel et al., 1997; Fig. 5E), we could also analyze the percentage of pH3 + cells in the G2 or M phase in respect to total pH3 + cells. NE-TgDyrk1A mice showed a significant increase in the density of pH3 + cells as compared to wild types (Figs. 5D and G, TGNE *vs.* WTNE genotype-treatment interaction $F(1,33) = 3.73$, $p = 0.06$; Bonferroni as *post-hoc*: $p < 0.05$). Moreover, a significantly higher proportion of pH3 + cells with a G2 phase staining pattern were found in NE-TgDyrk1A DG (Figs. 5F and G, TGNE *vs.* WTNE genotype-treatment interaction $F(1,16) = 4.84$, $p < 0.05$; Bonferroni as *post-hoc*: $p < 0.001$), indicating that more proliferating cells in TgDyrk1A remain in the G2 phase. Importantly, although EE did not modify cell cycle progression in wild type mice, EE produced an increase in the proportion of pH3 + cells with a staining pattern suggestive of M phase at the expense of a reduction of cells in G2 phase in TgDyrk1A mice (Figs. 5F and G, TGEE *vs.* TGNE Bonferroni as *post-hoc*: $p < 0.01$), thus normalizing the G2 and M proportion with respect to wild types.

Interestingly, while most of the pH3 + proliferating cells in wild type mice were placed in the SGZ (Fig. 5H) where the proliferative niches are normally located, a significantly higher proportion of pH3 + cells were detected in GCL of NE-TgDyrk1A (Fig. 5H, TGNE *vs.* WTNE genotype-treatment interaction $F(1,33) = 16.46$, $p < 0.001$; Bonferroni as *post-hoc*: $p < 0.001$). This altered pH3 + cell positioning mainly involved cells with a G2 phase staining pattern (Fig. 5I, TGNE *vs.* WTNE genotype-treatment interaction $F(1,16) = 19.89$, $p < 0.001$; Bonferroni as *post-hoc*: $p < 0.001$). EE also reduced the proportion of pH3 + cells abnormally located in the GCL of DG in TgDyrk1A mice (Fig. 5H, TGEE *vs.* TGNE Bonferroni as *post-hoc*: $p < 0.001$) and the specific alteration of G2 phase pH3 + cell localization (Fig. 5I, TGEE *vs.* TGNE Bonferroni as *post-hoc*: $p < 0.001$).

Pharmacological DYRK1A kinase activity normalization restores proliferative alterations in TgDyrk1A mice

Given that one month of EE normalized DYRK1A kinase overdosage in TgDyrk1A hippocampus and rescued alterations in cell cycle exit and progression of proliferating cells in TgDyrk1A, in the next experiment we explore if the pharmacological DYRK1A kinase activity inhibition could also rescue the altered proliferation and cell cycle phase progression of progenitor cells in the TgDyrk1A DG. We took advantage of epigallocatechin-3-gallate (EGCG), a potent and safe non-competitive DYRK1A kinase inhibitor (Adayev et al., 2006). In an independent experiment, DYRK1A kinase activity was measured in the hippocampus. One month of oral treatment with EGCG normalized the levels of DYRK1A kinase activity in TgDyrk1A hippocampus (Fig. 4I, TG EGCG *vs.* TGNE genotype-treatment interaction $F(1,32) = 5.41$ $p < 0.05$; Bonferroni as *post-hoc*: $p < 0.01$), confirming the EGCG inhibition of DYRK1A kinase activity *in vivo*.

Interestingly, EGCG significantly reduced the density of ki67 + cells (Figs. 4G and H lower panel, TG EGCG *vs.* TGNE Bonferroni as *post-hoc*: $p < 0.05$) and normalized the proportion of proliferating cells exiting cell cycle in TgDyrk1A mice (Figs. 5B and C lower panel, TG EGCG *vs.* TGNE Bonferroni as *post-hoc*: $p < 0.001$). EGCG also normalized the density of pH3 + cells (Figs. 5D and G, TG EGCG *vs.* TGNE Bonferroni as *post-hoc*: $p < 0.05$) and significantly restored the percentage of cells in G2 phase (Figs. 5F and G, TG EGCG *vs.* TGNE Bonferroni as *post-hoc*: $p < 0.05$) that were less detected in the GCL of TgDyrk1A DG (Fig. 5I, TG EGCG *vs.* TGNE Bonferroni as *post-hoc*: $p < 0.05$). Taken together, these results indicate that cell proliferation and cell cycle alterations of TgDyrk1A might be DYRK1A kinase activity-dependent.

EE rescued premature migration of newly formed cells in the adult DG in TgDyrk1A mice

The alterations in the localization of pH3 + proliferating cells detected in NE-TgDyrk1A mice, suggested that Dyrk1A overdosage could affect the migrating behavior of newly generated hippocampal cells. Newly formed cells in the subgranular zone (SGZ) of the DG migrate into the granular cell layer (GCL) to become granule neurons. In this experiment, mice received 4 BrdU injections and were sacrificed 24 h, 1 week or 1 month after the last injection (Fig. 6A). The percentage of BrdU + cells in the GCL *vs.* total BrdU + cells in the DG was analyzed. In wild type mice the proportion of BrdU + cells present in the GCL progressively increased starting from the first week of cell life, when immature neurons initiated the migration to their final location (Kempermann et al., 2003). Conversely, NE-TgDyrk1A mice presented a significantly higher percentage of BrdU + cells located in the GCL already 24 h after the last BrdU injection (Figs. 6B and C, TGNE *vs.* WTNE genotype-treatment interaction $F(1,20) = 3.83$, $p = 0.06$; Bonferroni as *post-hoc*: $p < 0.001$) that was also detected one week after BrdU injections as compared to wild types (Fig. 6B, genotype-treatment interaction $F(1,19) = 1.71$, $p = 0.206$; genotype effect $F(1,19) = 4.29$, $p < 0.05$). These results suggested that TgDyrk1A newborn cells undergo premature migration to the GCL. However, NE-TgDyrk1A mice showed a significantly reduced percentage of surviving BrdU + cells in the GCL one month after the last BrdU injection, as compared to wild type mice (Figs. 6B and D, TGNE *vs.* WTNE genotype-treatment interaction $F(1,21) = 3.203$, $p = 0.08$; Bonferroni as *post-hoc*: $p < 0.001$). Moreover, the abnormal localization of proliferating cells in TgDyrk1A mice did not arise from an atypical disposition of the precursor cells, since no differences in the localization of GFAP + Sox2 + cells were detected between genotypes (data not shown). Since EE recovered the abnormal proportion of pH3 + cells localized in the GCL of TgDyrk1A mice, we also evaluated the effect of EE on the migration along the DG of newly formed cells. In the same line, EE prevented the premature migration to the GCL of newly born cells observed in TgDyrk1A, being the differences statistically significant 24 h (Figs. 6B

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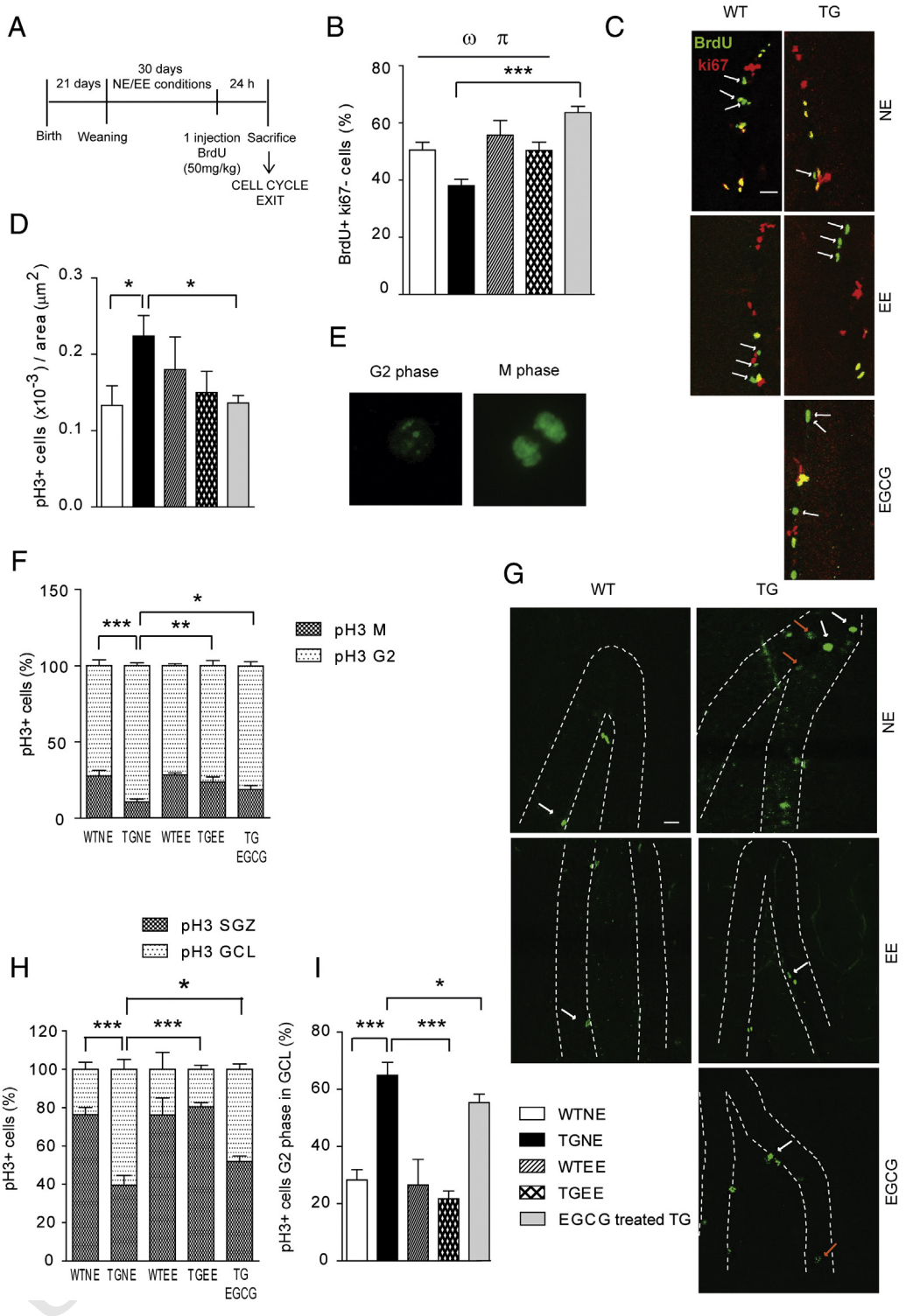

Fig. 5. Cell cycle of neuronal progenitors in the DG. (A) Experimental schedule used to analyze the cell cycle exit. (B) Percentage of BrdU+ cells negative for ki67 twenty-four hours after one BrdU injection in wild type (WT) and transgenic (TG) mice reared under non-enriched (NE) or enriched (EE) conditions and EGCG treated TG mice. (C) Representative confocal micrographs showing BrdU and ki67 cells. Scale bar = 20 μm. Arrows show BrdU+ ki67− cells (cells exiting cell cycle). (D) Average density of pH3+ cells. (E) Representative confocal micrographs showing pH3+ cells in the G2 and M phase of the cell cycle. (F) Percentage of pH3+ cells with a staining pattern suggestive of G2 or M phase. (G) Representative confocal micrographs showing pH3+ cells. Scale bar = 20 μm. White arrows represent pH3+ cells suggestive of M phase. Orange arrows show pH3+ cells suggestive of G2 phase. (H) Percentage of pH3+ cells in the subgranular zone (SGZ) and granular cell layer (GCL). (I) Percentage of pH3+ cells with a nuclear pattern suggestive of G2 phase detected in the GCL. WTNE n = 4–8; TGNE n = 4–8; WTEE n = 4–8; TGEE n = 4–8; TG EGCG n = 4–6. Data are represented as mean ± SEM. Two-way ANOVA genotype effect ω $p < 0.05$; treatment effect π $p < 0.05$. Bonferroni as *post-hoc* * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and C, TGEE *vs.* TGNE Bonferroni as *post-hoc*: $p < 0.05$) and 1 month (Figs. 6B and D, TGEE *vs.* TGNE Bonferroni as *post-hoc*: $p < 0.0001$) after the last BrdU injection, reaching NE-wild type levels

Thus, the activity-dependent regulation of cell cycle progression in TgDyrk1A by EE leads also to rescuing effects on migration of newborn cells through the GCL.

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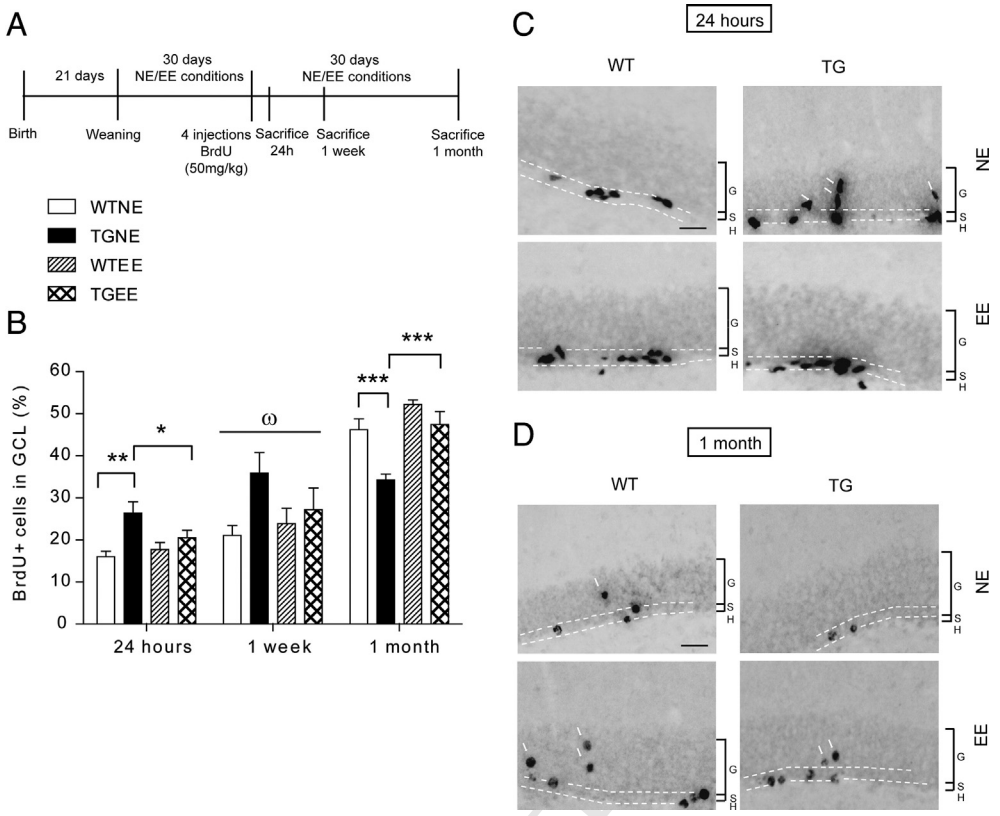

Fig. 6. Migration of newly formed cells in the DG. (A) Experimental schedule used to analyze cellular migration and survival in the DG under non-enriched (NE) or enriched (EE) conditions. (B) Percentage of BrdU+ cells localized in the GCL of the DG in wild type (WT) and transgenic (TG) mice reared under NE or EE conditions. (C–D) Representative photomicrographs of BrdU+ cell localization at (C) 24 h and (D) 1 month after BrdU injections. Arrows represent BrdU+ cells localized in the GCL. Scale bar = 20 μm. WTNE n = 6; TGNE n = 6; WTEE n = 6; TGEE n = 6. Data are represented as mean ± SEM. Two-way ANOVA genotype effect ω $p < 0.05$. Bonferroni as *post-hoc* * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. In C and D: G: granular cell layer; S: subgranular zone; H: hilus.

EE rescued the reduced survival of newly born cells in the adult DG in TgDyrk1A mice

One critical aspect of adult neurogenesis is that, while large numbers of new neurons are born in the DG, only a fraction of those survive. In fact, some studies postulate that only those cells surviving after two weeks will mature and integrate in the circuits (Kempermann et al., 2003), a process regulated by EE (Kempermann et al., 1997, 2003).

In accordance with the previous experiment, Dyrk1A overexpression could be affecting cell survival. Following the protocol described before (Fig. 6A), we counted the number of BrdU+ cells throughout the DG upon repeated BrdU injections at different time points (24 h, 1 week, and 1 month). As described elsewhere we found a progressive reduction of the density of newly generated cells in the DG in both genotypes. However, while no significant differences between genotypes were observed in the density of BrdU+ cells after 24 h of BrdU injections under NE conditions (Figs. 7A and B), at one week NE-TgDyrk1A mice showed a marked reduction in the density of BrdU+ cells compared to wild types (genotype-treatment interaction $F(1,23) = 1.46$, $p = 0.24$; genotype effect $F(1,23) = 19.03$, $p < 0.001$) that were also detected one month after the last BrdU injection (genotype-treatment interaction $F(1,25) = 0.02$, $p = 1$; genotype effect $F(1,25) = 46.92$, $p < 0.0001$). Accordingly, while 80% of newly formed cells survived one week in wild type mice, this percentage was only 40% in NE-TgDyrk1A mice. However, the percentage of these cells that survive at 1 month was the same in wild type and TgDyrk1A mice, suggesting that Dyrk1A dependent effects on survival are mainly operative during the first week of life of newborn cells.

EE significantly increased the density of BrdU+ cells in wild types and also in TgDyrk1A mice at all time points studied after BrdU injections (Figs. 7A and B, EE effect at 24 h: treatment effect $F(1,20) = 9.25$, $p < 0.01$; 1 week: treatment effect $F(1,23) = 21.63$, $p < 0.0001$; 1 month: treatment effect $F(1,25) = 49.01$, $p < 0.0001$). EE animals had similar ratios of increased surviving cells in both genotypes (1 week: ~20% and 1 month: ~10%), so that TgDyrk1A mice survival of newborn cells reached the levels of NE-wild type mice.

As changes in survival could be related to altered apoptosis, we analyzed caspase3+ cells (an effector of caspase 9 that has been previously related with Dyrk1A (Laguna et al., 2008)). We observed a significant increase in the density of caspase3+ cells in NE-TgDyrk1A mice (Figs. 7C and D, genotype-treatment interaction $F(1,12) = 2.85$, $p = 0.12$; genotype effect $F(1,12) = 8.88$, $p < 0.01$). However, the final number of granule cells in the DG of transgenic mice was not affected (Fig. 7E). In concordance with the increased density of surviving newly formed cells upon EE conditions, we detected a reduced density of caspase3+ cells in EE mice as compared to NE ones (Figs. 7C and D, treatment effect $F(1,12) = 11.2$, $p < 0.01$).

We next evaluated whether the alterations in survival in NE-TgDyrk1A mice could affect neuronal differentiation by analyzing the percentage of BrdU+ cells expressing doublecortin (DCX) or calretinin, two markers of immature and postmitotic neurons, one week after the last BrdU injection. At this stage, NE-TgDyrk1A mice showed a reduction in the percentage of newly generated cells expressing DCX (Figs. 8A and C) and calretinin, although it only reached statistical significance in the case of calretinin+ cells (Fig. 8B, genotype-treatment interaction $F(1,12) = 0.56$, $p = 0.46$; genotype effect $F(1,12) = 3.66$, $p < 0.05$). Taken together, these results suggested that neuronal differentiation might be impaired or delayed in TgDyrk1A newly generated cells. Importantly, the recovering effects of EE in TgDyrk1A mice on cell proliferation, cell cycle progression, migration and survival had an impact on the proportion of newly generated cells differentiating towards a neuronal phenotype. Indeed, EE increased the percentage of one week BrdU+ DCX+ (Figs. 8A and C, genotype-treatment interaction $F(1,12) = 0.47$,

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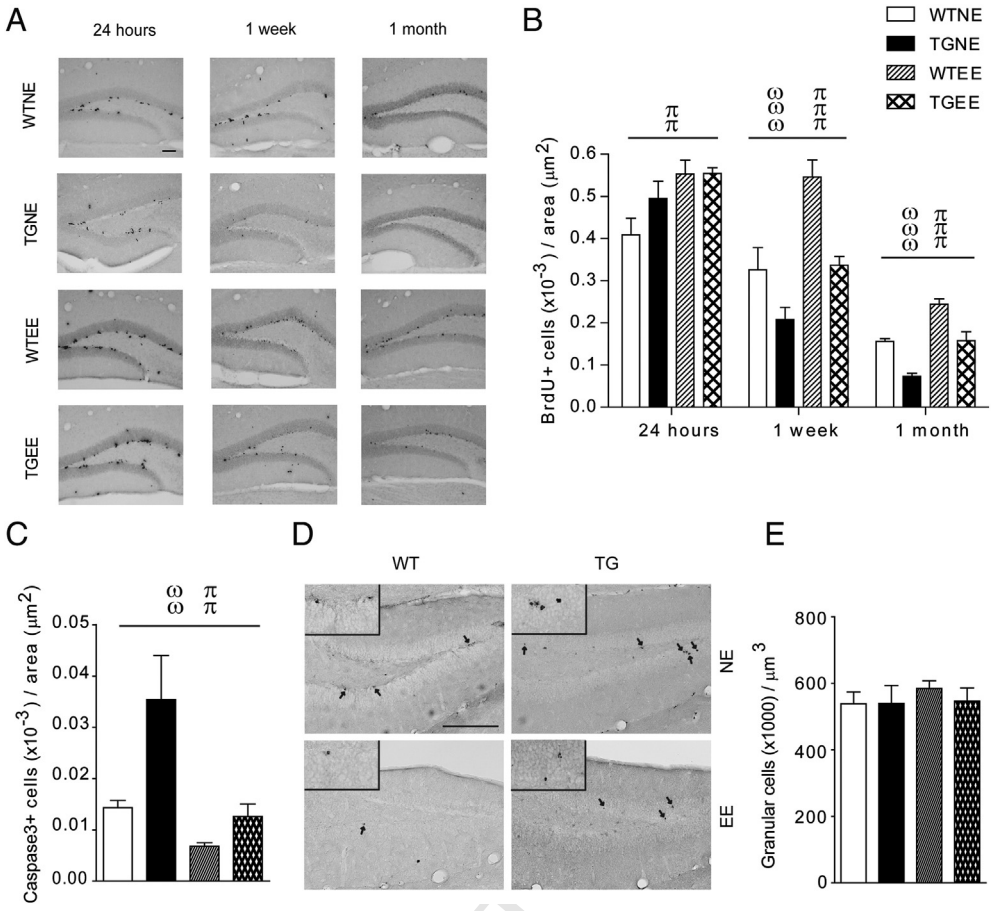

Fig. 7. Survival and cell death of newly born granule cells. (A) Representative photomicrographs of BrdU+ cells at 24 h, 1 week and 1 month after BrdU injection in wild type (WT) and transgenic (TG) mice reared under non-enriched (NE) or enriched (EE) conditions. Scale bar = 100 μm. (B) Average density of BrdU+ cells in the DG at 24 h, 1 week and 1 month after BrdU injection. (C) Average density of caspase 3+ cells. (D) Representative photomicrographs of caspase 3+ cells. Scale bar = 500 μm. Arrows represent caspase3+ cells. (E) Granule cell density in the DG. WTNE n = 4–6; TGNE n = 4–6; WTEE n = 4–6; TGEE n = 4–6. Data are represented as mean ± SEM. Two-way ANOVA genotype effect ωω $p < 0.01$; ωωω $p < 0.001$; treatment effect ππ $p < 0.01$; πππ $p < 0.001$.

$p = 0.50$; treatment effect $F(1,12) = 5.91$, $p < 0.05$) and BrdU+ calretinin+ surviving cells (Fig. 8B, treatment effect $F(1,12) = 12.92$, $p < 0.01$) in both genotypes being the proportion completely rescued in transgenic mice. We also quantified the number of BrdU+ cells expressing NeuN one month after BrdU injections, age in which newly formed cells acquire their final cell fate. NE-TgDyrk1A mice showed significant reduced density of BrdU+ NeuN+ cells (Fig. 8D, genotype-treatment interaction $F(1,21) = 0.125$, $p = 1$; genotype effect $F(1,21) = 28.23$, $p < 0.0001$) that was rescued by EE (Fig. 8D, treatment effect $F(1,21) = 11.74$, $p < 0.01$). However, the percentage of cells differentiating into neurons (BrdU+ NeuN+ cells in respect to the total surviving cells) was not affected by genotype or rearing conditions (Fig. 8E), indicating that neuronal fate was not influenced by Dyrk1A overexpression or EE. Interestingly, NE-TgDyrk1A mice showed a significant higher percentage of BrdU+ GFAP+ cells (Fig. 8F, TGNE *vs.* WTNE genotype-treatment interaction $F(1,18) = 27.103$, $p < 0.001$; Bonferroni as *post-hoc*: $p < 0.001$) that was restored by EE (Fig. 8F, TGEE *vs.* TGNE Bonferroni as *post-hoc*: $p < 0.0001$). These results suggested that Dyrk1A overexpression shifted newly generated cells towards a glial fate, a process that is sensitive to environmental stimuli.

EE rescued the reduced dendritic arborization and functional integration of new neurons in the adult DG in TgDyrk1A mice

Once the newly formed cells migrate to their final location in the GCL, they rapidly start to develop dendritic projections through the molecular layer. We investigated if Dyrk1A overexpression would affect the development of dendritic arborization in DCX+ cells using digital reconstruction techniques (Fig. 9A).

No differences were observed in the area or perimeter of the soma of the DCX+ cells between genotypes (data not shown). However, NE-TgDyrk1A DCX+ neurons showed a significant reduction of dendritic length compared to wild types (Figs. 9A and B, TGNE *vs.* WTNE genotype-treatment interaction $F(1,80) = 7.098$, $p < 0.01$; Bonferroni as *post-hoc*: $p < 0.01$). Dendritic branching complexity was also affected in TgDyrk1A immature neurons that presented less nodes than wild type ones (Figs. 9A and C, TGNE *vs.* WTNE genotype-treatment interaction $F(1,80) = 2.16$, $p < 0.09$; Bonferroni as *post-hoc*: $p < 0.001$), indicating poor arborization. EE significantly increased the total dendritic length (Figs. 9A and B, WTEE *vs.* WTNE Bonferroni as *post-hoc*: $p < 0.001$; TGEE *vs.* TGNE Bonferroni as *post-hoc*: $p < 0.001$) and number of nodes (Figs. 9A and C, WTEE *vs.* WTNE Bonferroni as *post-hoc:* $p < 0.001$; TGEE *vs.* TGNE Bonferroni as *post-hoc*: $p < 0.001$) in both genotypes, indicating that newly formed neurons were sensitive to environmental stimuli as was previously described (van Praag et al., 2000). Importantly, in TgDyrk1A EE achieved a complete normalization of the abnormal dendritic microarchitecture.

Surviving newborn neurons develop dendritic branching to interact with neurons of the preexisting network and to become functional. We analyzed the functional integration of surviving newborn cells co-immunostaining BrdU and cFos, an immediate early gene whose expression has been correlated with neuronal firing. In basal conditions, the percentage of double BrdU+ cFos+ cells in respect to total BrdU+

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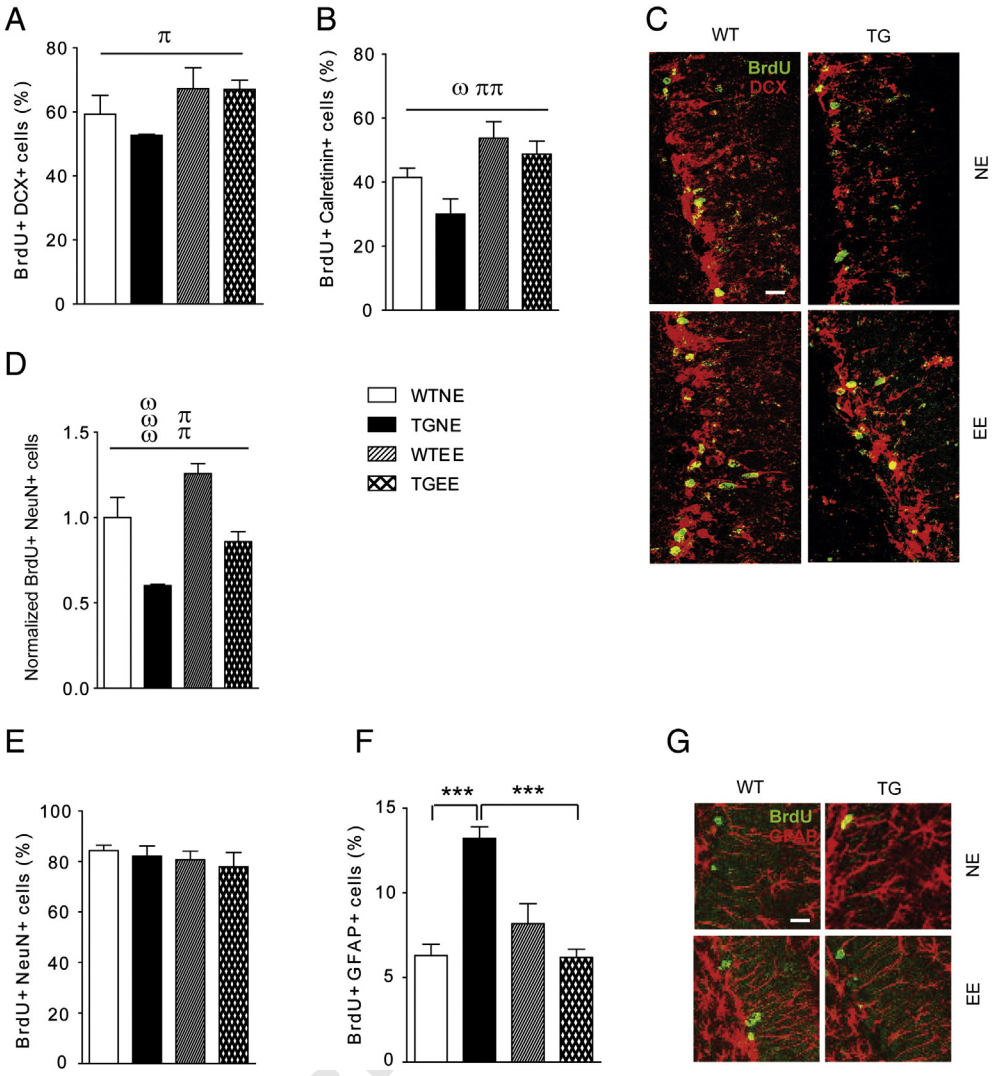

Fig. 8. Cell fate of newly born granule cells. (A–B) Percentage of BrdU+ cells expressing (A) DCX or (B) calretinin one week after BrdU injections. (C) Representative confocal micrographs showing BrdU and DCX cells. Scale bar = 20 μm. (D) Average density of BrdU+ NeuN+ cells in the DG one month after BrdU injections normalized respect to WTNE mice. (E–F) Percentage of BrdU+ cells expressing (E) NeuN and (F GFAP after 1 month of the last BrdU injection. G) Representative confocal micrographs showing BrdU and GFAP cells. Scale bar = 20 μm. WTNE n = 4–6; TGNE n = 4–6; WTEE n = 4–6; TGEE n = 4–6. Data are represented as mean ± SEM. Two-way ANOVA genotype effect ω $p < 0.05$; ωωω $p < 0.001$; treatment effect π $p < 0.051$; ππ $p < 0.01$. Bonferroni as *post-hoc* *** $p < 0.001$.

cells was low in wild type DG (≈1.5%), and absent in NE-TgDyrk1A DG (Fig. 9E, TGNE *vs.* WTNE genotype-treatment interaction $F(1,16) = 7.41$, $p < 0.05$; Bonferroni as *post-hoc*: $p < 0.01$). EE was able to increase the percentage of BrdU+ cFos+ cells in the TgDyrk1A mice restoring basal activation to wild type levels (Fig. 9E, TGEE *vs.* TGNE Bonferroni as *post-hoc*: $p < 0.001$), but had no effect on the number of basal BrdU+ cFos+ cells in wild types.

Since the adult-generated granule cells are mainly active during spatial memory tasks, we examined whether Dyrk1A overexpression specifically altered the integration of newly born neurons (NeuN+) in those functional memory circuits. To this end, mice reared in EE/NE conditions received four injections of BrdU and were tested one month later in a learning paradigm (Morris water maze, MWM) (Fig. 9D, and see Materials and methods section). They were sacrificed one hour after the last MWM session (probe trial) and the number of BrdU+ cFos+ NeuN+ cells was analyzed. NE-TgDyrk1A mice showed a reduced percentage of newly formed neurons that were activated upon spatial learning, compared to wild type (Fig. 9F, genotype-treatment interaction $F(1,28) = 0.754$, $p = 0.394$; genotype effect $F(1,28) = 5.799$, $p < 0.05$), suggesting an impaired integration of newborn cells in the preexisting spatial learning functional circuits. Upon hippocampal-dependent spatial stimulation, EE significantly increased the proportion of activated newly generated neurons (BrdU+ NeuN+ cFos+ cells) in both genotypes (Fig. 9F, treatment effect $F(1,28) = 25.86$, $p < 0.001$), rescuing the impairment observed in transgenic mice. EE could lead to an improvement in the circuitry functionality favoring the performance in hippocampal-dependent spatial learning tasks.

Importantly, after the spatial learning task the proportion of BrdU+ neurons expressing cFos in both genotypes was increased with respect to basal non-learning conditions, supporting the idea that newly generated cells are involved in activity-dependent learning processes (Koehl and Abrous, 2011; Mongiat and Schinder, 2011).

Discussion

In Down syndrome (DS), produced by trisomy of chromosome 21, the "gene dosage" hypothesis predicts that overexpression of a single gene is sufficient to affect multiple brain functions. Among those, *Dyrk1A* is a strong candidate, which overexpression recapitulates DS hippocampal learning and memory deficits (Ahn et al., 2006; Altafaj et al., 2001) and neuronal architecture disruption (Martinez de Lagran et al., 2012). It has also been reported that Dyrk1A is involved in

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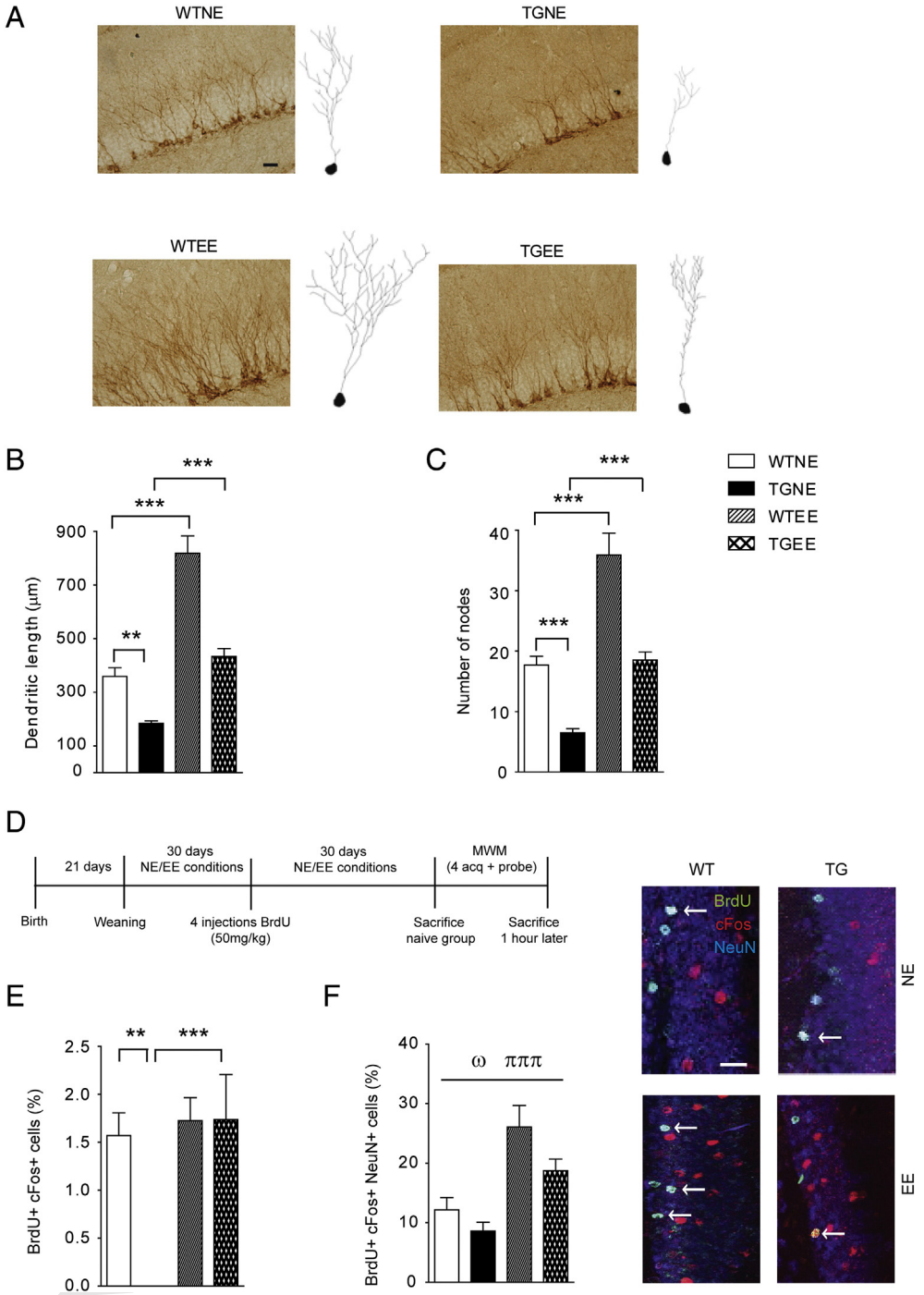

Fig. 9. Dendritic morphology and activation upon learning of newborn hippocampal neurons. We analyzed the morphology (A,B,C) and activation pattern (D,E,F) of hippocampal newborn cells to determine their functional integration in the circuits. (A) Representative photomicrographs of DCX+ cells in the DG and digital reconstruction using the Neurolucida software in wild type (WT) and transgenic (TG) mice reared under non-enriched (NE) or enriched (EE) conditions. Scale bar = 20 μm. (B) Total dendritic length and (C) number of nodes per neuron were quantified in DCX+ cells of the DG. The number of neurons analyzed was 20 per genotype and condition. (D) Experimental schedule used to analyze activation of newly formed neurons. (E) Percentage of BrdU+ cells expressing cFos in the naive group that was not trained in the spatial learning task. (F) Percentage of BrdU+ cells co-expressing cFos and NeuN one hour after the probe session in the MWM (left panel) and representative confocal photomicrographs (right panel). Scale bar = 20 μm. Arrows represent BrdU+ cFos+ NeuN+ cells. WTNE n = 6–8; TGNE n = 6–8; WTEE n = 6–8; TGEE n = 6–8. Data are represented as mean ± SEM. Two-way ANOVA genotype effect ω $p < 0.05$; treatment effect πππ $p < 0.001$. Bonferroni as *post-hoc* ** $p < 0.01$; *** $p < 0.001$.

neuronal proliferation and differentiation during embryogenesis (Aranda et al., 2011; Tejedor and Hammerle, 2011). However, its role in adult hippocampal neurogenesis, one of the activity-dependent neuronal plasticity mechanisms underlying learning and memory impairment in DS remained unknown.

We here provide for the first time evidence that *Dyrk1A* overexpression impairs adult neurogenesis in the DG underlying cognitive impairments in TgDyrk1A mice, similar as the ones observed in trisomic mice and DS individuals. Taken into account that *Dyrk1A* is a dosage-sensitive gene, we here reported that normalizing DYRK1A kinase activity through environmental and pharmacological treatments we rescued completely hippocampal adult neurogenesis impairments in transgenic mice. Our results potentiate the importance of targeting Dyrk1A to improve therapeutical strategies for DS.

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Specifically, *Dyrk1A* overdosage induces alterations in cell proliferation, cell cycle progression and cell cycle exit of DG proliferating cells with consequences in neuronal survival and migration. Because some deficits in hippocampus-dependent learning tasks have been associated with abnormalities on adult neurogenesis (Aimone et al., 2010), we could speculate that the activity-dependent adult neurogenesis disruptions observed in TgDyrk1A could underlie hippocampal learning deficits (Altafaj et al., 2001). In support of this assumption, the functional integration of newly born cells in learning and memory networks was altered in Dyrk1A overexpressing mice. Moreover, pharmacological inhibition of DYRK1A kinase activity using EGCG was able to rescue the cognitive phenotype in both TgDyrk1A and trisomic mice (De la Torre et al., in press) and is able to correct some of the adult neurogenesis deficits in transgenic mice. Interestingly environmental enrichment (EE), which has a rescuing effect on cognition and on adult neurogenesis in TgDyrk1A, was able to normalize DYRK1A kinase activity in the hippocampus.

Nowadays, early intervention programs are the only effective non-pharmacological therapy to improve hippocampal-dependent cognitive impairments in DS children (Bonnier, 2008; Mahoney et al., 2006). Therefore, in order to resemble these early stimulating programs but in mice, we reared mice under EE conditions after weaning (P21), as this age corresponds to the developmental stage at which DS individuals start receiving interventional therapies. In this line, our results showing a complete recovery of learning and memory deficits in TgDyrk1A mice upon EE are in concordance with previous studies in trisomic mouse models (Dierssen et al., 2003; Martinez-Cue et al., 2002). EE is known to improve hippocampal-dependent learning and memory in mice (Nithianantharajah and Hannan, 2006) through the modulation of neuronal structural plasticity mechanisms including adult neurogenesis due to changes in the expression of genes involved in neuronal structure, synaptic signaling and plasticity (Rampon et al., 2000). Our results showing that one-month EE normalizes DYRK1A kinase activity in TgDyrk1A hippocampus indicate that *Dyrk1A* is a dosage-sensitive gene susceptible to environmental changes. As memory storage is believed to occur as a result of changes in neuroplasticity mechanisms, it is considered likely that enrichment increases plasticity.

Adult neurogenesis in the DG has become a widely studied neuronal plasticity process contributing to hippocampal learning and memory (for review see (Deng et al., 2010; Pons-Espinal et al., 2013)). One hallmark of adult neurogenesis is its sensitivity to physiological stimuli at almost every stage, from proliferation of neural precursors, to development, maturation, integration and survival of newborn neurons (Zhao et al., 2008). Our results showed that one month of EE increased neuronal survival, dendritic complexity and the number of surviving cells activated after spatial learning in both wild type and transgenic mice. However, in TgDyrk1A mice, EE was also able to rescue cell cycle alterations, normalizing cell cycle exit probably because EE cells adequately progressed from G2 to M phase. Previous studies have demonstrated that EE also rescued adult neurogenesis deficits and cognitive alterations in trisomic mice (Chakrabarti et al., 2011). Therefore, since EE normalized DYRK1A overdosage in TgDyrk1A hippocampus indicates that some of the recovery effects are Dyrk1A kinase-dependent. Reinforcing this idea, the rescue of proliferation and cell cycle alterations in TgDyrk1A mice using a DYRK1A kinase inhibitor (EGCG) confirmed their dependence on DYRK1A kinase activity. Previous reports already had suggested that Dyrk1A overexpression might disturb cell proliferation (reviewed in (Tejedor and Hammerle, 2011)) during the embryonic period. Concretely, gain of function of Dyrk1A leads to neuronal proliferation arrest, while its loss of function causes over proliferation and cell death (Hammerle et al., 2011). Consistent with these previous observations, we observed reduced number of BrdU+ cells at 2 hour post injection in the adult DG of TgDyrk1A mice, indicating reduced proliferation rate. This was not due to a reduction of the number of progenitor cells, since no differences were observed in the numbers of type 1 (GFAP+ Sox2+ cells) or type 2 (GFAP− Sox2+) cells, nor to reduced recruitment of either neural precursor to enter the cell cycle (ki67+ Sox2+ cells). Although previous work had shown reduced density of type 1 (GFAP+ Sox2+) cells in the subependymal zone of adult *Dryk1A* ± mice (Ferron et al., 2010), our observation does not conflict with these experiments since *Dyrk1A* is a dosage-sensitive gene, which gain- or loss-of-function may produce different consequences in different neurogenic niches, and thus a strict comparison is not possible.

Despite the number of TgDyrk1A BrdU+ cells at 2 hour post injection (in S-phase of the cell cycle) was reduced in transgenic mice, the total number of proliferating ki67+ cells (endogenous marker expressed in all phases of the cell cycle except the resting phase) was increased, and the proportion of cells exiting cell cycle was reduced. Taken together, these results suggested an impairment of cell cycle progression, as supported by the increased proportion of pH3+ cells in the G2 phase. Thus, we conclude that in the adult TgDyrk1A hippocampus, progenitor cells arrest in G2 phase. This is in accordance with the high levels of cyclin B1, involved in G2/M cell cycle phase transition, detected in a YAC transgenic mouse model overexpressing *Dyrk1A* (YAC152F7; Branchi et al., 2004). Moreover, DS fetuses and newborn Ts65Dn mice, which also overexpress DYRK1A, present a higher number of proliferating cells in G2 phase and a reduced number of cells in M phase in the DG (Contestabile et al., 2007). In fact, Dyrk1A has also been related to G1-S phase transition during embryogenesis in different organisms (Hammerle et al., 2011; Park et al., 2010; Yabut et al., 2010) and Ts65Dn shows impairment in this transition due to decreased expression of Skp2 leading to cell cycle elongation (Contestabile et al., 2009). Thus, the possibility of a reduction of the S phase length or an elongation of the G1 phase leading to less progenitor cells entering the S phase (BrdU+ cells two hours after injection) should also be considered. All together our experiments further support the involvement of Dyrk1A in the regulation of the cell cycle and indicate for the first time that Dyrk1A overexpression could affect cell cycle progression in the adult DS dentate gyrus.

In addition, we found that after 24 h of cumulative BrdU incorporation, TgDyrk1A mice present higher density of BrdU+ cells as compared to wild types. This result is in accordance with the idea that newly formed cells in TgDyrk1A get stacked in the cell cycle and less proportion of them are exiting the cell cycle. In our experiment newly generated TgDyrk1A cells prematurely migrate to the granular cell layer (GCL). Remarkably, most of BrdU+ cells found at 24 h in the GCL of transgenic mice were in G2 cell cycle phase. However, one week after the last BrdU injection in TgDyrk1A mice we detected a marked reduction of the number of BrdU+ cells in the DG: while 80% of BrdU+ cells survived in wild type mice, only 40% did so in transgenic mice. However, late survival (between 1 week and 1 month) was similar in both genotypes indicating that Dyrk1A overexpression affects survival only during the first week of life of newborn cells when premature migration is taking place in TgDyrk1A mice.

In adult organisms, neurons born in the SGZ migrate into the GCL to become granule neurons and to functionally integrate in the preexisting network (van Praag et al., 2002). Once the newborn cells have migrated into the GCL, those that survive after two weeks would mature, developing dendrites that synapse with preexisting hippocampal granule cells (Kempermann et al., 2003). Interestingly, we detected reduced percentage of BrdU+ cells expressing immature neuronal markers (DCX+ and calretinin+ cells), suggesting that Dyrk1A overexpression is impairing or delaying neuronal differentiation. Accordingly, the surviving TgDyrk1A immature cells had reduced dendritic complexity, similar to adult pyramidal neurons of Ts65Dn mice and DS individuals (Dierssen et al., 2003; Ferrer and Gullotta, 1990; Marin-Padilla, 1976). These newborn neurons were less capable of being activated in basal conditions, thus suggesting that the reduced complexity of the dendritic tree is probably affecting the functional integration in the preexisting network. Moreover, the number of newborn neurons that are specifically activated upon hippocampal-dependent spatial learning was also reduced in transgenic mice. Mounting evidence supports contributions of

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adult-born neurons to hippocampal function (Deng et al., 2010; Ramirez-Amaya et al., 2006) and thus, our results suggest that the decrease activation of newborn granule transgenic neurons could contribute to the impairment observed in hippocampal visual–spatial tasks in DS.

In conclusion, we have shown that *Dyrk1A* overdose underlies hippocampal–neuronal plasticity deficits in DS and that targeting DYRK1A kinase activity excess either pharmacologically or using environmental stimulation in the adult can correct adult neurogenesis defects. Therefore, understanding the function of Dyrk1A expands our knowledge regarding the regulation of adult neurogenesis in the hippocampus and reinforces the idea that adult neurogenesis alterations driven by increased DYRK1A kinase activity are sensitive to environmental stimulation. These observations suggest that normalization of DYRK1A kinase activity would potentiate environmental therapies for DS cognitive improvement.

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Conflict of interest statement

None declared.

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Epigallocatechin-3-gallate, a Dyrk1A inhibitor, rescues cognitive deficits in Down syndrome mouse models and in humans

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Key words: cognition; Down syndrome; DYRK1A; Epigallocatechin gallate; homocysteine

Abstract

Trisomy for human chromosome 21 results in Down syndrome, which is among the most complex genetic perturbations leading to intellectual disability. Accumulating data suggest that overexpression of the dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A (DYRK1A), is a critical pathogenic mechanisms in the intellectual deficit. Here we show that the green tea flavonol epigallocatechin-3-gallate (EGCG), a DYRK1A inhibitor rescues the cognitive deficits of both segmental trisomy 16 (Ts65Dn) and transgenic mice overexpressing *Dyrk1A* in a trisomic or disomic genetic background, respectively. It also significantly reverses cognitive deficits in a pilot study in Down syndrome individuals with effects on memory recognition, working memory and quality of life. We used the mouse models to ensure that EGCG was able to reduce DYRK1A kinase activity in the hippocampus and found that it also induced significant changes in plasma homocysteine levels, which were correlated with Dyrk1A expression levels. Thus, we could use plasma homocysteine levels as an efficacy biomarker in our human study. Our study is a good example of the use of mouse models back to back with clinical studies. We conclude that EGCG is a promising therapeutic tool for cognitive enhancement in Down syndrome, and its efficacy may depend of Dyrk1A inhibition.

Introduction

Health benefits of the green tea catechins on the prevention of cardiovascular diseases [1], cancer chemoprevention [2], infective [3], and as neuroprotectors in neurodegenerative disease [4] have been postulated. Nevertheless, their in vivo effectiveness and molecular mechanisms are difficult to elucidate and remain a challenging task. Nevertheless, the safety of green tea extracts and of its main active cathechin, the flavonol epigallocatechin gallate (EGCG) should easy the translation from in vitro and animal models to human clinical trials. Recently, the effectiveness of (EGCG) on the promotion of adult hippocampal neurogenesis has been reported [5] and invites its application in clinical neurodevelopmental and neurodegenerative disorders. In this context a previous report also suggested the beneficial effects of EGCG in animal models of Down syndrome (DS) [6].

Trisomy for human chromosome 21 (HSA21) results in Down syndrome (OMIM 190685) and is one of the most common human chromosomal disorders. While the trisomy affects every tissue, in DS reduced cognitive ability is among the most limiting features [7,8]. Even though there is certainly a contribution of non-coding regions to the phenotype, it is likely that the dosage imbalance of some individual genes on HSA21 directly contributes to some phenotypes. Thus, normalizing expression levels or the function of critical genes could prevent or reverse the deleterious effects of gene overdose. *DYRK1A* is a plausible candidate gene to explain some DS phenotypes [reviewed in 9,10]. It is localized in the DS critical region of the HSA21 [11,12] and encodes for a dual kinase phosphorylating both threonine/serine and tyrosine residues. In mice dosage imbalance of *Dyrk1A in vivo* affects brain structure and learning and

memory [13,14]. Reduced *Dyrk1A* expression in heterozygous mice produces microcephaly [15], and compromises neuritogenesis [16] leading to reduced length and complexity of dendritic branches. Heterozygous mice also show visuospatial learning and memory deficits. Similarly, *Dyrk1A* overexpression in transgenic mice (TgDyrk1A) leads to cognitive impairment and less complex dendritic trees [17,18]. Intriguingly, cognition impairment and dendritic tree alteration in TgDyrk1A recapitulates that of Ts65Dn mice, a DS mouse model bearing in trisomy 88 of 161 classical protein coding genes present on HSA21, [19,20,21]. This suggests Dyrk1A as the underlying cause and points its normalization as potential DS therapy.

EGCG is a potent and selective inhibitor of DYRK1A activity [22]. Previous work showed that prenatal EGCG treatment could partially rescue brain alterations in transgenic pups overexpressing *Dyrk1A* and was able to normalize the levels of some synaptic plasticity related proteins in the hippocampus of adult Dyrk1A transgenic mice suggesting possible cognitive effects [6]. We here have explored for the first time if EGCG could rescue the cognitive alteration in adult DS mice and in a pilot study in DS subjects. We have used trisomic and transgenic mice to determine the possible effects of short-term EGCG treatment in improving cognitive function, in particular on memory deficits in adults and to identify biomarkers of EGCG efficacy to normalize Dyrk1A activity that could then be used in patients. Then, we have carried out a randomized, double-blind, placebo-controlled pilot study evaluating the safety of EGCG and preliminarily its clinical effects in young adults with DS. We conclude that EGCG rescues cognitive deficits in trisomic (Ts65Dn) mice and DS individuals possibly through normalizing DYRK1A activity.

Materials and methods

1. Preclinical pharmacological studies

Mouse models

Transgenic mice overexpressing Dyrk1A (TgDyrk1A) were obtained as previously described [13]. Female B6EiC3Sn a/A-Ts (1716) 65Dn (Ts65Dn) mice were obtained from Jackson Laboratories (Bar Harbor, ME) and bred with euploid B6C3BF1 males [23]. Young adult (3 months of age) male mice were randomly distributed in four groups (TgDyrk1A or Ts65Dn and their corresponding wild-type littermates, on placebo or EGCG). Of interest for the present work, Dyrk1A over dosage in TgDyrk1A yielded similar levels of overexpression than those detected in both the foetal DS brains and the partial trisomic Ts65Dn model [24]. We used the following transgenic (TgDyrk1A) groups: WT non-treated n = 13; TG (TgDyrk1A)-non treated n = 16; WT-EGCG n = 16; TG-EGCG n= 14. For the trisomic (Ts65Dn) we used: WT-non-treated n = 19; Ts65Dn-non-treated n = 14; WT-EGCG n = 22; Ts65Dn-EGCG n = 14.

Ethical statement

Animals were bred and housed under SPF standard environmental conditions, and all experimental procedures were performed in compliance with animal welfare policies and approved by the local ethical committee (Comité Ético de Experimentación Animal del PRBB (CEEA-PRBB); procedure numbers MDS-08-1060P1 and JMC-07-1001P1-MDS). All met local (Spanish law 5/1995 and decrees 214/97, 32/2007; French Ministry of Agriculture law 87/848) and European (EU directives 86/609 and 2001-486) regulations and the Standards for Use of Laboratory Animals n° A5388-01 (NIH). The CRG is authorized to work with genetically modified organisms (A/ES/05/I-13 and A/ES/05/14). The

research personnel involved in the experiments were granted the official accreditation to perform the reported experiments.

EGCG administration

Mice were administered epigallocatechin-3-gallate (EGCG) in drinking water for one month. EGCG solution was prepared freshly from a green tea extract [Mega Green Tea Extract, Lightly Caffeinated® (0.8% caffeine) Life Extension®, USA; EGCG content of 326.25 mg per capsule] every 3 days (EGCG concentration: 90 mg/mL for a dose of 2-3 mg per day). To test EGCG cognitive effects, TgDyrk1A or Ts65Dn and their corresponding wild-type littermates, on placebo or EGCG, were tested in a hippocampal-dependent learning using a water maze (WM; see [13,25]) and a novel object recognition task.

Learning and memory tests

Water maze

To test the effect of EGCG on hippocampal-dependent spatial cognition, mice were trained in a water maze (WM) after being administered EGCG in drinking water during 30 days. TgDyrk1A or Ts65Dn and their corresponding wild-type littermates, on placebo or EGCG were tested. The water maze consisted of a circular pool filled with tepid water (19°C) opacified by addition of white non-toxic paint. A white escape platform was located 1 cm below the water surface in a fixed position. White curtains with affixed black patterns surrounded the maze to provide an arrangement of spatial cues. Mice learned the position of the hidden platform in 4 training (acquisition) trials per day during 5 or 6 days, depending on the strain [see 9], until the animals reached the asymptotic performance levels (best execution level). In each trial, mice were placed at one of the starting locations in random order and were allowed to swim until they

located the platform. Mice failing to find the platform within 60 s were placed on it for 20 s and were returned to their home cage at the end of every trial. To assess the reference memory a probe session was performed 24 h after last acquisition session. In this session, the platform was removed and mice were allowed to swim for 60 s. All the trials were recorded and traced with an image tracking system (SMART, Panlab, Spain). Escape latencies, swimming speed, percentage of time spent in each quadrant of the pool were monitored and computed. Reference memory was quantified in the probe session comparing the amount of time mice spent in the target quadrant versus the average of the three other quadrants of the pool.

Novel object recognition

Mice were tested for learning and memory deficits in the novel object recognition task [25]. This task is based on the innate tendency of rodents to differentially explore novel objects (NO) over familiar ones (FO). TgDyrk1A or Ts65Dn and their corresponding wild-type littermates, on placebo or EGCG, were given a habituation session in a circular arena for 30 min on a single day. In the training session 24 h later, mice were placed in the centre of the arena and allowed to explore 2 identical objects during 10 min. Following the training period, the rodent was removed from the environment for a delay period which ranged from 1 to 24 hours, depending on the type of memory being tested in each strain. After the delay, the rodent is returned to the bin, where a new one replaced one of the original objects. For each mouse, the objects were randomly assigned as either familiar or novel. Also the location of the NO (left or right) is counterbalanced between groups. The exploration time for the familiar (TF) and the new object (TN) during the test phase was recorded. Memory was

operationally defined by the percentage of discrimination index for the novel object (DI) as the proportion of time that animals spent investigating the novel object minus the proportion spent investigating the familiar one in the testing period [% Discrimination Index, DI = [(Novel Object Exploration Time – Familiar Object Exploration Time) / Total Exploration Time] x 100].

Biochemical analysis

DYRK1A activity

Kinase activity of DYRK1A protein was determined from hippocampus (6 mice per group) according to previously published protocol [26].

Plasma biomarkers of Dyrk1A activity: homocysteine

The effect of EGCG on plasma homocysteine (Hcy) was evaluated since Dyrk1A expression is correlated with plasma Hcy in Down syndrome [27] Plasma total Hcy (tHcy), defined as the total concentration of Hcy after quantitative reductive cleavage of all disulfide bonds, was determined using a fluorimetric high-performance liquid chromatography method [28]. We also checked if chronic EGCG administration was able to significantly modify Dyrk1A activity in brain samples of TgDyrk1A transgenic mice.

2. Pilot clinical trial

Participants

Thirty-one young adults with DS aged 14 to 29 years old were enrolled from a large cohort of outpatients of the “Fundació Catalana Síndrome de Down” (FCSD, Barcelona).

Exclusion criteria

Subjects with neurological disease other than DS, relevant medical disease, co-morbid mental disorder or currently taking any treatment that could interfere

with cognitive function or alter any key biomarkers and biochemical parameters analysed in the study were excluded. Other common exclusion criteria applied to all the participants were: a) having suffered from any major illness or undergoing major surgery in the last three months before the study; b) regular medication in the month preceding the study. Exceptions were made for single doses of symptomatic medication administered up to the week preceding the trial; c) current ingestion of vitamin supplements or catechins or AINE in the two weeks preceding the study; d) history of gastrointestinal, hepatic, renal or any other problems that may alter absorption, distribution, metabolism, or excretion of the drug; e) subjects under vegetarian diet; f) practice of physical exercise for more than 2 hours per day or energy consumption of more than 3.000 kcal per week.

Randomisation and masking

The study design was randomized, double blind and parallel groups. Subjects were randomly assigned to EGCG (n = 15; same preparation as for animal models) or placebo (n = 16, identical hard gelatine capsules containing brown sugar).

Ethical statement

The study was approved by the local ethics committee (CEIC-Parc de Salut Mar ,EGCG/DYRC1A/DS/IMIM/1, ClinicalTrials.gov Identifier: NCT01394796) and conducted in accordance with the ethical standards of Helsinki Declaration. Participants, parents and/or legal guardians (in case of legal incapacitation) were informed of the ensuing protocol and gave their written informed consent before enrolment.

EGCG administration

Participants assigned to the treatment group or placebo received one or two daily capsules depending on body weight, with a mean EGCG oral dose of 9 mg/kg/day (range 6.9-12.7). During the enrolment visit, participants received dietary recommendations to avoid food supplemented with folic acid and raw green vegetables. Medical, biochemical and neuropsychological explorations were performed at the Hospital del Mar Medical Research Institute (IMIM), and consisted of four evaluations during a 6 months period: baseline, after 1 and 3 months of treatment, and 3 months after treatment discontinuation. Twenty-nine participants completed the 6 months trial: 13 of the EGCG group (two withdrawals) and 16 of the placebo group. Two participants experienced side effects: one in the EGCG group presented increased excitability that required withdrawal of treatment and one in the placebo-treated group required dose reduction due to abdominal pain (from 2 to 1 capsule per day) but continued in the study. Another subject was excluded due to non-reliable compliance of EGCG administration (Table 1S; CONSORT flow diagram; http://www.consort-statement.org/, supplementary materials).

Neuropsychological testing

A comprehensive neuropsychological battery was administered assessing psychomotor speed, attention, episodic memory, executive functions, and visuomotor precision (see Table 2). Tests were presented in a fixed order to allow adequate inter-trial intervals on episodic memory measurements. Parallel versions available for episodic memory and executive function tests were used during the follow-up assessments, to control for learning effects. Parents and

caregivers completed scales of functional ability in daily living, adaptive behaviour and quality of life. Concerning computerized tests, only adult versions of the selected tests from the Cambridge Neuropsychological Test Automated Battery (CANTAB) were used.

Qualitative data on treatment effects

A brief semi-structured self-made interview was conducted with parents at the end of the 6 month trial with they aim to obtain feedback on the performance of the study and to collect qualitative data on their subjective impressions on several parameters of interest related with treatment effects: notion of group assignment (treatment/placebo) before breaking the blind, presence or absence of relevant changes along the study (functional, cognitive, behavioural, clinical, or others) and specific changes observed.

Biochemical analysis

Blood samples (25 mL) were collected after 10-14 h fasting for general biochemical analyses. tHcy concentrations were determined by micro particle enzyme immunoassay (MEIA, Abbott Laboratories, Abbott Park, Ill, USA). Serum aspartate aminotransferase (AST/GOT) and alanine transaminase (ALT/GPT), glucose, total cholesterol and triglycerides were analysed by standard enzymatic methods. LDL-cholesterol was calculated by the Friedewald formula whenever triglycerides were <300 mg/dL. Oxidized LDL (oxLDL) in plasma was measured by a sandwich ELISA procedure (oxLDL, Mercodia, Uppsala, Sweden). Plasma glutathione peroxidase (GSH-Px) activity was measured using cumene hydroperoxide as glutathione oxidant (Ransel RS 505, Randox Laboratories, Crumlin, UK). Treatment compliance and bioavailability were analysed measuring plasma EGCG by HPLC/MS.

Statistical analysis

For mice experiments, we assessed significant differences using two-way ANOVA followed by Bonferroni/Dunnet post hoc tests. Treatment effect on plasma Hcy was analyzed using two-way (genotype*treatment) ANOVA followed by Bonferroni/Dunnet post hoc test. Differences were considered significant at $p<0.05$. Spearman correlation analyses of Hcy levels and Dyrk1a levels were first performed separately on wild type and Ts65Dn mice ($p < 0.1$ was considered significant); the two groups were therefore analysed together. Data were expressed as mean ± SEM. The SPSS statistical software was used for the analysis.

For DS individuals, a descriptive analysis of the socio-demographic, clinical and biochemical parameters of both groups (EGCG and placebo) at baseline was performed by means of absolute and relative frequencies in the case of categorical variables, as well as mean and standard deviation measures for quantitative ones. Treatment effect after three months was estimated for clinical, biochemical and cognitive variables within the framework of ANCOVA models. To avoid a possible bias due to group differences at baseline, these models included both baseline values and gender as covariates. Additionally, the intellectual disability level (mild/moderate vs. severe) was included in the models for the cognitive variables. We calculated 95% confidence intervals for the treatment effect. The statistical software package R (The R Foundation for Statistical Computing) was used for all the analyses.

Results

EGCG rescues cognitive deficits in Ts65Dn and TgDyrk1A mice

In the Morris water maze, although non-treated TgDyrk1A mice overexpressing Dyrk1A in an otherwise disomic genetic background showed a slight, though non-significant impairment in visuo-spatial learning and memory, their performance was significantly improved by EGCG (Fig. 1A, ANOVA repeated measures, treatment effect, $p < 0.05$). Poor learning strategies, assessed as percentage of time spent in the periphery of the pool (thigmotaxia), were also observed in non-treated TgDyrk1A (Fig. S1A, ANOVA with Bonferroni, $p < 0.01$), that were rescued by EGCG (Fig. S1A, ANOVA with Bonferroni, $p < 0.01$). Finally, non-treated TgDyrk1A showed impaired reference memory (Fig. 1C, ANOVA followed by post-hoc Bonferroni analysis, $p < 0.01$) that was again improved by EGCG (Fig. 1C, ANOVA followed by post-hoc Bonferroni analysis, $p < 0.05$). Conversely, EGCG did not modify the performance of wild-type mice (Fig.1A).

To ensure that the effect of EGCG was relevant for DS, we tested the treatment in the Ts65Dn partial trisomic mouse model. Ts65Dn mice showed spatial learning deficits (Fig. 1B, ANOVA repeated measures, genotype effect: $p<0.0001$), and a marked thigmotactic behaviour during the acquisition sessions (Fig. S1B, ANOVA repeated measures, genotype effect: $p < 0.0001$), that were rescued by EGCG (Fig. 1B and S1B, ANOVA repeated measures treatment effect, $p < 0.05$) without affecting performance in wild-types. In the probe test, Ts65Dn mice showed again a thigmotactic strategy (Fig. 1D, ANOVA with

Bonferroni $p < 0.001$) that was recovered by EGCG treatment (Fig. 1D, ANOVA with Bonferroni, $p < 0.05$).

EGCG also rescued the object recognition memory impairment exhibited by Ts65Dn and TgDyrk1A mice in the novel object recognition test. Both Ts65Dn and TgDyrk1A mice showed a clear deficit in novelty recognition as shown by a significantly lower recognition index (Fig. 2A and 2B, two-way ANOVA genotype x object interaction with Bonferroni as post-hoc: WT non treated vs. TgDyrk1A non treated $p < 0.01$; WT non treated vs. Ts65Dn non treated $p < 0.01$). EGCG significantly restored novel object recognition in both transgenic (Fig. 2A, two-way ANOVA genotype x treatment interaction with Bonferroni as post-hoc: TgDyrk1A non treated vs. TgDyrk1A EGCG $p < 0.01$) and Ts65Dn (Fig. 2B, $p < 0.01$). Interestingly, EGCG impaired novel object recognition in wild type mice (Fig. 2A, WT non treated vs. WT EGCG: two-way ANOVA genotype-treatment interaction with Bonferroni as post-hoc $p < 0.01$).

Dyrk1A activity and plasma biomarkers

DYRK1A kinase activity was increased in the hippocampus of TgDyrk1A mice (Fig. 3A, one-way ANOVA $p < 0.01$) due to Dyrk1A overexpression. EGCG normalized DYRK1A kinase activity in transgenic animals but it did not induce significant modification in wild type mice (Fig. 3A, two-way ANOVA genotype-treatment interaction with Bonferroni as post-hoc $p < 0.01$).

Hcy in plasma were correlated with Dyrk1A brain levels (Fig. 3D; Spearman correlation $Rho = 0.73$; $p = 0.027$). In basal conditions, a decrease of Hcy plasma levels was detected in TgDyrk1A and Ts65Dn mice (Fig. 3B and 3C), as

has also been described in DS patients [29]. One month of treatment with EGCG normalized Hcy plasma levels of both TgDyrk1A and Ts65Dn to untreated wild type levels although the effect only reached statistical significance in trisomic mice (Fig. 3B and 3C, two-way ANOVA genotype-treatment interaction with Bonferroni as post-hoc: TgDyrk1A $p = 0.06$; Ts65Dn $p < 0.01$).

Pilot Clinical Trial

Twenty-nine participants (Table 1) balanced for gender (51.7% male and 48.2% female) and age (22.2 ± 4.2 years in EGCG and 20.6 ± 2.2 in placebo) between treatment groups, were enrolled. Both groups had similar school attendance in special or standard education centres (14.86 ± 2.6 vs. 15.38 ± 2.4 for EGCG and placebo group respectively). Average IQ was similar for both groups, within the range of moderate intellectual disability (IQ = 45.9 ± 7.8 in EGCG vs. 42.4 ± 6.4 in placebo). EGCG group showed a higher rate of individuals with trisomy 21 (76·9%) against placebo group (68·7%). In addition, one 1 individual had translocation (7·6%) in the EGCG group whereas 1 individual was mosaic (6·2%) in the placebo group. Down syndrome genetic variations of six individuals remained unknown, 2 (15·3%) in the EGCG group and 4 (25%) in the placebo group.

. The clinical and biochemical evaluation revealed no significant differences between groups in AST (GOT) or ALT (GPT) values after three months of treatment (Table 2S). Plasma concentrations of cholesterol (placebo 173.7 ± 26.0 vs. EGCG 153.8 ± 22.0 mg/dL, $p=0.02$), and LDL-cholesterol (111.6 ± 24.8 vs. 88.9 ± 22.1 mg/dL, $p = 0.014$) were significantly reduced after three months

of EGCG treatment. No treatment effects were observed on triglycerides, HDL-cholesterol, glucose, and GSH-Px activity.

Although plasma EGCG concentrations did not differ between genders or time of evaluation (94.4 ± 45.2 vs. 89.4 ± 49.3 ng/mL, first vs. third month), a significant increase of Hcy was detected after one month of treatment, (Treatment effect at one month $p = 0.024$, 9.7 ± 1.5 vs. 11.6 ± 2.6, paired Student's t test) remaining below the critical value (15-20 μM) that was maintained along treatment, and returned to baseline after treatment discontinuation.

Table 3S displays EGCG effects on neuropsychological performance adjusted for gender, IQ, and baseline values. We found significant effects of EGCG on episodic memory and working memory after three months of treatment. EGCG-treated individuals showed a significantly higher percentage of correct answers in visual memory recognition (Immediate Pattern Recognition Memory (PRM)-percentage correct; $p = 0.04$) compared to placebo (Figure 4). A tendency to significant differences was also found for working memory performance between EGCG and placebo groups, the former showing a lower error rate (Spatial Span backwards total errors; $p = 0.08$). Also, the analyses showed a trend to significant EGCG effects on psychomotor speed (MOT) and improved social functioning on the Kidscreen-27 quality of life index (Social Support & Peers; $p = 0.05$; Table 4S) compared to placebo. After three months of treatment discontinuation, the effects declined and percentage of correct answers in the immediate PRM decreased returning to baseline measures (Figure 4). In addition, the descriptive analysis on treatment effects collected from the parents at the end of the trial (Table 5S) showed that most parents

correctly guessed the treatment assignment, and parents with EGCG treated children reported a broad range of changes, referring specific positive effects, whereas most parents of placebo condition reported no relevant changes.

Discussion

The aim of the present study was to investigate the potential benefits of normalizing the activity of DYRK1A; a kinase overdosed in DS on cognitive performance in mouse models and human DS individuals. We here report improvement of cognitive function both in DS mouse models and humans treated with EGCG, a specific inhibitor of DYRK1A. We also took advantage of the animal models to proof the use of Hcy plasma levels, as a biomarker of hippocampal DYRK1A activity. Our study supports the use of DYRK1A inhibitors, and specifically of EGCG, as promising therapeutic tools for cognitive enhancement in Down syndrome.

In previous work, we observed that EGCG could partially rescue some brain morphological alterations in transgenic pups overexpressing *Dyrk1A* [6] and normalized the levels of some synaptic plasticity related proteins in the hippocampus of adult *Dyrk1A* transgenic mice. However, in this initial work, the mice tested only overexpressed *Dyrk1A* in an otherwise disomic genetic context, and besides we did not know the possible cognitive effects, if any. In the present study we used again mice with single overexpression of *Dyrk1A* in a disomic genetic context (TgDyrk1A) but they were adult, and we also included in the analysis a partial trisomic well-established DS model, the Ts65Dn mice [23]. Our results indicate that one month of oral administration of EGCG rescues the hippocampal-dependent learning deficit in both TgDyrk1A and

Ts65Dn without affecting the performance of wild types. Besides a general effect on hippocampal dependent learning, inadequate learning strategies (thigmotaxia) were reduced in EGCG-treated Ts65Dn and TgDyrk1A mice. Thus, we can conclude that EGCG was able to rescue the phenotypes derived of Dyrk1A overexpression in transgenic mice and in the partial trisomy model, Ts65Dn. This suggested that the pathological effect driven by Dyrk1A overexpression is relevant for DS, and that Dyrk1A is a strong candidate for therapy. Our previous work [30] already demonstrated that functional cortico-striatal defects could be corrected through the specific inhibition of Dyrk1A expression using adenoviral vectors (AAVshDyrk1A) in TgDyrk1A mice [31], but here we show that also kinase activity normalization in adult mice can rescue the cognitive phenotypes. The next step was to ensure that the treatment with EGCG attained DYRK1A kinase activity normalization and to find a plasma biomarker that could be then used in human. We first showed that EGCG normalized DYRK1A kinase activity in the hippocampus (Fig. 3A), in TgDyrk1A at the same dose regime that improved hippocampal-dependent tests. However, we needed a plasma biomarker that could proof the decrease of DYRK1A kinase activity upon treatment with EGCG in humans. Our previous studies showed that over-expression of *Dyrk1A* is related to decreased Hcy plasma concentrations in Tg*Dyrk1A* and Ts65Dn mice suggesting that Hcy could be a suitable biomarker correlated with Dyrk1A in Down syndrome [27]. We here demonstrated a significant correlation between Hcy in plasma and brain levels of Dyrk1A (Fig. 3D) in Ts65Dn mice. Ts65Dn mice showed decreased Hcy plasma levels similar to what has been described in Down syndrome individuals [29]. One month of treatment with EGCG normalized the

reduced levels of plasma Hcy of both TgDyrk1A and Ts65Dn to untreated wild type levels. These experiments served as a proof of concept that the learning deficits caused by *Dyrk1A* overexpression could be rescued by Dyrk1A activity normalization and that plasma Hcy levels could be used as a biomarker.

We then explored the effects of EGCG on cognitive performance in young adults with DS, paying special attention to hippocampal function. Concretely, besides other measures (see Results section) we used a visual recognition task that measured visuo-perceptual processing, the weakest component of visual memory in DS individuals. This measure has proven to be sensitive to hippocampal functioning, in particular of perirhinal cortex, which is critically involved in object recognition memory [32-35] and is reduced in size in DS [36]. EGCG treated individuals showed higher accuracy in visual memory recognition [37,38] and spatial working memory [39], suggesting a positive effect of this compound also on the prefrontal system, in particular ventromedial, ventrolateral and dorsolateral cortices. Overall, the results from this pilot study are consistent with EGCG effects found in TgDyrk1A and Ts65Dn mice and tentatively indicate that short-term EGCG treatment is able to induce beneficial effects on cognition in young adults with DS, probably acting on distributed hippocampal-prefrontal functional networks supporting memory abilities.

EGCG administration also induced positive effects on quality of life and social functioning. This was detected by using objective measures such as the Kidscreen battery, but also in a parent/caregivers survey in which subjective positive impressions of mild cognitive enhancement and/or improved behavioural control over a wide array of cognitive/behavioural skills were reported in EGCG treated individuals. Interestingly, while parents reported

improved verbal cognitive abilities, that are less preserved in DS, and considered one of the landmarks of global intellectual impairment in these individuals [40,41] and a significant impact of EGCG cognitive enhancement on everyday living, the neuropsychological testing could not detect these effects. Such discrepancy could respond to a low sensitivity of the functional assessment tools used.

One important aspect was safety and toxicity, since DS individuals may be more sensitive than euploids to some adverse effect. EGCG has been demonstrated to be a safe substance after repeated administration in humans. No alteration of hepatic function markers (AST and ALT) was observed. Animal studies have reported EGCG to be involved in redox cycling and quinone formation having both antioxidant and pro-oxidative activities and being able to induce oxidative stress [42]. We found no increase of biomarkers of oxidative stress (oxLDL and GSH-Px activity), but a reduction of lipid oxidation in subjects treated with EGCG. The improvement on the oxidative/antioxidative status combined with a healthy lipid profile, shown by the total cholesterol and LDL cholesterol concentrations can be considered beneficial.

Importantly, as for mice, in humans the biochemical results allowed to establish a clear relationship between memory improvement and Hcy levels, suggesting a direct dependence of cognitive improvement on Dyrk1A activity. Moreover, after three months of treatment discontinuation EGCG cognitive effects decline along with a parallel decrease in plasma Hcy levels, suggesting that Dyrk1A activity normalization induced cognitive changes through temporary neuronal changes. This is important since Dyrk1A has been related to synaptic plasticity in the brain that may lead to stable structural modifications in neural circuits

[15,16,18]. This has functional consequences, since Down syndrome learning and memory problems become considerably more noticeable through childhood and adolescence and appear to be related to an inability to ‘stabilize’ information that is acquired, a problem in memory consolidation that is a function of the hippocampal system (see [10]. Consolidation of labile information storage requires the translation of short-lasting biochemical synaptic events into more stable functional and structural changes. This has been shown in trisomic mouse models of DS and also inTgDyrk1A and is proposed to underlie the reduced stability of learning in the human phenotype (see [11] for review). By normalizing Dyrk1A activity, EGCG is possibly favouring learning through these mechanisms, and thus it could be speculated that it could be especially beneficial if paired with interventions that also increase plasticity, such as cognitive stimulation.

In conclusion, we here demonstrate the positive effects of EGCG on learning and memory deficits of DS mouse models and humans, thus supporting further research on the use of EGCG as a potential therapeutic agent for improving cognitive deficits in young DS adults. Homocysteine plasma concentrations are proposed as a good biomarker of efficacy of the treatment.

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Conflict of Interest Statement

Authors declare no conflict of interests in the present manuscript

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Table 1. Sociodemographic characteristics and clinical parameters at baseline

	EGCG (n = 13)	**Placebo (n = 16)**
Age	22.2 (4.2)	20.6 (2.2)
Sex		
Female	6 (46.2%)	8 (50.0%)
Male	7 (53.8%)	8 (50.0%)
Education (years)	14.86 (2.6)	15.38 (2.4)
Intellectual disability level		
Mild/Moderate	8 (61.5%)	5 (31.2%)
Severe	5 (38.5%)	11 (68.7%)
Intellectual Quotient (IQ)		
K-BIT (standardized	45.9 (7.8)	42.4 (6.4)

score)		
DS genetic variations		
Trisomy 21	10 (76.9%)	11 (68.7%)
Mosaic	0	1 (6.2%)
Translocation	1 (7.6%)	0
Unknown	2 (15.3%)	4 (25.0%)
Weight	60.1 (13.3)	56 (10.9)
Body Mass Index (BMI)	25.4 (4.0)	23.2 (3.8)

Results are presented as mean (standard deviation) for continuous variables and absolute frequency (relative frequency) for categorical variables.

Figures Legends

Figure 1. Effects of EGCG on performance in the visuo-spatial learning and memory task in TgDyrk1A and Ts65Dn mouse models. (A, B) Time to reach the platform (escape latency) during learning sessions in (A) TgDyrk1A and (B) Ts65Dn. (C, D) Percentage of time spent in the periphery (thigmotaxis) of the pool during the probe session in (C) TgDyrk1A and (D) Ts65Dn. Data are represented as mean ± SEM. Genotype effect * $p<0.05$, *** $p < 0.001$; treatment effect # $p<0.05$.

Figure 2. Effect of the EGCG treatment in the Novel object recognition test. (A, B) Percentage of discrimination index during the test session in (A)

TgDyrk1A and (B) Ts65Dn mice. Data are represented as mean ± SEM. Two-way ANOVA with Bonferroni * $p<0.05$, ** $p < 0.01$.

Figure 3. Effect of the EGCG treatment on Dyrk1A activity and plasma homocysteine levels. (A) DYRK1A kinase activity was normalized after one moth of EGCG treatment in the hippocampus of TgDyrk1A mice. Plasma homocysteine levels were normalized to wild type levels in TS65Dn (C) (Two way ANOVA genotype x treatment, followed by Bonferroni/Dunnet post hoc $p < 0.01$) and the same tendency was detected in TgDyrk1A (B) mice, although it did not reach statistical significance ($p = 0.06$). (D) Brain Dyrk1A protein level is negatively correlated with plasma homocysteine level in Ts65Dn ($p = 0.027$).

Figure 4. Positive subclinical effects of short-term EGCG treatment on episodic memory. Longitudinal analyses of EGCG treated individuals and placebo group for memory recognition [Pattern Recognition Memory (PRM) immediate recall percentage correct]. The figure shows 95% confidence interval of the mean score at each evaluation.

Supplementary Materials
Table 1S. CONSORT diagram

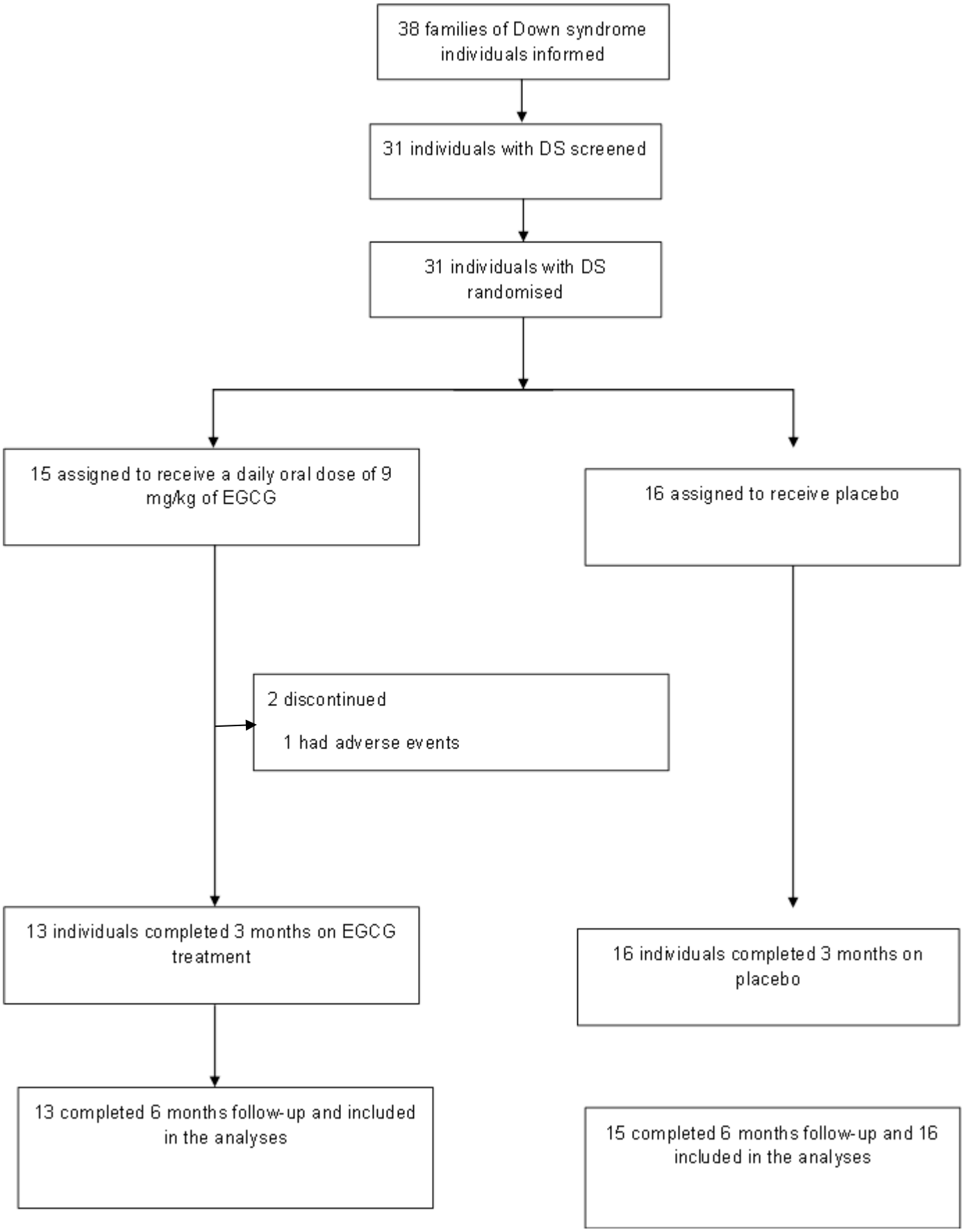


Table 2S. Descriptive values for clinical and biochemical parameters at baseline and after three months of treatment

	EGCG intervention		Placebo intervention			
	Baseline	**3 months**	**Baseline**	**3 months**	**Treatment effect**	***p*-value**
Weight (Kg)	60.1 (13.3)	59.6 (12.4)	56.0 (10.9)	56.3 (11)	-0.56; [-1.48; 0.35]	0.22
Homocysteine	9.7 (1.5)	10.8 (2.2)	8.8 (2.3)	9.8 (2.4)	0.26; [-1.11; 1.62]	0.70
AST (IU/L)	23.6 (3.2)	23.7 (4.1)	23.0 (6.5)	23.2 (6.6)	0.00; [-3.42; 3.43]	0.99
ALT (IU/L)	21.1 (5.6)	22.7 (7.5)	22.3 (12)	21.1 (8.9)	1.36; [-4.41; 7.13]	0.63
Glucose (mg/dL)	88.2 (5.5)	85.9 (6)	89 (3.7)	88 (6.3)	-1.67; [-6.03; 2.69]	0.44
Total cholesterol mg/dL)	165 (26.8)	153.8 (22)	175.1 (24.5)	173.7 (26)	-12.34; [-22.20; -2.49]	**0.02**
HDL cholesterol (mg/dL)	51.8 (8.9)	51.6 (10.3)	51.1 (8.7)	49.1 (6.8)	1.99; [-1.70; 5.69]	0.28
LDL cholesterol (mg/dL)	98.6 (24.4)	88.9 (22.1)	110.8 (19)	111.6 (24.8)	-11.48; [-19.94; -3.02]	**0.01**
Triglycerides (mg/dL)	73 (34.4)	66.3 (30.4)	66.3 (16)	63.4 (17.1)	-1.19; [-14.58; 12.20]	0.86
Oxidised LDL (U/L)	44.8 (18.5)	39.5 (15.3)	50 (13.6)	44.6 (12.6)	-2.24; [-11.37; 6.90]	0.62
GSH-Px activity (U/L)	638.4 (58.4)	618.1 (48.2)	615.2 (84.4)	619.7 (99.1)	-23.61; [-57.39; 10.17]	0.16

Estimated treatment effect (95% CI and p-values, ANCOVA models) adjusting for gender, IQ, and baseline scores. A positive sign indicates a larger average value under EGCG treatment compared to placebo. BMI: Body Mass Index; AST: aspartate aminotransferase; ALT: alanine transaminase; HDL: high-density lipoprotein; LDL: low-density lipoprotein; GSH-Px: glutathione peroxidase; EGCG: epigallocatechin-3-gallate.

Table 3S. Effect of EGCG treatment on neuropsychological performance after three months of administration

	Treatment effect		
	Estimate	**95% CI**	***p*-value**
Attention (forward recall)			
Verbal Span Digits: Total score	-0.13	[-0.88; 0.62]	0.73
SSP Visual Span: Span length	0.46	[-0.51; 1.43]	0.33
SSP Visual Span: Total error	1.48	[-1.69; 4.66]	0.35
Psychomotor Speed			
MOT: Mean error	1.62	[-0.69; 3.94]	0.16
MOT: Mean latency	-124.62	[-410.97; 161.72]	0.38
Working Memory (backward recall)			
Verbal Span Digits: Total score	-0.54	[-1.32; 0.24]	0.17
SSP Visual Span: Span length	-0.43	[-1.49; 0.62]	0.40
SSP Visual Span: Total errors	-2.18	[-4.67 0.30]	**0.08**
Memory			
FULD: Total A1-A5	2.12	[-1.77; 6.01]	0.27
FULD: Delayed recall	-0.22	[-1.60; 1.15]	0.74
PAL: Stages completed	0.08	[-0.69; 0.85]	0.83
PAL: Total errors adjusted	-4.38	[-32.06; 23.31]	0.75
PRM: Immediate recognition	13.56	[0.35; 26.76]	**0.04**
PRM: Delayed recognition	0.08	[-12.14; 12.30]	0.99
Executive Functions			
McCarthy: Word Fluency	-1.25	[-4.67; 2.17]	0.46
Word Fluency (animals/1min)	0.09	[-1.94; 2.13]	0.93
IED: Stages completed	-0.11	[-1.46; 1.25]	0.87
IED: Total errors (adjusted)	-0.86	[-29.95; 28.22]	0.95
Visuomotor coordination			
Purdue: Preferred hand	-0.51	[-1.90; -0.88]	0.46
NEPSY visuomotor: Total time	-1.00	[-30.76; -28.77]	0.95
NEPSY visuomotor: Total error	5.02	[-12.37; 22.41]	0.56

Treatment effect (95% CI and p-values from ANCOVA models) adjusting for gender, IQ, and baseline scores. A positive sign indicates a increased average value under EGCG treatment compared to placebo. EGCG: epigallocatechin-3-gallate.

Table 4S. Effect of EGCG treatment on functional ability, adaptive behaviour and quality of life outcomes after three months of administration

	Treatment effect		
	Estimate	**95% CI**	***p*-value**
Adaptive behaviour & functional ability			
ICAP: Motor ability	0.59	[-2.23; 3.41]	0.67
ICAP: Social/communication	1.83	[-1.99; 5.64]	0.33
ICAP: Autonomy in daily living	-3.50	[-8.87; 1.86]	0.19
ICAP: Life in Community	0.91	[-3.25; 5.07]	0.66
ICAP: Total Score	-0.06	[-9.09; 8.97]	0.99
Disrupting behaviours			
ICAP: Range of behaviours	0.07	[-1.54; 1.69]	0.93
ICAP: Frequency (mean score)	-0.71	[-1.71; 0.29]	0.16
ICAP: Severity (mean score)	0.13	[-0.49; 0.75]	0.67
Quality of Life (parents)			
Kidscreen-27: Physical well-being	-1.18	[-6.26; 3.91]	0.64
Kidscreen-27: Psychological well-being	0.30	[-1.42; 2.01]	0.73
Kidscreen-27: Autonomy & parents	3.27	[-2.47; 9.02]	0.25
Kidscreen-27: Peers & social support	5.18	[-0.54; 10.90]	**0.05**
Kidscreen-27: School environment	1.37	[-7.38; 10.13]	0.70

Estimated treatment effect (95% CI and p-values from ANCOVA models) adjusting for gender, IQ, and baseline scores. A positive sign indicates a larger average value under EGCG treatment compared to placebo. EGCG: epigallocatechin-3-gallate.

Table 5S. Descriptive analysis on the qualitative data provided by parents at the end of the study period on blind guess of treatment assignment, presence or absence of relevant changes along the trial and nature of the changes observed.

	Study Group assignment	
	EGCG (n=13)	**Placebo (n=16)**
Subjective notion of group assignment		
EGCG	9 (69.23%)	3 (18.75%)
Placebo	4 (30.76%)	11 (68.75%)
Unknown	0	2 (6%)
Changes along the trial		
Presence	10 (76.92%)	4 (25.0%)
Absence	3 (23.07%)	12 (75.0%)
Positive/Negative changes		
Positive	9	3
Negative	1	1
Positive specific effects [a]		
Cognition (enhancement)		
Unspecific enhancement	1	1
Attention-concentration	3	
Language (verbal speech)	1	
Memory and learning	1	1
Executive functions (reasoning)	1	
Behaviour (improvement)		
Behavioural control	2	
Mood	1	1
Maturity/Self confidence	2	1
Clinical parameters		
Weight Loss	4	
Muscle mass increase	1	
Negative specific effects [a]		
Behavioural		
Heightened activation	2	

Results are presented as absolute frequency (relative frequency) for categorical variables. [a] Specific changes in the EGCG group (n=10) and in placebo group (n=4). EGCG: epigallocatechin-3-gallate

Figure S1. Effects of EGCG on thigmotactic behaviour in the visuo-spatial learning task in TgDyrk1A and Ts65Dn mouse models. (A, B) Percentage of time spent in the periphery (thigmotaxis) of the pool during the acquisition sessions in (A) TgDyrk1A and (B) Ts65Dn. Data are represented as mean ± SEM. Genotype effect ** $p<0.01$, *** $p < 0.001$; treatment effect # $p<0.05$, ## $p<0.01$.

Figure 1

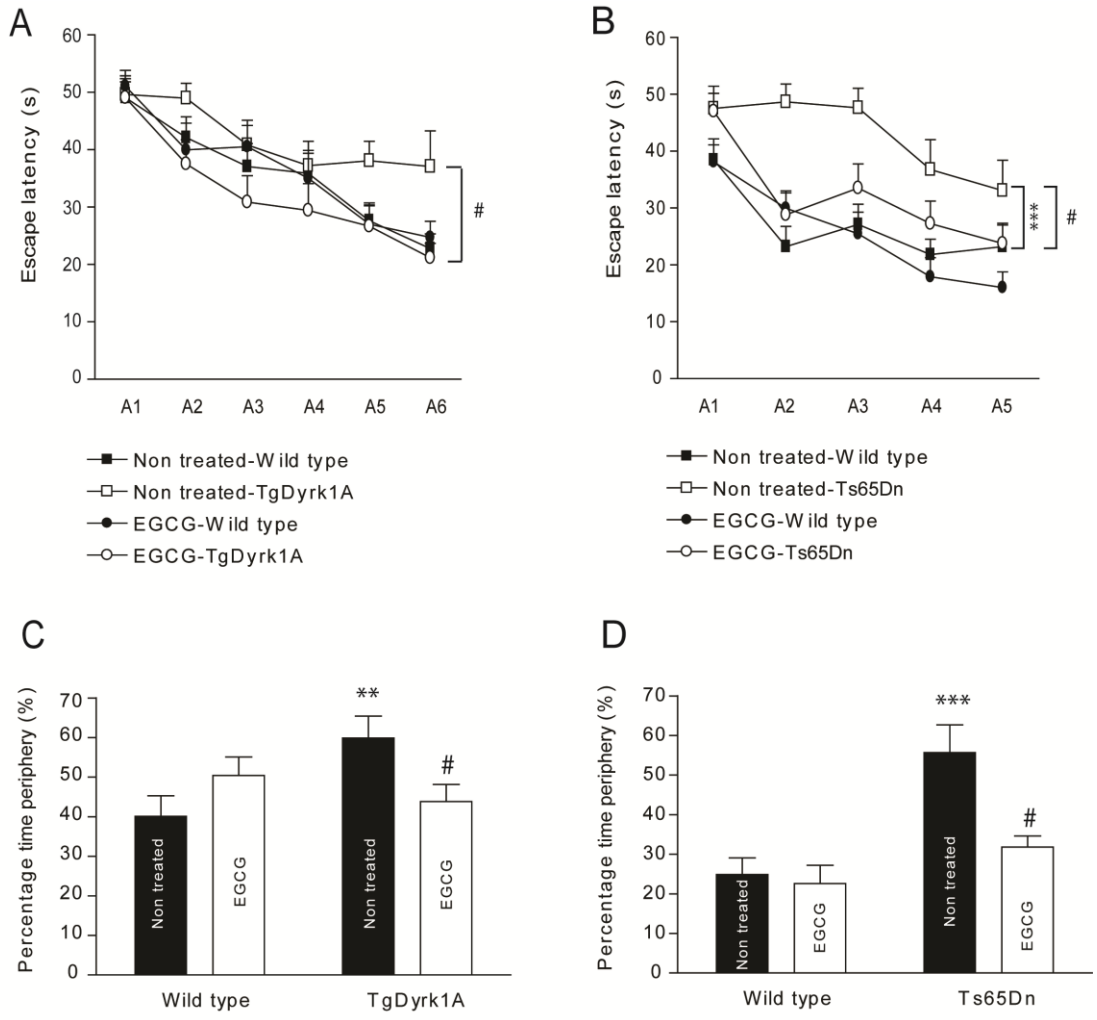

A
Escape latency (s)
60
50
40
30
20
10
0
A1
A2
A3
A4
A5
A6
#
Non treated-Wild type
Non treated-TgDyrk1A
EGCG-Wild type
EGCG-TgDyrk1A
B
Escape latency (s)
A1
A2
A3
A4
A5
***
#
Non treated-Wild type
Non treated-Ts65Dn
EGCG-Wild type
EGCG-Ts65Dn
C
Percentage time periphery (%)
70
60
50
40
30
20
10
0
**
#
Non treated
EGCG
Non treated
EGCG
Wild type
TgDyrk1A
D
Percentage time periphery (%)
***
#
Non treated
EGCG
Non treated
EGCG
Wild type
Ts65Dn

Figure 2

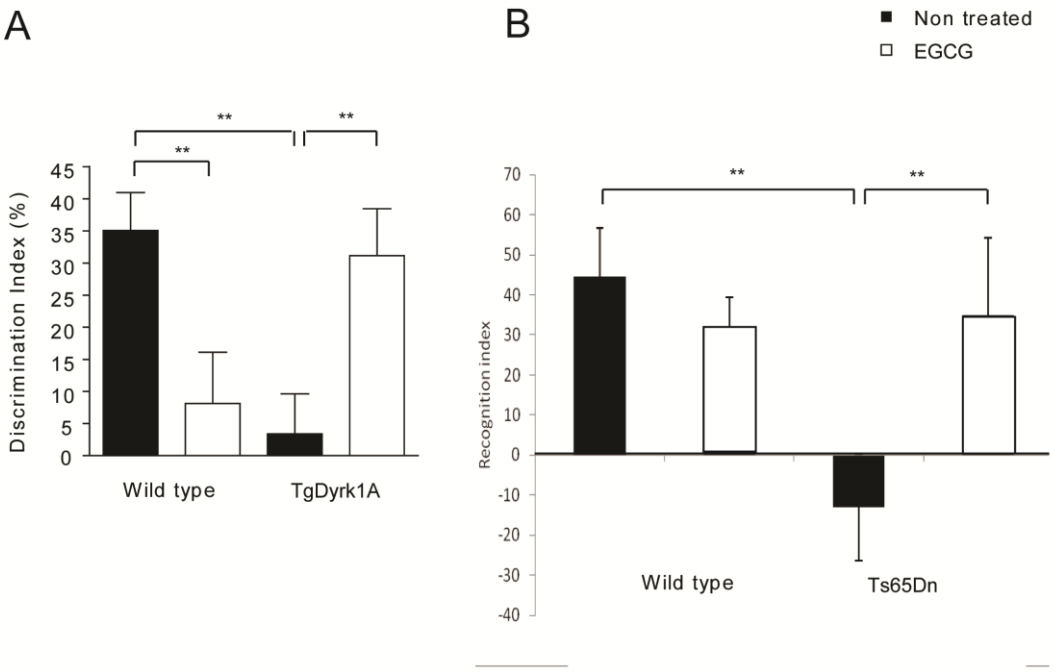

A
B
Non treated
EGCG
Discrimination Index (%)
45
40
35
30
25
20
15
10
5
0
**
**
**
Wild type
TgDyrk1A
Recognition index
70
60
50
40
30
20
10
0
-10
-20
-30
-40
**
**
Wild type
Ts65Dn

Figure 3

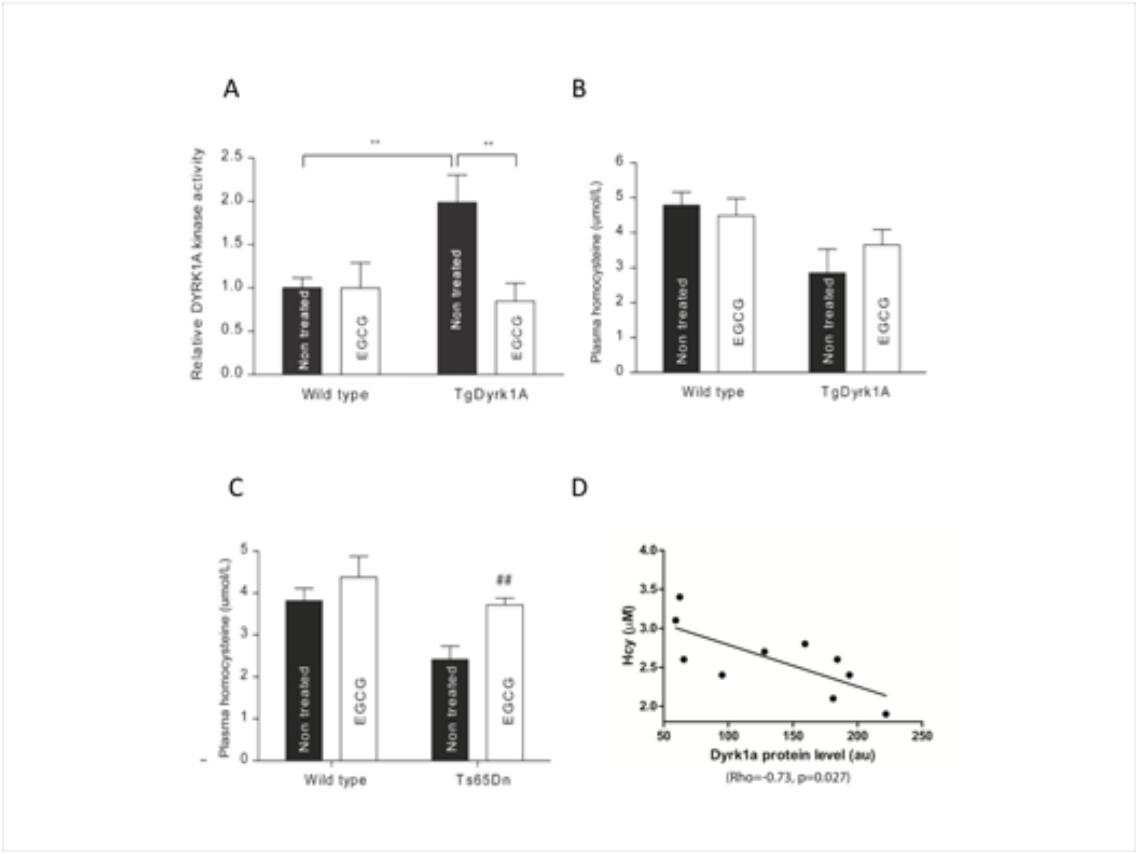
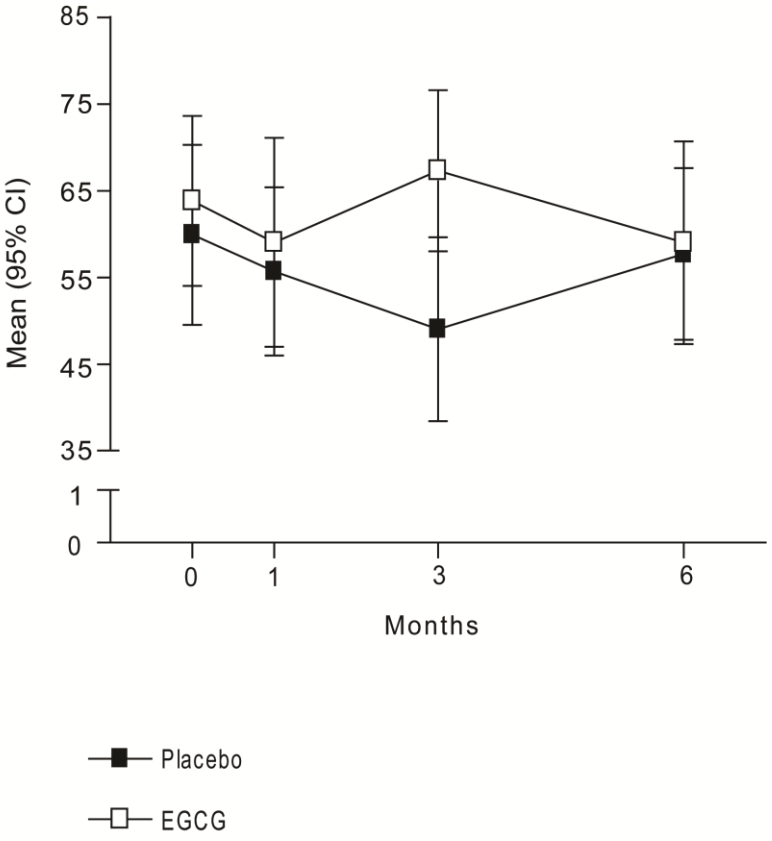
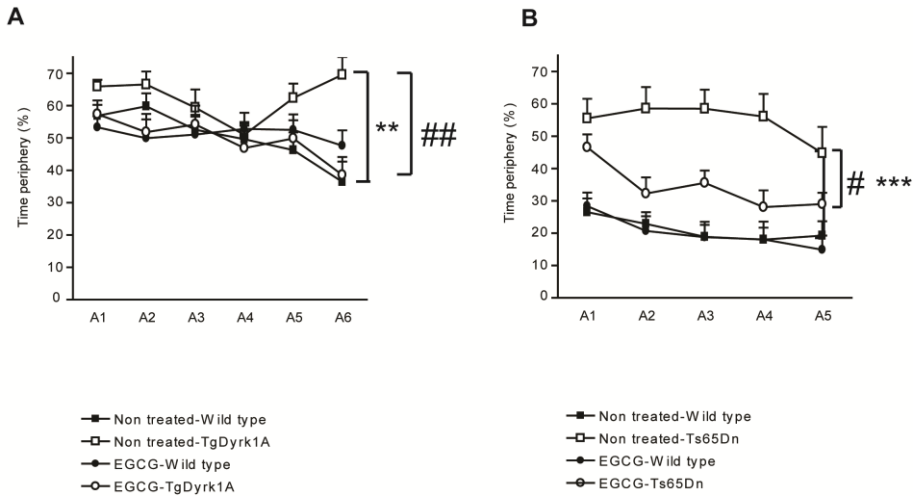

A
B
C
D
Relative DYRK1A kinase activity
Plasma homocysteine (umol/L)
Non treated
EGCG
Wild type
TgDyrk1A
Ts65Dn
##
Hcy (µM)
Dyrk1a protein level (au)
(Rho=-0.73, p=0.027)

Figure 4



Supplementary Figure 1



Pons-Espinal M, Martinez de Lagran M, & Dierssen M. **Functional implications of hippocampal adult neurogenesis in intellectual disabilities.** Amino Acids. 2013; 45(1): 113-131.