“Study of the neuroprotective and neurogenic actions of synthetic micromolecules in animal models of neurodegeneration”.

«Μελέτη των νευροπροστατευτικών-νευροαναγεννητικών δράσεων νέων συνθετικών μικρομορίων σε ζωικά μοντέλα νευροεκφύλισης».

Dissertation

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Περίληψη

Αν και μέχρι πρότεινως θεωρείτο ότι δεν υπάρχει ικανότητα αναγέννησης του νευρικού ιστού στον ενήλικο νευρικό σύστημα, κατά τη διάρκεια των τελευταίων δεκαετιών υπάρχουν δεδομένα που υποστηρίζουν την γέννηση και λειτουργική ενσωμάτωση νέων νευρώνων σε συγκεκριμένες περιοχές του εγκεφάλου όπως η υποκοιλιακή ζώνη και η οδοντωτή έλικα του ιπποκάμπου. Μελέτες σε διάφορα ζωικά μοντέλα έχουν καταδείξει γενετικούς, περιβαλλοντικούς και φαρμακολογικούς παράγοντες που ελέγχουν τη διαδικασία της νευρογένεσης στον ενήλικο οργανισμό. Δυσλειτουργία αυτού του συστήματος έχει εμπλακεί στην παθογένεση νευροεκφυλιστικών ασθενειών όπως η ασθένειες του Χαντινγκτον, Αλζχέιμερ και Παρκινσον καθώς και νευροψυχιατρικών καταστάσεων όπως η κατάθλιψη. Επιπρόσθετα δεδομένα συνηγορούν στην ιδέα ότι φαρμακολογική επαγωγή της νευρογένεσης μπορεί επηρεάσει την έναρξη και εξέλιξη συγκεκριμένων δυσλειτουργιών του εγκεφάλου όπως στη περίπτωση τράυματος στο νευρικό ιστό και στη κατάθλιψη. Για το λόγο αυτό η σύγχρονη φαρμακολογία ψάχνει για νέες μικρομοριακές ουσίες που μπορούν να διαπεράσουν τον αιματεγκεφαλικό φραγμό και είναι ασφαλείς στη κλινική χρήση, με την ικανότητα να αυξάνουν τη ενήλικη νευρογένεση προς θεραπεία νευεκφυλιστικών ασθενειών και σχετιζόμενων με το στρες.

Η φινγκολιμόδη είναι το πρώτο εκ του στόματος χορηγούμενο φάρμακο που πήρε έγκριση για τη θεραπεία της υποτροπιάζουσας διαλείπουσας μορφής της πολλαπλής σκλήρυνσης, μια απομυελινωτική αυτοάνοσης αιτιολογίας νόσο του ΚΝΣ. Δρα μέσω ενεργοποίησης 3 υπότυπων του S1P υποδοχέα που ανήκουν στην οικογένεια των GPCRs μιμούμενο τις δράσεις του ενδογενούς S1P (sphingosine-1-phosphate). Ο μηχανισμός που εμπλέκεται στις θεραπευτικές δράσεις της φινγκολιμόδης έγκειται στην παρατεταμένη ενεργοποίηση του S1P1 υποδοχέα στην επιφάνεια των ενεργοποιημένων T cells και συνεπακόλουθη ενδοκυττάρωσή του, κάτι που συμβάλλει σε εγκλωβισμό αυτών στους λεμφαδένες. Παρολ' αυτά, οι υποδοχείς του S1P εντοπίζονται σε όλα τα κύτταρα του ΚΝΣ, ενώ συστηματική χορήγηση της φινγκολιμόδης έχει αποδειχθεί ότι επιφέρει νευροπροστατευτικές δράσεις και αύξηση της έκφρασης του νευροτροφικού παράγοντα BDNF από τα νευρικά κύτταρα. Οι ενδείξεις αυτές για τις πιθανές δράσεις της φινγκολιμόδης στο ΚΝΣ μας έκαναν να αναρωτηθούμε αν θα μπορούσε να έχει κάποια επίδραση στο φαινόμενο της ενήλικης

νευρογένεσης. Πράγματι, πράγματι χορήγηση του φαρμάκου in vivo και in vitro αύξησε το ρυθμό πολλαπλασιασμού και την επιβίωση των πρόδρομων νευρικών κυττάρων ενώ οι δράσεις αυτές σε κυτταρικό επίπεδο συσχετίστηκαν με βελτίωση της απόδοσης ποντικών σε ένα τεστ συμπεριφοράς που φαίνεται να αναπαριστά το λειτουργικό ρόλο της ενήλικης νευρογένεσης που είναι ο διαχωρισμός περιβάλλοντων.

Η διυδροεπιανδροστερόνη (DHEA) είναι ένα το πιο άφθονο νευροστεροειδές που παράγεται στη περιφέρεια απο τα επινεφρίδια και της γονάδες ενώ επίσης παράγεται τοπικά στον εγκέφαλο. Μεταβολικά προιόντα της DHEA είναι τα οιστρογόνα και τα ανδρογόνα. Τα ποσά αυτής μειώνονται δραματικά κατά τη γήρανση και έχουν σχετισθεί με την εμφάνιση νευροεκφυλιστικών ασθενειών όπως η νόσος Αλζχέιμερς. Η DHEA έχει δειχθεί πως αυξάνει τη νευρογένεση σε in vitro και in vivo μοντέλα ενώ αναστέλλει την επιβλαβή δράση των γλυκοκορτικοειδών και ενισχύει την επαγωγική δράση των αντικαταθλιπτικών.

Τα τελευταία χρόνια στο εργαστηριό μας έχει καταδειχθεί η νευροπροστατευτική δράση της DHEA μέσω της ενεργοποίησης ενδοκυττάριων μονοπατιών που έχουν σαν τελικό αποτέλεσμα την επαγωγή έκφρασης αντιαποπτωτικών πρωτεινών όπως η Bcl-2. Επίσης, η DHEA φαίνεται να μιμείται την δράση του Νευρικού αυξητικού παράγοντα (NGF) μετά απο πρόσδεση και ενεργοποίηση των υποδοχέων του μειώνοντας την αποπτωση που προκαλείται απο αποστέρηση απο NGF σε νευρώνες που εξαρτούν την επιβίωσή τους απο αυτόν όπως φάνηκε απο πειράματα σε πρωτογενείς καλλιέργιες αισθητικών νευρώνων και κατα την ανάπτυξη στην επιβίωση αισθητικών νευρών των γαγγλίων της ραχιαίας ρίζας στα *ngf* -/- ποντίκια.

Παράλληλα, το εργαστήριό μας έχει αναπτύξει συνθετικά μόρια της DHEA που διατηρούν τις νευροπροστατευτικές δράσεις της DHEA αλλά στερούνται της ικανότητας μεταβολισμού προς ανδρογόνα και οιστρογόνα. Τα μόρια αυτά ώς μικρομοριακά και λιπόφιλα έχουν την ικανότητα να διαπερνούν τον αιματοεγκεφαλικό φραγμό και μπορούν να χορηγηθούν περιφερικά χωρίς το κίνδυνο εμφάνισης ορμονοεξαρτώμενων δυσλειτουργιών.

Στο δεύτερο μέρος της διδακτορικής μου διατριβής επικεντρώθηκα στη διερεύνηση της πιθανής δράσης των μορίων αυτών και συγκεκριμένα ενός εξ αυτών με την ονομασία BNN27 στη νευρογένεση στον ενήλικο ιππόκαμπο κατα τη γήρανση και την εμφάνιση της νόσου του Αλζχέιμερ. Το BNN27 φάνηκε ανίκανο να αυξήσει τη νευρογένεση σε ποντικούς αγρίου τύπου ούτε να αναστείλει την μείωση του

πολλαπλασιασμού των πρόδρομων νευρικών κυττάρων κατά τη γήρανση. Παρολ' αυτά το BNN27 ήταν ικανό να αναστείλει τη μείωση στο ρυθμό νευρογένεσης στα 5XFAD ποντίκια, τα οποία φέρουν 5 μεταλλάξεις που έχουν συνδεθεί με την οικογενή μορφή της νόσου Αλζχέιμερ. Στα ίδια διαγονιδιακά ποντίκια το BNN27 είναι ικανό να μειώσει την ατροφία των χολινεργικών νευρώνων και τη συσσώρευση του αμυλοειδούς επηρεάζοντας θετικά πολλά στοιχεία της παθολογίας της νόσου Αλζχέιμερ.

Στο τρίτο και τελευταίο μέρος της διατριβής μου μελέτησα το τρόπο με τον οποίο επιδρά η τοπογραφία του μικροπεριβάλλοντος των νευρικών κυττάρων και συγκεκριμένα αυτή που δημιουργείτε από διατάξεις μικροκόνων ελλειπτικής διατομής από πυρίτιο, στον προσανατολισμό της νευραξονικής αναγέννησης και της κυτταρικής μετανάστευσης. Φαίνεται ότι το συγκεκριμένο είδος τοπογραφίας το οποίο χαρακτηρίζεται από μη συνεχόμενη γεωμετρία παρόλ'αυτά περιέχει ένα στοιχείο ανισοτροπίας που είναι η κεντρική άξονα της ελλειπτικής διατομής των μικροκόνων, μπορεί να κατευθύνει τη προσονατολισμό των νευραξόνων του περιφερικού νευρικού συστήματος καθώς και τη μετανάστευση κυττάρων Schwann, τα κύτταρα της γλοίας που περιβάλλουν τους άξονες στη περιφέρεια. Η μελέτη της συγκεκριμένης τοπογραφίας μπορεί να συμβάλλει στη δημιουργία μικροροικούν κατασκευών, νευροαισθητήρων για ταυτόχρονη διέγερση και καταγραφή αποκρίσεων νευρονικών δικτύων αλλά και στην ανάπτυση θεραπευτικών λύσεων για επαγωγή και προσανατολισμό της νευραξονικής αναγέννησης σε περιπτωση τραύματος στο νευρικό ιστό.

ABSTRACT

The adult mammalian brain possesses only a limited capacity for regeneration under pathological conditions that cause neuronal cell loss. However, the identification of endogenous adult neural stem cells (aNSCs) that can proliferate and differentiate into functional neurons, unveil new intriguing possibilities towards restoration of brain function in case of a disease. aNSCs that lie mainly in the area of the dentate gyrus (DG) and in the subventricular zone (SVZ) are generated continuously and after they differentiate they get integrated to the existing circuit. These cells are responsive to intrinsic as well as external stimuli that affect their proliferation, their survival and differentiation. Such molecules are neurotrophins such as Nerve growth factor (NGF) and Brain derived neurotrophic factor (BDNF), as well as other growth factors such as hormones, which are also responsible for the patterning of nervous system during development. Increasing neurogenesis with genetic or pharmacological manipulation improves performance on various cognitive tasks while it has been recently shown that there are specific types of functions of the DG that require the integration of new neurons such as pattern separation.

Neurogenesis has been implicated in the pathophysiology of many neurodegenerative and neuropsychiatric diseases. Alterations in neurogenesis have been observed in various animal models of Alzheimer's disease (AD) and have been linked to accumulation of aβ amyloid and the development of neuroinflammation. On the other hand stimulating adult hippocampal neurogenesis in these models either by means of environmental enrichment, physical exercise or with pharmacological ways reduces amyloid pathology and improves cognitive function. Finally, adult neurogenesis is dramatically reduced during ageing and pharmacological agents that succeed to restore this decline were also effective to alleviate age-related memory deficits.

Nevertheless, the clinical use of many molecules that have been shown to possess neurogenic properties is often problematic either because of their peptidic nature and their inability to cross the blood brain barrier or because they accompanied by off target adverse effects. For this reason, modern pharmacology is seeking of small lipophilic molecules, which can mimic the actions of endogenous growth factors and show good availability to the CNS as well as a good safety profile.

In this study I have tested the neurogenic potential of two small molecules that belong to two different classes. One of them is fingolimod that has been approved for the

treatment of multiple sclerosis based on its ability to inhibit the egress of lymphocytes from lymphoid nodes, thus preventing its migration to the site of inflammation. It acts through activation of 5 subtypes of S1P receptors that belong to GPCR superfamily but also as an intracellular messenger itself by regulating the expression of many genes such as BDNF and other growth factors. However, S1P is also synthesized *de novo* in the CNS and S1P receptors are abundantly expressed in various regions and by a plethora of cell types in the CNS and the therapeutic effects of fingolimod have been tested for a wide range of CNS disorders, from neurodegenerative to neuropsychiatric conditions. Intrigued by the role of S1P during development but also in the maintenance of an intact nervous system I sought to determine if there are any effects of the -already in clinical use- agonist of S1PRs, fingolimod in regulation of adult neurogenesis, which may contribute to its beneficial actions. My results show that fingolimod can induce the proliferation and survival of adult hippocampal progenitors *in vivo* and *in vitro* through activation of MAPK pathway and induction of BDNF while it affects behavior that have been associated with the functional role of adult neurogenesis.

The other molecule that it was tested for its ability to induce adult neurogenesis was a synthetic analogue of the endogenous neurosteroid Dehydroepiandrosterone (DHEA), called BNN27. DHEA is synthesized in the periphery from the adrenal glands, but also is synthesized *de novo* and secreted locally in the CNS in high concentration. DHEA has been shown to promote neuronal survival in various models of neurodegeneration both *in vivo* and *in vitro* through divergent mechanisms and recent data from our laboratory support the idea that some of these effects are mediated by activation of NGF receptors (namely TrkA and $p75^{NTR}$). However, the therapeutic use of DHEA is compromised because it widely affects the endocrine system by its own or after conversion to other steroid hormones with possible tumorogenic action. In particular breast, endometrial, or prostate cancers have been associated with disturbances on estrogens and androgen levels. Moreover the exact actions of DHEA cannot be distinguished from its metabolites. BNN27, has been developed by modifications of DHEA structure in order to sustain its neuroprotective properties and avoid its further metabolism to androgens and estrogen. In this way, BNN27 lacks of the possible tumorogenic potential of the parent molecule, DHEA. In order to test whether BNN27 could reproduce the anti-apoptotic effects of DHEA mediated by activation of NGF receptors' initiated signaling, we used cultures of sympathetic

neurons that are known to be dependent on NGF for their survival as well as NGF null mice embryos that exhibit massive cell death in the DRGs during the embryonic development. BNN27 was found efficient to reduce apoptosis due to NGF deprivation in both cases. Based on the neuroprotective actions of BNN27 I further sought to investigate if this molecule could support neurogenesis in the adult rodent hippocampus. BNN27, was not effective in inducing either proliferation or survival of neural stem cells in the area in WT mice nor did it reversed neurogenic deficits in old animals. However, long-term administration of BNN27 ameliorated the neurogenic and cholinergic deficits in an animal model of amyloidosis, the 5XFAD mice, while it also reduced the amyloid burden.

One the other part of this study I investigated the effects of microconically patterned silicon substrates on the behavior of peripheral nervous system neurons and glial cells. Directed axonal outgrowth is necessary for nerve regeneration after an injury but also in a broad range of applications in neuroscience such as construction of microfluidic devices or neuronal interfaces. Among the factors that control the orientation of a regrowing axon are cellular cues and substrate topography. Based on their design, different topographies that have been tried so far to manipulate cell growth, migration, and differentiation of various cell types could be described by the following geometries: continuous and discontinuous. These geometries could be further classified based on the directionality as anisotropic, in which cues are provided along a single axis or isotropic topographies which are uniform in all directions, providing cues along multiple axesExamples of continuous topographies are photolithographically fabricated grooved silicon substrates or electrospun polymer fibers at parallel or random orientation, while discontinuous geometries include silicon or gold pillars or posts.

Recently the bioengineering lab in IESL in FORTH developed and characterized micropatterned silicon culture substrates fabricated with the use ultra-fast pulsed laser structuring. Upon increasing laser fluence surface roughness is also increased and acquires anisotropic geometrical characteristics. More specifically as we pass from flat silicon to surfaces that display a higher degree of roughness, substrates are comprised of microcones with elliptical shape and specific orientation. Surface roughness and wettablity of the micropatterned Si substrates, influence fibroblast adhesion as well as differentiation capacity of PC12 cells as response to NGF. At the

framework of our collaboration we aim to investigate the effects of substrate of topography in cell outgrowth and morphology of PNS populations.We showed that cultured Schwann cells and sympathetic neurons of SCGs migrate or grow their axons along the major axis of microcones in parallel alignement. Moreover when Schwann cells are present they seem to drive the growing axons as it was revealed in coculture studies of the populations examined as well as in whole DRG explant cultures a classic model to study neurite outgrowth and Schwann cell migration.

Our study demonstrates for first time that a discontinuous topography could drive the directional outgrowth of neurons and glial cells of the peripheral nervous system if it contains at least a feature of anisotropy which is the elliptical shape of the microcones. This distinct inherent property of our microstructures combined with the conductance of the material, provides a useful system to explore and control neuronal functions and subsequent network characteristics.

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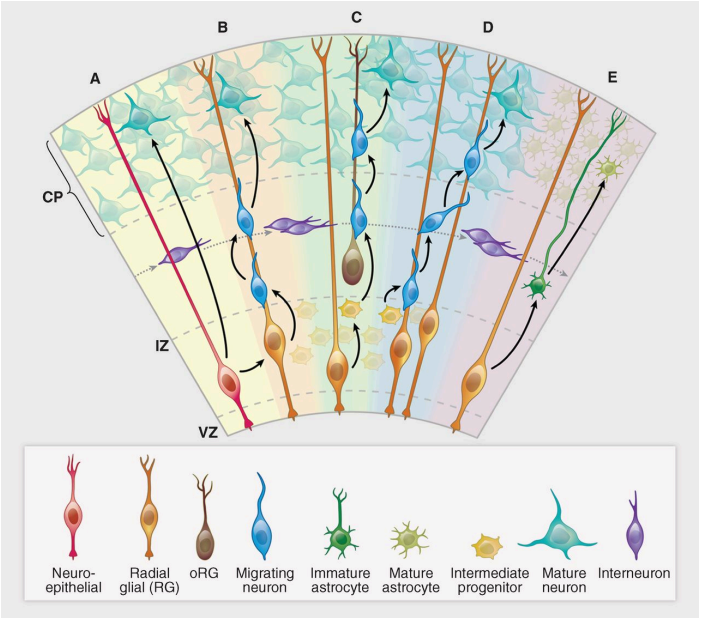
INTRODUCTION

Neurogenesis in Brain Development

The mammalian nervous systems is characterized by a unique cellular heterogeneity that serves its complex functions, such as perception and cognition. Divergent neuronal cell populations of the Central Nervous System (CNS) arise from a pool of neuroepithelial cells (NECs), of the neural tube, through a complicated process of self renewal, migration and differentiation. These cells that possess the capacity to multiply themselves through division in order to generate neurons as well as 2 types of macroglial cells in the CNS, the oligodendrocytes and astrocytes, have been characterized as «Neural stem cells» (McKay et al., 1997; Gage et al., 2000; Gotz et al., 2005).

Early in embryogenesis and before neurogenesis at embryonic day 8 (E8) in mice, the neural tube consists of both multipotent stem cells that can give rise to neurons oligodendrocytes and astrocytes, as well as restricted progenitors (Kalyani et al., 1997 and 1998). The identity of a terminally differentiated neurons is finally determined by spatiotemporal expression of cell type specification genes that, in turn, are regulated by various morphogens such as fibroblast growth factors (FGFs), retinoic acid (RA), Sonic hedgehog (Shh) and bone morphogenetic proteins (BMPs). At the same time neural crest stem cells give rise to all cell populations of Peripheral Nervous System (PNS) and are likely to, also, derive from the NECs themselves (Stemple et al., 1992). At this stage and until E10, NECs initially undergo symmetric division in order to expand their population, which means that they produce two identical daughter stem cells. Later on, however, and as they lose their epithelial characteristics they divide asymmetrically producing one progenitor cells that will become neuron and a new type of stem cell called radial glial cell (RGC). During this period the neuroepithelium transforms to a tissue with multiple layers and cells with stem cell characteristics are restricted to a thin layer called ventricular zone (VZ) overlaying the ventricle. RGCs express various astroglial markers such as the astrocyte specific glutamate transporter (GLAST), the Ca2+- binding protein S100β, glial fibrillary acidic protein (GFAP), vimentin and brain-lipid-binding protein (BLBP). RGC continue to divide asymmetrically to form terminally differentiated neurons as well as new RGC and basal progenitors that further differentiate to neurons after a round of symmetric division (Miyata et al., 2001; Noctor et al., 2001). However, RGC retain another role in the process of neurogenesis which is the proper migration of newborn neurons during brain

development (Barry et al., 2014). During radial migration progenitors of excitatory glutamatergic neurons in the VZ exit the cell cycle and move perpendicular to the VZ surface along a chain or radial glial cells in order to reach their final destination forming the outer layers of the cortex. In contrast tangential type of migration refers to the movement of neurons in parallel to VZ and does not involve any guidance from RGCs. In this case progenitors are born in the ganglionic eminence and are destined to become GABAergic interneurons.



Schematic representation of radial and tangential migration during cortical development. CP, cortical plate; IZ, intermediate zone; VZ, ventricular zone. The VZ early in development has a thickness of ~10 cell bodies (50 to 100 μm). The CP ranges in thickness from two to three cell bodies at the earliest stages of development, eventually forming a mature cerebral cortex that is 2 to 4 mm thick.

Poduri et al., 2013

Once migration is complete and structures have formed, axons and dendrites begin to grow to their mature size and shape. Moreover newly formed neurons start to make synapses in order to communicate with other neurons or target tissue. At this point, also, an important portion of neurons die through apoptosis by a physiological process called programmed cell death due to failure to compete for chemicals provided by targets. All these events are precisely controlled by molecules secreted by surrounding cells like neurotrophins that guide the growing axon, promote synaptogenesis and regulate the survival of neurons.

Neurogenesis in the adult brain.

Until nearly twenty years ago a central dogma in the field of neuroscience, regarding the development of the nervous system during adulthood, was that established by the prominent histologist Ramon y Cajal «..Everything may die, nothing may be regenerated. It is for the science of the future to change, if possible, this harsh decree..». Since then a lot of things have changed in our view about the regenerative capacity of the adult nervous system and a milestone to this direction was the identification of endogenous neural stem cells in the adult mammalian brain. This was attributed to Joseph Altman, who in 1960 used a radioactive thymidine analog which is incorporated to the DNA of cells that undergo mitosis, to show with autoradiography that indeed there are proliferative cells in the adult hippocampus, an area responsible for learning and memory (Altman and Das 1965). While the lack of specific markers at that period did not allow Altman to demonstrate that these cells become neurons, it was Michel Kaplan who 10 years later combined Altman's technique with electron microscopy and showed that the cells that incorporated the radiolabeled thymidine acquire a neuronal phenotype. Indeed, we now known that thousand of neurons are born every day in specific areas of the brain of the adult brain, while a considerable number of others is also lost. Moreover, the use of more advanced technics, like the use of confocal microscopy permitted the tracing of these cells, especially after the introduction of another thymidine analog (BrdU) that can be immunohistochemically detected, along with their phenotypic characterization. The first who made a link between neurogenesis and behavior was Fernando Nottebohm who showed fluctuations in the volume of brain areas responsible for acquisition of seasonal song learning in male canaries (Alvarez-Buylla et al., 1988; Nottebohm et al.,1989), an event that was also correlated with the levels of the steroid Testosterone. Since then however they have been discovered a myriad of intrinsic factors and environmental stimuli that control the phenomenon of adult neurogenesis. Finally, the pharmacological or genetic manipulation of adult neural stem cells fate allowed the thorough investigation of their functional role.

In the adult mammalian brain there are two distinct areas of proliferation of neural stem cells and these are the dentate gyrus (DG) of the hippocampus and the

subventricular zone (SVZ). In this study I focused on the role of two small lipophilic molecules in adult hippocampal neurogenesis.

Cell types, lineage, and architecture of the germinal zone in the adult dentate gyrus.

As was firstly mentioned by Altman, new neurons are continuously generated in area of hippocampus from mice to humans. These are originated from self-renewing and multipotent adult neural stem cells (NSCs) residing in the subgranular zone (SGZ) of DG. SGZ is considered as a two cells layer underlying the Granular cell layer (GCL) of the DG and it is formed by cells migrating from the VZ of DG at the late stages of gestation. There are 2 types of NSCs in this area with regards to their proliferative properties as well as their phenotype and can be distinguished both morphologically but also in terms of the expression profile of specific protein markers. Type 1 is referred to Glial Fibrillary Acidic Protein (GFAP) positive astrocytes with stem cell characteristics. These could be distinguished based on their orientation to radial astrocytes (rA) reminiscent of radial glial cells that appear during development, and horizontal astrocytes (hA). Regarding their morphology rAs possess round or triangular cell body with thin lamellae tangentially oriented along the SGZ while hAs have more elongated soma and no radial projection, but instead they extend branches parallel to the SGZ (Seri et al., 2004). Both types of astrocytes express also markers of undifferentiated NSCs such as the transcription factor of SRY-related HMG box family (Sox2) as well as the intermediate filament proteins Nestin, Vimentin, which however, are common markers of all types of ependymal cells as well. Recently, prominin1 (CD133) has emerged as a reliable marker that characterizes GFAP+ radial and horizontal NCSs in the adult Subependymal zone (SEZ) and DG and allows their in vivo tracing or their prospective isolation for in vitro experiments (Beckervordersandforth et al.,2010 and 2014; Walker et al., 2014). Type 1 NSCs regardless of their morphology comprise a rather quiescent population under normal conditions despite the fact that hA cells display a degree of proliferation, which is dependent on notch signaling (Lugert et al., 2010). Asymmetric slow divisions of Type 1 cells give rise to Transit amplifying progenitors (TAPs) which can differentiate to either neurons, astrocytes or oligodendrocytes and finally according to a recent view they become mature astrocytes themselves (Encinas et al., 2011). There are

several types of TAPs, which can be identified on the basis of morphology and marker expression (Suh et al. 2007; Lugert et al. 2010). These cells have short processes parallel to the SGZ and they perform a tangential migration. They usually proliferate in clusters in close proximity with blood vessels. The major difference between type 2A, type 2B, and type 3 cells is that type 2B cells were initially identified in Nestin-GFP reporter mice and expression of the immature neuronal marker doublecortin (DCX) in Nestin-GFP+ cells defines the transition between type 2A and type 2B, whereas Nestin-negative type 3 cells express DCX only (Dhaliwal and Lagace 2011). The Sox2 trascription factor is still expressed by type 2 cells, before their transition to late stage TAPs (type 2B) or neuroblasts (type 3) while another transcription factor belonging to bHLH superfamily, the Ascl1 (or MASH1) is thought to maintain the neuronal precursors in the DG in an undifferentiated state (Pleasure et al., 2000; Kim et al., 2007,2011) and is expressed mainly by type 2A TAPs that will become granule cell neurons in the DG. Finally, there is down-regulation of DCX and up-regulation of calretinin and NeuN as immature neurons differentiate into mature glutamatergic granule neurons.

Maturation of adult-born neurons in DG

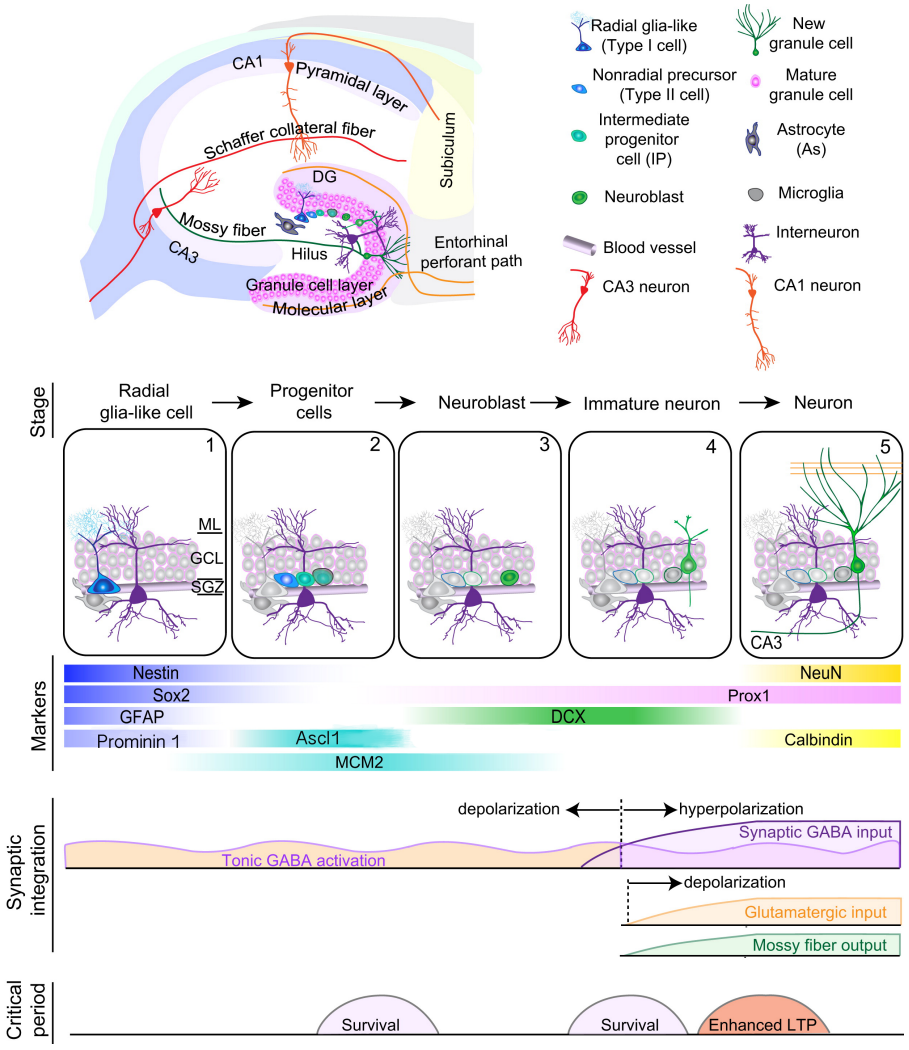
The initial maturation stage of NSCs occurs during the first week of age where new born dentate granule cells (DGCs) are considered to be functionally "silent": they have no connection to other neurons in the network and they do not fire typical action potentials (Esposito et.al., 2005; Ambrogini et.al., 2004;Wang et. al., 2000). Regulated by the local network activity, new neurons migrate short distance to reside the inner granule cell layer of DG (Deng et.al., 2010). Physiologically, these new born cells lack much of the neuronal machinery, since they are characterized by extremely high resistance and low capacitance (due to their size) (Aimone et.al., 2010). A key player of neuronal maturation is the excitatory GABAergic input (possibly dendritic) that new cells receive (Esposito et.al., 2005; Ge et.al.,2000), because of their high intracellular concentration of Cl^- (Aimone et.al., 2010). This GABAergic modulation is essential for the neurite outgrowth, migration during the early maturation stage (Aimone et.al., 2010).

Until the end of the second week after their birth, the adult-born DGCs become more neuron-like by acquiring polarization: their dendrites extend towards the molecular

layers and axons form the mossy fibers by extending through the hilus towards CA3 (Zhao et.al., 2006). Yet, because of their higher membrane resistance and distinct firing properties, immature neurons differ from their mature counterparts (Esposito et.al., 2005). At this time point, (in consistence with the absence of dendritic spines) adult-born neurons do not receive glutamatergic inputs (Zhao et.al., 2006) whereas GABAergic inputs from local interneurons still exist (Esposito et.al., 2005). The timing of the next stage of maturation (begins around the 16^{th} day) coincides with the formation of dendritic spines (Zhao et.al., 2006) and the increased plasticity of mossy fibers, which begin to make synapses on downstream CA3 neurons (Toni et.al., 2008). Now, another key player, NMDA, is necessary for the maturation and survival of new neurons (Tashiro et.al., 2006), reflecting the onset of glutamatergic input received by the new DGCs (Esposito et al., 2005) and thus the involvement of NMDA-related processes e.g., plasticity (Blish & Gollingrindge, 1993). This time window corresponds with the gradual transition of GABA, from being depolarizing molecule, to becoming inhibitory neurotransmitter (Ge et.al., 2006). Adult-born neurons that are 4-6 weeks of age, are more excitable and exhibit higher degrees of synaptic plasticity compared to their mature counterparts (Ge et.al., 2007), due to their increased membrane resistance and depolarized potentials (Esposito et.al., 2005), whereas their action potentials have kinetic characteristics that resemble those of mature DGCs (Esposito et.al., 2005).

Newly generated neurons in SGZ are structurally and functionally mature in 6–8 weeks (Van Praag et al. 2002; Zhao et al. 2006) after their birth, however not all of the neurons that are born survive to reach this stage. Only a proportion of proliferating cells will find their way and get integrated to granule cell circuitry, while other will die through apoptosis in this, showing that the phenomenon of adult neurogenesis is a dynamic process like the one that occurs during development by elimination of excess neurons. The question about the exact age when this selection takes place was addressed by Dayer A. et al., 2003 who found that there is a loss of 50% of BrdU-labeled cells over a 22-day period between day 6-28 of BrdU incorporation. Taking into consideration that BrdU keeps labeling daughter cells of dividing NSCs for up to 4 days before it is completely diluted the elimination process lasts about 3 weeks. It was, moreover, discovered lately that resident activated microglia in the physiological brain is responsible for phagocytotic cell death of the majority of late NCSs and early postimitotic neurons during the initial period of 4 days while the rest of dying neurons

are lost in a later stage by other means (Sierra A. et al., 2010). After this critical period, newborn neurons in GCL have been shown to survive for at least 8 months in rodents (Altman and Das et al., 1965), 12 weeks in the macaque (Gould et al., 2001), and 2 years in humans (Eriksson et al., 1998).



Schematic representation of the distinct morphological stages of an adult born GCL neuron maturation. (Adopted from Ming G. et al.,2011)

Modulation of adult neurogenesis

Enviromental influence.

A lot of intrinsic and extrinsic stimuli are know to influence the capacity the adult brain to produce new neurons throughout the lifespan. The first proof came from the work of Kempermann, G., et al., (1997), which showed that mice grown in an enriched environment have increased neurogenesis and larger volume of hippocampal granule cell layer. Since however environmental enrichment affects several parameters such as learning but also physical activity van praag et al., 1999 tried to dissect the precise role of these different factors. He found that voluntary running of mice in a wheel was efficient in improving both proliferation and survival of NSCs in the DG. Meanwhile, it has been discovered ageing was negatively correlated with the number of proliferating cells in the same area while later it was shown that running is capable reverse this decline (Kuhn et al., 1996, Praag et al., 2005). Apart from ageing, another important factor that negatively regulates the phenomenon of neurogenesis is chronic stress which can be mimicked by the administration of the adrenal steroid, corticosterone (Gould et al., 1992). On the other hand, antidepressant treatment with classic monoamine reuptake inhibitors, like fluoxetine, increases both proliferation and survival of new neurons and counteracts the effects of stress in both neurogenesis and depressive behavior (Santarelli et al., 2003) in mice. The progenitor population which responds to the mitogenic action of fluoxetine is the type 2 TAPs (Encinas et al., 2006). Moreover, the necessity of SGZ neurogenesis for the behavioral effects of antidepressants was addressed by using X-ray irradiation which depletes all the actively proliferating cells in the hippocampus. However, not all of antidepressants seem to act by regulating neurogenesis while their effects may vary depending on the experimental design and the behavioral tests that were examined (Zhao et al., 2008). A recent study which sheds light to the exact role of neurogenesis propose that adult born neurons are necessary for homeostasis of glucocorticoid levels of the brain regulating stress response, emphasizing the role of hippocampus in the control of hypothalamic-pituitary-adrenal (HPA) axis (Snyder et al., 2011).

While increasing adult hippocampal neurogenesis improves performance of experimental animals in various memory tasks, which will be discussed in another chapter, learning procedure per se have a multifaceted effect on the phenomenon of neurogenesis. Spatial learning that involves training in water maze platform increases apoptosis of immature neurons while it increases both survival of more mature newborn neurons and proliferation of their progenitors in a later stage, creating with the latter a "neurogenic reserve" for possible demands in the future (Dupret et al., 2007; Kempermann et al., 2008). Finally, Bruel-Jungerman and colleagues found that long-term potentiation (LTP), the cellular correlate of "learning," induced neurogenesis in the adult hippocampus in vivo (Bruel-Jungerman et al., 2006)

Activity and major neurotransmitter systems

The DG as a structure receives inputs from different regions in the brain while it participates in the intra-hippocampal circuit. The perforant pathway from the entorhinal cortex is the major input to the hippocampal DG (Amaral and Lavenex, 2007). From layer 2 of entorhinal cortex (EC), projection neurons extend their axons toward both the DG and the CA3 areas. Granule cells of the DG project to CA3 pyramidal neurons extend axonal projections called Shaffer collateral toward the CA1 pyramidal neurons. Then, CA1 pyramidal neurons signal back to neurons of the entorhinal cortex. The DG itself contains both the excitatory interneurons (mossy cells) and the inhibitory interneurons (for example, basket cells) which interact with neural stem cells in the area that they are interconnected with the previously described tri-synaptic hippocampal network. In turn, EC receives afferents from the cingulate gyrus, the temporal cortex, the orbital cortex, and the amygdala. Therefore, the neural activities at these association cortices may affect the process of hippocampal neurogenesis which themselves are regulated by neurotrasmitters GABA and Glutamate. A recent work tried to delineate the role or GABA released from Parvalvumin (PV) positive interneurons in the DG, in neural stem cell properties using optogenetic stimulation PV+ cells or experience dependent activation of the DG granule cells. This revealed that GABA mediates the quiescence of radial glial Like cells (RGLs) through the $\gamma 2$ subunit of $GABA_A$ receptor when there is increased activity of the PV+ interneurons. However while GABA decrease proliferation of

NSCs, at the same time it has been shown that increases neuronal differentiation acting on type-b cells which are excited in response to GABA increasing the release of intracellular Ca++ levels (Tozuka et al., 2005). On the other hand, as it was mentioned before, the DG is directly innervated by excitatory glutamatergic fibers from entorhinal cortex. Disruption of the perforant pathway results in increased neurogenesis, implicating Glutamate in regulation of NSCs proliferation and survival. In consistence with this, administration of NMDA decreases neurogenesis while blockage of NMDA receptor by injection of a competitive antagonist rapidly increases neurogenesis. Thus NMDA receptors seems to mediate the effects of Glutamate in neurogenesis (Cameron et al., 1995,1997).

Another source of inputs to the hippocampus and specifically to the DG are the cholinergic fibers of neurons lying on the septum of the basal forebrain (composed mainly of medial septal nuclei and diagonal band of Broca) (Amaral and Lavenex et al., 2007). Recent studies have shown that lesion in the medial septum cholinergic system negatively affects the proliferation of NSCs/neuronal progenitor cells (Cooper-Kuhn et al., 2004, Van der Borght et al., 2005), and that administration of acetylcholine esterase (AChE) inhibitors promotes NSC/neuronal progenitor cell proliferation (Mohapel et al., 2005; Narimatsu et al., 2009). In addition to the previous reports indicating a role of Achetylocholine (Ach) in the regulation of adult hippocampal neurogenesis, AchE was able to restore neurogenesis in aged mice on which the production of new neurons is diminished. (Itou et al., 2011).

Finally, the hippocampal formation receives monoaminergic ascending pathways (serotoninergic and noradrenergic) (Amaral and Lavenex, et al., 2007) that have been observed to penetrate the granular cell layer of the DG. We have discussed earlier that classic monoaminergic antidepressants are the most common pharmacological agents that increase neurogenesis. Apart from that there is also direct evidence supporting a role of these neurotransmitters in regulation of neurogenesis. For instance Inhibition of 5-HT synthesis or lesions in raphe nucleus decreases BrdU incorporation in the DG while studies with selective agonists of 5-HT receptor of serotonin revealed that Subtype 5-HT_{1A} and 5-HT_{2A} are important for the effects in the DG (Banasr et al., 2004, Brezun et al., 1999).

Growth factors

Neurotrophic factors are molecules that define cell fate of various neuronal populations during development of the peripheral and central nervous system. Brain derived neurotrophic factor (BDNF) is a key regulator of synaptic plasticity hippocampal dependent learning and memory during adulthood. Moreover it is upregulated in response to environmental enrichment, physical exercise and antidepressant treatment, while it is strongly suppressed by chronic stress and in depression (Murakami et al., 2005; Lang et al., 2004; Shimizu et al., 2003). Since there has been an obvious correlation between the levels of BDNF and neurogenesis in all of the aforementioned conditions it was raised the question if there is a causative relation between these events. The first link of neurogenesis with BDNF expression was made from Jaewon Lee et al., 2002 who showed that BDNF +/- mice had reduced levels of proliferation and survival of NSC's in the DG compared to WT mice and this was reversed by caloric restriction. Moreover, local infusion of BDNF to hippocampus resulted in increased number of newborn granule cells to the dentate gyrus as well as to other areas such as the hylus (Scharfman et al., 2005). In addition to this, the effects of BDNF in neurogenesis, have been shown to be responsible for the behavioral effects of chronic fluoxetine treatment (Ii et al., 2008) providing a mechanistic alternative to the monamine hypothesis for the action of classic antidepressants. However, although the contribution of BDNF in the process of neurogenesis is quite clear the exact receptor that mediates its actions is rather controversial (Bath et al., 2013). While TrkB, as the classic survival receptor for BDNF in mature neurons, was initially implicated in this action (Tervonen et al., 2006; Donovan et al., 2008; Li et al., 2008), $p75^{NTR}$ gained a lot of attention later on as an important player in adult neurogesis and probably mediator of this neurotrophin effects in NSCs (Young et al., 2007; Bernabeau et al., 2010; Colditz et al., 2010; Catts et al., 2009; Shi et al., 2013). A more recent study however proposed a different mechanism by which BDNF exerts its proneurogenic and antidepressant actions, which is the stabilization of γ2 subunit-containing $GABA_A$ receptors at the cell membrane, leading in this way to persistent reduction in neuronal excitability (Vithlani et al., 2013).

Nerve Growth Factor (NGF) is the first neurotrophin identified and its role in the development of the nervous system as well in neurodegeneration will be discussed extensively in another chapter. As far as neurogenesis is concerned little is known about the role of NGF in regulation of adult neurogenesis compared to its relative neurotrophin BDNF. However, it has been reported that NGF promotes the survival of neural stem cells in young but not aged rats and this was correlated with enhanced cholinergic activation in the hippocampus (Frielingsdorf et al., 2007). This action was attributed to the increase in the levels of Ach secreted to hippocampus by basal forebrain cholinergic afferents in response to NGF infusion. Moreover, NGF was shown to rescue cholinergic and neurogenic deficits in a mouse model of Hundington's disease (Yu et al., 2013). On the other, NGF was recently shown to affect neural stem cells in a cell autonomous manner since AD11 mice, a transgenic knock in strain, in which the expression of a recombinant antibody leads to a chronic postnatal neutralization of endogenous NGF show reduced levels of proliferation and survival of NSCs of the SVZ and produce less number of neurospheres *in vitro* (Scardigli et al., 2014). Again, by the two cognate receptors of NGF the pan neurotrophin receptor $p75^{NTR}$ appears to be most probably the one that mediates its neurogenic effects (Young et al., 2007, Bernabeau et al., 2010; Colditz et al., 2010, Catts et al., 2009; Shi et al., 2013). Consistent with this notion is the fact that while the two neurotrophins BDNF and NGF have the same potential to affect neurogenesis BDNF appears to be more important since it is the more abundantly expressed neurotrophin in the CNS.

Epidermal Growth Factor (EGF) responsive cells are the neurosphere forming cells of embryonic and adult brain. The receptor of EGF (EGFR) a type of tyrosine kinase receptor is expressed by activated multipotent neural stem cells and rapidly dividing neural progenitors. EGF acts as a mitogen for type C cells in the SVZ but high concentrations of EGF result in conversion of these cells to highly proliverative glial like cells with tumorigenic characteristics (Doetsch et al., 2002). Only a 3 day intracerebroventricular (ICV) administration of EGF can restore the proliferative capacity of aged mice to the levels of young (Jin et al., 2003).

Vascular endothelial growth factor (VEGF) is a hypoxia-induced angiogenic protein that exhibits neurotrophic and neuroprotective properties (Meirer et al., 2001). Given

that neurogenesis occurs in close proximity to blood vessels and that clusters of dividing cells contain endothelial precursors, VEGF may constitute the link between neurogenesis and angiogenesis. This is supported by the observation that ICV administration of VEGF can stimulates cell proliferation in the rodent SVZ and SGZ (Jin et al., 2002) which is associated with an upregulation of cyclin D1, Cyclin E and cdc25 (Zhu et al., 2003).

Hormones

Adrenal corticosteroids are from the first factors identified to suppress neurogenesis (Cameron et al., 1994; Gould et al., 1994). Corticosterone belongs to glucocorticoids that are released to blood circulation after activation of the (Hypothalamo – pituitary – adrenal) HPA axis by stress (McEwen et al., 2001). Suppression of corticosterone secretion by adreanalectomy (adx) results in increased mitotic activity of NSCs in the DG whereas proliferation in SVZ remains unaltered (Rodriguez et al., 2001). The cognate receptor of glucorticoids (GR) is expressed in the DG however its expression is more robust in mature granule cells rather than neural progenitors (Cameron et al., 1993). Studies on the mechanism of corticosterone induced inhibition of NSCs proliferation revealed a crucial reduction of type D cyclins expression which is mediated by activation of GR (Sundberg et al., 2006). Finally, other non cell autonomous mechanisms of the actions of corticosteroids might be involved, since prolonged exposure to glucocorticoids augments glutamate accumulation in the hippocampus (Stein-Behrens et al., 1994).

Sex differences have been observed in the basal levels of neurogenesis in the DG and have been positively correlated with the amount of sex steroids, like estrogens in the circulation. This is also confirmed by the fact that females showed increased proliferative activity during the estrus compared to diestrus period. However, most of these neurons fail to survive for longer periods and eventually differences are not evident 2 weeks after BrdU administration, between male and female rats as well between different phases of estrus cycles (Tanapat et al., 1999). In contrast to this other studies have reported lower cell proliferation in the presence of high estrogen levels, though in different species (Galea et al., 1999, Ormerod et al., 2001). In accordance, Short-term (6–7 days) ovariectomy dramatically reduces cell proliferation

in the dentate gyrus of adult female rats compared to sham-operated females in proestrus (Tanapat et al., 1999). On the other hand, long-term ovariectomy (3–4 weeks) does not appear to reduce cell proliferation (Lagace et al., 2007, Tanapat et al., 2005) or short-term cell survival (1 week) compared to sham-operated females, indicating the potential presence of a compensatory mechanism by which levels of neurogenesis are returned to normal levels post-ovariectomy. ERa and ERb receptors are expressed on NSCs derived from embryonic but not from adult brain, and influence their proliferative and differentiating capacity. Moreover, while ER activation seems to have a positive impact to the proliferative activity of NSCs the effects are opposite in the presence of EGF. These results imply that there is a more complex mechanism underlying the effects of this hormone on neurogenesis. (Brannvall et al., 2002).

Androgens have also implicated in the regulation of adult neurogenesis in several species (Rasika et al., 1994; Louissaint et al., 2002) without affecting progenitor proliferation from testosterone implantation or castration experiments. Since testosterone is metabolized to estrogens, the hypothesis of an indirect action of this hormone through its conversion to estradiol was tested. However, only testosterone as well as another metabolite of it, Dehydroandrosterone, but not estradiol was able to increase survival of cells on the DG (Spritzer et al., 2007). Androgen receptors are localized in neural stem cells from adult SVZ and could mediate the effects observed (Brannvall et al., 2005). However, testosterone implants have been also shown to increase the levels of growth factors such as BDNF and VEGF that could also influence the survival rate of neural progenitors (Rasika et al., 1999; Louissaint et al., 2002).

Neurosteroids

The term neurosteroid refers to a class of cholesterol derivatives which are locally synthesized in the brain, have been shown to have trophic effects on neurons and glial cells and to modulate the activity of a variety of neurotransmitter receptors and ion channels, including $GABA_A$, NMDA and N- and L-type Ca2+ channels. There is also accumulating evidence that they exert neurogenic actions in the hippocampus while

they influence learning and memory processes (Schumacher et al., 1997, Charalampopoulos et al., 2008) through neuromodulation or prevention of apoptotic cell death. It has been proposed that pregnenolone sulfate (Preg – S) could have a positive impact on cognitive performance during senescence through elevation of neurogenesis in the hippocampus, an effect that involves modulation of $GABA_A$ receptors (Mayo et al., 2005). Moreover, it was able to protect newborn neurons loss and reverse spatial memory deficits in APP/PS1 mouse model of Alzheimer's disease (Xu et al., 2012). A similar effect has been reported for allopregnenolone which was found to reverse neurogenic and cognitive deficits in another transgenic mouse with AD pathology, the 3xTgAD (Wang et al., 2009).

The neurogenic potential of DHEA, have been also tested in several studies, but the results are conflicting so far. When DHEA was administered for 2 weeks in the form of a subcutaneously implanted pellet in rats, both proliferation and survival of newborn neurons was increased in young and aged animals while it the inhibitory effect of corticosterone was antagonized. The dose that was used achieves plasma levels of DHEA equivalent with that after a subcutaneous injection of 40mg/kg (Karishma et al., 2002). However in another study of the same group DHEA pellet failed to increased neurogenesis in unchallenged with corticosterone rats, however it was able to enhance the neurogenic capacity of Fluoxetine. In this case, though, the pellet was the half size of that used in the aforementioned study (Pinnock et al., 2009). Yet in another study with mice, DHEA was efficient to increase neurogenesis and depressive phenotype of olfactory bulbectomized (OBX) mice. This action was shown to be dependent on Sigma-1 receptor (Moriguchi et al., 2013). Moreover DHEA had direct actions on proliferation and differentiating capacity of human neural stem cells through NMDA and Sigma-1 receptors (Suzuki et al., 2003).

Neurogenesis in brain disease.

Except for various endogenous and environmental factors, certain acute brain lesions and pathological conditions, such as stroke, brain trauma, and seizures, are also known to trigger cell proliferation in the DG but also to induce neurogenesis (Parent et al., 2003; Kokaia et al., 2006; Lichtenwalner and Parent 2006; Miles and Kernie 2006) in normally non – neurogenic as in striatum in both rodents and humans.

Ectopic production of new neurons seems to occur by direct conversion of resident astrocytes (Ernst et al., 2015, Magnusson et al., 2014, Darsalia et al., 2005) indicating an endogenous response for self-repair. However, it remains unclear both the functional significance of this phenomenon or its potential therapeutic use. For instance, although ablation of NSCs does not increase lesion size nor worsens motor symptoms following MCAO, a mouse model of stroke, it significantly affects the ability for learning and memory suggesting that neurogenesis contributes to the recovery of cognitive function after ischemic brain injury (Sun et al., 2013).

Moreover, alterations in neurogenesis have been reported for almost all the classical neurodegenerative diseases

Parkinson's disease is a disorder that is characterized by loss of dopaminergic neurons mainly in the area of Substantia Nigra (SN) leading to dyskinesia and rigidity. In humans manifestation of the disease is associated with accumulation of a-synuclein oligomers. Mouse models that overexpress the human form of a-synuclein have shown reduced rates of neurogenesis (Winner et al. 2004). This is consistent with evidence from other experimental models that used toxins such as 1-methyl-4-phenyl-1,2,3,6- tetrahydropyridine (MPTP) or 6 OH-DA to selectively destroy dopaminergic neurons (Honglinger et al., 2004; Winner et al., 2006). However other studies have increased dopaminergic neurogenesis despite the decrease in proliferation rate of NSCs (Yamada et al., 2004, Winner et al., 2006). The new neurons have been detected in the olfactory bulb (OB), which is the place where neural progenitors of the SVZ migrate. On the other hand, while there are a few studies supporting the appearance of newborn dopaminergic neurons in the SN after a lesion (Yoshimi et al. 2005, Zhao et al. 2003) a lot of others have failed to replicate these results (Kay and Blum 2000; Mao et al. 2001; Lie et al. 2002; Frielingsdorf et al. 2004; Steiner et al. 2006) which was attributed to different methodological approaches for evaluation of co-localization of BrdU with neuronal markers.

Huntington's disease (HD) is an autosomal dominant neurodegenerative disease caused by CAG trinucleotide repeat expansion in the gene of huntingtin. Clinical manifestations of the disease are progressive involuntary movement, bradykinesia, cognitive decline and psychiatric syndromes. In cellular level it is characterized by

neuronal damage in medium spiny neurons of the neostriatum and secondarily of the cortex. Neurogenesis has been found to be decreased in two mouse models of HD, the R6/1 and R6/2 (Gil et al., 2005; Van der Borght and Brundin 2007) and this was correlated with decreased performance in spatial learning tasks (Murphy et al., 2000; Smith et al. 2006). However, since a lot of changes occur at the biochemical level in these mice including alterations in the expression of receptors and certain growth factors such as BDNF and in the levels of endocrine hormones it is difficult to conclude if the decrease in neurogenesis is responsible for the behavioral deficits observed or if this it is another hallmark of the disease itself (Smith et al., 2005).

Azheimer's disease (AD) is one of the most common causes of dementia in humans. The majority of the cases concern the late onset sporadic form of the disease while there are rare, familial, early- onset autosomal dominant forms of Alzheimer's disease (FAD) that are caused by mutations in genes encoding amyloid precursor protein (APP), presenilin-1 (PS1) and presenilin-2 (PS2). Neuropathological signs include neuronal and synaptic loss due to the development of neurofibrillary tangles, composed of aggregated hyperphosphorylated tau, and amyloid deposition, composed of aggregated Aβ. The small Aβ oligomeric species rather than its amyloid counterpart are considered to be the toxic culprit in the disease (Goedert et al., 1991). The disease is also accompanied by extensive neuroiflammation, which is manifested with activation of resident microglia and proliferation of astrocytes. The areas that are mostly affected are associative cortices, hippocampus and basal forebrain.
In order to study the molecular mechanisms contributing to the disease a variety of mouse models have been developed based on the increase of the levels of Aβ42 neurotoxic peptide compared to non-toxic Aβ40 fragment. This is accomplished by overexpression of mutant forms of APP, the precursor form of Aβ42, or PS1, which is the catalytic component of γ-secretase, the enzyme that cleaves APP. A commonly used AD model, are the triple transgenic mice (3XTg-AD) bearing mutation in 3 genes (APP, PS1 and tau). In this it has been shown decreased proliferation which was directly associated with the presence of Ab plaques (Rodriguez et al., 2008).

The overall conclusion arising from studies in different transgenic mouse models is that adult neurogenesis is compromised in AD and precedes neuronal loss; However, conflicting results emerged in many cases depending on the use of PS1, PS2 or

different APP single mutations knock-ins. In addition to this, bromodeoxyuridine (BrdU) regimens, doses, time points analyzed after BrdU treatment, genetic backgrounds of the mice, and regions investigated vary considerably in all of the studies contributing in the discrepancies between them (Lazarov & Marr, 2010; Marlatt & Lucassen, 2010). Another important factor is the age tested. A recent study that tried to delineate the temporal dynamics in adult hippocampal neurogenesis in neurodegenerative diseases used the model of prion disease and samples from humans with Alzheimer's disease or Creuzfeldt-Jacob's disease (Gomez-Nikola et al., 2014). The authors suggest that neurogenesis is increased in the progression of the disease and partially counteract the effects of chronic neurodegeneration preserving hippocampal function albeit this is not sufficient to compensate for the extensive neuronal loss. Moreover, the initial increase in proliferation will exhaust the self-replacement mechanism, which might finally lead to impairment in neurogenesis at later stages of the disease, which is also consistent with previous human studies.

Functional role of adult neurogenesis.

As I presented above adult neurogenesis is a sensitive system to environmental changes and is implicated in various pathological conditions however its physiological role has not fully elucidated. In order to dissect the function of the continuous production of new neurons in the adult brain two strategies have been employed: either depletion of neural stem cells or expansion of their population and investigation of the consequence in behavior and regeneration capacity after an insult in the CNS. Methods that have been used to eliminate neurogenesis were the administration of the antimitotic agent methylazoxymethanol acetate (MAM), irradiation or genetic ways. Most studies revealed differences in some but not all hippocampus involving memory tasks (Shors et al., 2002) reflecting deficits mainly in long term rather than short-term retention of spatial information (Deng et al., 2009 Snyder et al., 2005). A common function of the DG that seems to rely on neurogenesis and it was first proposed by computational models is discrimination between similar stimuli whether these contain spatial information or temporally adjacent events (Aimone et al., 2006). This process is called pattern separation and ensures that new memories and old memories do not interfere with one another. In

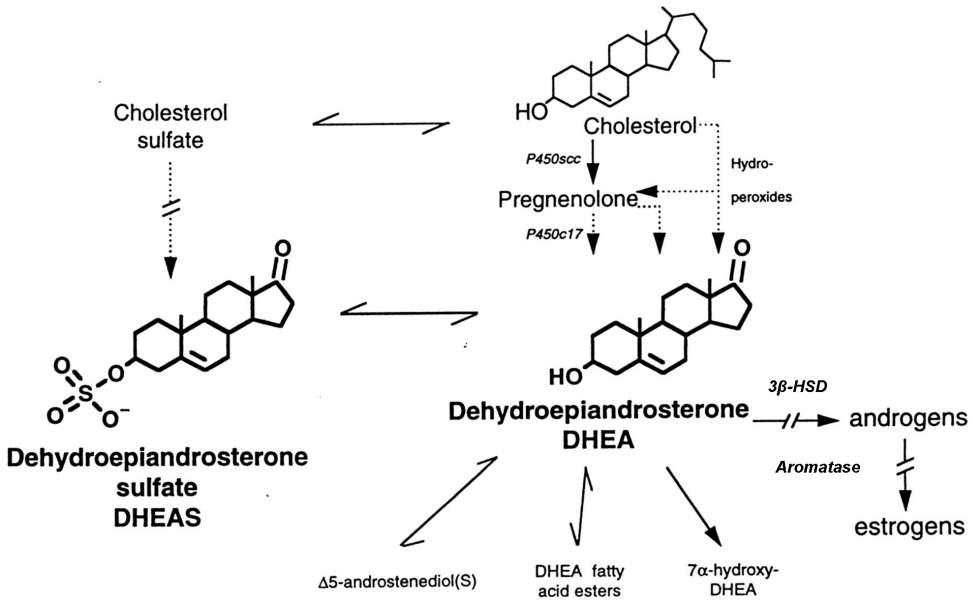
this context newly added neurons serves as substrate for encoding of novel features while old neurons are responsible for encoding features similar to familiar memories (Aimone and Gage, 2011). Experimental data confirmed that adult hippocampal neurogenesis is a necessary and sufficient phenomenon for spatial pattern separation (Clelland et al. 2009; Creer et al., 2010). Moreover, worst performance in a spatial discrimination task in aged mice, have been shown to be correlated with decreased cell genesis in the DG and running was unable to reverse reduction in both of them (Creer et al., 2009). In addition to the previous, another two studies demonstrated that ablating dentate neurogenesis impairs while increasing survival of newborn neurons by conditional deletion of a proapoptotic gene improves performance on contextual fear discrimination learning task (Tronel et al., 2010, Sahay et al., 2011) that involves association of contextual information with an unconditional, a fearful stimuli. Based on the above observations from animal models, about the role of DG neurogenesis in pattern separation, it has been recently proposed that deficits in neurogenesis could result in this case in overlapping of contextual representations of fearful events in humans (Kheirbek et al., 2009). Given also the role of adult neurogenesis in the control of emotional behavior such as exploration, anxiety and stress responses (Surget et al. 2011; Schloesser et al., 2010), the previous assumption suggests that disorders like Post traumatic stress disorder (PTSD) could arise from a DG dysfunction and is manifested with generalized fear and increased anxiety. One the other hand a more recent study showed that increased rate of neurogenesis occurring in young age or after pharmacological manipulation helps forgetting fearful memories by facilitating fear extinction, a process that requires remodeling of the hippocampal circuit.

Finally, a possible role of impaired neurogenesis in the pathophysiology of depression have widely discussed in the literature because of the fact that common antidepressant treatments increase neurogenesis in a timecourse that coincides with the first manifestation of their antidepressant effects, which usually takes 2 - 4 weeks in humans (Sapolsky M. et al., 2004). However, studies trying to find a causal relationship between stress neurogenesis and development of depression have failed so far since suppression neurogenesis does not seem to produce depressive phenotype in mice (Malberg and Duman, 2003, Santarelli et al., 2003 and Vollmayr et al., 2003). A very recent study though provided evidence that intact neurogenesis is needed in order to balance the levels of glucocorticoids after a stressful event (Snyder et al.,

2011).

Microneurotrophins

As it was mentioned before, the term neurosteroids refers to steroid hormones that are produced locally in the nervous system by neurons and glial cells, after metabolism of circulating precursor molecules but they can also be synthesized de novo from cholesterol. (McEwen et al., 1994; Baulieu et al., 1998; Schumacher et al., 1999). The main neurosteroids include pregnenolone (PREG) and pregnenolone sulfate (PREGS), progesterone (PROG), allopregnanolone (ALLO), dehydroepiandrosterone (DHEA), DHEA sulfate (DHEAS), Testosterone (T) and Estradiol. (E2). These have been shown to regulate brain function in an autocrine and paracrine manner via membrane or nuclear receptors (Baulieu, 1998; Baulieu et al., 2001; Plassart-Schiess and Baulieu, 2001; Singh, 2006). However, it is often very difficult to distinguish the actions of each one of them, since they produce numerous active metabolites in vivo.



Baulieu E , and Robel P PNAS 1998;95:4089-4091

Brain DHEA(S) metabolism.

or 17α-hydroxylase ; 3β- HSD, 3β-Hydroxysteroid dehydrogenase ; 5α-R, 5α-Reductase ; 17β-HSD, 17β- Hydroxysteroid dehydrogenase ; The biosynthesis of PREG and DHEA might proceed from cholesterol either through the classical pathway involving successively cytochromes P450scc and P450c17, or by an alternative pathway involving the intermediacy of sterol and/or steroid

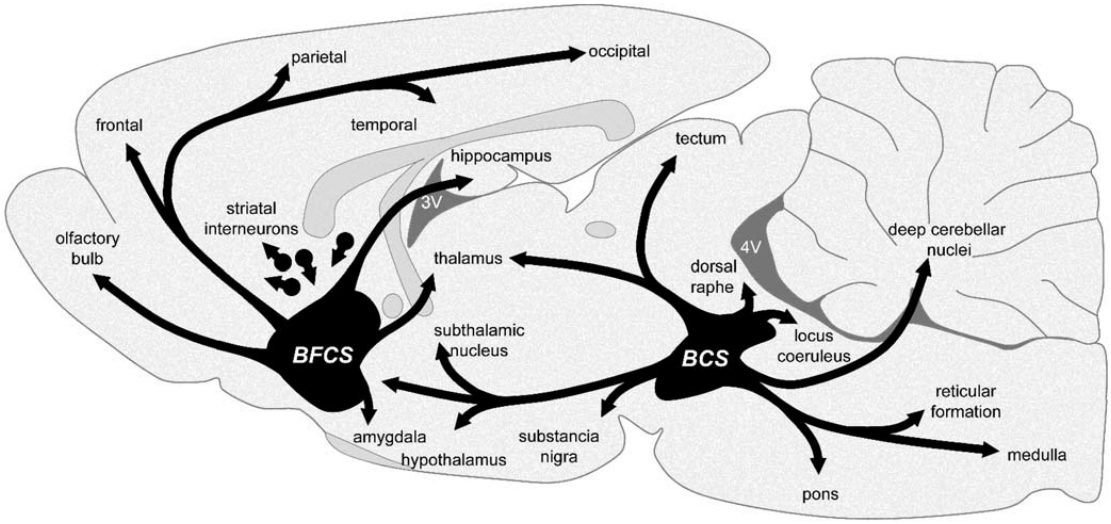
hydroperoxides. DHEA is reversibly converted to DHEAS and is the precursor of several metabolites, including sex steroid hormones.

DHEA and its sulfate derivative DHEAS is the most abundant steroid in blood stream of humans and are produced mainly from adrenal glands. Brain concentration of these steroids is even higher, implying that there is a significant amount of DHEA synthesized locally in the brain while a proportion of it should be derived from the periphery after crossing the blood brain barrier (BBB) (Lacroix et al., 1987). In rodents, plasma levels of DHEA are very low since adrenal glands are incapable to produce this steroid, however it is biosynthesized in the brain in sufficient amounts (Maninger et al., 2009). The limiting enzyme for the production of DHEA, P450c17, is localized in many areas of the human and rodent brain including the hippocampus, in the spinal cord as well as in the peripheral nervous system such as in dorsal root ganglia (DRGs) from early developmental stages (Compagnone NA 1995, Marcus D. 2012).

The amount of circulating and brain DHEA and its metabolite's DHEAS has been found to be substantially reduced during aging and in patients with alzheimer's disease (Maringer et al., 2009). This led to the exploration of a potential therapeutic use of this steroid in neurodegenerative diseases. DHEA is generally considered to exert neuroprotective actions in the brain, modulate neurotransmitter release and improve synaptic plasticity and cognition in rodents (Vallee et al., 2001, Hajszan et al., 2004). These effects are mediated by a wide range of membrane initiated and intracellular mechanisms including modulation of $GABA_A$, NMDA and sigma subtype 1 (σ1) receptors. However no one of the currently known classical nuclear hormone receptors is involved in these actions (Nicole Maringer 2009). Recently our lab discovered new mediators of DHEA neuroprotective action namely the NGF receptors, TrkA and p75NTR. DHEA binds to both receptors with high affinity, at the nanomolar concentration (Kd), and activates their downstream signaling pathways such as mitogen activated protein kinase (MAPK) prosurvival pathway and this of RHO kinase pathway respectively (Lazaridis et al., 2011). The aforementioned effects were tested in, neural crest derived, PC12 cells that express both of the receptors and have been widely used to study the effects of NGF on differentiation and survival as well as in NNR5 cell line that express only p75NTR. DHEA was proved capable to reduce apoptosis after serum deprivation in these cells only in the presence of TrkA

while the effect was abolished when TrkA was deleted with the use of SiRNAs for the receptor or in the case of NNR5 cells. In addition, DHEA protected NGF dependent population of the peripheral nervous system such as DRGs and Superior cervical ganglia (SCGs) from apoptosis after NGF withdrawal both *in vivo* and *in vitro*.

In accordance with our hypothesis, other reports in the literature support a neurotrophic action of DHEA similar with that of NGF. DHEA as well as NGF support Ach secretion from basal forebrain neurons to the hippocampus. (Olivier et al., 2006; Auld et al., 2001). These neurons express the two cognate receptors of NGF, which appears to have a physiological role in maintenance of its cholinergic activity but not its survival (Crowley et al., 1994). Basal forebrain cholinergic system is responsible for a variety of cognitive functions through afferents that sends to hippocampus and cortex and consists one of the first areas that are affected in AD and during ageing (Terry et al., 2003)



Schematic diagram of cholinergic systems in a rodent brain, adapted from Woolf (1991). The two major cholinergic systems composed of projection neurons are represented, the basal forebrain cholinergic system (BFCS) and the brainstem cholinergic system (BCS), together with the cholinergic interneurons of the striatum. The BFCS and the BCS encompass the Ch1-4 and Ch5–6 groups, respectively.

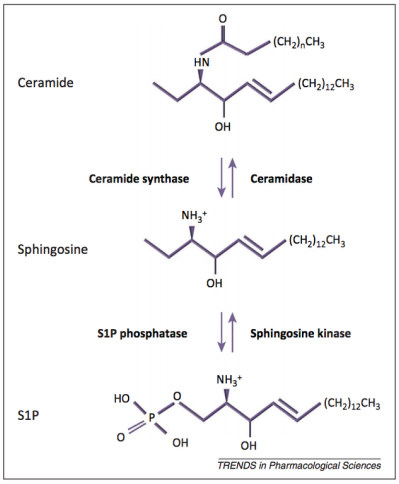
DHEA's neuroprotective properties, mediated through different mechanisms described above, as well as its small size and lipophilic nature make it an ideal candidate drug for the treatment of various neurodegenerative conditions. However, DHEA therapeutic use is compromised because it widely affects the endocrine system

by its own or after conversion to other steroid hormones with possible tumorogenic effects. In particular breast, endometrial, or prostate cancer has been associated with disturbances on estrogens and androgen levels (Henderson et al., 2000, Takahashi et al., 2006).

To overcome these obstacles we chemically modified DHEA structure at positions C3 or C17 in order to develop molecules devoid of androgenic and estrogenic action that still retain its neuroprotective properties. A library of 10 DHEA derivatives was tested for their efficacy to protect PC12 cells from serum free induced apoptosis and 3 of them, compound 20,23 and 27 were proved efficient to significantly reduce apoptosis almost at the levels of parent molecule DHEA. In order to evaluate the carcinogenic potential of these molecules the expression levels of ER regulated genes was checked after treatment with compounds of human carcinoma cell lines. The 3 compounds that were chosen did not display any agonistic effect on ER receptors or induce the proliferation of these cells (Calogeropoulou et al., 2008). Aiming to assess the potential NGF mimetic effects of our synthetic compounds we focused on one of them, called BNN27 ((R)-3β,21- dihydroxy- 17R,20-epoxy-5-pregnene), which proved to be the most potent, and at the same time the most soluble in hydrophilic diluents, making it easy to handle in *in vivo* experiments. Binding assays on HEK293 cells transfected with the cDNA of the specific receptor for each neurotrophin (TrkA, TrkB, TrkC) as well as of the pan-neurotrophin receptor $p75^{NTR}$ revealed that BNN27 selectively interacts with NGF receptors, TrkA and p75NTR (Ki: 1.86±0.4nM and 3.9±1.2nM respectively) and shows lesser affinity for receptors of BDNF and NT3. In accordance to these results BNN27 selectively induced phosphorylation of TrkA receptor and promotes release of RhoGDI from $p75^{NTR}$ decreasing the activity of RhoA as it happens after binding of NGF. Moreover, BNN27 was proved capable of decreasing apoptosis of sympathetic neurons after NGF deprivation *in vitro* but also of DRGs of E13.5 *ngf -/-* embryos that are dependent on NGF at this age for their survival. However, BNN27 was unable to promote survival of these populations for longer time points or induce differentiation of PC12 cells and this was attributed to transient activation of MAPK pathway in response to BNN27 compared to sustained activation of the pathway after binding of NGF to TrkA (Pediaditakis et al., 2015).

S1P Agonists: Biology and Pharmacology

S1P is produced by the sphingosine kinase – dependent phosphorylation of sphingosine, a lipid mediator, which is the metabolic product of ceramide that in turn derives after hydrolysis of membrane sphingomyelin.



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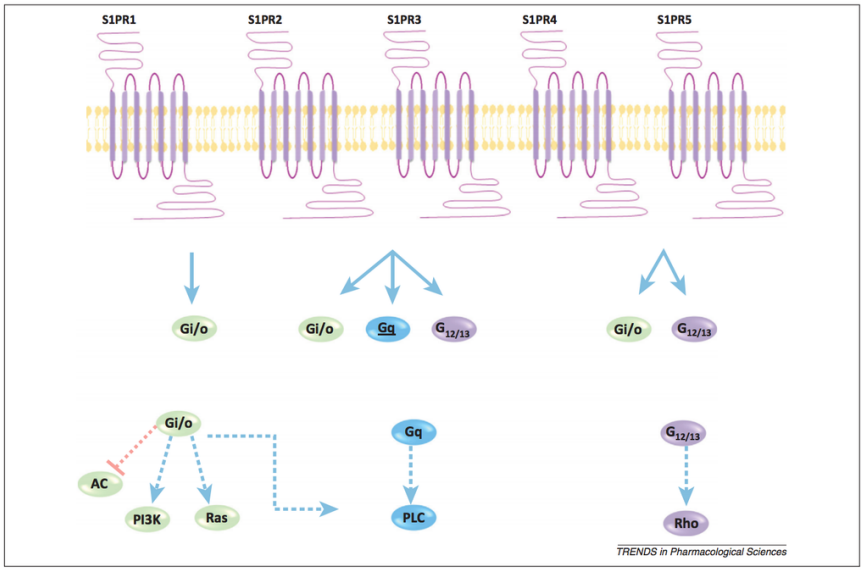
Cellular levels of S1P are low and tightly controlled by the balance between its synthesis and degradation. S1P acts through 5 types of S1P receptors (S1P1-5) which belong to the class of G protein coupled receptors (GPCRs) while it is also known to act as an intracellular messenger itself (Hait et al., 2009).

The S1PR1 subtype is coupled exclusively with the Gi/o type of alpha subunit and hence inhibits adenylyl cyclase, resulting in a reduction in cAMP production. S1PR1 subtype activation results in an increase in intracellular calcium concentration, while can stimulate pathways involving the rat sarcoma (Ras) family of small GTPases and extracellular signal-regulated kinase (ERK) to enhance proliferation and can activate the phosphatidylinositide 3-kinase (PI3K)/Akt (protein kinase B) pathway to inhibit apoptosis. Moreover, this receptor subtype activates the PI3K/Rac pathway to promote cytoskeletal rearrangement and cellular migration (Hannun et al.,2008). Similar to S1PR1s, the S1PR2–5 receptors couple with Gi/o but also with G12/13 to enhance Rho activation and hence inhibit Rac. The S1PR2 and S1PR3 couple additionally with Gq to activate phospholipase C (PLC) (Kumlesh et al., 2013). Thus, this receptor family can elicit many cellular responses in a temporal and spatial manner that is dependent on the receptor expression pattern in each cell type.



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In this way S1P has been implicated in vascular development and the physiological function of immune and cardiovascular system acting on endothelial cells as well as on lymphocytes and myocytes respectively.

CNS actions

Apart from its actions in the periphery, S1P is also synthesized de novo in the CNS and S1P receptors are abundantly expressed (Blondeau et al., 2007, Beer et al., 2000, Deogracias et al., 2012) in various brain regions and by a plethora of cell types including oligodendrocytes, neurons and astrocytes as well as by their progenitors regulating basic functions as their proliferation, survival and differentiation (Hurst et al., 2008, Stuart et al., 2009, Coelho et al., 2007, Colombaioni et al., 2004). Moreover, S1P signaling has been found to be important for proper neural development and normal neural function (Herr et al., 2007, Mizugishi et al., 2005).

Particularly, S1P1 receptor has been found adjacent to lateral ventricles of mouse whereas S1P increased GTPγS binding through activation of G proteins in SVZ, implicating a role of S1P in neurogenesis (McGiffert et al., 2002, Waeber et al., 1999). The latter is also indicated by embryonic lethality of Sphk1 KO and S1P1 null mice characterized by decreased mitosis and increased apoptosis rate during development of the nervous system (Mizuguishi et al., 2005). In addition, S1P induced the proliferation and morphological changes of embryonic hippocampal neural progenitors in cultures via ERK signaling (Harada et al., 2004) while S1P

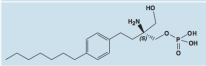
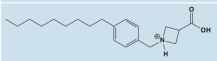
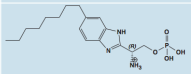
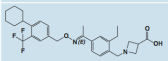
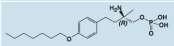
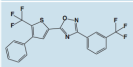
receptors are also expressed in rat and human embryonic neural stem cells and are functionally coupled to Gi and Gq proteins (Hurst et al., 2008, Stuart et al., 2009).
Finally, levels of S1P in the spinal cord are increased following injury (62) and it has been found to possess a chemo-attractant role through s1p1 activation for neural stem/ progenitor cells, which have been reported to migrate towards sites of injury in the CNS.
Particular attention has been given to alterations in S1P signaling at different stages of Multiple Sclerosis (MS), the devastating demyelinating disorder of the CNS. Compared with control individuals, patients with MS have been reported to have a lower content of sphingomyelin in their white matter (Wheeler et al., 2008) but an increased level of S1P in their cerebrospinal fluid (Kulakowska et al., 2010). Moreover, S1P1 and S1P3 expression have been shown to be elevated in reactive astrocytes in chronic MS lesions (Doorn et al., 2010) or under proinflammatory conditions (Fischer et al., 2011). An important role of S1P1 in the control of reactive astrogliosis has been also suggested from studies in experimental models of MS. More specifically genetic deletion of S1P1 from astrocytes or treatment with its functional antagonist Fingolimod, produced a reduction of the elevated S1P levels occurring in animals challenged by EAE (Choi et al., 2011) and correlated with the progression of the disease.

S1P receptors agonists

There are several well-characterized agonists and antagonists of S1PR; however, most compounds have been directed toward modulating the activity of S1P1.
FTY720 (Fingolimod/Gilenya; Novartis) is the prototypical S1PR agonist and was approved by the U.S. Food and Drug Administration as a first line oral therapy for relapsing-remitting multiple sclerosis (MS) (Chun et al., 2010, Brinkmann et al., 2010). Although FTY720 acts as an agonist at picomolar to nanomolar concentrations on S1P1 and S1P3-5, it also acts as a functional antagonist for S1P1 by inducing endocytosis and degradation of this receptor (Mandala et al., 2002, Brinkmann et al., 2002). However sustained internalization of S1P1 receptor is responsible for the inhibition of Lymphocyte egress and its concomitant sequestration in lymphoid

organs preventing in this way their infiltration to the CNS that causes the damage of myelin in Multiple Sclerosis.

Agonists of S1P receptors

S1P		S1PR1–5 agonist
pFTY720		S1PR1, 3, 4, 5 agonist
Phosphonate		S1PR1, 3, 4, 5 agonist
Azetidine		S1PR1, 5 agonist
Benzimidazole		S1PR1, 4, 5 agonist
BAF312		S1PR1, 5 agonist
AFD(R)		S1PR1 agonist
SEW2871		S1PR1 agonist

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Direct CNS effects of the S1P receptor modulator, Fingolimod.

Apart from its immunomodulatory effects, fingolimod can easily penetrate the BBB and it is well documented the last few years that it affects, as its parent molecule S1P, various CNS cell populations exerting neuroprotective and neurorestorative actions. In animal models of MS such as the relapsing–progressive EAE model in mice, prophylactic and therapeutic fingolimod treatment inhibited subsequent relapses and axonal loss in the spinal cord, and facilitated motor recovery while lower dose than 0,3mg/kg as well as short administration is not efficient, even it produces a rapid and substantial lymphopenia. (Foster et al., 2007).

Moreover, fingolimod was proven insufficient to suppress EAE when initiated at a very late stage of the model (after 4 months), during the non-relapsing, secondary advanced progressive stage, after accumulation of significant neurological deficits, which suggests that immunosuppression is not sufficient to confer protection from demyelination and neurodegeneration (Al-Izki et al., 2011). The same opinion is proposed from another study in which fingolimod was found to be effective in reducing oligodendrocyte and axonal loss in the corpus callosum in the cuprizone model of demyelination, an effect that was independent from its peripheral action on lymphocytes (Kim et al., 2011).

Clinical data support also the notion that fingolimod could afford neuroprotection since Phase III clinical studies revealed that patients who received fingolimod therapy showed reduced brain volume loss (BVL) compared to those that received IFNβ-1a, a common drug that is prescribed for the treatment of MS (Barkhof et al., 2014; Calabresi et al., 2014; Kappos et al., 2010).

Fingolimod was also found effective to preserve neuronal function and reduce injury in other animal models of neurodegeneration. Experimental studies in mice and rats have shown therapeutic efficacy of fingolimod after ischemic brain injury (Czech et al., 2009; Shichita et al., 2009; Hasegawa et al., 2010; Pfeilschifter et al., 2011a,b; Wei et al., 2011; Kraft et al., 2013) with doses from 0.25 to 1 mg/kg applied systemically. The beneficial effects on lesion size and functional outcome in stroke models were accompanied by a reduction in cleaved caspase-3 and TUNEL positive neurons in the infarcted brain areas such, (Hasegawa et al., 2010), and an activation of pro-survival pathways such as extracellular signal-regulated kinase (ERK1/2), protein kinase B/Akt kinase (PKB/Akt) and Bcl-2 upregulation (Hasegawa et al., 2010; Wei et al., 2011).

Disturbances in the levels of S1P have been linked to the development of AD mostly from clinical studies revealing altered ratio of ceramide to S1P correlated to elevated ASM and acid ceramidase (AC) along with a reduction in activity of Sphk1 and Sphk2(Brunkhorst et al., 2014). In addition to this S1P receptor modulation by fingolimod emerges as a promising therapeutic approach in AD models reducing both the production (Takasugi et al., 2013) and the neurotoxicity (Asle-Rousta et al., 2013; Doi et al., 2013; Hemmati et al., 2013;Fukumoto et al., 2014) of Aβ peptide.

Apart from these studies there is a growing amount of evicence that fingolimod also affects mood and cognition conferring potential therapeutic action in neuropsychiatric disorders. The first study indicating these properties of fingolimod was that of Deogracias et al., 2011 who showed that this drug is capable to increase the production of the neurotrophic factor BDNF and relief motor symptoms in an animal model of RETT syndrome, the Mecp2 KO mice which are characterized by decreased levels of BDNF. The authors have associated these effects with activation the of MAPK pathway in cortical neurons expressing S1P1 receptors. However, S1P as well

as its analog fingolimod –p have also intracellular targets acting as histone deacetylase (HDAC) inhibitors regulating epigenetically the expression of many transcription and growth factors. (Hait et al., 2011 and 2014). In this way fingolimod was found to facilitate deletion of fear memories and proposed as a potential therapeutic agent in post-traumatic stress disorder (PTSD). Consistent with the previous findings is a recently published study that fingolimod confers antidepressant-like activity by inhibiting deacetylase activity, which partially attributed to the increased levels of neurogenesis on stressed animals (Nuzzo et al., 2015).

Neurogenesis as a therapeutic target for neurodegenerative diseases.

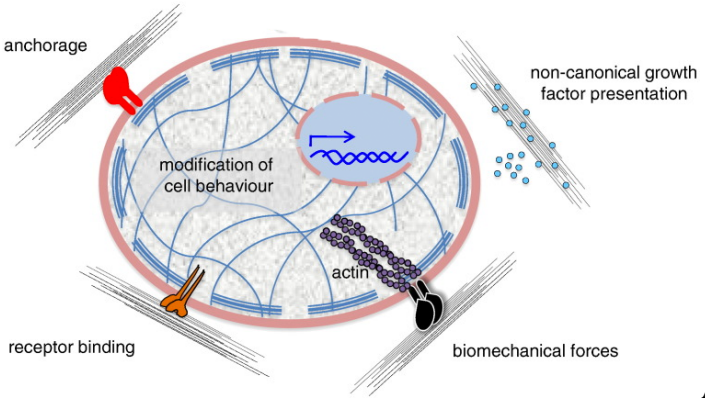
Although considerable progress has been made in the understanding of molecular mechanisms underlying neurodegenerative events efforts to develop efficient drugs for devastating diseases such as Alzheimer's disease have been failed so far. Moreover, almost all of neurodegerenarive conditions are characterized my decline in cognitive functions with mood regulation and memory being severely affected. The discorvery of adult hippocampal neurogenesis, which consists one of the major form of plasticity during adulthood, raised new hopes for therapeutic intervention. After an injury in the CNS neurotrophic factors are released from neighboring cells providing trophic support and triggering neurogenesis. These factors are mainly large proteins responsible for patterning of the nervous system during development such as BDNF NGF and FGF2. Unfortunately, however, the therapeutic use of these molecules is limited due to difficulties in peripheral administration to patients. For this reason there has been an attempt to discover new micromolecular substances that mimic their neurotrophic actions and can be easily used in clinical practice.

Extracellular matrix controls cell behavior.

Except for secreted and soluble factors neural stem cells and newborn neurons receive signals from their surrounding microenvironment that regulate its basal functions, defined as the extracellular matrix (ECM). This is a complex mixture consisting of fibrous proteins (such as collagen) and other macromolecules (glycosaminoglycans, proteoglycans, cell-binding adhesive glycoproteins) that are organized in a formation characterized by distinct biophysical, mechanical and biochemical properties according to each tissue (Hines 2009). ECM plays an important role both during

development and in regeneration after tissue injury contributing to stem cell pool maintenance, differentiation and proper migration to their destination.

From a biochemical point of view, the ECM displays both direct and indirect signaling properties, since it can act directly by binding cell surface receptors or by non-canonical growth factor presentation (Gattazzo et al., 2014). However, physical properties such as rigidity, porosity, topography and insolubility dictate the assembly of ECM proteins with specific spatial distribution in an intermediate 2D to 3D microenviroment providing topographical cues in micro and nanoscale that influence cell shape and movement (Singh et al. 2010).



Adapted from Gattazzo et al., 2014

The role of topography.

In vitro, it has been shown from early on that cells respond to this kind of topographical features (Harrison 1912), while it was Paul Weiss in 1945 that described the «contact guidance» effect to explain the tendency of the cells to orient themselves along anisotropic features of the surface (such as fibers or ridges). *In vivo*, there are a number of instances in the developing nervous system in which cells migrate along tracts of glial cells or oriented ECM fibers. For example, during cortical development, cortical neurons are guided along radial glial cells (Rakic et al., 1972; O'Rourke et al., 1995). The migrating neurons wrap around the radial glia, which act as both the scaffold and the source of new neurons (Malatesta et al., 2008). In a similar manner, migrating neuroblasts of the external granule layer of the cerebellum follow already existing axon tracts (Hynes et al., 1986). Interestingly, when granule neurons from the cerebellum were cultured with astroglial fibers from the hippocampus, and hippocampal neurons with astroglial fibers from the cerebellum, migration of each type of neuron was identical to that in the homotypic culture,

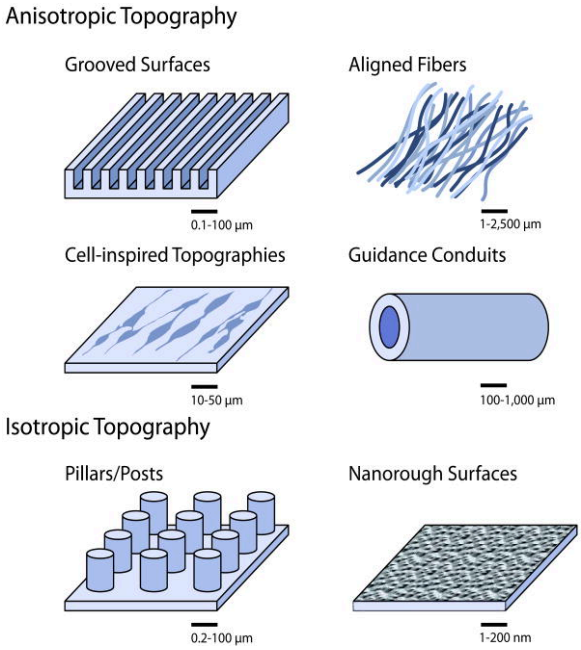
indicating that the glial fibers may provide a generic pathway for neuronal migration *in vivo* (Hatten et al., 1990). In contrast to this, a different mode of migration has been observed in the adult brain. There neuroblasts from the SVZ move in chains to the OB using blood vessels as scaffolds instead of radial glial cells although these are present along the RMS (Lois et al., 1996; Bovetti et al., 2006)

Another phenomenon observed in the developing nervous system is that of guidance by guidepost cells, specific cells in the embryonic environment which serve as intermediate locations during axon pathfinding (Palka et al., 1992). Though this may not be a case of contact guidance in a continuous surface it depicts that axons may grow in intervals, responding to a local cue with a structural change before stopping to seek out the next permissive local cue. As will be discussed in the next section, axons can be guided in vitro by discontinuous structures on the order of the size of a single cell, indicating that the idea of guidance by contact with guidepost structures may be broadly applicable for navigating axons.

Contact guidance has been also observed during peripheral nerve regeneration when after a nerve transection Schwann cells proliferate and get oriented in parallel chains in order to form longitubinal structure containing also ECM, called the band of Büngner, that serves as a scaffold guiding the regenerating axon and providing trophic support (Torigoe et al., 1996).

Types of topographies that has been tested so far *in vitro*.

The development of microfabrication techniques over last 20 years, including electrospinning, self-assembly of materials, and lithography based methods, enabled as to generate artificial scaffolds with desirable characteristics in order to study *in vitro* the effects of specific topographical cues on various cell functions. In all of these studies two major types of topographies have been implemented so far based on the phenomenon of contact guidance and guidepost effect that were presented above. These are continuous anisotropic topographies including grooved surfaces, aligned fibers, cell-inspired surfaces and guidance conduits, or discontinuous isotropic topographies like nanorough surfaces or patterned with pillars or posts.



Hoffman-Kim et al., 2011

Anisotropic continuous

Microgrooved topography studies have revealed that neurite response to topography in terms of alignment or outgrowth increases with increasing groove depth from around 0.2 to 4 μm (Miller et al., 2002; Walsh et al., 2005; Hirono et al., 1988), where no effect is observed on feature sizes less than 200 nm. Grooved surfaces with heights in the sub-micron to micron range showed that dorsal root ganglia (DRG) neurons interacted with both grooves and plateaus. However, increasing groove height resulted in both increased alignment and increased restriction of cell somata and neurites onto either the groove or the plateau, reducing crossing between features separated by a step (Miller et al., 2002). In a similar vein adult rat hippocampal neural stem cells align their neurites on the direction of grooves 44 μm high (Recknor et al., 2006). The position of the neuronal somata is important in this case because it determines the angle at which an approaching neurite contacts the feature (a wall or edge). Thus when axons react the edge of the platform or groove from a perpendicular angle, they tended to stay straight and cross the feature, whereas axons approaching the feature from a more parallel angle tending to turn through the relatively small angle required to align to the feature. This was made clear by a study showing that cortical neurons cultured in relatively swallowed grooves they were either aligned with them or cross over them according to the angle of the axon that was approaching them (Li et al., 2005).

Distance between anisotropic topographical cues has also been shown to play a role in directed neurite guidance. Both groove floors and plateaus present distinct topographical cues to cells, and can vary in width from the subcellular (nanometer and single-micron) to the cellular (tens of microns) to the supracellular (up to hundreds of microns) scales. Both sympathetic and sensory neurons have aligned neurites to grooves of 300 nm depth, with groove widths varying from 100-400 nm and plateaus varying from 100-1600 nm, and tended to grow on the ridge edges and plateaus rather than in the grooves. Of note is also that the smallest patterns with 100 nm features did not guide as well as the larger width patterns (Johansson et al., 2006).

Though the vast majority of studies using grooved substrates report parallel alignment of neuronal cell bodies, neurites, or glial cells other types of guidance have been reported as well. In particular, it has been demonstrated that submicron grooves can elicit perpendicular contact guidance in CNS neuroblasts (Nagata et al., 1993) and rat hippocampal cells (Gomez et al., 2007; Rejnicek et al., 2007), in a manner dependent on surface feature sizes. Neurites undergoing this form of contact guidance extended at an angle perpendicular to the features, crossing over adjacent grooves and ridges rather than extending parallel to a single feature. When a cellular extension reaches a linear topographical discontinuity, it can either cross it or follow it. It appears that this choice depends both on substrate dimension and cell type, and it may be that cellular processes of a certain type resist conforming to features which are too closely spaced and too deep, and instead cross between adjacent features, resulting in perpendicular contact guidance. This particular phenomenon has also been reported in vivo (Ono et al., 1997), as well as in 2D and 3D, microexplant and organotypic cerebellar cultures (Nagata et al., 2006).

Aligned fibers of different material is another popular type of surface topography that has been used to control orientation of neurite outgrowth and guide cell migration. This type of topography resembles structures the in vivo environment of the mature nervous systems such as CNS white matter tracts in the spinal cord. Nerve guidance channels with longitudinally oriented tracks serving as scaffold for axon regeneration resembling the bands of Büngner that are formed after nerve transection has also been used (Yu and Shoichet 2005). In vitro, the adhesion and outgrowth of DRG neurons was shown to be increased on these scaffolds, especially in the presence of Laminin -

derived peptides. In another *in vitro* guidance channel study, DRG explants were cultured on polypropylene filaments inside a PVC hollow fiber membrane, and the effect of filament diameter on neurite outgrowth and SC migration was measured (Wen et al., 2006). Fibers ranged from supracellular (100 to 500 μm) to cellular (30 μm) to subcellular (5 μm) in diameter, and an inverse relationship between filament diameter and both SC migration and neurite outgrowth was found. In addition, when fibers were uncoated, SCs migrated ahead of neurite outgrowth, indicating that SCs may be establishing a pathway for neurite growth, but when fibers were coated with Laminin or Fibronectin, the neurites actually grew past the Schwann cells, indicating that with permissive ECM support, neurite growth may be independent of Schwann cell migraton. Finally, another study explored the effects of electrospun fibers of polyethersulfone with different dimensions on the behavior of adult NSCs, and they found that fibers of small dimension (283 nm) promoted oligodendrocyte specification, whereas larger fibers (749 nm) increased neuronal differentiation.

Isotropic discontinuous

Respectively, according to the guide post effect that was explained above, a number of studies have shown that neurites follow interrupted guidance cues with a high degree of fidelity (kriparamanan et al., 2006). In this case the outgrowth of neurites remain patterned but it is extended in every direction and that is why this type of geometry is characterized as isotropic. Hippocampal neurons plated on surfaces with 1 μm tall pillars either 0.5 or 2 μm in diameter and arranged in a grid 1.5 to 4.5 μm apart aligned to pillar geometries, moving between adjacent pillars (Dowell-Mesfin et al., 2004). The neurites tended to span the smallest distance between pillars, aligning either at 0° or 90°, with the highest alignment with the larger pillars at the smallest spacing. As the spacing between pillars increased, the fidelity of alignment decreased, and at 4.5 μm spacing, the distribution of neuronal arbor was similar to that found on a flat surface. In addition, at an early time in culture (1 day), the neurons on the pillar topographies, unlike the flat surfaces, had a single presumed axon forming. This indicates that guidepost-like topography may facilitate neuronal differentiation.

Surfaces with varying heights that are less than 1 micron different are often considered to be nanostructured, or nanorough. These surfaces have been used with a variety of cell types and surface chemistries, and a range of responses has been

observed in terms of cellular adhesion, proliferation, and morphology(Martinez et al., 2009). Roughness is often measured in terms of an average feature size, called R_a. Some studies of neurons on globally isotropic surfaces with nanoscale features have been performed, with mixed results. One study showed that nanotextured titanium nitride films with R_a values from 1.3 to 5.6 nm reduced attachment of primary hippocampal neurons relative to Poly-D-Lysine (PDL) coated glass (Cyster et al., 2004). Another study found that embryonic primary cortical neuron adhesion and viability was affect in a unimodal fashion by nanotextured silicon with R_a from 18 to 204 nm, where an intermediate value of R_a=64 nm promoted an optimal response, and both higher and lower roughness reduced this response (Khan et al., 2005). In addition, local nanoroughness on the order of growth cones promoted PC12 extension and neuronal gene expression patterns in the absence of NGF (Tamplenizza et al., 2013).

Finally, One approach to elicit neuronal guidance and reconstruct an in vivo-like environment is to co-culture neurons with other cells, with and without additional topographical guidance features. Cells by themselves provide both biochemical and topographical cues to regenerating axons. For example, aligned monolayers of Schwann cells can direct neuronal outgrowth to follow the direction of alignment even in the absence of other topographical cues (Thompson et al., 2006). In addition, Schwann cells can be guided by topography themselves, and like neurites appear to be sensitive to the depth of anisotropic groove topography (Hsu et al., 2006), leading to studies which indicate that there is a synergy between the cues presented by cells and cues presented by topography. When Schwann cells were preseeded onto grooved scaffolds of 15 μm depth, over 60% of them aligned on 50 and 100 μm width grooves, while over 90% aligned on 2-20 μm grooves. These patterned cultures which presented DRG neurons with both cellular and geometric topographical cues were able to orient the neurites in the direction of the imposed topography and the aligned Schwann cells (Leitz et al., 2006).

Cell types such as meningeal cells and astrocytes have also been used to direct neurite outgrowth in vitro, though they tend to be associated with inhibiting regrowth in the injury environment (Fawcett et al., 1999). In particular, outgrowth of both postnatal and adult DRG neurons aligned to monolayers of meningeal cells which were preplated on substrates with anisotropic nanoscale roughness (Walsh et al., 2005),

from 250 nm to 1 μm in depth. As expected, neurites did align to topography without the intervening cell layer, and increased topography depth in conjunction with meningeal cells elicited increased alignment. However, the meningeal cells appeared to provide some additional cue beyond their topography, as the neurites showed enhanced orientation and growth when presented with the intermediate cell layer, with the effect more pronounced with younger neurons. Similarly, DRG neurons were guided by astrocyte monolayers which had been oriented by an electric field (Alexander et al., 2006). Interestingly, the same response could be seen on fixed aligned astrocytes, indicating that the astrocytes are not necessarily providing active biochemical cues. Further, astrocytes have also been shown to promote neuronal differentiation and alignment. When neural progenitor cells were plated on microgrooved surfaces with and without an intermediate layer of astrocytes, though the progenitor cells could orient to both surfaces, only in the presence of astrocytes, grooves induced increased differentiation into cells expressing neuronal markers (Recknor et al., 2009).

From these studies, it seems that guidance cues from cells are able to provide some information which geometric topography alone cannot, but it is not clear if this information is primarily biochemical or topographical. For instance, polymer replicas of Schwann cells aligned in 2D surfaces though were able to support the oriented growth of DRG neurites depicting that biochemical cues are not necessary for this functions (Richardson et al., 2011).

AIM OF THE STUDY

The adult mammalian brain possesses only a limited capacity for regeneration under pathological conditions that cause neuronal cell loss. However, the identification of adult neural stem cells that can proliferate and differentiate into functional neurons, unveil new intriguing possibilities towards restoration of brain function in case of a disease. In this direction, there are two main strategies that have been tried. One is the transplantation of either exogenous neural stem cells from donor organism or induced pluripotent stem cells from the same organism. Nevertheless in most of the cases these attempts have failed because of the inadequate growth of the transplanted cells in the host environment. In the contrary, endogenous neural stem cells that lie mainly in the area of the dentate gyrus (DG) and in the subventricular zone (SVZ) are generated continuously and get integrated to the existing hippocampal circuit getting involved in its function as mediator of memory formation and consolidation. These cells are responsive to intrinsic as well as extrincic stimuli that affect their proliferation, their survival and differentiation. Such molecules are neurotrophins as Nerve growth factor (NGF) and Brain derived neurotrophic factor (BDNF), as well as other growth factors such as hormones, which are also responsible for the patterning of nervous system during development. Nevertheless, the therapeutic use of these molecules is often compromised due to their polypeptide nature and their inability to cross the blood brain barrier or because they produce off target undesirable effects. Thus there is the need for clinically relevant, small lipophilic molecules, which can mimic the actions of endogenous growth factors and show good availability to the CNS.

In the first part of this study I have tested the pro-neurogenic potential of two small molecules that belong to two different classes.

The first of them is fingolimod, an oral drug that has been approved for the treatment of multiple sclerosis based on its ability to inhibit the egress of lymphocytes from lymphoid nodes, thus preventing its migration to the site of inflammation. Fingolimod mimics the actions of sphingosine-1-phosphate a metabolic product of membrane sphingolipids, that is produced by phosphorylation of sphingosine by sphingosine kinase (SphK1 and Sphk2) and acts through activation of 5 subtypes of S1P receptor

that belong to GPCR superfamily but also as an intracellular messenger itself. Furthermore, the enzyme for conversion of fingolimod to its active metabolite fingolimod-p it is expressed in the nucleus where the latter binds to histodeacytylace (HDAC) regulating the expression of many genes such as BDNF and other growth factors.

Endogenous S1P is mainly implicated in the development of cardiovascular system and regulation of lymphocyte trafficking under inflammation. However, in the past few years there has been a growing amount of evidence about a specific role of this lipid as well as its analog, fingolimod, in regulation of various cell functions inside the CNS affecting its development and the maintenance of its integrity.

Taking this into consideration the potential therapeutic effects of Fingolimod have been tested for a wide range of CNS disorders, from neurodegenerative to neuropsychiatric conditions. Fingolimod was found effective to ameliorate cognitive decline and apoptosis in the hippocampus of aβ amyloid injected mice and reduced its levels in an animal model of Alzheimers disease (AD) (Asle rousta et al., 2013,Takagushi et al., 2013). Short-term treatment with fingolimod increased the levels of neurotrophic factor BDNF in the brain conferring neuroprotection and alleviating the motor symptoms in an animal model of RETT syndrome (Deogracia et al., 2012).

In addition to this, inhibition of HDAC by fingolimod, facilitated extinction of fear memories (Hait et al., 2014) and conferred antidepressant effects in stressed mice (Nuzzo et al., 2015).

Since neurogenesis consists one of the major mechanisms of brain plasticity, affecting mood and cognition, we hypothesized that some of the above actions could be attributed to increased neurogenesis after treatment with fingolimod.

Using the thymidine analogue BrdU to trace neural stem cells, I have observed an increase in the proliferative capacity as well as in the net production of new neurons in the area of Dentate gyrus of the Hippocampus after administration of fingolimod in young adult and adult mice. However this was not evident for older, middle aged mice. Moreover, immunohistochemical detection of S1P1 the most prominent subtype that mediates the actions of fingolimod in the periphery and CNS, with various markers of NSC lineage revealed expression of the latter in rapidly dividing

progenitors expressing the neural precursor cell specific transcription factor mash1 and DNA replication licensing factor (Mcm2), marker of proliferation. In order to make a mechanistic exploration of this action in the molecular level I used neurosphere cultures of neural stem/ precursor cells (NS/PCs) isolated from P7 mouse hippocampus. In specific, the area of DG was dissected and after dissociation cells were cultured for 2-3 passages in the presence of EGF/FGF in order to form neurospheres. Postnatal hippocampal NS/PCs express S1P1 receptor in vitro as was detected with immunocytochemistry and western blot and when fingolimod-p, which is the active metabolite of fingolimod in vivo, applied to the cultures increased their proliferative activity. Furthermore, the use of pertussis toxin (PTX) (an uncoupler of Gi protein from the enzyme adenylate cyclase) or a specific inhibitor of MEK1 (U0126) completely abolished the proliferative effects of fingolimod-p indicating that these are dependent on activation of S1P1 and subsequent induction of MAPK pathway. Furthermore, fingolimod-p was proved efficient to induce stimulation of ERK1/2 kinase in a PTX-sensitive manner. In addition to the above effects fingolimod-p significantly reduced apoptosis after growth factor withdrawal in vitro and this action was attributed to increased levels of BDNF as a monoclonal a-BDNF antibody restored apoptosis rate to basal levels. Finally the effects I observed in neurogenesis were correlated with improved performance in an adult neurogenesis related task, which is the context discrimination after fear conditioning.

In a second part of this study I focused on the neuroprotective and neuroregenerative actions of another small synthetic molecule, an analogue of the endogenous neurosteroid Dehydroepiandrosterone (DHEA). DHEA is a neurosteroid that is synthesized in the periphery from the adrenal glands, but also is synthesized de novo and secreted locally in the CNS in high concentration. During lifetime though, the circulating concentrations of DHEA both in plasma and CSF decline with age and this reduction has been correlated with the development of various neurodegenerative diseases such as senile dementia. DHEA has been shown to promote neuronal survival in various models of neurodegeneration both in vivo and in vitro through divergent mechanisms most of which implicate modulation of GABAA NMDA and SIGMA-1 receptors on which DHEA binds with low affininty (Maninger et al., 2009). For instance DHEA protect cells in the hippocampus from NMDA induced excitotoxicity by upregulating GABAA receptors (Kimonides et al., 1998, Belelli et

al., 2005). DHEA, also, has been reported to exert neurogenic action on adult hippocampus mediated via NMDA and Sigma-1 receptors (Suzuki et al., 2004). Moreover, it antagonizes the neurotoxic effects of glucocorticoids, although it is not known if GR receptor is directly implicated (Kimonides et al., 1999, Karishma et al., 2002).

Finally, recent data from our laboratory support the view that DHEA binds to both NGF receptors (TrkA and p75) activating intracellular pathways to promote survival of neurons. DHEA was shown to compensate NGF withdrawal in primary cultures of sympathetic neurons that express both of the receptor will reduced cell death in vivo in NGF-/- mice at embryonic day E13.5 in the area of dorsal root ganglia (DRG). In accordance with our hypothesis, other reports in the literature support a neurotrophic action of DHEA similar with that of NGF. DHEA as well as NGF support Ach secretion from basal forebrain neurons to the hippocampus. (Olivier et al., 2006; Auld et al., 2001). These neurons express the two cognate receptors of NGF, which appears to have a physiological role in maintenance of its cholinergic activity but not its survival (Crowley et al., 1994) Basal forebrain cholinergic system is responsible for a variety of cognitive functions through afferents that sends to hippocampus and cortex and it is from the first areas that are affected in AD and ageing.

DHEA's neuroprotective properties, mediated through different mechanisms described above, as well as its small size and lipophilic nature make it an ideal candidate drug for the treatment of various neurodegenerative conditions. However, DHEA therapeutic use is compromised because it widely affects the endocrine system by its own or after conversion to other steroid hormones with possible tumorogenic action. In particular breast, endometrial, or prostate cancer has been associated with disturbances on estrogens and androgen levels (Henderson et al., 2000; Takahashi et al., 2006). Moreover the exact actions of DHEA cannot be distinguished from its metabolites.

BNN27, has been developed by modifications of DHEA structure in order to sustain its neuroprotective properties and avoid its further metabolism to androgens and estrogen. In this way, BNN27 lacks of the possible tumorogenic potential of the parent molecule, DHEA.

In order to test the efficacy of BNN27 to reproduce the anti-apoptotic effects of DHEA, the neural crest derived PC12 cell line expressing functional TrkA receptors was used as well as primary neurons, the superior cervical ganglia sympathetic

(SCG's) neurons. BNN27 was found capable to reduce apoptosis in PC12 cells after serum deprivation as well as in SCG's in culture after NGF withdrawal. Also, In Vivo administration of BNN27 to pregnant mothers compensated for lack of NGF in E13,5 ngf null embryos.

Both NGF and DHEA have been reported in the literature to induce adult hippocampal neurogenesis. Given the aforementioned role of neurogenesis as a potential therapy for neurodegenerative disease, especially these that are accompanied by deterioration of cognitive function, we sought to test the pro-neurogenic capacity of our synthetic DHEA analogue BNN27. To this aim I followed the previously described protocol that was established for the evaluation of the neurogenic properties of fingolimod.

BNN27, was not effective to induce either proliferation or survival of neural stem cells in the area of DG in WT mice in any of the ages tested (3,7, and 12 months). However, BNN27 was proved capable to restore neurogenesis in an animal model of amyloidosis, which is the 5XFAD mice, when administered in the form of subcutaneous pellet prior to the development of any Aβ pathology (at 1,5 months of age). These effects were correlated with reduction in the number of Aβ plaques at the end of the 1,5 month treatment period compared to control animals, albeit no significant differences were detected in activation of microglia in the same area. In addition to this, BNN27 was effective to restore atrophy of basal forebrain cholinergic neurons observed in 5XFAD compared to WT littermates.

In the final part of this study I was interested in the development of new culture platforms in order to orient neuronal outgrowth and cell migration. A variety of methods have employed so far in the literature in order to tailor surfaces of different types of biomaterials from soft ones such as collagen to more rigid like silicon in order to manipulate cell function of neural cells and NSCs *in vitro*. To date, this has been achieved with the aid of micro- and nanofabrication techniques giving rise to various anisotropic topographies, either in the form of continuous or discontinuous structures. The growing field of bioengineering is aiming at understanding the way specific characteristics of the microenviroment can yield cell responses in order to harness this knowledge for the development of *in vivo* scaffolds that promote tissue regeneration. Moreover, it enables the construction of microfluidic devices where neuronal cells can grow in a controllable manner making feasible the recapitulation of

in vivo physiology in a dish with wide application in neuroscience (compartmentalized cultures) or pharmacology (investigation of the pharmacological actions and pharmacokinetic profiles of novel molecules) (Park et al., 2009; Taylor et al., 2005). Finally, topologically defined artificial networks allows for the development neurochips and biosensors reading electrical signals produced by the cells (Voelker and Fromherz 2005).

In this study we propose a currently unexplored geometry of a 3D culture substrate for neuronal cell growth comprising discontinuous subcellular microstructures with anisotropic geometrical cross-section in the form of parallel oriented elliptical microcones (MCs). This was made possible by ultra-short pulsed laser processing. Using increasing laser fluence, three types of micropatterned Si surfaces, which exhibit different geometrical characteristics (denoted as low, medium and high roughness substrates, respectively), have been fabricated and characterized as to surface morphology, wetting properties and surface chemistry. These three micropatterned Si substrates together with the unpatterned flat Si have been applied to *in vitro* cell cultures. The cell models used included, peripheral nervous system (PNS) populations like sympathetic neurons of SCGs, DRGs and Schwann cells as well as NSCs of the embryonic cortex. The ability of each substrate to promote guidance of neuronal outgrowth and cell migration was evaluated by using immunofluorescence. The specific interaction of living cells with the underlying structures and the effects of the imposed topography in cell shape were also investigated using SEM analysis.

MATERIALS AND METHODS

Animals.

Male C57/BL6 mice and *ngf* +/− mice that were obtained from the Jackson Laboratory and maintained on C57BL/6 background were kept on a 12h light/dark cycle (lights on at 07.00a.m.) with *ad libitum* access to food and water. Animals were habituated to housing conditions for one-week prior the beginning of the experimental procedures. Animal experimentation received the approval of Veterinary Directorate of Prefecture of Heraklion, Crete and was carried out in compliance with Greek Government guidelines and the guidelines of our ethics committee. Genotyping was performed on tail DNA using the following primers: NGFKOU2 (5′CCG TGA TAT TGC TGA AGA GC3′), NGFU6 (5′CAG AAC CGT ACA CAG ATA GC3′), and NGFD1 (5′TGT GTC TAT CCG GAT GAA CC3′). Genomic PCR reactions containing the 3 primers were incubated for 32 cycles at 95°C (30 s)/59°C (30 s)/72°C (1 min).

Fabrication and characterization of micropatterned Si substrates

Single crystal n-type Silicon (1 0 0) wafers were subjected to laser irradiation in a vacuum chamber evacuated down to a residual pressure of 10^{-2} mbar. A constant sulfur hexafluoride (SF_6) pressure of 500 Torr was maintained during the process through a precision microvalve system. The irradiating laser source was constituted by a regenerative amplified Ti:Sapphire (λ = 800 nm) delivering 150 fs pulses at a repetition rate of 1 kHz. The sample was mounted on a high-precision X–Y translation stage normal to the incident laser beam. The laser fluence used in these experiments was in the range 0.68–1.50 J/cm^2. After laser irradiation, micro-structured surfaces were morphologically characterized by scanning electron microscopy (SEM), as previously described (Simitzi et al., 2014). SEM analysis was performed on a JEOL 7000 field emission scanning electron microscope with an acceleration voltage of 15 kV. An image processing algorithm (ImageJ, National Institutes of Health, Bethesda, MD, USA) was implemented in order to determine the topological characteristics of the MCs, including height (h), base major (b) and minor (b′) axes, interspike distance (c) and density (D) from top, side-view and cross-sectional SEM images.

Substrate coating

In some cases, substrates were immersed in 0.01% type I collagen (Sigma Aldrich Chemie GmbH, Munich, Germany) or Poly-D-Lysine (PDL- Sigma Aldrich Chemie GmbH, Munich, Germany) for 2 h at room temperature (RT) and washed in PBS prior to culture initiation. Electron microscopy analysis showed that protein deposition conformed to the underlying topography of the substrates and did not bridge spikes (data not shown). The coated or non-coated substrates were directly placed in 48-well cell culture plates (SARSTEDT, Neurberg, Germany).

The substrates were sterilized in an autoclave and further used for the *in vitro* assays. For each case, flat Si substrates have been used as a control.

Tissue processing and immunofluoresence.

C57/Bl6 mice treated as mentioned in the text were euthanized with pentobarbital and trans-cardially perfused with saline followed by 4% Paraformaldehyde (PFA, Sigma) in PBS. The brains were dissected and post-fixed overnight in the same solution at 4^{o}C. After thorough washing in PB 0,1M brains were cryoprotected by being immersed in 30% sucrose solution in PB 0,1 M for 24h at 4^{o}C until they sunk and were frozen in isopentane at -40^{o}C. 40μM coronal sections were cut in the dorsoventral axis of hippocampus (from bregma -1.34mm to -3.80) and stored in cryoprotective medium (30% glycerol/30% ethylene glycol in 50 mM phosphate buffer) at -20 °C until they processed for immunofluoresence.

For *in vivo* experiments in *ngf* +/- embryos, mice heterozygous for the NGF gene disruption were interbred to obtain mice homozygous for the disrupted gene. The pregnant mothers were treated daily with an intraperirtoneal injection of BNN27 (100 mg/kg/day) or vehicle (4.5% ethanol in 0.9% saline) starting from the third day after gestation until day E13,5 or E17,5. At the end of the treatment mothers were euthanized by cervical disocation and embryos were collected and fixed overnight in a solution of 4% PFA, 15% Picric Acid, 0.05% GA in phosphate buffer 0.1 at 4°C. The next day embryos washed in 0.1 M phosphate buffer and cryoprotected by being immersed in a 30% sucrose solution in PB 0,1M at 4°C until they sink. Finally, embryos were frozen in OCT in isopentane at −40°C. The samples were sectioned (16 μm) in a freezing microtome and mounted onto Superfrost plus slides (Menzel-Glaser

J1800AMNZ). Slides were post fixed for 10 min in cold acetone and left to dry for 10 min at room temperature and stored at -20 until later use.

NS/PCs cultures.

The hippocampi of postnatal day 7 (P7) C57/BL6 mice were digested for 30min in accutase solution (Sigma) at 37^{o}C. After mechanical dissociation cells were plated at a density of 5x10^{4} *per* ml into uncoated T25 culture flasks in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (Sigma) supplemented with 1% B27 (InVitrogen), L-glutamine 2mM (Gibco), D-glucose 0.6%, primocin 100μg/ml (InVitrogen), in the presence of 20 ng/ml FGF2 (R&D) and EGF (R&D) and allowed to form neurospheres. Cells were passaged every 5th day by dissociating neurospheres into single cells with accutase (Sigma).

NS/PCs proliferation and survival assay.

For the proliferation assay, passages 1 to 3 of primary neurospheres were dispersed to single cell suspension and the last were plated in Poly-D-Lysine/Laminin coated dishes, and stayed for 24h in complete medium. The day after cells deprived of EGF/FGF for 3h and then exposed for 24h to 1 μM fingolimod-p in the presence or absence of 300 ng/ml Pertussis Toxin (PTX) or 10 μM of ERK1/2 inhibitor U0126 that were added 30min or 3h before drug addition. At the end of the treatments, 10 mM BrdU was applied to the cultures for 16h in order to label all actively proliferating cells before they were fixed and stained against BrdU. For the assessment of the effects of fingolimod on survival of NPCs after growth factors withdrawal, cells were plated as above and cultured for 72h in EGF/FGF-free medium in the presence or absence of 1 μM fingolimod-p, with or without the addition of 10 μg/ml anti-BDNF neutralizing antibody. Finally, cells were fixed and stained with in situ cell death detection labeling kit TUNEL (Roche) according to the manufacturers instructions.

Superior Cervical Ganglia Neuronal Cultures

Superior cervical ganglia (SCG) were dissected from newborn (P0–P1) rat pups and dissociated in 0.25% trypsin (Gibco, 15090) for 30 min at 37°C. After dissociation SCG neurons were re-suspended in RPMI culture medium 1640 containing 3% fetal bovine serum (FBS), 100 units/ml penicillin, 0.1 mg/ml streptomycin, 10mM antimitotic agent FdU with uridine, and 50 or 100 ng/ml NGF (Millipore, 01-125). Cells were plated on collagen coated 24-well plates and cultured for 7 d prior to use. For NGF withdrawal experiments, cells were washed twice with NGF free medium and culture for 48h in medium lacking NGF and containing anti-NGF antibody at 1:500 dilution (Millipore, AB1526). At the end of the treatment period neurons were immunostained against NF200kD and TUNEL assay (Roche) was conducted according to manufacturer instructions in order to stain apoptotic neurons.

Immunofluoresence.

Free-floating sections or NS/PCs attached in Poly-D-Lysine/Laminin and fixed with 4% PFA solution for 15 min, were washed twice with 0.1 M PB pH 7.4 and blocked in 5% BSA in 0,3% Triton X-100 in PBS (PBST) for 1h at RT before they were incubated with primary antibodies in 0,3% PBST overnight at 4^{o}C. The day after sections or cells were washed twice with PBS and incubated with Alexa Fluor chromophore conjugated secondary antibody (1:1000 dilution, InVitrogen) in 0,3% PBST for 1h at RT. Finally, sections were washed twice again with PBS and mounted onto slides using antifade reagent with DAPI (InVitrogen). For detection of BrdU-labeled nuclei, specimens have been previously incubated in 2N HCl at 37^{o}C, followed by a 10min rinse in 0.1M sodium tetraborate pH 8.5 and 2 rinses with PBS before blocking step.

Immunocytochemistry

After removal of the medium, cells were fixed with 4% paraformaldehyde in PBS 0.1M for 30 minutes, blocked for 1h at RT in PBS 0.3% Triton X-100 containing 5% Donkey serum and incubated overnight at 4^{o}C with primary antibodies in blocking buffer. Schwann cells were stained with anti-S100b (1:500 Abcam, Rab Mab ab52642), while neurons were stained with anti-NF200kD (1:10000 Abcam, Chicken

polyclonal ab4680). Additionally, fibronectin, which is expressed by non-neuronal cells was detected using an anti-rat fibronectin antibody (1:100 Millipore, rabbit polyclonal Ab2047). After 3 washes with PBS, specimens were incubated for 1h at RT with Alexa Fluor 546 goat anti-rabbit IgG (MoBiTec A-11010) or Alexa Fluor TM 488 goat anti-mouse IgG (MoBiTec A-11001) secondary antibodies, diluted 1:500 in PBST. The cells were washed and nuclei counterstained with 4 0,6-diamidino-2phenylindole dihydrochloride (DAPI; 1:1000) or TOPRO (1:5000). The substrates were mounted on coverslips with antifade reagent (Invitrogen).

Results were evaluated with a Laser Scanning Spectral Confocal Microscope (Leica TCS *SP2)*.

Scanning electron microscopy (SEM)

The morphology of the cells growing on the patterned surfaces was analyzed by SEM. After culture termination, the cells were washed with 0.1M sodium cacodylate buffer (SCB) and then incubated in the same solution for 15 min. After repeating this step twice, the cells were fixed using 2% glutaraldehyde, 2% formaldehyde in 1% SCB fixative buffer for 1 h at 4°C. All surfaces were then washed twice (for 15 min each time) with 1% SCB at 4°C, dehydrated by immersion in serially graded ethanol solutions (50–100%) and incubated for 15 min in dry 100% ethanol. Prior to electron microscopy examination, the samples were sputter-coated with a 10 nm gold layer (Humme Technics Inc, Alexandria, Virginia USA). SEM analysis used a JEOL 7000 field emission scanning electron microscope at an acceleration voltage of 15 kV.

Quantitative evaluation with image processing

Quantitative information (regarding cell number, neurite length, etc.) was assessed using an image processing algorithm (ImageJ).

Orientation of silicon cone morphology

Top view electron microscopic images of the micropatterned Si substrates were taken digitally and opened with ImageJ. Images were inverted, thresholded (using the same threshold value for all images) and the major axis for each spike has been graphically selected. The orientation angle of the spike was measured as the angle between the major axis of the ellipse and the vertical axis of the image plane (Oy). Its values ranged between 0^o, signifying a perfectly aligned spike, to 90^o for a vertical spike.

Only the absolute values have been considered, therefore no discrimination between leftward or rightward divergence from the Oy axis was taken into account. This was repeated for all spikes in each image and stored in an Excel spreadsheet. Using the frequency function for the set of angle values and a given set of bins (0-10^o, 10-30^o, 30-60^o and 60-90^o), the values occurring in each defined interval were counted. At least 60 spikes for each roughness type have been used for the analysis.

Schwann cell outgrowth orientation on the micro-patterned Si substrates

To determine Schwann cell orientation on the different micro-patterned silicon substrates, the cell nucleus orientation was measured, according to respective literature [26-28]. Elliptical-shaped cells were outlined by hand and the major and minor axis of each cell ellipse was manually traced. The angle of the cell nucleus was then determined using the major axis of the cell ellipse. The orientation angle of the nucleus was measured as the angle between the major axis of the ellipse and the vertical axis of the image plane (Oy). Its values ranged between 0^o, signifying a perfectly aligned cell nucleus, to 90^o for a vertical cell nucleus. Only the absolute values have been considered, therefore no discrimination between leftward or rightward divergence from the Oy axis was taken into account. This was repeated for all the nuclei in each image. The values from all fields of views were inserted in a spreadsheet. The orientation of the cell nuclei with respect to the y-axis was binned into 30◦ intervals ranging from 0^o to 90^o to generate an orientation frequency, f, for the i_{th} interval i=1,2,3 (using the Frequency Function). The orientation angle has been calculated from at least 500 cell nuclei for each roughness type.

Axonal orientation on the micro-patterned Si substrates

Assuming each axon (stained with anti-NF) connecting two somatas (stained with DAPI) as a vector, the orientation angle of this vector has been calculated and used as an index of the axonal outgrowth orientation. The orientation angle of each axon was measured as the angle between the axonal vector and the vertical axis of the image plane. Its values ranged between 0^o, signifying a perfect alignment to 90^o for a vertical outgrowth. Only the absolute values have been considered, therefore no discrimination between leftward or rightward divergence from the Oy axis was taken into account. This was repeated for all the axons in each image. The values from all

fields of views were inserted in a spreadsheet. The orientation of the axons with respect to the y-axis was binned into 30◦ intervals ranging from 0^o to 90^o to generate an orientation frequency, f, for the i_{th} interval i=1,2,3 (using the Frequency Function). This procedure was repeated for n experiments (to get the standard error). The orientation angle has been calculated from at least 300 axons for each roughness type.
Axonal thickness of NF positive axons was measured from fluorescence microscopy images of high magnification (x 63 objective). For each axon the average thickness of the values in at least two points has been calculated. The axonal thickness was binned into 3 intervals, ranging from 0 to >4 μm, to generate an orientation frequency, f, for the i_{th} interval i=1-3 (using the Frequency Function). The axonal fasciculation has been calculated from at least 80 axons for each roughness substrate type.

Cell counts and quantification.

For quantification of BrdU+ cells in the hippocampus, one every six adjacent sections was chosen and processed for immunofluorescence against BrdU, based on a modified unbiased stereology protocol (Malberg et al., 2000, Rossi et al.,2006). The number of BrdU+ cells was then counted under x40 magnification under a fluorescent microscope (Leica DMLB equipped with a DC300 F camera) at the area of (granular cell layer) GCL and SGZ (defined as a two cell layer in the borders of GCL, omitting these in the outermost focal plane, for a total of 10 sections and the average number of cells was estimated. The mean was then multiplied with the total number of sections (approximately 60) in order to estimate the total number of cells per DG.
To measure the total volume of DG, the area of GCL was outlined and computed using images in every 6^{th} adjacent section for a total of 10 sections in photos taken by a confocal fluorescence microscope (Leica TCS SP2). Subsequently, and according to the Cavalieri principle, the areas summed and multiplied with the intersection distance and average section thickness to estimate the entire DG volume in mm^3.
For *in vivo* co-localization experiments confocal analysis under x63 oil lenses was conducted. The percentage of double labeled cells for the each marker was estimated by counting the cells in the DG in three sections of total 5 mice.

In order to quantify apoptotic neurons in embryos caspase-3+ cells inside the area of the DRG were counted in 12-14 sections being 160μM apart from one another across

most of the rostrocaudal extent of the spinal cord and the mean of apoptotic cells per sections was estimated.

For *in vitro* experiments, the number of BrdU or TUNEL positive cells in each culture was counted using an objective (×32) from 8-10 visual fields for every condition. DAPI-stained cells represent the total cell number. Finally, the percentage of BrdU+/DAPI+ or TUNEL+/DAPI+ or TUNEL+/ NF200kD cells was normalized to the control condition.

Numbers of cells (Schwann cells and sympathetic neurons) that have been grown on the micro-patterned silicon substrates were determined by counting cell nuclei stained with DAPI. Nuclei number was assessed with ImageJ v1.36, using the "Cell Counter" plugin. The results represent the means of at least three different experiments (n= ~30 fields of view for each roughness type and time of culture).

Schwann cells

Schwann cell primary cultures were prepared from sciatic nerves of P2-P4 rat pups. Upon dissection, nerves were enzymatically triturated in a Trypsin 0.25% and 1% collagenase Type 1 (Sigma C0130) in HBSS solution for 30min at 37^{o} C. The enzymes were deactivated by adding Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Biosera, Sussex, UK) to the cells and the sciatic nerves were mechanically dissociated into single cell suspensions and plated to dishes. Before the first passage, Schwann cells were purified from contaminating fibroblasts using anti- thy1.1 antibody (AbD Serotec) and rabbit complement (Calbiochem) according to Brockes et al. [25]. Then 10^5 cells were seeded onto micropatterned silicon scaffolds and cultured for 4 – 6 days until fixation for further analysis, with medium replacement every 2 days. In some experiments, PDL coating has been used. Schwann cells had a purity of more than 90%, and in these primary cultures only S100-positive Schwann cells were evaluated.

Co-culture of Dissociated SCGs and Schwann cells

Schwann cells were cultured on the micro-patterned Si substrates, as described above, for 2 days. Then, dissociated SCGs were seeded to the monolayer of Schwann cells and cultured for an additional time period of 4 days in RPMI medium containing 10% FBS, 1%p/s supplemented with 100ng/ml NGF.

DRG whole explant culture.

Mouse embryos (embryonic day (E) 13.5) were dissected from pregnant mice. DRGs from the lumbar region (L1–L4) were collected in cold PBS, then seeded onto the micro-structured Si substrates (2 – 3 DRG per substrate) and incubated in growth medium (DMEM)–F12, (GIBCO/Life Technologies 22320-022) supplemented with 10% FBS and 100ng/ml NGF. Explants were incubated 3-5 days *in vitro* (DIV) until fixation for further analysis.

Western blot analysis and ELISA

Cells were harvested in lysis buffer (50 mM Tris-HCl, 0.15 M NaCl, 1% Triton-X100, pH 7.4) supplemented with protease inhibitors (1 mM PMSF and 1 g/ml aprotinin). Total protein was quantified with BCA protein kit (Pierce) and 30 μg of total protein was loaded and run on sodium dodecyl sulfate-polyacrylamide gel. The proteins were then transferred to a nitrocellulose membrane and blocked in a solution of 5% BSA and 0.1% Tween-20 in TBS (TBS-T) for 1h followed by incubation with the primary antibody in blocking solution overnight at 4^{o}C. The next day, the membranes washed 3 times in TBS-T and incubated in anti-rabbit secondary antibody conjugated to horseradish peroxidase (1:5000) in blocking solution for 1h. Finally, the membranes washed again in TBS-T and incubated for detection with enhanced chemiluminescence (ECL) substrate (Pierce).

For BDNF expression in hippocampal NS/PCs each protein-sample was quantified with specific mouse BDNF ELISA kit (Boster Immunoleader) according to manufacturers instructions and normalized to pg/mg of total protein. Then, relative BDNF expression was normalized to the control conditions in each experiment.

Antibodies and reagents.

The following primary antibodies and dilutions were used: rat anti-BrdU (1:300, Abcam); rabbit anti caspase -3 antibody (1:250, cell signaling); rabbit polyclonal anti-EDG1, (1:100, Abcam); rabbit monoclonal anti-EDG1 (1:1000, Abcam); goat anti-DcX,(1:150, Santa Cruz); mouse anti-Mash1 (1:50, BD Pharmingen); chicken anti-GFAP (1:1000, Chemicon); goat anti - Mcm2 (1:100, santa Cruz); mouse anti-NeuN (1:200, Chemicon) ; chicken anti-NF200kD (1:10.000, abcam); rabbit polyclonal anti-p-p44/42 and p44/42 MAPK (1:1000, Cell Signaling), rabbit anti – TrkA (1:100, Millipore).

The following reagents were used in proliferation and survival assay: fingolimod, fingolimod–phosphate (Novartis, BASEL), BNN27 pellets releasing 10/mg/kg day were custom made by Innovative Research of America. Human BDNF (Novus Biologicals), Anti-BDNF (mab#9 Developmental Studies Hybridoma Bank), U0126 (Sigma), Pertussis Toxin (Sigma), NGF (MILLIPORE), Anti – NGF (Millipore, AB1526).

Contextual fear discrimination.

Male mice, 3 months old, received either a vehicle or fingolimod treatment for 14d, once a day, 0.3mg/kg/day, i.p. On the 15th day, mice (one at a time) were placed in the fear conditioning chamber (MedAssociates, Inc), which was controlled through a custom-made interface connected to the computer. After 7min of habituation to the conditioning chamber, each mouse received one mild electrical foot-shock (600ms, 0.75 mA), and remained in the chamber for another 5min.[20] The following day (testing day), mice were returned to the training chamber using the same context (context A) for 3min. Subsequently, they were removed from the chamber, and after 1h of rest, they were placed in the same chamber in which the context was modified by the addition of olfactory auditory and visual cues (mild scent on the walls, open fan, and red floor) for another 3min. Mice in the no–shock group followed the same protocol as above, but did not receive any electrical foot-shock during the training day. The freezing behaviour was analyzed manually using the J-Watcher software (http://www.jwatcher.ucla.edu/). Context discrimination ratio is defined as (Freezing in context A - Freezing in context B)/(Freezing in context A + Freezing in Context B).

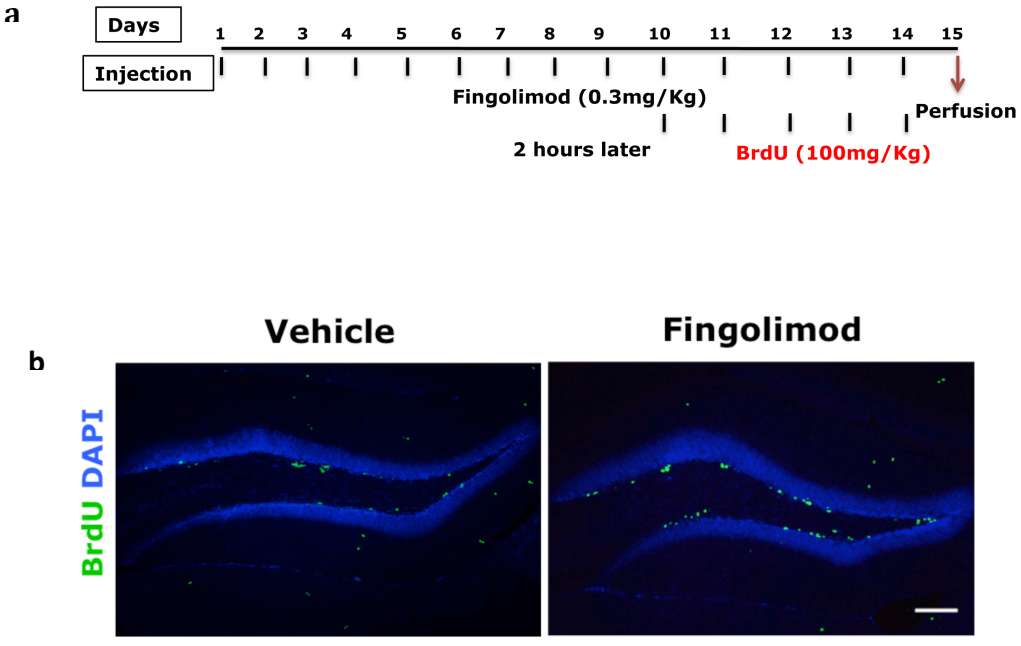
Statistical analysis

Statistical analysis was performed using Graphpad Prism software or Microsoft Excel. Statistical significance was assessed by unpaired two-tailed Student's t-tests, ANOVA followed by Fisher's predicted least-square difference post hoc tests when necessary or 2-way-ANOVA with fingolimod treatment and age as the factors followed by Bonferroni post hoc tests when appropriate. Statistical significance was established at $p \leq 0.05$.

RESULTS

Fingolimod induces adult neurogenesis in young but not in aged mice.

In order to test the effect of chronic exposure to Fingolimod on the proliferation rate of NP/SC cells *in vivo*, we administered a therapeutic dose of Fingolimod (0,3mg/kg/day) for 14 days and the thymidine analog BrdU (100mg/kg) for the last five days of the treatment. Stereological counting of BrdU+ cells in the area of SGZ of the dentate gyrus revealed that fingolimod significantly increased the number of proliferating cells in 3 months old mice, compared to the untreated control animals (2831 ± 138 vs. 2295 ± 103 BrdU+ cells; n=7, mean ± SD, $p<0.05$) (Figure 1a,b) after a 2 week treatment. Since neurogenesis dramatically declines during ageing (Kuhn et al., 1996) we would like to further test if the pro-neurogenic properties of Fingolimod are retained in older ages. The stimulatory effect of fingolimod on proliferation of NS/PCs was present in the 7 month old mice (1368 ± 86 Vs 939± 78 BrdU+ cells; n=6, mean ± SD $p<0.05$) of the DG as compared to the untreated controls taking the vehicle only. (Figure 1b,h). However, fingolimond did not have any effect on the proliferation of NS/PCs in the 12 months old animals (378± 125 BrdU+ cells vs. 495 ± 64 BrdU+ cells; n=7, mean ± SD) compared to the controls of the same age (Figure 1b,h).



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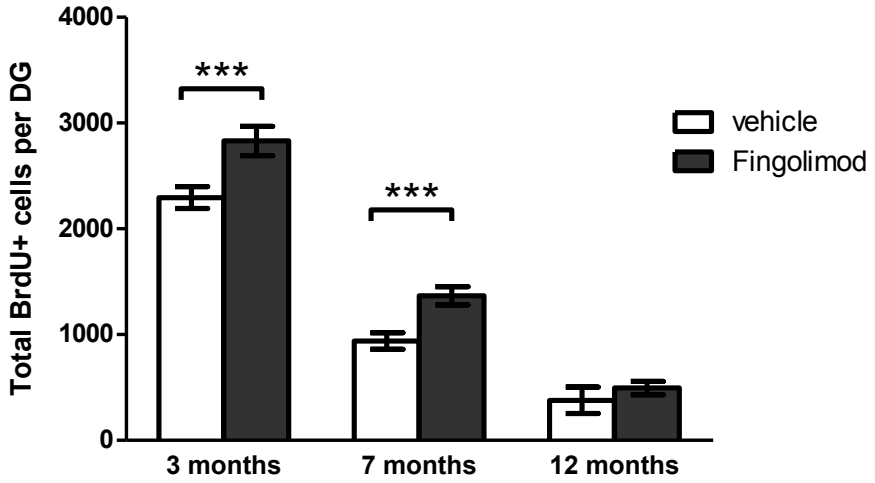
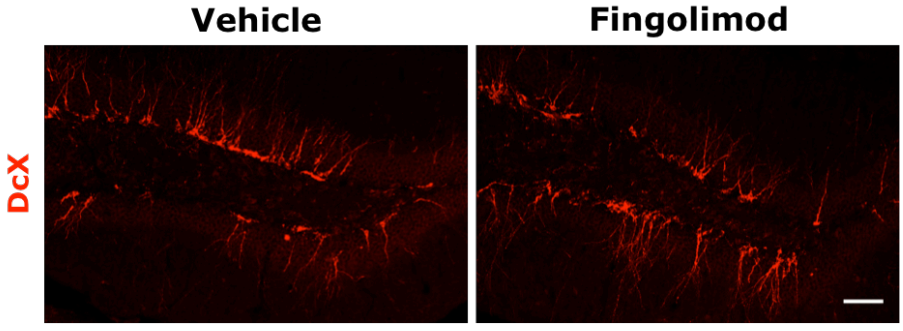


Figure 1: (a) Illustrative graph that shows the exact time of administration of BrdU and fingolimod in animals for the assessment of proliferation of NSCs. **(b)** Coronal section of dorsal DG from 3 months old C57/BL6 mice injected with Fingolimod or vehicle daily for 14d, co-injected with BrdU for the last 5 days. Sections were immunostained for BrdU (green) and counterstained with DAPI. **(c)** Quantification of BrdU+ cells in SGZ in Fingolimod or vehicle treated mice of different ages(mean ± SD; n = 6; * P<0.05; ***P<0.02).

As a next step we estimated the total number of immature neurons, stained with microtubule protein marker DcX, in the SGZ and GZ of the hippocampus in the same samples, in order to investigate the effects of the drug in the production of new neurons. 2-way ANOVA analysis of DCX+ cell number showed significant effects of age and treatment and an interaction between age and treatment as above ($p<0.0001$) suggesting that the animals respond differently to the treatment in relation to their age. Bonferroni post-hoc analysis also showed that the difference in the number of BrdU+ cells between treated and untreated animals at 3 and 7 months remains significant ($p<0.001$) which is not the case for middle aged animals (12 months of age).

a



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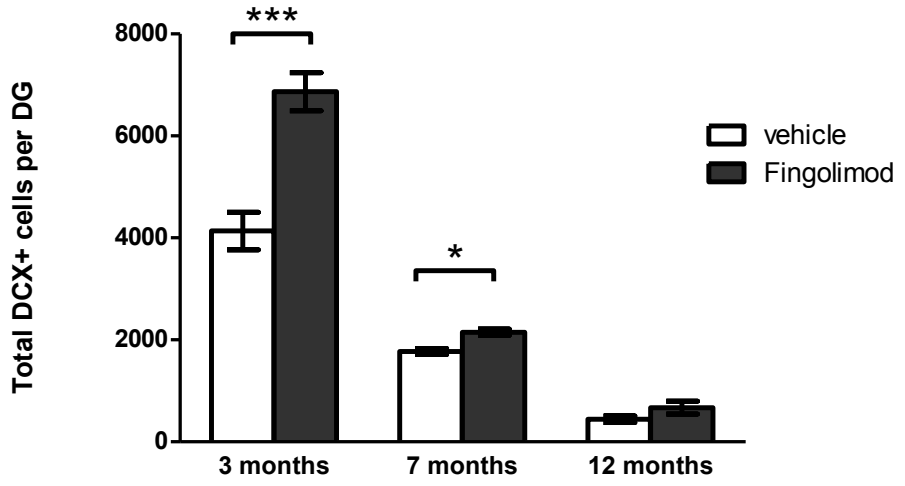


Figure 1: (a) Immunofluoresence staining of DG section of mice treated with either fingolimod or the vehicle for 14 days. **(b)** Quantification of DcX+ cells in SGZ in Fingolimod or vehicle treated mice of different ages (mean ± SD; n = 6; * P<0.05; ***P<0.02).

evaluation of NP/SC survival, as well as the number of BrdU+ NSCs that survived after 21 days of administration (1475 ± 65, n=7 vs. 1102 ± 53; n=6, mean ± SD, p<0.05) (Figure 1c,d). Most of the BrdU+ cells were also stained positive for NeuN, suggesting a neuronal phenotype (Figure 2f). The increase of the number of new neurons in the area of the DG following Fingolimod treatment was also checked by staining with Doublecortin (DcX) (6866 ± 372 vs. 4135 ± 368; 2150 ± 25 DcX+ cells Vs. 1770 ± 60 and 670 ± 126 Vs 443 ± 64 DcX+ cells, n=7, mean ± SD, p<0.05) (Figure 1g,h).

However, from the pool of proliferative neural stem cells lying in the SGZ of the DG, only a limited number survive and mature to become a fully differentiated neuron, while a substantial portion of them are eliminated during the first 2 weeks of their life through programmed cell death (Cameron & McKay, 2001). Aiming to test if fingolimod treatment, also affects, the survival of NS/Pcs the thymidine analog Brdu was administered for the first 5 days of a 3 week treatment period in a different set of experiments. Fingolimod was proved capable to increase the number BrdU+ cells, NS/PC cells that survived after 21 days of administration (1475 ± 65, n=7 vs. 1102 ± 53; n=6, mean ± SD, p<0.05) compared to vehicle treated animals (Figure 3).

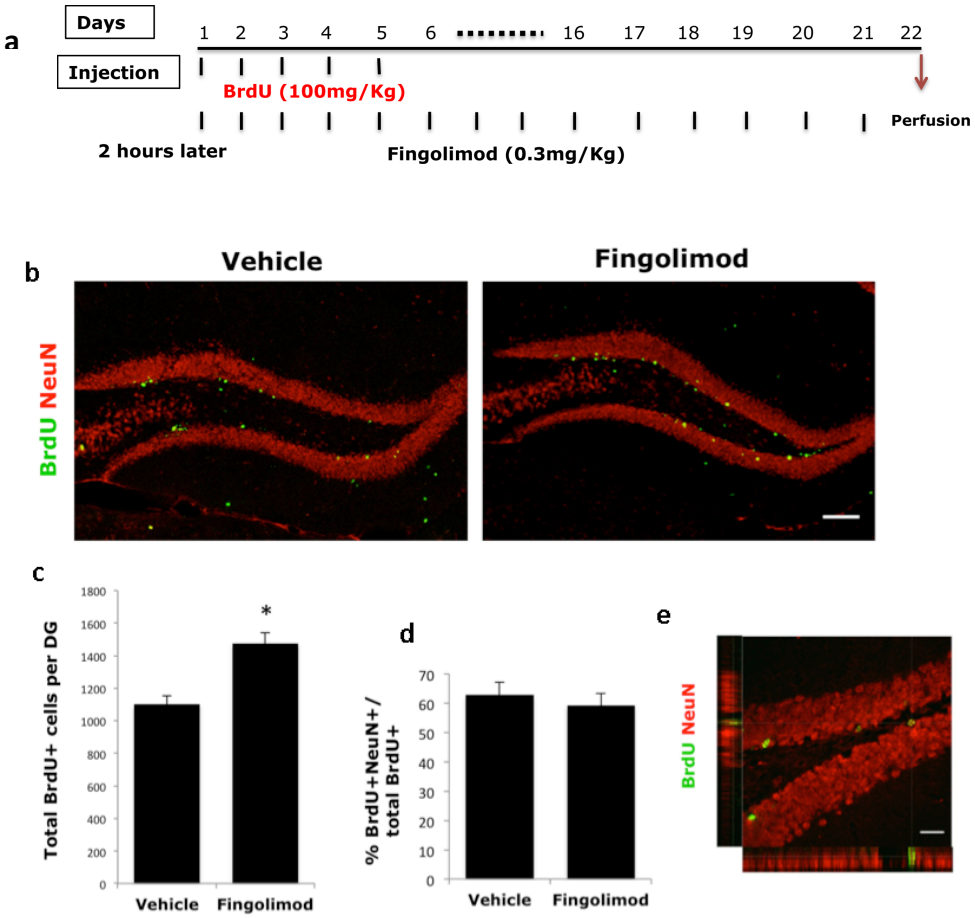


Figure 3: Coronal section of dorsal DG from 3 months old C57/BL6 mice injected with Fingolimod or vehicle daily for 21 days, co-injected with BrdU for the first 5 days. Sections were immunostained for BrdU (green) and NeuN (red). (c) Higher magnification of a confocal image of BrdU and NeuN stained cells in SGZ. (d) Quantification of BrdU+ cells in SGZ and GCL in Fingolimod or vehicle treated 3 months old mice (mean ± SD; n = 6; *P < 0.05 vs vehicle). (e) Percentage of BrdU+/NeuN+ cells in the DG of Fingolimod or vehicle treated mice.

Most of the BrdU+ cells were also stained positive for NeuN, suggesting that they acquired a neuronal phenotype (Figure 3 c,d). The fact that this percentage was not significantly altered following fingolimod treatment indicates that the increase in the number of BrdU+ cells reflects an increase in the net production of new neurons.

S1P1 receptor is expressed in neural progenitor cells.

S1P1 is the most prominent, from s1p receptor subtypes, in mediating the effects of fingolimod in the immune system as well as in the CNS (Groves et al., 2013). For this reason I sought to delineate its expression pattern in different cell types of NSC lineage, by performing immunohistochemical experiments of S1P1 receptor with

several well-established markers of adult NSCs. S1P1 was not detectable in GFAP positive cells in the DG which means that it is not expressed by quiescent slowly divided neural stem cells but, there was a strong co-localization with the transcription factor Mash1, that is known to be expressed by transit amplifying NPCs. Moreover, S1P1 staining was also observed in few DcX positive neuroblasts. Most importantly, almost all S1P1 positive cells were labeled for the DNA replication licensing factor (MCM2) marker of proliferation (Figure 2a). These findings suggest that expression of S1P1 in the SGZ is restricted to rapidly dividing neural progenitors. The above results were confirmed *in vitro* in cultures of postnatal hippocampal neural stem cells that were grown in the presence of EGF. Western blot analysis and immunofluorescence staining for S1P1 receptor revealed that it is abundantly expressed by these cells. More specifically, the expression was more pronounced in the center of hippocampal neurospheres, while all the cells were positive for Mash1.

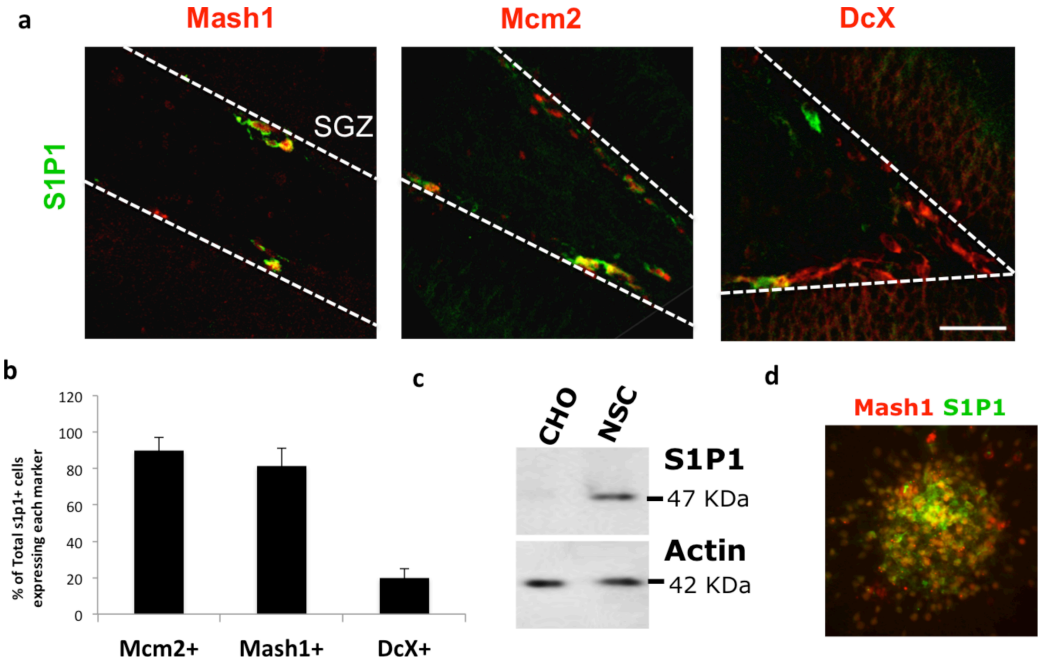


Figure 3: (a) S1P1, DcX, Mash1 and Mcm2 staining in the DG of 3 months old C57/BL6 mice. **(b)** Quantification of the percentage of S1P1+ cells stained for the indicated marker. **(d)** Western blot analysis of S1P1 expression in neurospheres derived from the respective area as indicated. **(e)** Immunohistochemical detection of S1P1 in postnatal hippocampal neurospheres. (*mean ± SD; n = 3*) scale bar, 37.5µM.

Fingolimod induced proliferation of NS/PCs in culture through activation of MAPK pathway.

In order to test if the mitogenic effects of Fingolimod that we observed *in vivo* are cell autonomous, meaning that the drug acts directly on NSCs, we used neurosphere cultures deriving from the hippocampus of C57/bl6 mice of postnatal day 7 (P7) because at this age NSC's of the marginal zones acquire for the first time characteristics of adult neural precursors while they yield the maximum number of cells due to their high proliferation rate. Isolated cells were grown in the presence of EGF/FGF to form neurospheres and after 1-3 passages they were plated in Poly-D-Lysine/Laminin-coated dishes, deprived of EGF for 3h and exposed for 24 h to 1 μM fingolimod-p, which is the active metabolite of fingolimod *in vivo*. At the end of the treatment 10mM BrdU were applied to the cultures for 16 h in order to label all actively proliferating cells. Quantification of the percentage of BrdU+ cells at the end of the treatment revealed that fingolimod-p significantly increased the proliferative activity of NP/CS (Figure 3a). Since induction of MAPK pathway - initiated from activation of cell surface receptors - has been widely implicated in the actions of both endogenous S1P as well as of fingolimod's on various cell functions we would like to test its involvement on the effects we observed. Pre-incubation of culture media with pertussis toxin, an uncoupler of Gi from the enzyme adenylate cyclase, or a specific inhibitor of MEK1 (U0126) completely abolished the proliferative effects of fingolimod-p in postnatal hippocampal NS/PCs (Figure 3b). Furthermore, fingolimod-p was proved efficient to induce stimulation of ERK1/2 MAPK kinase in a pertussis sensitive manner (Figure 3C). The data above indicate that activation of S1P1 receptor and subsequent stimulation of MAPK pathway is responsible for the mitogenic effects of Fingolimod-p on postnatal neural stem cells.

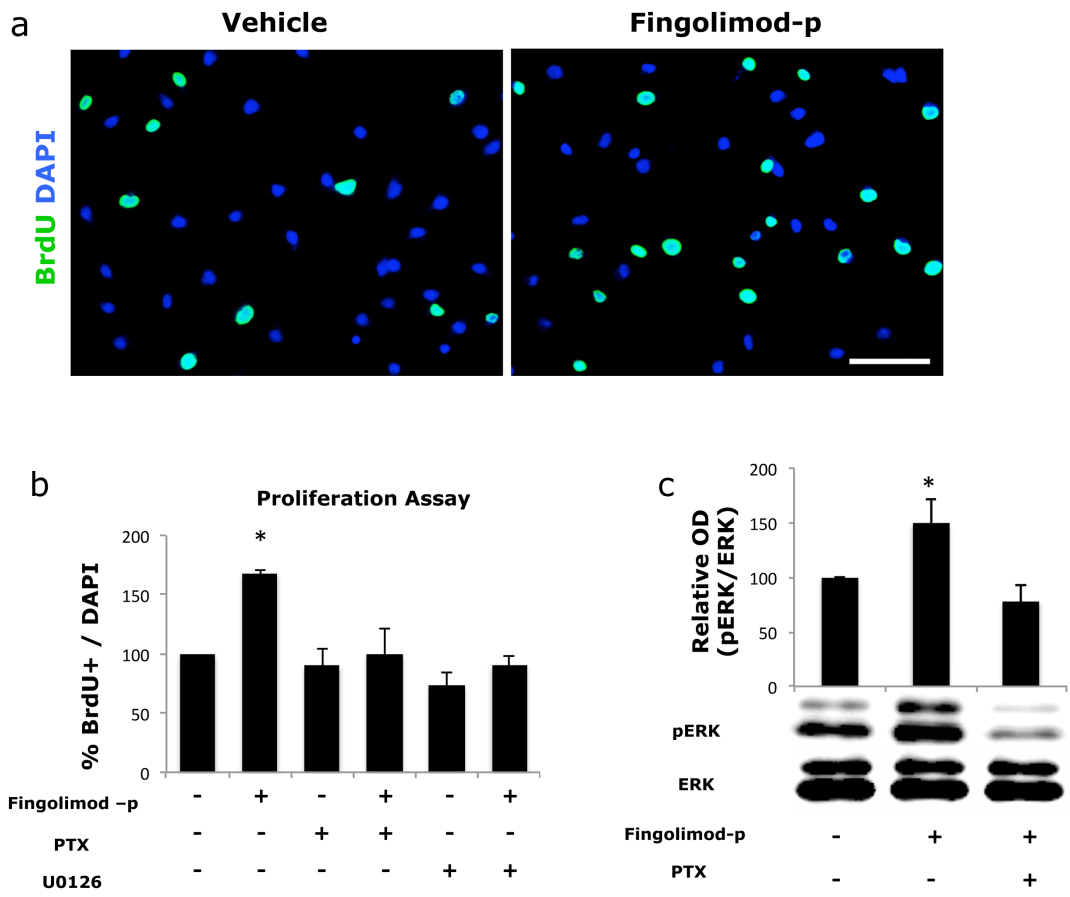


Figure 4: (a) BrdU staining of hippocampal neural stem cells after they have been treated for 24h as indicated in the absence of growth factors. Scale bar: 100 μM and **(b)** Percentage of BrdU+/DAPI+ (*mean ± SD; n = 3; *P < 0.05 vs vehicle treated cells*) **(c)** Phosphorylation of ERK1/2 after 10 min exposure of NS/PCs culture in growth factor free medium in the presence or absence of 1μM fingolimod–p after they have been pre-incubated or not with PTX. Western blot analysis was performed and relative optical density (OD) of phosphorylated ERK 1/2 (pERK) to this of total ERK 1/2 (tERK) was estimated and normalized to control values (*mean ± SD; n = 3; *P < 0.05 vs vehicle-treated cells*).

Fingolimod affected the survival of NS/PC cells via an induction of BDNF expression.

BDNF as we discussed above is a major factor that supports the growth of neurons during development of the nervous system and regulates synaptic plasticity and survival of newborn neurons during adulthood. Recent findings have shown that treatment with fingolimod increases the expression of BDNF in neurons *in vitro* and *in vivo*, promoting thus their survival (Deogracias et al., 2012). On the other hand

NSCs are capable of secreting BDNF themselves.25,26 In accordance to the already known actions in neuronal cells I showed using elisa quantification of BDNF in postnatal hippocampal neural stem cells in culture increased production of this neurotrophin after 24h of treatment with 1μM fingolimod-p. The use of MEK inhibitor U0126 did not block this effect (Figure 4b). In addition to this, fingolimod-p was able to decrease apoptosis rate of NS/PC after growth factor withdrawal for 72h (Figure 4c), as it was revealed by TUNEL staining (Figure 4a). The prosurvival effects of fingolimod were attenuated by the use of an anti-BDNF neutralizing antibody showing that increased BDNF expression after fingolimod treatment is sufficient to protect these cells from apoptosis after growth factor withdrawal.

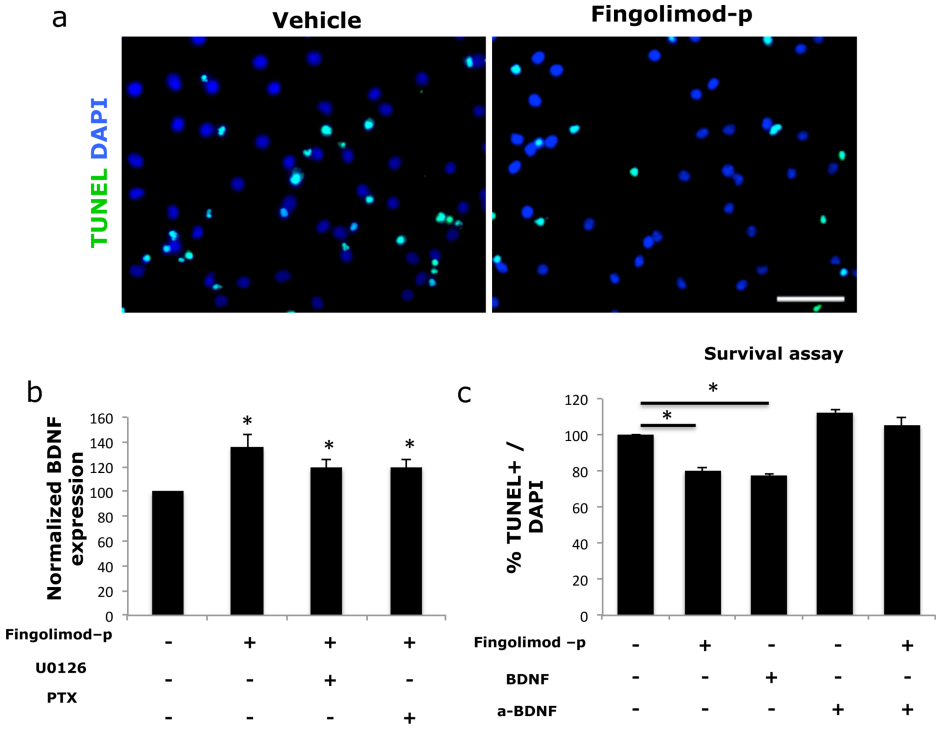


Figure 5: (a) TUNEL staining of hippocampal neural stem cells after 72h treated as indicated in the absence of growth factors. (b) BDNF protein levels quantified by ELISA in lysates of hippocampal NSC cultures treated with 1 μM fingolimod–p or not in the presence or absence of U0126 or perts toxin for 24h (mean ± SD; n = 3; *P < 0.05 vs vehicle treated cells). (c) Percentage of TUNEL+/DAPI+ (mean ± SD; n = 3; *P < 0.05, vs vehicle treated cells). Scale bar: 50μM

Fingolimod treatment improved contextual fear memory.

We next wanted to investigate if elevated neurogenesis by fingolimod treatment could be correlated to a behavioral outcome such as an improvement of memory function. In order to test this, we used a context discrimination paradigm, a cognitive task that has been shown to reflect the functional role of adult hippocampal neurogenesis. According to this, mice were placed in the training chamber and received a mild foot shock. The next 2 days mice were returned either to the same context (context A) or to a slightly modified one (context B) and they were evaluated for their freezing behavior. Mice that have been treated with Fingolimod for 2 weeks displayed significantly increased discrimination ratio (Figure 5c) (-0,05 ± 0,03 Vs 0,17 ± 0,05, N=13, mean ± SEM, $p<0.05$) and less freezing in context B (Figure 5a) (32,7% ± 3,5 vs 22,6% ± 3,7 % freezing, N=13, mean ± SEM, $p<0.05$) compared to context A during the first day post training. Fingolimod treatment did not affect freezing neither in context A or context B (Figure 5b) in non-shocked animals.

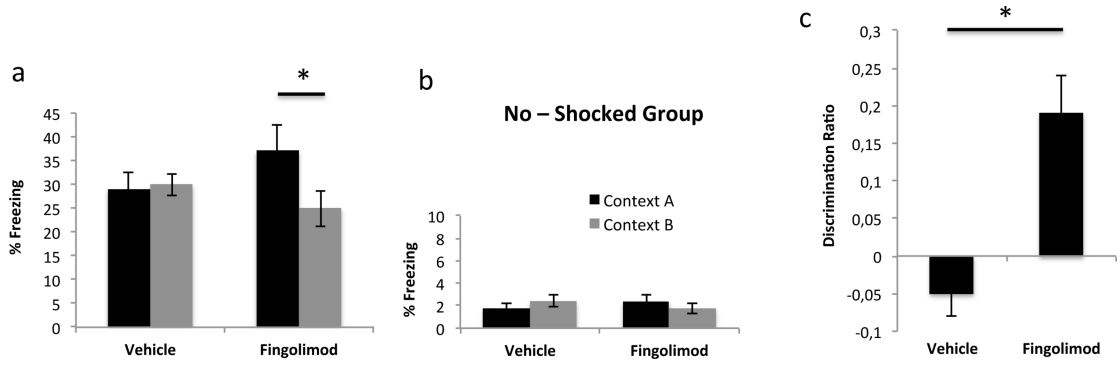


Figure 6 : (a) Percent of time freezing in context A (black bars) and context B (grey bars) one day after fear conditioning, for mice that were treated with either vehicle or Fingolimod for 14 days. (b) Percentage of freezing in context A and B for mice that have not received shock during the training day. (c) Discrimination ratio, which represent (freezing in context A – freezing in context B) to (freezing in context A + freezing in context B) (mean ± SEM; n = 13; *P < 0.05, t-test compared with vehicle).

BNN27 reduces apoptosis after NGF deprivation in neurons *in vitro* and *in vivo.*

The NGF mimetic properties of the DHEA analog, BNN27, were tested in NGF dependent neuronal populations such as SCGs and DRGs of the peripheral nervous

system for which NGF is necessary for their proper development during late embryogenesis and early post-natal life (Crowley et al., 1994; Smeyne et al., 1994). Sympathetic neurons were isolated from superior cervical ganglia of P1 rats and cultured in the presence of 100ng/ml NGF for 7 days to form an extensive network. The antimitotic agent FDU was also added to the medium in order to eliminate any non-neuronal cell in the culture. After 1 week cells were washed and switched to medium without NGF containing also a monoclonal anti-NGF neutralizing antibody with or without the addition of 100nM BNN27. 48h later cells were fixed the rate of apoptosis was quantified using TUNEL staining. BNN27 significantly reduced apoptosis compared to anti-NGF condition from 63%±3.6 to 43±3.2. Moreover, the anti-apoptotic effects of BNN27 were abolished with the addition of a selective TrkA inhibitor (64%±3.17).

The percentage of apoptotic neurons was correlated also with the levels of phosphorylated form of the proapoptotic protein JNK (Jun- amino- terminal kinase) after 18h of NGF deprivation. JNK, a kinase of MAPK pathway is activated in NGF dependent populations after in absence of the neurotrophin phosphorylating downstream c- JUN, triggering in this way apoptosis by upregulating the expression of pro- apoptotic genes (Estus et al., 1994, Palmada et al., 2002).

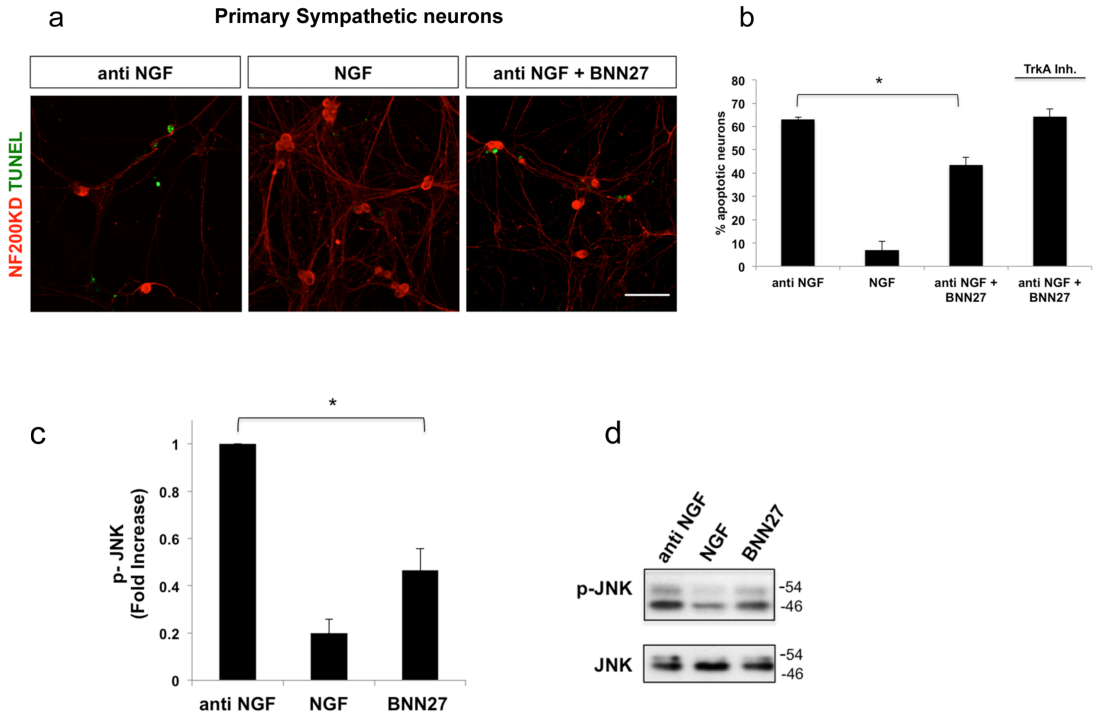


Figure 7: Sympathetic neurons, from superior cervical ganglia (SCG) isolated from P1 rats were cultured for 7 days in the presence of NGF (100 ng/ml), then they washed and cultured for 48h in either in the same medium or in NGF-free medium in the presence of a NGF polyclonal neutralizing antibody with or without 100 nM BNN27 and in the absence or the presence of TrkA inhibitor. Neurons were stained with an antibody against NF200kD (Red) and with TUNEL (Green). Over 300 neurons were counted in six to seven randomly selected optical fields and the percentage of TUNEL+ , apoptotic neurons for each condition is presented as mean from three (3) separate experiments (*P<0.01 relative to anti-NGF condition). C. In different cultures treated in the same way the activity of JNK was assessed by western blot analyses, 18 h after NGF deprivation. Scale bar represents 100 μM

BNN27 was also capable to delay apoptosis in DRGs of NGF null mice embryos when administered to pregnant mother from day E6.5 until E13.5. At this age, TrkA starts to abundantly been expressed in a subpopulation of sensory neurons of the DRG, which becomes dependent on NGF for their survival (Crowley et al., 1994) and as a result, it is observed a dramatic loss of these neurons in NGF null mice through induction of programmed cell death (White et al., 1996). BNN27 administered to pregnant mothers heterozygous for the deletion of NGF resulted in less caspase-3 positive neurons in the DRGs of *ngf null* embryos at E13,5 compared to homozygous mice for NGF deletion that were not treated with BNN27 during gestation. However, at E17,5 most of TrkA positive neurons have been lost despite the presence of BNN27.

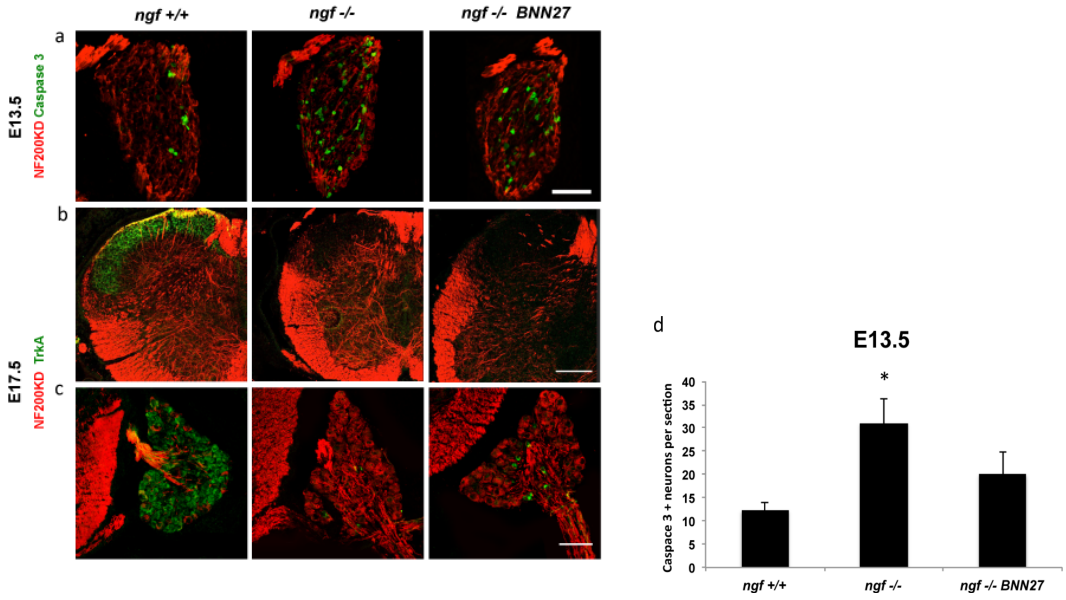


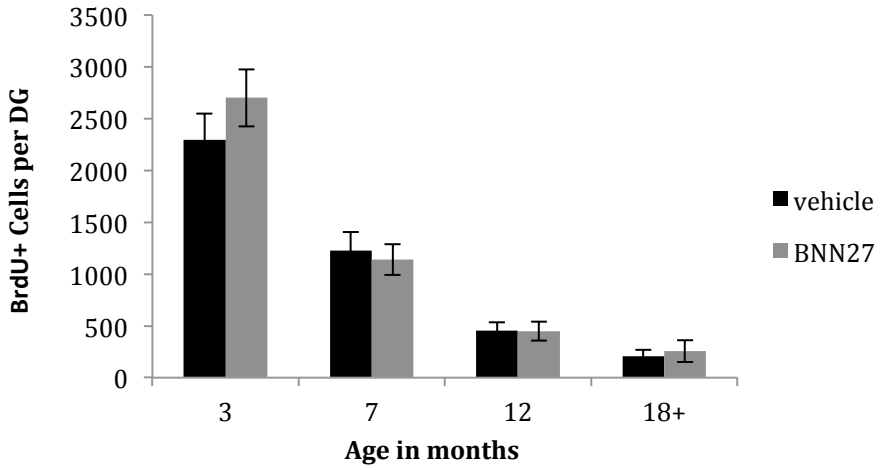
Figure 8: Mice heterozygous for NGF deletion were crossed and pregnant mothers were treated from E6.5 until the indicated embryonic day with either saline or 100mg/kg BNN27 per day. (a) Caspace-3 (green) positive neurons in the area of DRGs from E13.5 embryos of the indicating genotype treated or

not with BNN27. (b) Trka staining (green) on DRGs of E13.5 and (c) E17.5 embryos for the respective genotype and treatment. (d) Number of caspase-3 positive neurons per section in E13.5 embryos treated or not with BNN27 (Mean ± SD; n= 7,* P< 0,05)

BNN27 does not induce neurogenesis in the adult dentate gyrus of C57/BL6 mice.

Both DHEA and NGF have been shown to promote the survival of newborn neurons in the adult hippocampus (Karisma et al., 2002, Frielingsdorf et al., 2007). In order to test the hypothesis that the synthetic neurosteroidal analogue BNN27 possesses any pro-neurogenic properties we administered the thymidine analog BrdU in groups of mice of different ages (from 3 to 18 months of age) for the last 5 days of a 2-week treatment period with either the vehicle or BNN27. Steorological counts of Brdu+ cells in the SGZ of the DG have revealed that BNN27 was not capable to increase the proliferation rate of adult neural stem cells in no one of the ages tested.

a



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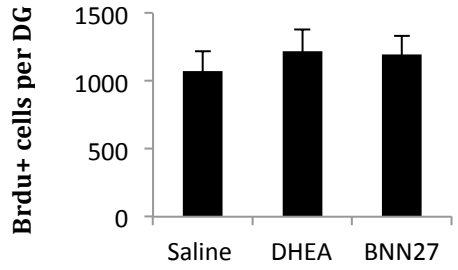


Figure 8: Total Brdu+ cells in the DG of C57/BL6 mice of the indicative age that were treated intraperitoneally for 14 days with either the vehicle (5% EtOH) or BNN27 and were also injected with

100mg/kg BrdU for the last 5 days (a) or the first 5 days (b). At the end of the treatment period mice were sacrificed and their brain processed for immunofluorescence for Brdu (Mean ± SD;n=7; P>0.05).

In addition to this, neither DHEA nor BNN27 were proven capable to increase the survival of adult NSCs of the SGZ, in 3 months C57/BL6 mice.

BNN27 attenuates neurogenic deficits in 5XFAD mice.

Altered neurogenesis is an early common manifestation of AD in both animal models and human patients (Mu and Gage 2011) and was associated with the accumulation of the neurotoxic Aβ amyloid. Pharmacological induction of hippocampal neurogenesis in this case might help to compensate for neuronal lose in this area and alleviate cognitive symptoms related to AD pathology. Despite the lack of efficacy of BNN27 to induce neurogenesis in the intact adult brain I wanted to further investigate if BNN27 was capable to restore neurogenesis under a neurodegenerative condition. To this aim I used a mouse model of amyloidosis observed in patients with AD, the 5XFAD mice, which carry 5 mutations linked with the familial form of the disease. These mice develop amyloid pathology, as well as neurogenic deficits (Moon et al., 2014), from 1,5 month of age accompanied by neuroiflammation and neuronal cell death in the area of subiculum, cortex and hippocampus at later stages (Oakley et al., 2006). BNN27 was administered in 1,5 months old 5XFAD mice and WT littermates in the form of subcutaneous implanted pellet that release 3 mg of the substance per day for 60 days. Control mice were also implanted a placebo pellet. Stereological quantification of DcX+ cells in the DG revealed significantly reduced number of newborn neurons in the SGZ of the DG in 5XFAD compared to WT mice of the same age. However, BNN27 almost reversed this reduction to the levels of WT mice albeit it did not have any effect in neurogenesis in intact mice as it was expected.

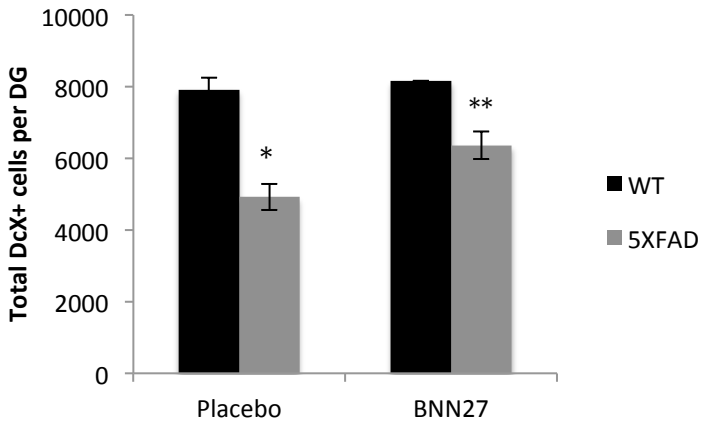


Figure 9: 5XFAD mice and WT littermates were implanted a BNN27 pellet releasing 10mg/kg/day or a placebo pellet at 1,5 months of age (before the manifestation of amyloid pathology) and were sacrificed 2 months later. DcX positive cells in the DG were stereologically counted in 10 section through the rostrocaudal axis of hippocampus and multiplied by the total number of sections per hippocampus to estimate the total number of cells. (Mean ± SD; n=5;*P<0.05, **P<0.05 Vs 5XFAD vehicle treated)

BNN27 rescues cholinergic atrophy in basal forebrain of 5XFAD mice.

Another hallmark of AD is the neurodegeneration of basal forebrain cholinergic system that projects to the hippocampus and cortex and has been linked to deterioration of cognitive functions that appear in subjects affected by the disease (Hefti et al., 1986, Auld et al., 2002). The cholinergic neurons in the area of Medial septum (MS) and Vendral Diagonal Band of Broca (VDB) express both of the cognate receptors of NGF (TrkA and $p75^{NTR}$) and their activation is responsible for the maintenance of the cholinergic phenotype of these neurons by regulating expression of ChAT (choline acetylransferase), the necessary enzyme for Acetylocholine biosynethesis, as well as for their proper functionality. Furthermore, blocking NGF signaling results to cholinergic neuron atrophy AD phenotype including development of amyloid pathology (Capsoni et al., 2011, chen et al., 1997) while administration of NGF to rodents and humans promotes regeneration of axotomized cholinergic neurons in this area and alleviates cognitive symptoms (Kordower et al., 1994, Tuszynski et al., 1990).

Taking into consideration the agonistic effects of BNN27 on NGF receptors I sought to examine if our synthetic analogue BNN27 possesses any possible neurotrophin-like

properties on this system. In addition to this, the neurogenic effects of NGF in adult hippocampus have been attributed to an increase in cholinergic activity (Frielingsdorf et al., 2007). Initially analyzing the phenotype 5XFAD mice I found that there is a significant reduction in the soma size of neurons in the area of MS stained against ChaT, compared to WT of the same age and BNN27 reversed this atrophy. However, more animals are needed in order to draw a safe conclusion.

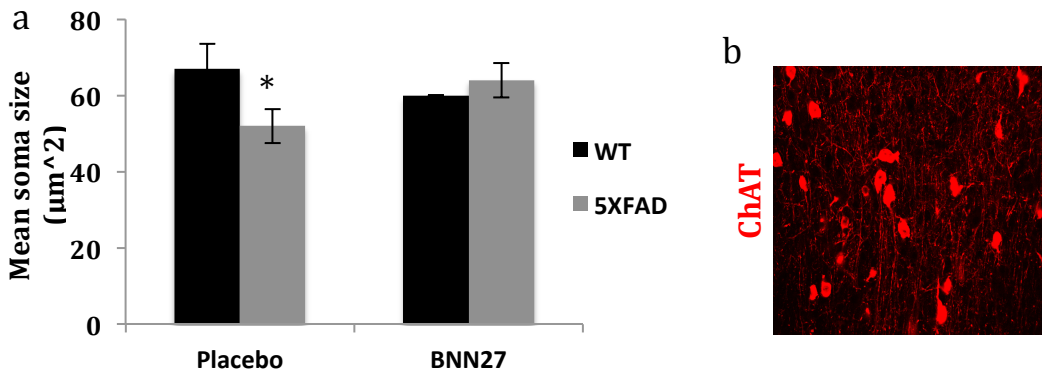


Figure 10: The mean area of somata of neurons in the area of medial septum stained against Chat **(b)** was estimated using image j in 4 sections been 200µM apart and the mean was calculated for its condition **(a)** (Mean ± SD; n=5,*P<0.05)

BNN27 reduces amyloid burden in 5XFAD mice.

Finally, aiming to further explore the potential therapeutic role of BNN27 in AD I sought to determine if treatment with BNN27 affects amyloid pathology in the 5XFAD mice. The aβ amyloid burden in the hippocampus was quantified by counting the number of plaques in the area after immunostaining with an antibody against aβ-amyloid. By 3,5 months of age at the end of the experiment 5XFAD mice had widespread plaques covering the area of the DG. Transgenic mice that were treated with BNN27, however, showed significant less number of plaques.

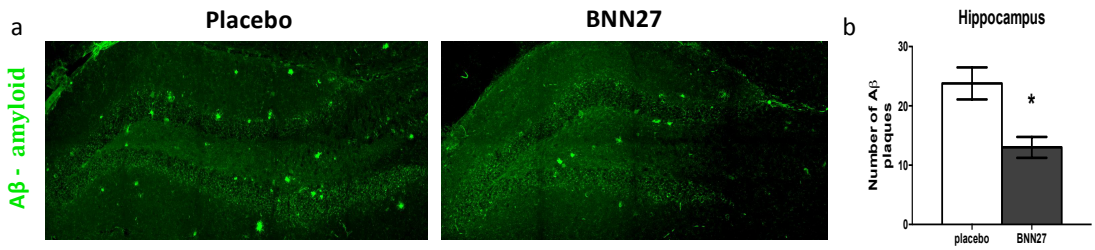


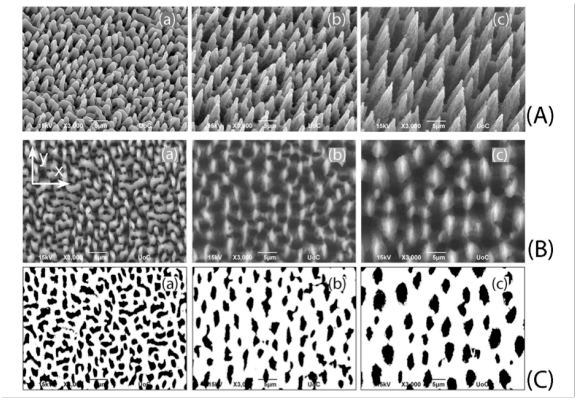
Figure 10: 5XFAD mice and WT littermates were implanted a BNN27 pellet releasing 10mg/kg/day or a placebo pellet at 1,5 months of age (before the manifestation of amyloid pathology) and were

sacrificed 2 months later. The number of plaques stained with a monoclonal antibody against $A\beta_{17-24}$ **(a)** were counted in 5 random sections throughout the rostrocaudal axis of the hippocampus of 5XFAD mice and the mean was estimated **(b)** (Mean ± SD; n=6,*P<0.05).

Characterization of microconically structured Si surfaces

Culture substrates were manufactured by ultra-short pulsed laser structuring of crystalline silicon (Si) wafers. This technique offers the advantage of patterning Si surfaces with periodic arrays of topographical features of microscale size (spikes), while offering high accuracy and reproducibility (Stratakis et al., 2012). Upon increasing the laser energy (fluence), three types of microconical morphologies exhibiting different geometrical characteristics (denoted as low, medium and high roughness substrates, respectively) were formed (Figure 1A).

Top SEM views revealed an arbitrary shaped cross-section of the microcones (MCs) at low fluences that became almost elliptical as the laser fluence increased (Fig. 1B). In order to determine the directionality of the attained microstructures, the orientation angle of each MC with respect to the vertical (y) axis of the image plane was statistically determined from SEM micrographs (Figure 1C-D). According to this analysis, low roughness Si substrates displayed a broad range of orientation angles (23, 26, 24 and 27% of the microcones had angles in the range of 0-10 ^{o}, 10-30^{o}, 30-60^{o} and 60-90^{o}, respectively). On the contrary, the majority of conical features in the medium and high roughness substrates (68% and 79 % respectively) were mostly showing a parallel alignment (oriented at an angle of 0-10^{o}) (Figure 1D). Therefore, although at the lower fluence the microstructures did not seem to follow a preferred orientation, the increase of laser energy resulted in a directional orientation of MCs, described as semiperiodical discontinuous topography with anisotropic features (the major axis of the MCs' elliptical cross-section are parallel aligned).



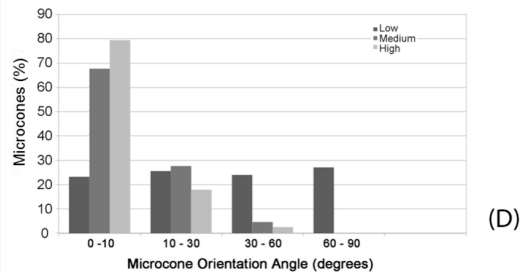


Figure 1: Quantitative evaluation of MCs directionality. (A) Scanning electron microscopy images (Tilted view) of micropatterned Si substrates of different roughness: low (a), medium (b) and high (c). (B) Scanning electron microscopy images (Top view) of micropatterned Si substrates of different roughness: low (a), medium (b) and high (c). (C) The same images after thresholding and binarizing. (D) MC directionality expressed in terms of the orientation angles' (frequency) distribution. The orientation angle of the MCs was measured as the angle between the major axis of the elliptical cross-section and the y-axis of the image plane. The number of features exhibiting an orientation angle value within a specific range is expressed as percentage of microcones. At least 60 spikes for each roughness type have been used for the analysis.

Based on this topographical model (i.e. ellipitical microcone), the specific geometrical characteristics of each structure have been accordingly calculated from SEM image analysis. Thus, increasing laser fluence, MCs' intercone distance correlated positively with height (R^2=0.947) and negatively with distance (R^2= 0.9839). MCs height and intercone distance varied from 1.26 and 2.59 μm in the low roughness structures to 8.63 and 6.50 μm in the high roughness structures, respectively, while MCs' density ranged from 10^6 to $10^7/cm^2$ [Table 1].

Table 1: Geometrical characteristics of the different substrates. *

Type of Roughness	**Density, D±STDEV (*$10^6/cm^2$)**	**Height, a ± STDEV (μm)**	**Base major axis, b± STDEV (μm)**	**Base minor axis, b´± STDEV (μm)**	**Interspike Distance, c± STDEV (μm)**
Low	9.75±1.54	1.26±0.28	1.96±0.35	1.11±0.20	2.59±0.72
Medium	5.01±0.19	3.76±0.42	3.57±0.43	1.98±0.34	4.71±0.37
High	2.50±0.26	8.63±1.17	7.25±0.72	3.78±0.77	6.50±0.84

* Geometrical characteristics of the different substrates used for this study, calculated out of scanning electron microscopy (SEM) images with the aid of image processing software (ImageJ). The mean values were calculated from at least ten individual measurements. The data were subjected to ANOVA followed by Tukey test for multiple comparisons between pairs of means. The difference in height between surfaces of low, medium and high roughness surfaces was highly significant ($p< 0.001$). The difference in base major axis and interspike distance between surfaces of low and high roughness surfaces was highly significant ($p< 0.001$).

Although MC roughness increase was correlated with surface hydrophobicity (Zorba et al., 2006), thermal treatment and/or subsequent protein coating rendered micropatterned surfaces superhydrophilic, while preserving the initial roughness (Simitzi et al., 2014). Therefore, surface topography was the sole variable parameter in the biological system studied here.

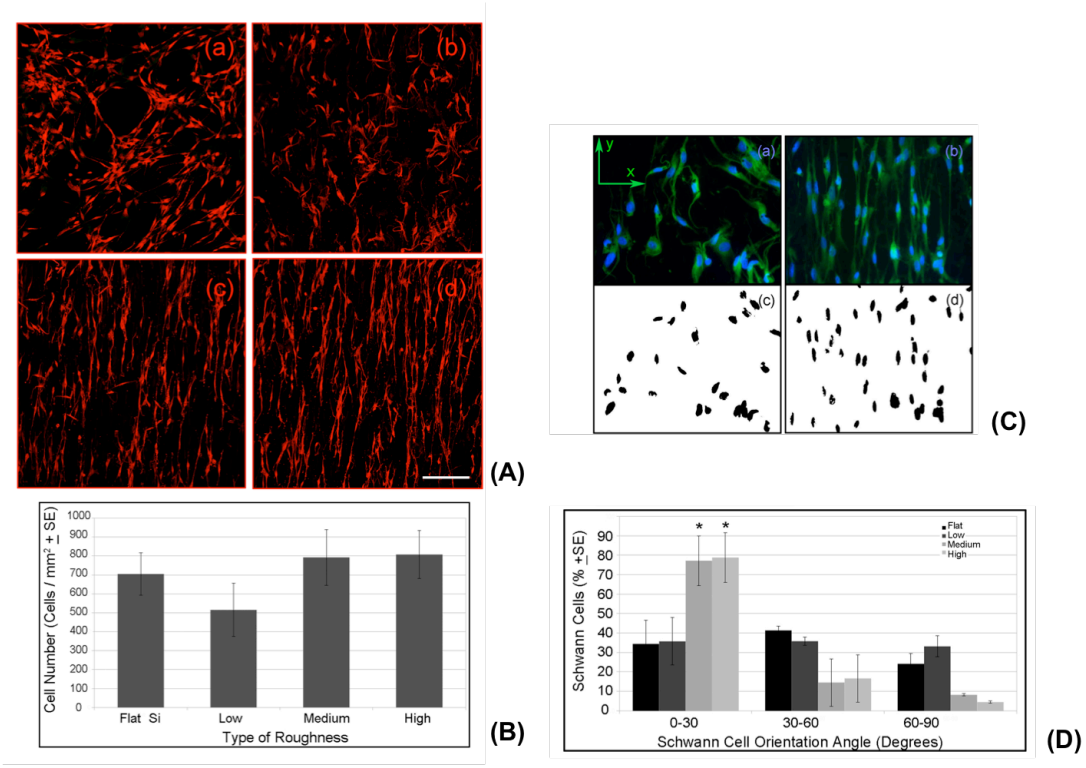


Figure 2: (A) Confocal microscopy images of S100b positive Schwann cells grown on different silicon substrates for 5 days of culture: Flat (a), low (b), medium (c) and high (d) roughness Si substrates. Scale bar: 150 μm. (B) Numbers of Schwann cells grown on flat Si and laser micropatterned Si substrates of different roughness for 5 days of culture. The results are expressed as cells/mm^2 ($\pm$ standard error of the mean, SE) and represent the means of 3 different experiments (n=30 fields of view for each roughness type). (C) Composite of fluorescence images showing both the entire Schwann cell body (Green) and its nucleus (Blue) on a micropatterned Si substrate of low (a) and high (b) roughness substrate. Thresholded and binarized images showing (c,d) was used for quantification of the orientation angle. (D) This was defined as the angle between the major axis of the ellipse of the nucleus and the vertical axis of the image plane. The number of cells exhibiting an orientation angle value within a specific range is expressed as percentage of cells $\pm$ standard error of the mean. The results represent the means of three different experiments. The significance level (p) was calculated using the student's t-test between flat and medium or high roughness Si substrates at the orientation angle range of 0-30 degrees (*: $p<0.05$).

Effect of surface roughness on Schwann cell growth and morphology

Previous studies have shown that microstructrured Si substrates could support growth and differentiation of PC12 cells, which represent a neuronal cell model terminally differentiating into neuron-like cells upon stimulation with nerve growth factor (Simitzi et a., 2014). In order to move into a more physiologically relevant system we used another type of a neural crest derived cells, the Schwann cells, which consist the glial cells of the PNS.

Primary Schwann cells isolated from embryonic sciatic nerves were seeded onto micropatterned Si substrates and upon culture termination cell growth was assessed both qualitatively (immunostaining with S100b antibody) and quantitatively (computer-based image analysis of DAPI nuclear staining). As shown in Figure 2 all three micro-patterned Si substrates could equally well support the growth of Schwann cells. A striking, however, observation was that the growth pattern of Schwann cells was markedly affected by the substrate microtopography. Although arbitrary cell growth takes place in the micropatterned surfaces at low fluences, cells tend to follow a preferential orientation during growth at substrates of medium and high roughness.
In order to quantify this phenomenon, the orientation of the cell nuclei was determined by measuring the angle between the major axis of the nuclear ellipse and the y-axis of the image plane (Figure 2C). According to this analysis, Schwann cells displayed a broad range of orientation angles on low roughness Si substrates (34.8, 34.8 and 32.4% of the cells were outgrown at angles of 0-30^{o}, 30-60^{o} and 60-90^{o}, respectively), a response similar to flat Si (34.4, 41.4 and 24.2% of the cells were outgrown at angles of 0-30^{o}, 30-60^{o} and 60-90^{o}, respectively). On the contrary, the majority of the Schwann cells on the medium and high roughness substrates were mostly aligned in parallel (77.2% and 78.8 % of the cells had an orientation angle of 0-30^{o} values, respectively) (Figure 2D). It was therefore clear that a directional cell outgrowth was dictated by the substrates of mid and high roughness. Interestingly, cell nucleus and cytoskeleton (Fig. 2C) were mostly aligned with the MCs' orientation, indicating that cells could sense the discontinuous directional topographical features at subcellular lengthscales.

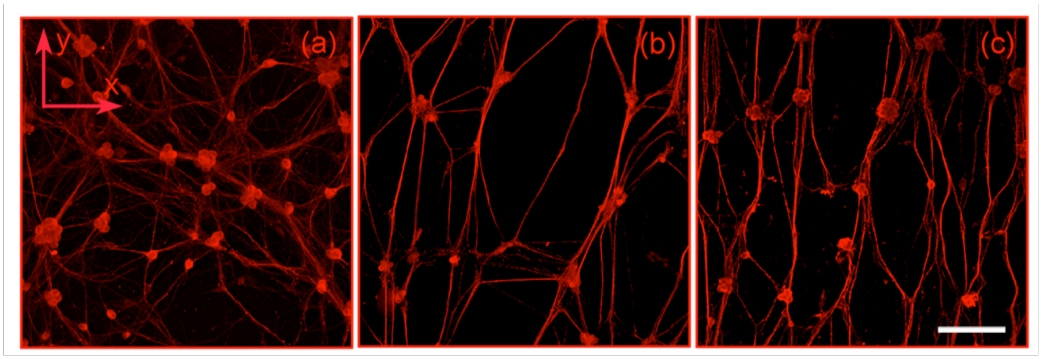
Effect of surface roughness on axonal growth and network formation of sympathetic dissociated neurons

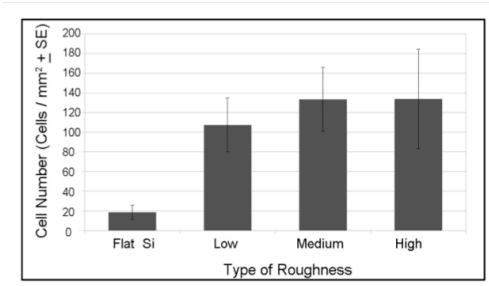
The effect of microconical topography on neurite outgrowth and network formation was evaluated using rat sympathetic neurons isolated from the superior cervical ganglia (SCG), which is the neuronal population arising from neural crest cells during development. In order to quantitatively evaluate cell outgrowth on the different substrates, scaffolds were stained with DAPI and the number of nuclei/mm^2 surface area was evaluated using image analysis (Figure 3B). While very few neurons were grown on the collagen-coated flat Si substrates, micropatterned Si substrates were able to support neuronal outgrowth.

More importantly, albeit sufficient neuronal outgrowth could be observed in all of the three micropatterned substrates (Fig. 3A) the axons on the low roughness substrates (Fig. 3Aa) were shown to grow randomly, whereas neurons on medium (Fig. 3Ab) and high (Fig. 3Ac) roughness substrates exhibited a directional growth giving rise to a network of parallel aligned axons. It should be noted that, although a limited number of neurons could grow onto micropatterned substrated that were not covered by a protein coating, the same effect of topography-guided axonal orientation was observed in this case as well (data not shown).

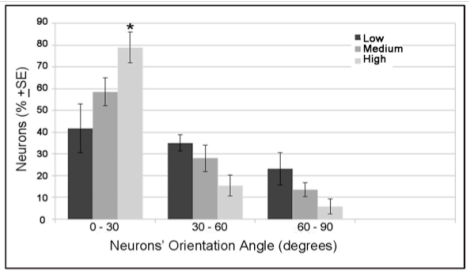
To further describe this oriented axonal outgrowth, the axon orientation angle, defined as the angle between the axonal vector and the y- axis of the image plane, has been measured (Figure 3C). Although the growth was very sparse, the neurons on the flat Si substrates were almost isotropically distributed (30.2, 35.2 and 34.6% of the neurons had an orientation angle of 0-30^o, 30- 60^o and 60- 90^o values, respectively). Neurons on the low roughness substrates showed a slight orientation preference at 0-30^o angles (41.7, 35.1 and 23.2% of the neurons had an orientation angle of 0-30^o, 30-60^o and 60- 90^o values, respectively). While neurons on medium roughness showed an increased preference to parallel alignment (58.5, 28.0 and 13.5 % of neurons had an orientation angle of 0-30^o, 30- 60^o and 60- 90^o values, respectively). Finally, the best conditions for directional growth were observed in the case of high roughness substrates (78.9, 15.3 and 5.8 % of neurons had an orientation angle of 0-30^o, 30- 60^o and 60- 90^o values, respectively). The results presented above indicate that neuronal outgrowth orientation was highly influenced by substrate morphology. It can be postulated that the development of the axon network is driven by the MCs preferential

orientation, which accordingly becomes more pronounced as the roughness increases. Conclusively, it appears that neurons as well as schwann cells can sense discontinuous directional topographical cues with sizes ranging at the subcellular lengthscale.





(B)



(C)

Figure 3: Effect of surface topography on neuronal growth. (A) Confocal microscopy images of neurofilament positive sympathetic neurons grown on low (a), medium (b) and high (c) roughness micropatterned Si substrates for 6 days. Scale bar: 150 µm. (B) Numbers of sympathetic neurons grown on collagen-coated flat and laser micropatterned Si substrates of different roughness for 6 days of culture (DOCs). The results are expressed as cells/mm^2 ($\pm$ standard error of the mean, SE) and represent the means of three different experiments. The significance levels (p) were calculated using ANOVA followed by Tukey test for multiple comparisons between pairs of means (n =30 fields of view for each roughness type and time of culture). The difference between surfaces each rough surface and the flat surface was highly significant ($p < 0.001$). (C) Quantitative evaluation of axonal outgrowth orientation. Axonal orientation is expressed in terms of the orientation angles' distribution. The orientation angle of each axon was measured as the angle between the axonal vector and the vertical axis of the image plane. The number of cells exhibiting an angle value within a specific range is expressed as percentage of cells $\pm$ standard error of the mean. The results represent the means of three different experiments. The significance level (p) was calculated using the student's t-test between low and high roughness Si substrates at the orientation angle range of 0-30 degrees (*: $p<0.05$).

Spatial relationship and type of interaction among neurons and the substrate.

SEM revealed that neurons were grown on top of the microcones. More specifically, they seem to grow in contact with the elliptical walls of the upper parts of the cones in high roughness substrates while they appear to contact only with the top of the tips. in low roughness. In accordance the majority of axons take the form of a “tensed rope” that crosses between the cones with only some neuritic extensions exploring the space between the spikes in the high roughness in contrast with low rougness substrates on which the axons grow randomly, some times even over the cones, forming a more messy network. Moreover of note is that while Schwann cells adopt their shape to the imposed topography acquiring a more splindle like morphology SCG neurons keep its round form.

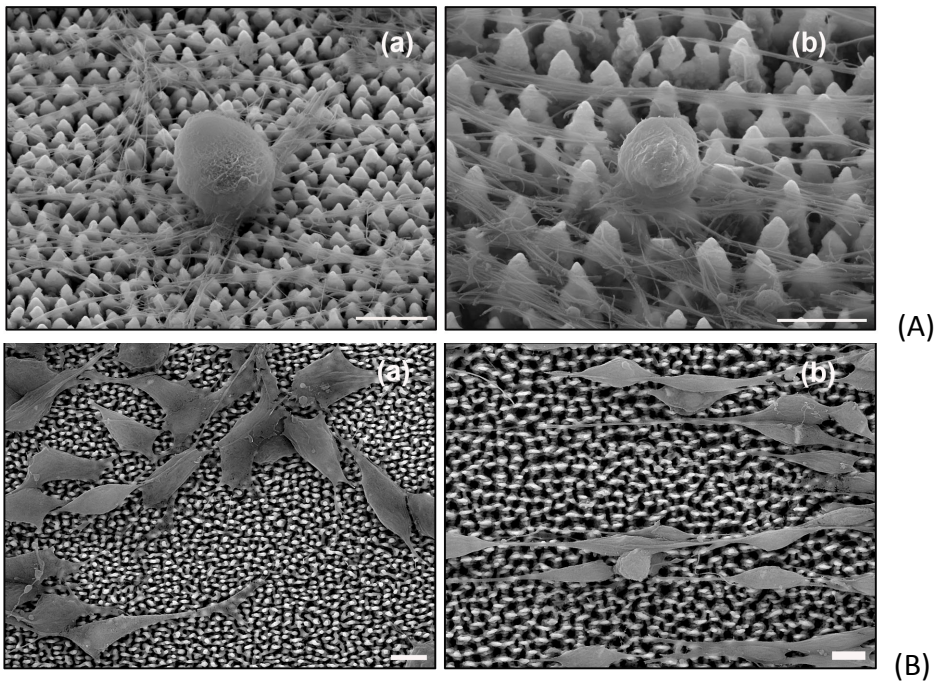


Figure 4 : Tilted views of scanning electron microscopy images of SCGs **(A)** on low **(a)** and high roughness **(b)** micropatterned substrates. Top views of scanning electron microscopy images of Schwann cells **(B)** on low **(c)** and medium **(d)** roughness micropatterned substrates. Scale bar: 10 μm.

Coculture of SCGs and Schwann cells on microconically structured Si surfaces.

It is well documented that during development or in response to injury, Schwann cell supports axonal outgrowth (Mahanthappa et al., 1996; Pearse et al., 2004). In order to examine if Schwann cells were capable to guide the neurite outgrowth of SCGs the latter were cultured on substrates pre-seeded with Schwann cells. As shown in Figure 5, Schwann cells exhibit a directional growth onto the anisotropic medium roughness substrates, while at the same time SCG neurons cultured on top of them followed their preferential orientation.

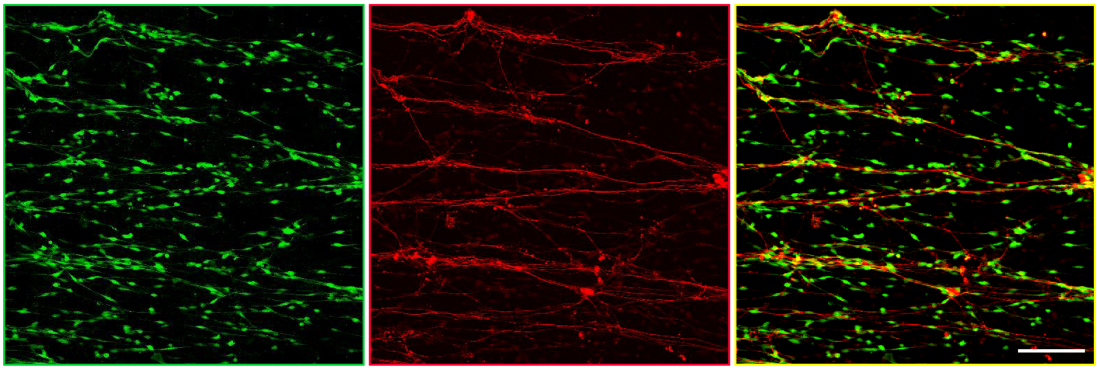


Figure 5: Effect of surface roughness on neuronal outgrowth. Confocal microscopy images of S100b positive Schwann cells (green) and Neurofilament positive sympathetic neurons (red) grown on medium roughness micropatterned Si substrates. Scale bar :150μm.

Effect of surface roughness on Schwann cell migration and neurite outgrowth in DRG explants.

In a similar vein, we studied the interaction among Schwann cells with neurons, grown in our substrates using in a naturally occurring coculture system, which is the whole DRG explant. In this case, flat Si surfaces could not support whole ganglion explant outgrowth with or without pre-coating, while laser micropatterned surfaces allowed DRG growth, even in the absence of protein coating.

On the low roughness substrates, however, the cells and the axons migrated away from the DRG explants in an isotropic manner (Figure 6a), while on medium and high roughness substrates, the Schwann cell migration and axon growth followed a directional orientation (Figure 6b). Moreover, Scanning electron and confocal microscopy analysis revealed that most of the axons were grown on top of underlying

Schwann cells that were oriented parallel to the anisotropic MCs (Figure 6c). It is therefore postulated that the elliptical MCs of medium and high roughness substrates dictate a virtual path to Schwann cells, which in turn guide axonal outgrowth.

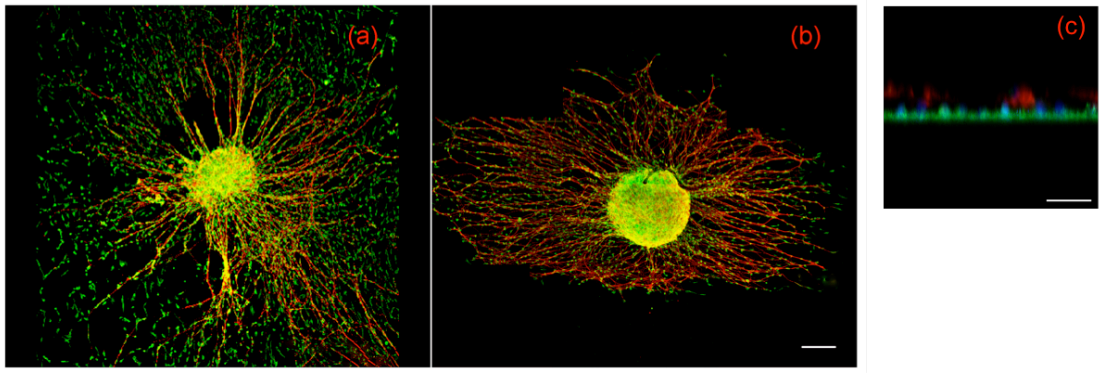


Figure 6: Confocal microscopy images of whole DRG explants on low (a) and high (b) roughness micropatterned Si substrates and (c) XZ optical section depicting the spatial relationship of the two cell types in Y axis. S100 positive Schwann cells and Neurofilament positive sympathetic neurons are labeled with green and red, respectively. Scale bar: 300 µm and 5 µm.

Schwann cells are known to modify their environment by synthesizing and secreting extracellular matrix (ECM) components, such as vitronectin, laminin and fibronectin (Bunge et al., 1978). Indeed, staining with a fibronectin-specific antibody showed that in a DRG explant grown on a micropatterned Si substrate of high roughness (Fig. 7A), axons developed within an interconnected network of Fn fibrils following, most of the times, their directional orientation (Figure 7B).

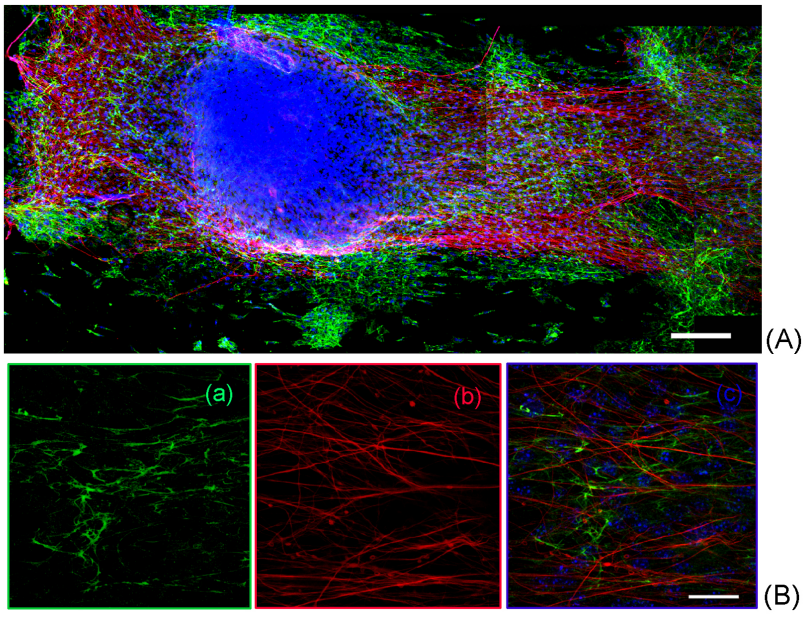


Figure 7: Fibronectin organization and axonal outgrowth on medium roughness micropatterned Si

substrates: **(A)** Representative montage of confocal microscopy images of Neurofilament (NF) positive sympathetic neurons (red), fibronectin (green) and nuclei (blue) on medium roughness micropatterned Si substrates. Scale bar: 150μm**. (B)** Higher magnification confocal microscopy images of Fibronectin (Fn) **(a)** and Neurofilament positive sympathetic neurons **(b)**. Co-staining of Fn, NF and TOPRO (c).

Effects of surface roughness on cortical NSCs

Neural stem cells have been also shown to respond to topographical cues of their microenviroment which have an impact in their morphology (Lim et al., 2010; McNamara et al., 2010) or its differentiation capacity. In order to test the influence of our substrates on an undifferentiated multipotent cell population we used neural stem cells of the embryonic cortex. Whole neurospheres were seeded to substrates of different roughness that were coated or not with adhesive proteins PDL/Laminin. After 1 week of culture in the presence of EGF and FGF neurospheres were alive but did not grow in substrates without coating (Figure 8A). However, in coated substrates neural stem cells started to migrate from the sphere capturing all the surrounding area independent of surface roughness though in high roughness (Figure 8B) they exhibited an oriented pattern of migration showing that embryonic cortical NSC cells sense the microscale topography of the microcones.

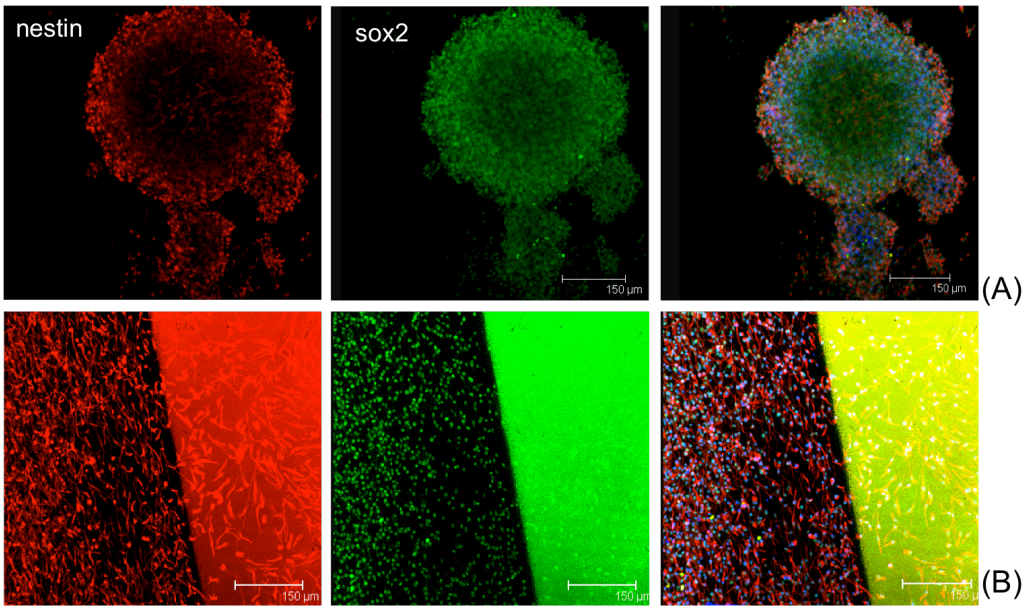


Figure 8: E13,5 cortical neural stem cells seeded on high rougness silicon substrates without **(A)** or with **(B)** the presence of PDL/Laminin coating and stained against markers of NSCs.

DISCUSSION

Despite the advances that have been made in the understanding of the molecular mechanisms underlying neurodegenerative diseases, the results of the attempts for the development of adequate therapeutics are so far discouraging. One of the reasons for this might be that current drugs, claiming to prevent cell death, are insufficient to compensate for the extensive neuronal loss observed in most of neurodegenerative conditions or they have been applied in a an irreversible stage of the disease. Neurogenesis, which is the ability of the adult brain to continuously generate new neurons during adulthood, in this case, could hold promise as a new therapeutic strategy for the treatment of neurological disorders. The new neurons arise from undifferentiated neural stem cells with the ability to proliferate that lie in specific regions of the murine as well as of human brain and have been found to respond to changes in cellular microenviroment under pathological conditions. Moreover, the proliferation and survival rate of neural stem cells that determines the number of new neurons that are produced is regulated by a plethora of intrinsic and extrinsic stimuli thus it is amenable to pharmacological manipulation.

However, molecules such as growth factors that have been proposed to promote neuroprotection and neuroregeneration in animal models usually yield adverse effects (Svensson et al., 2008, Takahashi et al., 1990) or they cannot be easily administered to patients so as to test their efficacy. For this reason, modern pharmacology is seeking for clinically relevant solutions such as small molecules that can easily penetrate the BBB and act on specific types of cells in the CNS.

Fingolimod is the first oral disease-modifying agent approved for multiple sclerosis, due to its ability to block the egress of autoreactive T cells from lymphoid nodes. Since this is a drug already in the market its safety profile is known and it is quite well tolerated in doses that are proposed (Brinkmann et al., 20100. Its mechanism of action lies on the sustained activation and internalization of S1P1 receptor on these cells. However, more recent evidence support the idea that fingolimod retains its functionality inside the CNS, where S1P1 receptor is expressed by many cell types, and more specifically that confers neuroprotection by increasing the secretion of BDNF which is a key regulator of brain plasticity during adulthood (deogracias et al., 2012). In a similar vein, I found that S1P1 is also expressed by adult NSCs of the dentate gyrus and fingolimod is able to increase both the proliferation and survival of

these cells. In accordance to this, another recent study has shown that isolated quiescent NSCs of the SVZ are rich in mRNA for receptors of S1P implicating a role for this sphingolipid in regulation of stem cell quiescence. Conclusively these findings lead us to speculate that fingolimod might force quiescent neural stem cells to enter the cell cycle and proliferate in response to sustained activation and internalization of S1P1. Indeed fingolimod-p the active metabolite of fingolimod *in vivo* was found efficient to induce stimulation of ERK, which is widely implicated in this process (Pages et al., 1999; Brunet et al., 1999). This action was sensitive to Pertussis toxin, which means that was mediated through activation of a GPCR receptor, coupled to a Gi protein, which probably is the S1P1. In contrast, PTX as well as the ERK1 inhibitor MEK1 did not abolish the effects of fingolimod on survival of NSCs which were blocked however by the use of an anti-BDNF neutralizing antibody. Since NSCs are known to secrete BDNF themselves (Barnabe-Heider et al., 2003), the latter may act as autocrine survival factor for these cells. This mechanism might involve intracellur targets and not the surface receptor since endogenous S1P as well as its analogue fingolimod-p binds to histone deacetylase HDAC1 and HDAC2, inhibiting their enzymatic activity and thus enhances histone acetylation. (Hait et al., 2011 & 14). This in turn results in induction of gene expression of various transcription and growth factors including BDNF.

Moreover, the cellular effects in the production of new neurons after treatment with fingolimod, were correlated to increased performance in a cognitive task which reflects the function of adult hippocampal neurogenesis. This was the context discrimination after fear conditioning that has been used to reveal the important role of DG in pattern separation, which is the ability to discriminate between similar subjects. Deficits in this function have been linked to generalization of emotions associated with a conditional stimulus, like the testing chamber in our case, after a stressful event, which was a mild footshock. This condition might also be the case in PTSD and increasing neurogenesis by pharmacological or environmental ways has been proposed as a therapeutic strategy for this disorder. According to this, fingolimod might be proved beneficial to people suffering from it. Interestingly this has also been proposed from another research group that which showed that fingolimod facilitates deletion of established fearful memories; an effect that has been associated to an epigenetic induction of brain plasticity mechanisms (Hait et al., 2014). In addition to the previous, another recent study has also suggested

neurogenesis related behavioural effects of fingolimod and specifically that confers antidepressant-like activity in stressed animals by inhibiting deacetylase activity (Nuzzo et al., 2015). Although the authors, in contrast to our results, did not detect any increase in neurogenesis in non-stressed animals, this might occur because of differences in the experimental design and particularly due to the fact that they examined neurogenesis 12 days after the end of fingolimod treatment.

In MS neurogenesis is upregulated at the first stages of the diseases albeit newborn neurons fail to survive for longer periods, as it has been observed for other neurodegenerative conditions. (Giannakopoulou et al., 2013; Chang et al., 2008; Gomez-nikola et al., 2014). In addition to this, MS is accompanied by cognitive deterioration due to neuronal loss and atrophy in various brain regions including the hippocampus. The manifestations of these deficits include memory loss and depression (Czerniawski et al., 2014; Siegert et al., 2005). In this context, fingolimod, due to its pro-neurogenic effects might be proved more useful in controlling these negative symptoms, compared to other therapies in MS. In addition to this, the above results suggest that fingolimod consists a drug candidate for alleviation of cognitive symptoms in other than MS neurodegenerative diseases.

In the other part of my work I occupied with investigation of potential neuroprotective and neurogenic properties of new steroidal molecules, called microneurotrophins. These novel and patented from our lab molecules, were synthesized based on C17-modifications on Dihydroepiandrosterone (DHEA) structure, an endogenous neurosteroid with well known neuroprotective properties (Charalampopoulos et al, 2004 and 2008). The DHEA analogues that were selected from a bigger library of molecules, retain the anti-apoptotic actions of DHEA but are devoid of the estrogenic and androgenic activity of DHEA, which compromise its clinical use due to the increased risk for the development of hormone-related carcinomas. Our team has previously published that DHEA mimics some of the actions of NGF after binding and activation of its cognate receptors, namely TrkA and p75NTR (Lazaridis et al., 2011).

In this study I investigated further the molecular mechanisms underlying the neuroprotective actions and the pro-neurogenic potential of one of these microneurotrophins, called BNN27, which has been found to be the most efficient in reducing apoptosis of PC12 cells after serum deprivation (Calogeropoulou 2009). Similarly to the parent molecule DHEA, BNN27 shows high binding affinity to both

TrkA and $p75^{NTR}$ (with a Ki of 1.86±0.4 nM and 3.9±1.2 nM respectively, n=6) and efficacy to activate TrkA and $p75^{NTR}$ initiated signaling pathways such as this of MAPK and RhoGDI kinases, respectively (Pediaditakis et al., 2015). Moreover, I showed using primary sympathetic neuron isolated from rat SCGs, that BNN27 was capable to protect NGF dependent populations *in vitro*, from NGF deprivation induced cell death, through activation of TrkA receptor. The antiapoptotic effects of BNN27 were also tested *in vivo*, in NGF null embryos, which exhibit massive cell death in the DRGs during embryonic days E13,5 to E17,5 when TrkA is expressed in these populations. BNN27 reduced apoptosis rate in the DRGs, as it was revealed after quantification TUNEL+ cells at day E13,5, but was incapable to support survival and finally rescue TrkA positive neurons when E17,5 embryos were examined. Indeed, NGF is physiologically secreted in axon terminals and is retrogradely transported to soma where it produces sustained activation of MAPK pathway and this is necessary for long-term survival of NGF responsive populations during development (Harrington et al., 2011). Moreover, sustained phosphorylation of ERK1/2 is responsible for differentiation of PC12 cells in response to NGF. BNN27 did only transiently stimulated erk1/2 phosphorylation in PC12 cells and thus was also unable to promote their differentitation (Data not shown). The specific "handicap" of BNN27 compared to NGF may arise from the way of interaction between the two molecules with TrkA or with the complex of TrkA with $p75^{NTR}$ and the stability of their association.

Since both NGF and DHEA has been reported to facilitate neurogenesis in the rodent hippocampus (Frielingsdorf et al., 2007; Karishma et al., 2002), I wondered if BNN27 is also effective to induce either proliferation or survival of NSCs in this area. However, BNN27 chronic administration did not increase proliferation neither survival of NSCs *in vivo*, in the area of DG of c57/BL6 mice nor did it reversed the decline of neurogenesis in older mice. The reason for which BNN27 failed to increase neurogenesis in these mice is unknown but we could speculate that they are implicated differences in the experimental protocols that were followed or differences in the mechanism of action of these molecules. In the unique report of exogenous administration of NGF to WT mice (Frielingsdorf et al., 2007) the increase in neurogenesis was correlated to enhanced Ach secretion from the basal forebrain neurons that project to hippocampus (Mohapel et al., 2005). Since NGF has a pivotal role in regulation of the basal forebrain cholinergic system function it would be very

interesting to test the NGF mimetic actions of BNN27 on this system although preliminary results show that BNN27 administration does not affect the soma size or ChaT expression in cholinergic neurons of this area. Therefore this might be a reason for the inability of BNN27 to increase the production of new neurons compared to NGF. On the other hand, studies investigating DHEA's capacity to induce neurogenesis in adult hippocampus has produced quite conflicting results. Prolonged administration of DHEA in the form of a subcutaneous pellet (100mg) and over the survival period increased the number of newborn neurons and antagonized the corticosterone induced suppression in neurogenesis (Karrisma et al., 2002). However in a follow up study the same group failed to detect any neurogenic effect of DHEA using a slightly smaller size of pellet although they found that it could act synergistically with antidepressant fluoxetine in increasing the proliferation of NSCs (Pinnock et al., 2009). In addition, a more recent study showed that a 2 week administration of a relevant dose to that we used (60mg/kg) reversed the neurogenic deficits of olfactory balbectomized mice bud it did not have any effect in neurogenesis in intact mice (Moriguchi et al., 2014). Similarly, in another study chronic DHEA treatment prevented the impairment of long term survival of newborn caused by Infusion of the toxic fragment of aβ-amyloid ($A\beta_{25–35}$) while it hardly increases neurogenesis in non-infused mice (Liang et al., 2010). Actually, another neurosteroid, allopregnenolone, administered to 3xTgAD mice, a model of AD characterized by overexpression of APP, reversed the reduction in the proliferation of NSCs in the DG but it was unable to further increase neurogenesis in no-TG animals (Chang et al., 2010).

There is an increasing amount of evidence supporting that elevation of neurogenesis in the hippocampus could be effective way to ameliorate cognitive impairment in AD and other neurodegenerative diseases. Thus, in order to test the possible therapeutic effects of microneurotrophin BNN27 in an animal disease model we chose the 5XFAD mice that bear five mutation linked with the familial form of alzheimer's disease and shows amyloid pathology and neuroinflammation in areas of cortex and the hippocampus from 1,5 months of age. Although it is generally accepted that neurogenesis is altered in AD, there have been a lot of discrepancies in the literature coming from studies either in animal models or post mortem human samples regarding whether neurogenesis is upregulated or downregulated. These controversies arise from the progression of the disease in each case, the time of observation and

most importantly the mutated genes in each transgenic line (Mu et al., 2011). A more meticulous investigation of the dynamics of neurogenesis during the time course of the disease revealed that there is an increase in production of newborn neurons early on, before any apparent manifestation of the disease and that it eventually declines in later stages, (Gomez-Nicola et al., 2014). The 5XFAD mice consist a very aggressive AD model because of the rapid accumulation of aβ-amyloid from a very young age and display reduced rate of neurogenesis at least at 6 months of age compared to WT littermates, as it was shown in a recent study (Moon et al., 2014). I implanted a BNN27 or placebo pellet at 1,5 month old 5XFAD mice and non-TG littermates and quantified the number of new neurons in DG stained against the microtubule protein DcX, when mice had reached 3 months of age. I firstly confirmed the reduction in neurogenesis between the two genotypes and I observed a significant reversal of this deficit in mice received the BNN27 pellet compared to placebo. The aforementioned observation was also correlated with a reduction in the number of aβ - amyloid deposits in the same group of mice. Both DHEA and NGF can increase the production and secretion of APP, thus switching the balance in favor of non-amyloidogenic route that produces soluble form of aβ-amyloid (Danenberg et al., 1996, Rossner et al., 1998). The latter can affect, when present in its aggregate form, NPCs' fate and survival through regulation of ca^{2+} homeostasis (Haughey et al., 2002). According to this idea BNN27 could exert beneficial effects in neurogenesis by reducing the levels of aβ-amyloid. However the mechanism by which this effect could be mediated and whether is similar with that of NGF needs further elucidation.

Aβ amyloid also binds to $p75^{NTR}$ thus inducing cell death of mature hippocampal neurons (Sotthibundhu et al., 2008). However cumulative evidence suggest that the function of $p75^{NTR}$ is totally different in NSCs. Activation of $p75^{NTR}$ has been shown to increase proliferation of NSCs (Young et al., 2007, Catts et al., 2008) and Binding of Aβ amyloid to $p75^{NTR}$ was proposed to be the reason for the increased production of new neurons during the first stages of amyloidosis in animal models overproducing Aβ amyloid such as the APP/PS1 transgenic mice while in later stages which however may result to depletion of NSCs in later stages (Sotthibundhu et al., 2009). Having this in mind it is unlikely BNN27 to reverse neurogenesis in 5XFAD mice through activation of $p75^{NTR}$ considering also the fact that small molecules agonist of $p75^{NTR}$ were found efficient to increase neurogenesis in WT mice (Shi et al., 2013).

Other receptors, such as σ1 have been implicated in the actions of DHEA in the CNS and thus it could be a possible target o BNN27. More specifically, the σ1 receptor antagonist NE100 completely prevented the protective effects of DHEA against the Aβ25–35-impaired long term survival of newborn neurons while the NMDAr channel blocker MK801 failed to affect the DHEA-action (Liang et al., 2010). Finally, as I mentioned before, GABA is an important regulator of adult neurogenesis (Claudio Giachino et al., 2013, Palloto et al., 2014) and DHEA has been shown to bind and modulate $GABA_A$ signaling (Majewska et al., 1990). The potential involvement of GABA receptors as mediator of BNN27 actions remains to be elucidated both in neurogenic as well as a neuroprotective molecule.

Conclusively, the scope of this study was the investigation of the possible neurogenic effects of two small lipophilic molecules, the one of which is already in clinical use,that can easily be administered as a therapeutic agent in a wide spectrum of neuropsychiatric diseases. As finglolimod is concerned, my results are consistent with convergent data indicating its neuro-restorative and regenerative capacity and support the idea that it may be beneficial for the control of negative symptoms in cognition that accompany neurodegenerative conditions, at least in young adults, also through regulation of adult neurogenesis. The ability of this substance to induce neurogenesis under pathological conditions such as stroke or AD it would be of great importance for its emergence as a novel theraupeutic approach for these disorders.

On the other hand BNN27 consists a novel molecule whose full repertoire of properties is now beginning to be unraveled. Although it seems to have agonistic effects on Trka and p75, as far as activation of specific downstream pathways are concerned, the exact degree of similarity with NGF is unclear. For instance BNN27 cannot support the long-term survival of NGF dependent populations indicating that it cannot substitute NGF in its basic physiological functions. Albeit BNN27 has been shown effective to prevent the reduction of neurogenesis in an animal model of AD and decrease amyloid burden when administered prophylactically, before the development of the disease. The exact mechanism underlying this effect as well as whether this effect could be related to a functional outcome such as prevention of cognitive deterioration in this mice remains to be elucidated.

The scope of the last part of this study was to examine the effects of a non-explored substrate topography on the behavior of nervous system cell populations. This is characterized by a discontinuous type of geometry, presenting however ever a feature of anisotropy which is the elliptical cross section of the microcones. The exploration of cell responses in specific topographical patterns could contribute to the development of 3D artificial niches that will support the growth of transplanted cell for tissue regeneration. Moreover the control of neuronal network morphology will enable the development of devices serving as neuronal interfaces.

Our microconical structured silicon surfaces have been proved efficient in supporting the oriented growth of a various cell types of the PNS as well as neural progenitors of the CNS, which is very important for the axon guidance during the regenerative process. In almost all of the cases though, the surface had to be functionalized first, with the use of adhesive proteins of the ECM such as collagen or PDL and laminin. Many parameters of the surface of materials, such as surface chemical groups and topography can affect cell adhesion (Corey et a., 2003). However, for Si it has been shown that the critical factor that affects the change of cell adhesion is the surface topography rather than chemical groups (Fan et al., 2002). More specifically the adhesion of substantia nigra cells is adverse on the polished Si wafer but is good enough on etched Si wafer with Ra in the range 20–70 nm. This is in agreement with previous reports that rough surfaces promote cell adhesion (Schakenraad et al.,1996). However in our case the height of the microcone was in the range of μM even in low roughness substrates. Only in the case of DRG explants the adhesion was increased with surface roughness without protein coating and this might happen because of the different lengthscale of whole ganglion which permit multiple contacts with surrounding microcones as it was revealed from SEM analysis. In addition to this the organotypic culture of DRG explant include many other cell types except for neurons such as Schwann cells which are known to secret components of the ECM like FN that facilitate contact with the substrate while the non neuronal cells themselves make a «carpet» on which neurons can grow.

Mechanical properties of the substrate are also an important issue in neuronal development and regeneration. Usually neurons of the CNS prefer to grow on soft materials resembling the elastic properties of the brain (Discher et al., 2005; Suter et al., 2011) however neurons of the periphery such as DRGs have better outgrowth with increased stiffness of the substrate (Koch et al., 2012). Neurites from DRG neurons

displayed maximal outgrowth on substrates with a Young's modulus of ~1000 Pa, whereas hippocampal neurite outgrowth is independent of substrate stiffness. Silicon has a Young's modulus of the order of Gpa thus it seems from our study that even more rigid substrates than those that were tested by Koch and colleagues could equally well support neurite outgrowth of DRGs. Stem cells are also sensitive to mechanical forces of their environment and it has been shown that solely substrate stiffness can trigger their differentiation towards a specific lineage (Engler et al., 2006). It appears that an increase in substrates modulus favors neuronal differentiation while an astrocytic phenotype is observed in softer materials (Saha et al., 2008). NCSs from the embryonic cortex showed very good growth in all the substrates and preferred orientation in high roughness substrates. However, we did not observe any sign of differentiation under our experimental conditions after 1 week in culture in the presence of growth factors EGF and FGF. It would be of interest for future study to test the capacity of our substrates to promote differentiation of NSCs under differentiation conditions after growth factor withdrawal.

Finally, many cells types that do not form extensive cell projections (e.g. fibroblasts, Schwann cells) have been shown to rearrange the shape of both their cytoskeleton and the nucleus showing a whole cell response to the imposed topography of artificial microstructured surfaces (Martinez et al., 2009). However neural cells have the unique ability to extend long protrusions that navigate the environment through the growth cone in order to reach their final target destination while the soma remains intact. In order to investigate the influence of our microconical structrured surfaces on cell shape of the different cell types tested in this study we performed SEM analysis of cultures of Schwann cells or SCG neurons. Our results show that Schwann cells transform their shape from polygonal to a more splindle-like that in order to get aligned to the underlying topography. On the other hand neuronal cell bodies remain spherical in shape though the axons extending from them acquire a specific orientation, which is the orientation of microcones elliptical cross section in high roughness substrates.

Future studies need to take advantage of the inherent properties of these substrates such as the preferred orientation and conductance of the material in order to develop devices that will provide the ability to investigate and monitor network responses under pharmacological or electrical stimulation.

REFERENCES

Aimone, J.B., Deng, W., and Gage, F.H. (2010). Adult neurogenesis: integrating theories and separating functions. Trends in cognitive sciences 14, 325-337.

Aimone, J.B., and Gage, F.H. (2011). Modeling new neuron function: a history of using computational neuroscience to study adult neurogenesis. The European journal of neuroscience 33, 1160-1169.

Al-Izki, S., Pryce, G., Jackson, S.J., Giovannoni, G., and Baker, D. (2011). Immunosuppression with FTY720 is insufficient to prevent secondary progressive neurodegeneration in experimental autoimmune encephalomyelitis. Multiple sclerosis 17, 939-948.

Alexander, J.K., Fuss, B., and Colello, R.J. (2006). Electric field-induced astrocyte alignment directs neurite outgrowth. Neuron glia biology 2, 93-103.

Altman, J., and Das, G.D. (1965). Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. The Journal of comparative neurology 124, 319-335.

Alvarez-Buylla, A., and Nottebohm, F. (1988). Migration of young neurons in adult avian brain. Nature 335, 353-354.

Asle-Rousta, M., Kolahdooz, Z., Oryan, S., Ahmadiani, A., and Dargahi, L. (2013). FTY720 (fingolimod) attenuates beta-amyloid peptide (Abeta42)-induced impairment of spatial learning and memory in rats. Journal of molecular neuroscience : MN 50, 524-532.

Auld, D.S., Mennicken, F., and Quirion, R. (2001). Nerve growth factor rapidly induces prolonged acetylcholine release from cultured basal forebrain neurons: differentiation between neuromodulatory and neurotrophic influences. The Journal of neuroscience : the official journal of the Society for Neuroscience 21, 3375-3382.

Banasr, M., Hery, M., Printemps, R., and Daszuta, A. (2004). Serotonin-induced increases in adult cell proliferation and neurogenesis are mediated through different and common 5-HT receptor subtypes in the dentate gyrus and the subventricular zone. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 29, 450-460.

Barkhof, F., de Jong, R., Sfikas, N., de Vera, A., Francis, G., Cohen, J., and on behalf of the, T.s.g. (2014). The influence of patient demographics, disease characteristics and treatment on brain volume loss in Trial Assessing Injectable Interferon vs FTY720 Oral in Relapsing-Remitting Multiple Sclerosis (TRANSFORMS), a phase 3 study of fingolimod in multiple sclerosis. Multiple sclerosis.

Barry, D.S., Pakan, J.M., and McDermott, K.W. (2014). Radial glial cells: key organisers in CNS development. The international journal of biochemistry & cell biology 46, 76-79.

Baulieu, E.E. (1998). [The decrease in the hippocampus of the neurosteroid pregnenolone sulfate is involved in memory deficit in the aged animal]. Comptes rendus de l'Academie des sciences Serie III, Sciences de la vie 321, 223-227.

Beckervordersandforth, R., Deshpande, A., Schaffner, I., Huttner, H.B., Lepier, A., Lie, D.C., and Gotz, M. (2014). In vivo targeting of adult neural stem cells in the dentate gyrus by a split-cre approach. Stem cell reports 2, 153-162.

Beckervordersandforth, R., Tripathi, P., Ninkovic, J., Bayam, E., Lepier, A., Stempfhuber, B., Kirchhoff, F., Hirrlinger, J., Haslinger, A., Lie, D.C., et al. (2010). In vivo fate mapping and expression analysis reveals molecular hallmarks of prospectively isolated adult neural stem cells. Cell stem cell 7, 744-758.

Beer, M.S., Stanton, J.A., Salim, K., Rigby, M., Heavens, R.P., Smith, D., and McAllister, G. (2000). EDG receptors as a therapeutic target in the nervous system. Annals of the New York Academy of Sciences 905, 118-131.

Belelli, D., and Lambert, J.J. (2005). Neurosteroids: endogenous regulators of the GABA(A) receptor. Nature reviews Neuroscience 6, 565-575.

Bernabeu, R.O., and Longo, F.M. (2010). The p75 neurotrophin receptor is expressed by adult mouse dentate progenitor cells and regulates neuronal and non-neuronal cell genesis. BMC neuroscience 11, 136.

Blondeau, N., Lai, Y., Tyndall, S., Popolo, M., Topalkara, K., Pru, J.K., Zhang, L., Kim, H., Liao, J.K., Ding, K., et al. (2007). Distribution of sphingosine kinase activity and mRNA in rodent brain. Journal of neurochemistry 103, 509-517.

Bovetti, S., De Marchis, S., Gambarotta, G., Fasolo, A., Perroteau, I., Puche, A.C., and Bovolin, P. (2006). Differential expression of neuregulins and their receptors in the olfactory bulb layers of the developing mouse. Brain research 1077, 37-47.

Brannvall, K., Korhonen, L., and Lindholm, D. (2002). Estrogen-receptor-dependent regulation of neural stem cell proliferation and differentiation. Molecular and cellular neurosciences 21, 512-520.

Brezun, J.M., and Daszuta, A. (1999). Depletion in serotonin decreases neurogenesis in the dentate gyrus and the subventricular zone of adult rats. Neuroscience 89, 999-1002.

Brinkmann, V., Billich, A., Baumruker, T., Heining, P., Schmouder, R., Francis, G., Aradhye, S., and Burtin, P. (2010). Fingolimod (FTY720): discovery and development of an oral drug to treat multiple sclerosis. Nature reviews Drug discovery 9, 883-897.

Bruel-Jungerman, E., Davis, S., Rampon, C., and Laroche, S. (2006). Long-term potentiation enhances neurogenesis in the adult dentate gyrus. The Journal of neuroscience : the official journal of the Society for Neuroscience 26, 5888-5893.

Brunet, A., Roux, D., Lenormand, P., Dowd, S., Keyse, S., and Pouyssegur, J. (1999). Nuclear translocation of p42/p44 mitogen-activated protein kinase is required for growth factor-induced gene expression and cell cycle entry. The EMBO journal 18, 664-674.

Brunkhorst, R., Vutukuri, R., and Pfeilschifter, W. (2014). Fingolimod for the treatment of neurological diseases-state of play and future perspectives. Frontiers in cellular neuroscience 8, 283.

Bunge, R.P., and Bunge, M.B. (1978). Evidence that contact with connective tissue matrix is required for normal interaction between Schwann cells and nerve fibers. The Journal of cell biology 78, 943-950.

Calabresi, P.A., Radue, E.W., Goodin, D., Jeffery, D., Rammohan, K.W., Reder, A.T., Vollmer, T., Agius, M.A., Kappos, L., Stites, T., et al. (2014). Safety and efficacy of fingolimod in patients with relapsing-remitting multiple sclerosis (FREEDOMS II): a double-blind, randomised, placebo-controlled, phase 3 trial. The

Lancet Neurology 13, 545-556.

Calogeropoulou, T., Avlonitis, N., Minas, V., Alexi, X., Pantzou, A., Charalampopoulos, I., Zervou, M., Vergou, V., Katsanou, E.S., Lazaridis, I., et al. (2009). Novel dehydroepiandrosterone derivatives with antiapoptotic, neuroprotective activity. Journal of medicinal chemistry 52, 6569-6587.

Cameron, H.A., and Gould, E. (1994). Adult neurogenesis is regulated by adrenal steroids in the dentate gyrus. Neuroscience 61, 203-209.

Cameron, H.A., McEwen, B.S., and Gould, E. (1995). Regulation of adult neurogenesis by excitatory input and NMDA receptor activation in the dentate gyrus. The Journal of neuroscience : the official journal of the Society for Neuroscience 15, 4687-4692.

Cameron, H.A., Woolley, C.S., and Gould, E. (1993). Adrenal steroid receptor immunoreactivity in cells born in the adult rat dentate gyrus. Brain research 611, 342-346.

Capsoni, S., Brandi, R., Arisi, I., D'Onofrio, M., and Cattaneo, A. (2011). A dual mechanism linking NGF/proNGF imbalance and early inflammation to Alzheimer's disease neurodegeneration in the AD11 anti-NGF mouse model. CNS & neurological disorders drug targets 10, 635-647.

Chang, A., Smith, M.C., Yin, X., Fox, R.J., Staugaitis, S.M., and Trapp, B.D. (2008). Neurogenesis in the chronic lesions of multiple sclerosis. Brain : a journal of neurology 131, 2366-2375.

Charalampopoulos, I., Remboutsika, E., Margioris, A.N., and Gravanis, A. (2008). Neurosteroids as modulators of neurogenesis and neuronal survival. Trends in endocrinology and metabolism: TEM 19, 300-307.

Chen, K.S., Nishimura, M.C., Armanini, M.P., Crowley, C., Spencer, S.D., and Phillips, H.S. (1997). Disruption of a single allele of the nerve growth factor gene results in atrophy of basal forebrain cholinergic neurons and memory deficits. The Journal of neuroscience : the official journal of the Society for Neuroscience 17, 7288-7296.

Chen, W.Y., Manson, J.E., Hankinson, S.E., Rosner, B., Holmes, M.D., Willett, W.C., and Colditz, G.A. (2006). Unopposed estrogen therapy and the risk of invasive breast cancer. Archives of internal medicine 166, 1027-1032.

Chen, Y., Kelton, C.M., Jing, Y., Guo, J.J., Li, X., and Patel, N.C. (2008). Utilization, price, and spending trends for antidepressants in the US Medicaid Program. Research in social & administrative pharmacy : RSAP 4, 244-257.

Choi, J.W., and Chun, J. (2013). Lysophospholipids and their receptors in the central nervous system. Biochimica et biophysica acta 1831, 20-32.

Choi, J.W., Gardell, S.E., Herr, D.R., Rivera, R., Lee, C.W., Noguchi, K., Teo, S.T., Yung, Y.C., Lu, M., Kennedy, G., et al. (2011). FTY720 (fingolimod) efficacy in an animal model of multiple sclerosis requires astrocyte sphingosine 1-phosphate receptor 1 (S1P1) modulation. Proceedings of the National Academy of Sciences of the United States of America 108, 751-756.

Chun, J., and Hartung, H.P. (2010). Mechanism of action of oral fingolimod (FTY720) in multiple sclerosis. Clinical neuropharmacology 33, 91-101.

Clelland, C.D., Choi, M., Romberg, C., Clemenson, G.D., Jr., Fragniere, A., Tyers, P., Jessberger, S., Saksida, L.M., Barker, R.A., Gage, F.H., et al. (2009). A functional role for adult hippocampal neurogenesis in spatial pattern separation. Science 325, 210-213.

Clelland, C.D., Choi, M., Romberg, C., Clemenson, G.D., Jr., Fragniere, A., Tyers, P., Jessberger, S., Saksida, L.M., Barker, R.A., Gage, F.H., et al. (2009). A functional role for adult hippocampal neurogenesis in spatial pattern separation. Science 325, 210-213.

Coelho, R.P., Payne, S.G., Bittman, R., Spiegel, S., and Sato-Bigbee, C. (2007). The immunomodulator FTY720 has a direct cytoprotective effect in oligodendrocyte progenitors. The Journal of pharmacology and experimental therapeutics 323, 626-635.

Coelho, R.P., Payne, S.G., Bittman, R., Spiegel, S., and Sato-Bigbee, C. (2007). The immunomodulator FTY720 has a direct cytoprotective effect in oligodendrocyte progenitors. The Journal of pharmacology and experimental therapeutics 323, 626-635.

Colditz, M.J., Catts, V.S., Al-menhali, N., Osborne, G.W., Bartlett, P.F., and Coulson, E.J. (2010). p75 neurotrophin receptor regulates basal and fluoxetine-stimulated hippocampal neurogenesis. Experimental brain research 200, 161-167.

Colombaioni, L., and Garcia-Gil, M. (2004). Sphingolipid metabolites in neural signalling and function. Brain research Brain research reviews 46, 328-355.

Compagnone, N.A., Bulfone, A., Rubenstein, J.L., and Mellon, S.H. (1995). Expression of the steroidogenic enzyme P450scc in the central and peripheral nervous systems during rodent embryogenesis. Endocrinology 136, 2689-2696.

Cooper-Kuhn, C.M., Winkler, J., and Kuhn, H.G. (2004). Decreased neurogenesis after cholinergic forebrain lesion in the adult rat. Journal of neuroscience research 77, 155-165.

Corey, J.M., and Feldman, E.L. (2003). Substrate patterning: an emerging technology for the study of neuronal behavior. Experimental neurology 184 Suppl 1, S89-96.

Creer, D.J., Romberg, C., Saksida, L.M., van Praag, H., and Bussey, T.J. (2010). Running enhances spatial pattern separation in mice. Proceedings of the National Academy of Sciences of the United States of America 107, 2367-2372.

Crowley, C., Spencer, S.D., Nishimura, M.C., Chen, K.S., Pitts-Meek, S., Armanini, M.P., Ling, L.H., McMahon, S.B., Shelton, D.L., Levinson, A.D., et al. (1994). Mice lacking nerve growth factor display perinatal loss of sensory and sympathetic neurons yet develop basal forebrain cholinergic neurons. Cell 76, 1001-1011.

Cyster, L.A., Parker, K.G., Parker, T.L., and Grant, D.M. (2004). The effect of surface chemistry and nanotopography of titanium nitride (TiN) films on primary hippocampal neurones. Biomaterials 25, 97-107.

Czech, B., Pfeilschifter, W., Mazaheri-Omrani, N., Strobel, M.A., Kahles, T., Neumann-Haefelin, T., Rami, A., Huwiler, A., and Pfeilschifter, J. (2009). The immunomodulatory sphingosine 1-phosphate analog FTY720 reduces lesion size and improves neurological outcome in a mouse model of cerebral ischemia. Biochemical and biophysical research communications 389, 251-256.

Czerniawski, J., and Guzowski, J.F. (2014). Acute neuroinflammation impairs context

discrimination memory and disrupts pattern separation processes in hippocampus. The Journal of neuroscience : the official journal of the Society for Neuroscience 34, 12470-12480.

Darsalia, V., Heldmann, U., Lindvall, O., and Kokaia, Z. (2005). Stroke-induced neurogenesis in aged brain. Stroke; a journal of cerebral circulation 36, 1790-1795.

Dayer, A.G., Ford, A.A., Cleaver, K.M., Yassaee, M., and Cameron, H.A. (2003). Short-term and long-term survival of new neurons in the rat dentate gyrus. The Journal of comparative neurology 460, 563-572.

Deng, W., Aimone, J.B., and Gage, F.H. (2010). New neurons and new memories: how does adult hippocampal neurogenesis affect learning and memory? Nature reviews Neuroscience 11, 339-350.

Deogracias, R., Yazdani, M., Dekkers, M.P., Guy, J., Ionescu, M.C., Vogt, K.E., and Barde, Y.A. (2012). Fingolimod, a sphingosine-1 phosphate receptor modulator, increases BDNF levels and improves symptoms of a mouse model of Rett syndrome. Proceedings of the National Academy of Sciences of the United States of America 109, 14230-14235.

di Nuzzo, L., Orlando, R., Tognoli, C., Di Pietro, P., Bertini, G., Miele, J., Bucci, D., Motolese, M., Scaccianoce, S., Caruso, A., et al. (2015). Antidepressant activity of fingolimod in mice. Pharmacology research & perspectives 3, e00135.

Discher, D.E., Janmey, P., and Wang, Y.L. (2005). Tissue cells feel and respond to the stiffness of their substrate. Science 310, 1139-1143.

Doetsch, F., Petreanu, L., Caille, I., Garcia-Verdugo, J.M., and Alvarez-Buylla, A. (2002). EGF converts transit-amplifying neurogenic precursors in the adult brain into multipotent stem cells. Neuron 36, 1021-1034.

Doi, Y., Takeuchi, H., Horiuchi, H., Hanyu, T., Kawanokuchi, J., Jin, S., Parajuli, B., Sonobe, Y., Mizuno, T., and Suzumura, A. (2013). Fingolimod phosphate attenuates oligomeric amyloid beta-induced neurotoxicity via increased brain-derived neurotrophic factor expression in neurons. PloS one 8, e61988.

Donovan, M.H., Yamaguchi, M., and Eisch, A.J. (2008). Dynamic expression of TrkB receptor protein on proliferating and maturing cells in the adult mouse dentate gyrus. Hippocampus 18, 435-439.

Dowell-Mesfin, N.M., Abdul-Karim, M.A., Turner, A.M., Schanz, S., Craighead, H.G., Roysam, B., Turner, J.N., and Shain, W. (2004). Topographically modified surfaces affect orientation and growth of hippocampal neurons. Journal of neural engineering 1, 78-90.

Drew, M.R., Denny, C.A., and Hen, R. (2010). Arrest of adult hippocampal neurogenesis in mice impairs single- but not multiple-trial contextual fear conditioning. Behavioral neuroscience 124, 446-454.

Dupret, D., Fabre, A., Dobrossy, M.D., Panatier, A., Rodriguez, J.J., Lamarque, S., Lemaire, V., Oliet, S.H., Piazza, P.V., and Abrous, D.N. (2007). Spatial learning depends on both the addition and removal of new hippocampal neurons. PLoS biology 5, e214.

Encinas, J.M., Michurina, T.V., Peunova, N., Park, J.H., Tordo, J., Peterson, D.A., Fishell, G., Koulakov, A., and Enikolopov, G. (2011). Division-coupled astrocytic differentiation and age-related depletion of neural stem cells in the adult

hippocampus. Cell stem cell 8, 566-579.

Engler, A.J., Sen, S., Sweeney, H.L., and Discher, D.E. (2006). Matrix elasticity directs stem cell lineage specification. Cell 126, 677-689.

Eriksson, P.S., Perfilieva, E., Bjork-Eriksson, T., Alborn, A.M., Nordborg, C., Peterson, D.A., and Gage, F.H. (1998). Neurogenesis in the adult human hippocampus. Nature medicine 4, 1313-1317.

Ernst, A., and Frisen, J. (2015). Adult neurogenesis in humans- common and unique traits in mammals. PLoS biology 13, e1002045.

Esposito, M.S., Piatti, V.C., Laplagne, D.A., Morgenstern, N.A., Ferrari, C.C., Pitossi, F.J., and Schinder, A.F. (2005). Neuronal differentiation in the adult hippocampus recapitulates embryonic development. The Journal of neuroscience : the official journal of the Society for Neuroscience 25, 10074-10086.

Estus, S., Zaks, W.J., Freeman, R.S., Gruda, M., Bravo, R., and Johnson, E.M., Jr. (1994). Altered gene expression in neurons during programmed cell death: identification of c-jun as necessary for neuronal apoptosis. The Journal of cell biology 127, 1717-1727.

Fischer, I., Alliod, C., Martinier, N., Newcombe, J., Brana, C., and Pouly, S. (2011). Sphingosine kinase 1 and sphingosine 1-phosphate receptor 3 are functionally upregulated on astrocytes under pro-inflammatory conditions. PloS one 6, e23905.

Fischer, I., Alliod, C., Martinier, N., Newcombe, J., Brana, C., and Pouly, S. (2011). Sphingosine kinase 1 and sphingosine 1-phosphate receptor 3 are functionally upregulated on astrocytes under pro-inflammatory conditions. PloS one 6, e23905.

Foster, C.A., Howard, L.M., Schweitzer, A., Persohn, E., Hiestand, P.C., Balatoni, B., Reuschel, R., Beerli, C., Schwartz, M., and Billich, A. (2007). Brain penetration of the oral immunomodulatory drug FTY720 and its phosphorylation in the central nervous system during experimental autoimmune encephalomyelitis: consequences for mode of action in multiple sclerosis. The Journal of pharmacology and experimental therapeutics 323, 469-475.

Frielingsdorf, H., Schwarz, K., Brundin, P., and Mohapel, P. (2004). No evidence for new dopaminergic neurons in the adult mammalian substantia nigra. Proceedings of the National Academy of Sciences of the United States of America 101, 10177-10182.

Frielingsdorf, H., Simpson, D.R., Thal, L.J., and Pizzo, D.P. (2007). Nerve growth factor promotes survival of new neurons in the adult hippocampus. Neurobiology of disease 26, 47-55.

Fukumoto, K., Mizoguchi, H., Takeuchi, H., Horiuchi, H., Kawanokuchi, J., Jin, S., Mizuno, T., and Suzumura, A. (2014). Fingolimod increases brain-derived neurotrophic factor levels and ameliorates amyloid beta-induced memory impairment. Behavioural brain research 268, 88-93.

Gage, F.H. (2000). Mammalian neural stem cells. Science 287, 1433-1438.

Galea, L.A., and McEwen, B.S. (1999). Sex and seasonal differences in the rate of cell proliferation in the dentate gyrus of adult wild meadow voles. Neuroscience 89, 955-964.

Gattazzo, F., Urciuolo, A., and Bonaldo, P. (2014). Extracellular matrix: a dynamic microenvironment for stem cell niche. Biochimica et biophysica acta 1840, 2506-

2519.

Ge, S., Goh, E.L., Sailor, K.A., Kitabatake, Y., Ming, G.L., and Song, H. (2006). GABA regulates synaptic integration of newly generated neurons in the adult brain. Nature 439, 589-593.

Ge, S., Pradhan, D.A., Ming, G.L., and Song, H. (2007). GABA sets the tempo for activity-dependent adult neurogenesis. Trends in neurosciences 30, 1-8.

George, O., Vallee, M., Le Moal, M., and Mayo, W. (2006). Neurosteroids and cholinergic systems: implications for sleep and cognitive processes and potential role of age-related changes. Psychopharmacology 186, 402-413.

Giannakopoulou, A., Grigoriadis, N., Bekiari, C., Lourbopoulos, A., Dori, I., Tsingotjidou, A.S., Michaloudi, H., and Papadopoulos, G.C. (2013). Acute inflammation alters adult hippocampal neurogenesis in a multiple sclerosis mouse model. Journal of neuroscience research 91, 890-900.

Gil, J.M., Mohapel, P., Araujo, I.M., Popovic, N., Li, J.Y., Brundin, P., and Petersen, A. (2005). Reduced hippocampal neurogenesis in R6/2 transgenic Huntington's disease mice. Neurobiology of disease 20, 744-751.

Goedert, M., Sisodia, S.S., and Price, D.L. (1991). Neurofibrillary tangles and beta-amyloid deposits in Alzheimer's disease. Current opinion in neurobiology 1, 441-447.

Gomez, N., Lu, Y., Chen, S., and Schmidt, C.E. (2007). Immobilized nerve growth factor and microtopography have distinct effects on polarization versus axon elongation in hippocampal cells in culture. Biomaterials 28, 271-284.

Gomez-Nicola, D., Suzzi, S., Vargas-Caballero, M., Fransen, N.L., Al-Malki, H., Cebrian-Silla, A., Garcia-Verdugo, J.M., Riecken, K., Fehse, B., and Perry, V.H. (2014). Temporal dynamics of hippocampal neurogenesis in chronic neurodegeneration. Brain : a journal of neurology 137, 2312-2328.

Gotz, M., and Barde, Y.A. (2005). Radial glial cells defined and major intermediates between embryonic stem cells and CNS neurons. Neuron 46, 369-372.

Gould, E., Vail, N., Wagers, M., and Gross, C.G. (2001). Adult-generated hippocampal and neocortical neurons in macaques have a transient existence. Proceedings of the National Academy of Sciences of the United States of America 98, 10910-10917.

Hait, N.C., Allegood, J., Maceyka, M., Strub, G.M., Harikumar, K.B., Singh, S.K., Luo, C., Marmorstein, R., Kordula, T., Milstien, S., et al. (2009). Regulation of histone acetylation in the nucleus by sphingosine-1-phosphate. Science 325, 1254-1257.

Hait, N.C., Wise, L.E., Allegood, J.C., O'Brien, M., Avni, D., Reeves, T.M., Knapp, P.E., Lu, J., Luo, C., Miles, M.F., et al. (2014). Active, phosphorylated fingolimod inhibits histone deacetylases and facilitates fear extinction memory. Nature neuroscience 17, 971-980.

Hajszan, T., MacLusky, N.J., and Leranth, C. (2004). Dehydroepiandrosterone increases hippocampal spine synapse density in ovariectomized female rats. Endocrinology 145, 1042-1045.

Harada, J., Foley, M., Moskowitz, M.A., and Waeber, C. (2004). Sphingosine-1-phosphate induces proliferation and morphological changes of neural progenitor cells.

Journal of neurochemistry 88, 1026-1039.

Hasegawa, Y., Suzuki, H., Sozen, T., Rolland, W., and Zhang, J.H. (2010). Activation of sphingosine 1-phosphate receptor-1 by FTY720 is neuroprotective after ischemic stroke in rats. Stroke; a journal of cerebral circulation 41, 368-374.

Hatten, M.E., and Mason, C.A. (1990). Mechanisms of glial-guided neuronal migration in vitro and in vivo. Experientia 46, 907-916.

Hefti, F., Hartikka, J., Salvatierra, A., Weiner, W.J., and Mash, D.C. (1986). Localization of nerve growth factor receptors in cholinergic neurons of the human basal forebrain. Neuroscience letters 69, 37-41.

Hemmati, F., Dargahi, L., Nasoohi, S., Omidbakhsh, R., Mohamed, Z., Chik, Z., Naidu, M., and Ahmadiani, A. (2013). Neurorestorative effect of FTY720 in a rat model of Alzheimer's disease: comparison with memantine. Behavioural brain research 252, 415-421.

Henderson, B.E., and Feigelson, H.S. (2000). Hormonal carcinogenesis. Carcinogenesis 21, 427-433.

Herr, D.R., and Chun, J. (2007). Effects of LPA and S1P on the nervous system and implications for their involvement in disease. Current drug targets 8, 155-167.

Herr, D.R., Fyrst, H., Creason, M.B., Phan, V.H., Saba, J.D., and Harris, G.L. (2004). Characterization of the Drosophila sphingosine kinases and requirement for Sk2 in normal reproductive function. The Journal of biological chemistry 279, 12685-12694.

Hoglinger, G.U., Rizk, P., Muriel, M.P., Duyckaerts, C., Oertel, W.H., Caille, I., and Hirsch, E.C. (2004). Dopamine depletion impairs precursor cell proliferation in Parkinson disease. Nature neuroscience 7, 726-735.

Hsu, S.H., Chen, C.Y., Lu, P.S., Lai, C.S., and Chen, C.J. (2005). Oriented Schwann cell growth on microgrooved surfaces. Biotechnology and bioengineering 92, 579-588.

Hurst, J.H., Mumaw, J., Machacek, D.W., Sturkie, C., Callihan, P., Stice, S.L., and Hooks, S.B. (2008). Human neural progenitors express functional lysophospholipid receptors that regulate cell growth and morphology. BMC neuroscience 9, 118.

Hurst, J.H., Mumaw, J., Machacek, D.W., Sturkie, C., Callihan, P., Stice, S.L., and Hooks, S.B. (2008). Human neural progenitors express functional lysophospholipid receptors that regulate cell growth and morphology. BMC neuroscience 9, 118.

Hynes, R.O. (2009). The extracellular matrix: not just pretty fibrils. Science 326, 1216-1219.

Hynes, R.O., Patel, R., and Miller, R.H. (1986). Migration of neuroblasts along preexisting axonal tracts during prenatal cerebellar development. The Journal of neuroscience : the official journal of the Society for Neuroscience 6, 867-876.

Itou, Y., Nochi, R., Kuribayashi, H., Saito, Y., and Hisatsune, T. (2011). Cholinergic activation of hippocampal neural stem cells in aged dentate gyrus. Hippocampus 21, 446-459.

Jin, K., Sun, Y., Xie, L., Batteur, S., Mao, X.O., Smelick, C., Logvinova, A., and Greenberg, D.A. (2003). Neurogenesis and aging: FGF-2 and HB-EGF restore neurogenesis in hippocampus and subventricular zone of aged mice. Aging cell 2,

175-183.

Jin, K., Zhu, Y., Sun, Y., Mao, X.O., Xie, L., and Greenberg, D.A. (2002). Vascular endothelial growth factor (VEGF) stimulates neurogenesis in vitro and in vivo. Proceedings of the National Academy of Sciences of the United States of America 99, 11946-11950.

Johansson, F., Carlberg, P., Danielsen, N., Montelius, L., and Kanje, M. (2006). Axonal outgrowth on nano-imprinted patterns. Biomaterials 27, 1251-1258.

Kalyani, A., Hobson, K., and Rao, M.S. (1997). Neuroepithelial stem cells from the embryonic spinal cord: isolation, characterization, and clonal analysis. Developmental biology 186, 202-223.

Kappos, L., Radue, E.W., O'Connor, P., Polman, C., Hohlfeld, R., Calabresi, P., Selmaj, K., Agoropoulou, C., Leyk, M., Zhang-Auberson, L., et al. (2010). A placebo-controlled trial of oral fingolimod in relapsing multiple sclerosis. The New England journal of medicine 362, 387-401.

Karishma, K.K., and Herbert, J. (2002). Dehydroepiandrosterone (DHEA) stimulates neurogenesis in the hippocampus of the rat, promotes survival of newly formed neurons and prevents corticosterone-induced suppression. The European journal of neuroscience 16, 445-453.

Kay, J.N., and Blum, M. (2000). Differential response of ventral midbrain and striatal progenitor cells to lesions of the nigrostriatal dopaminergic projection. Developmental neuroscience 22, 56-67.

Keirstead, H.S., Morgan, S.V., Wilby, M.J., and Fawcett, J.W. (1999). Enhanced axonal regeneration following combined demyelination plus schwann cell transplantation therapy in the injured adult spinal cord. Experimental neurology 159, 225-236.

Khan, S.P., Auner, G.G., and Newaz, G.M. (2005). Influence of nanoscale surface roughness on neural cell attachment on silicon. Nanomedicine : nanotechnology, biology, and medicine 1, 125-129.

Kheirbek, M.A., and Hen, R. (2013). (Radio)active neurogenesis in the human hippocampus. Cell 153, 1183-1184.

Kim, H.J., Miron, V.E., Dukala, D., Proia, R.L., Ludwin, S.K., Traka, M., Antel, J.P., and Soliven, B. (2011). Neurobiological effects of sphingosine 1-phosphate receptor modulation in the cuprizone model. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 25, 1509-1518.

Kimonides, V.G., Spillantini, M.G., Sofroniew, M.V., Fawcett, J.W., and Herbert, J. (1999). Dehydroepiandrosterone antagonizes the neurotoxic effects of corticosterone and translocation of stress-activated protein kinase 3 in hippocampal primary cultures. Neuroscience 89, 429-436.

Kimura, A., Ohmori, T., Ohkawa, R., Madoiwa, S., Mimuro, J., Murakami, T., Kobayashi, E., Hoshino, Y., Yatomi, Y., and Sakata, Y. (2007). Essential roles of sphingosine 1-phosphate/S1P1 receptor axis in the migration of neural stem cells toward a site of spinal cord injury. Stem cells 25, 115-124.

Koch, D., Rosoff, W.J., Jiang, J., Geller, H.M., and Urbach, J.S. (2012). Strength in the periphery: growth cone biomechanics and substrate rigidity response in peripheral and central nervous system neurons. Biophysical journal 102, 452-460.

Kokaia, Z., Thored, P., Arvidsson, A., and Lindvall, O. (2006). Regulation of stroke-induced neurogenesis in adult brain--recent scientific progress. Cerebral cortex 16 Suppl 1, i162-167.

Kordower, J.H., Winn, S.R., Liu, Y.T., Mufson, E.J., Sladek, J.R., Jr., Hammang, J.P., Baetge, E.E., and Emerich, D.F. (1994). The aged monkey basal forebrain: rescue and sprouting of axotomized basal forebrain neurons after grafts of encapsulated cells secreting human nerve growth factor. Proceedings of the National Academy of Sciences of the United States of America 91, 10898-10902.

Kraft, P., Gob, E., Schuhmann, M.K., Gobel, K., Deppermann, C., Thielmann, I., Herrmann, A.M., Lorenz, K., Brede, M., Stoll, G., et al. (2013). FTY720 ameliorates acute ischemic stroke in mice by reducing thrombo-inflammation but not by direct neuroprotection. Stroke; a journal of cerebral circulation 44, 3202-3210.

Kriparamanan, R., Aswath, P., Zhou, A., Tang, L., and Nguyen, K.T. (2006). Nanotopography: cellular responses to nanostructured materials. Journal of nanoscience and nanotechnology 6, 1905-1919.

Kuhn, H.G., Dickinson-Anson, H., and Gage, F.H. (1996). Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation. The Journal of neuroscience : the official journal of the Society for Neuroscience 16, 2027-2033.

Kulakowska, A., Zendzian-Piotrowska, M., Baranowski, M., Kononczuk, T., Drozdowski, W., Gorski, J., and Bucki, R. (2010). Intrathecal increase of sphingosine 1-phosphate at early stage multiple sclerosis. Neuroscience letters 477, 149-152.

Lacroix, C., Fiet, J., Benais, J.P., Gueux, B., Bonete, R., Villette, J.M., Gourmel, B., and Dreux, C. (1987). Simultaneous radioimmunoassay of progesterone, androst-4-enedione, pregnenolone, dehydroepiandrosterone and 17-hydroxyprogesterone in specific regions of human brain. Journal of steroid biochemistry 28, 317-325.

Lagace, D.C., Fischer, S.J., and Eisch, A.J. (2007). Gender and endogenous levels of estradiol do not influence adult hippocampal neurogenesis in mice. Hippocampus 17, 175-180.

Lang, U.E., Hellweg, R., and Gallinat, J. (2004). BDNF serum concentrations in healthy volunteers are associated with depression-related personality traits. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 29, 795-798.

Lavenex, P., Banta Lavenex, P., and Amaral, D.G. (2007). Postnatal development of the primate hippocampal formation. Developmental neuroscience 29, 179-192.

Lazaridis, I., Charalampopoulos, I., Alexaki, V.I., Avlonitis, N., Pediaditakis, I., Efstathopoulos, P., Calogeropoulou, T., Castanas, E., and Gravanis, A. (2011). Neurosteroid dehydroepiandrosterone interacts with nerve growth factor (NGF) receptors, preventing neuronal apoptosis. PLoS biology 9, e1001051.

Lazarov, O., and Marr, R.A. (2010). Neurogenesis and Alzheimer's disease: at the crossroads. Experimental neurology 223, 267-281.

Lee, J., Duan, W., and Mattson, M.P. (2002). Evidence that brain-derived neurotrophic factor is required for basal neurogenesis and mediates, in part, the enhancement of neurogenesis by dietary restriction in the hippocampus of adult mice. Journal of neurochemistry 82, 1367-1375.

Li, Y., Luikart, B.W., Birnbaum, S., Chen, J., Kwon, C.H., Kernie, S.G., Bassel-Duby, R., and Parada, L.F. (2008). TrkB regulates hippocampal neurogenesis and governs sensitivity to antidepressive treatment. Neuron 59, 399-412.

Lichtenwalner, R.J., and Parent, J.M. (2006). Adult neurogenesis and the ischemic forebrain. Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism 26, 1-20.

Lie, D.C., Dziewczapolski, G., Willhoite, A.R., Kaspar, B.K., Shults, C.W., and Gage, F.H. (2002). The adult substantia nigra contains progenitor cells with neurogenic potential. The Journal of neuroscience : the official journal of the Society for Neuroscience 22, 6639-6649.

Lietz, M., Dreesmann, L., Hoss, M., Oberhoffner, S., and Schlosshauer, B. (2006). Neuro tissue engineering of glial nerve guides and the impact of different cell types. Biomaterials 27, 1425-1436.

Lim, S.H., Liu, X.Y., Song, H., Yarema, K.J., and Mao, H.Q. (2010). The effect of nanofiber-guided cell alignment on the preferential differentiation of neural stem cells. Biomaterials 31, 9031-9039.

Lois, C., Garcia-Verdugo, J.M., and Alvarez-Buylla, A. (1996). Chain migration of neuronal precursors. Science 271, 978-981.

Louissaint, A., Jr., Rao, S., Leventhal, C., and Goldman, S.A. (2002). Coordinated interaction of neurogenesis and angiogenesis in the adult songbird brain. Neuron 34, 945-960.

Lugert, S., Basak, O., Knuckles, P., Haussler, U., Fabel, K., Gotz, M., Haas, C.A., Kempermann, G., Taylor, V., and Giachino, C. (2010). Quiescent and active hippocampal neural stem cells with distinct morphologies respond selectively to physiological and pathological stimuli and aging. Cell stem cell 6, 445-456.

Magnusson, J.P., Goritz, C., Tatarishvili, J., Dias, D.O., Smith, E.M., Lindvall, O., Kokaia, Z., and Frisen, J. (2014). A latent neurogenic program in astrocytes regulated by Notch signaling in the mouse. Science 346, 237-241.

Mahanthappa, N.K., Anton, E.S., and Matthew, W.D. (1996). Glial growth factor 2, a soluble neuregulin, directly increases Schwann cell motility and indirectly promotes neurite outgrowth. The Journal of neuroscience : the official journal of the Society for Neuroscience 16, 4673-4683.

Malatesta, P., Appolloni, I., and Calzolari, F. (2008). Radial glia and neural stem cells. Cell and tissue research 331, 165-178.

Malberg, J.E., Eisch, A.J., Nestler, E.J., and Duman, R.S. (2000). Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. The Journal of neuroscience : the official journal of the Society for Neuroscience 20, 9104-9110.

Mandala, S., Hajdu, R., Bergstrom, J., Quackenbush, E., Xie, J., Milligan, J., Thornton, R., Shei, G.J., Card, D., Keohane, C., et al. (2002). Alteration of lymphocyte trafficking by sphingosine-1-phosphate receptor agonists. Science 296, 346-349.

Maninger, N., Wolkowitz, O.M., Reus, V.I., Epel, E.S., and Mellon, S.H. (2009). Neurobiological and neuropsychiatric effects of dehydroepiandrosterone (DHEA) and DHEA sulfate (DHEAS). Frontiers in neuroendocrinology 30, 65-91.

Mao, L., Lau, Y.S., Petroske, E., and Wang, J.Q. (2001). Profound astrogenesis in the striatum of adult mice following nigrostriatal dopaminergic lesion by repeated MPTP administration. Brain research Developmental brain research 131, 57-65.

Marlatt, M.W., and Lucassen, P.J. (2010). Neurogenesis and Alzheimer's disease: Biology and pathophysiology in mice and men. Current Alzheimer research 7, 113-125.

Martinez, E., Engel, E., Planell, J.A., and Samitier, J. (2009). Effects of artificial micro- and nano-structured surfaces on cell behaviour. Annals of anatomy = Anatomischer Anzeiger : official organ of the Anatomische Gesellschaft 191, 126-135.

Mayo, W., Lemaire, V., Malaterre, J., Rodriguez, J.J., Cayre, M., Stewart, M.G., Kharouby, M., Rougon, G., Le Moal, M., Piazza, P.V., et al. (2005). Pregnenolone sulfate enhances neurogenesis and PSA-NCAM in young and aged hippocampus. Neurobiology of aging 26, 103-114.

McEwen, B.S., Cameron, H., Chao, H.M., Gould, E., Luine, V., Magarinos, A.M., Pavlides, C., Spencer, R.L., Watanabe, Y., and Woolley, C. (1994). Resolving a mystery: progress in understanding the function of adrenal steroid receptors in hippocampus. Progress in brain research 100, 149-155.

McGiffert, C., Contos, J.J., Friedman, B., and Chun, J. (2002). Embryonic brain expression analysis of lysophospholipid receptor genes suggests roles for s1p(1) in neurogenesis and s1p(1-3) in angiogenesis. FEBS letters 531, 103-108.

McKay, R.D. (1999). Brain stem cells change their identity. Nature medicine 5, 261-262.

McNamara, L.E., McMurray, R.J., Biggs, M.J., Kantawong, F., Oreffo, R.O., and Dalby, M.J. (2010). Nanotopographical control of stem cell differentiation. Journal of tissue engineering 2010, 120623.

Meirer, R., Gurunluoglu, R., and Siemionow, M. (2001). Neurogenic perspective on vascular endothelial growth factor: review of the literature. Journal of reconstructive microsurgery 17, 625-630.

Miles, D.K., and Kernie, S.G. (2006). Activation of neural stem and progenitor cells after brain injury. Progress in brain research 157, 187-197.

Miyata, T., Kawaguchi, A., Okano, H., and Ogawa, M. (2001). Asymmetric inheritance of radial glial fibers by cortical neurons. Neuron 31, 727-741.

Miyoshi, G., Bessho, Y., Yamada, S., and Kageyama, R. (2004). Identification of a novel basic helix-loop-helix gene, Heslike, and its role in GABAergic neurogenesis. The Journal of neuroscience : the official journal of the Society for Neuroscience 24, 3672-3682.

Mizugishi, K., Yamashita, T., Olivera, A., Miller, G.F., Spiegel, S., and Proia, R.L. (2005). Essential role for sphingosine kinases in neural and vascular development. Molecular and cellular biology 25, 11113-11121.

Mizugishi, K., Yamashita, T., Olivera, A., Miller, G.F., Spiegel, S., and Proia, R.L. (2005). Essential role for sphingosine kinases in neural and vascular development. Molecular and cellular biology 25, 11113-11121.

Mizugishi, K., Yamashita, T., Olivera, A., Miller, G.F., Spiegel, S., and Proia, R.L. (2005). Essential role for sphingosine kinases in neural and vascular development. Molecular and cellular biology 25, 11113-11121.

Moghaddam, B., Bolinao, M.L., Stein-Behrens, B., and Sapolsky, R. (1994). Glucocorticoids mediate the stress-induced extracellular accumulation of glutamate. Brain research 655, 251-254.

Mohapel, P., Leanza, G., Kokaia, M., and Lindvall, O. (2005). Forebrain acetylcholine regulates adult hippocampal neurogenesis and learning. Neurobiology of aging 26, 939-946.

Moon, M., Cha, M.Y., and Mook-Jung, I. (2014). Impaired hippocampal neurogenesis and its enhancement with ghrelin in 5XFAD mice. Journal of Alzheimer's disease : JAD 41, 233-241.

Moriguchi, S., Shinoda, Y., Yamamoto, Y., Sasaki, Y., Miyajima, K., Tagashira, H., and Fukunaga, K. (2013). Stimulation of the sigma-1 receptor by DHEA enhances synaptic efficacy and neurogenesis in the hippocampal dentate gyrus of olfactory bulbectomized mice. PloS one 8, e60863.

Mu, Y., and Gage, F.H. (2011). Adult hippocampal neurogenesis and its role in Alzheimer's disease. Molecular neurodegeneration 6, 85.

Murakami, S., Imbe, H., Morikawa, Y., Kubo, C., and Senba, E. (2005). Chronic stress, as well as acute stress, reduces BDNF mRNA expression in the rat hippocampus but less robustly. Neuroscience research 53, 129-139.

Murphy, K.P., Carter, R.J., Lione, L.A., Mangiarini, L., Mahal, A., Bates, G.P., Dunnett, S.B., and Morton, A.J. (2000). Abnormal synaptic plasticity and impaired spatial cognition in mice transgenic for exon 1 of the human Huntington's disease mutation. The Journal of neuroscience : the official journal of the Society for Neuroscience 20, 5115-5123.

Nagata, I., Kawana, A., and Nakatsuji, N. (1993). Perpendicular contact guidance of CNS neuroblasts on artificial microstructures. Development 117, 401-408.

Nagata, I., Ono, K., Kawana, A., and Kimura-Kuroda, J. (2006). Aligned neurite bundles of granule cells regulate orientation of Purkinje cell dendrites by perpendicular contact guidance in two-dimensional and three-dimensional mouse cerebellar cultures. The Journal of comparative neurology 499, 274-289.

Narimatsu, N., Harada, N., Kurihara, H., Nakagata, N., Sobue, K., and Okajima, K. (2009). Donepezil improves cognitive function in mice by increasing the production of insulin-like growth factor-I in the hippocampus. The Journal of pharmacology and experimental therapeutics 330, 2-12.

Noctor, S.C., Flint, A.C., Weissman, T.A., Dammerman, R.S., and Kriegstein, A.R. (2001). Neurons derived from radial glial cells establish radial units in neocortex. Nature 409, 714-720.

Nottebohm, F. (1989). From bird song to neurogenesis. Scientific American 260, 74-79.

O'Rourke, N.A., Sullivan, D.P., Kaznowski, C.E., Jacobs, A.A., and McConnell, S.K. (1995). Tangential migration of neurons in the developing cerebral cortex. Development 121, 2165-2176.

O'Sullivan, C., and Dev, K.K. (2013). The structure and function of the S1P1 receptor. Trends in pharmacological sciences 34, 401-412.

Oakley, H., Cole, S.L., Logan, S., Maus, E., Shao, P., Craft, J., Guillozet-Bongaarts, A., Ohno, M., Disterhoft, J., Van Eldik, L., et al. (2006). Intraneuronal beta-amyloid aggregates, neurodegeneration, and neuron loss in transgenic mice with five familial Alzheimer's disease mutations: potential factors in amyloid plaque formation. The Journal of neuroscience : the official journal of the Society for Neuroscience 26, 10129-10140.

Ono, K., Shokunbi, T., Nagata, I., Tokunaga, A., Yasui, Y., and Nakatsuji, N. (1997). Filopodia and growth cones in the vertically migrating granule cells of the postnatal mouse cerebellum. Experimental brain research 117, 17-29.

Ormerod, B.K., and Galea, L.A. (2001). Reproductive status influences cell proliferation and cell survival in the dentate gyrus of adult female meadow voles: a possible regulatory role for estradiol. Neuroscience 102, 369-379.

Pages, G., Guerin, S., Grall, D., Bonino, F., Smith, A., Anjuere, F., Auberger, P., and Pouyssegur, J. (1999). Defective thymocyte maturation in p44 MAP kinase (Erk 1) knockout mice. Science 286, 1374-1377.

Palka, J., Whitlock, K.E., and Murray, M.A. (1992). Guidepost cells. Current opinion in neurobiology 2, 48-54.

Palmada, M., Kanwal, S., Rutkoski, N.J., Gustafson-Brown, C., Johnson, R.S., Wisdom, R., and Carter, B.D. (2002). c-jun is essential for sympathetic neuronal death induced by NGF withdrawal but not by p75 activation. The Journal of cell biology 158, 453-461.

Parent, J.M. (2003). Injury-induced neurogenesis in the adult mammalian brain. The Neuroscientist : a review journal bringing neurobiology, neurology and psychiatry 9, 261-272.

Park, J.W., Kim, H.J., Byun, J.H., Ryu, H.R., and Jeon, N.L. (2009). Novel microfluidic platform for culturing neurons: culturing and biochemical analysis of neuronal components. Biotechnology journal 4, 1573-1577.

Pearse, D.D., Pereira, F.C., Marcillo, A.E., Bates, M.L., Berrocal, Y.A., Filbin, M.T., and Bunge, M.B. (2004). cAMP and Schwann cells promote axonal growth and functional recovery after spinal cord injury. Nature medicine 10, 610-616.

Pinnock, S.B., Lazic, S.E., Wong, H.T., Wong, I.H., and Herbert, J. (2009). Synergistic effects of dehydroepiandrosterone and fluoxetine on proliferation of progenitor cells in the dentate gyrus of the adult male rat. Neuroscience 158, 1644-1651.

Pitson, S.M., and Pebay, A. (2009). Regulation of stem cell pluripotency and neural differentiation by lysophospholipids. Neuro-Signals 17, 242-254.

Pitson, S.M., and Pebay, A. (2009). Regulation of stem cell pluripotency and neural differentiation by lysophospholipids. Neuro-Signals 17, 242-254.

Plassart-Schiess, E., and Baulieu, E.E. (2001). Neurosteroids: recent findings. Brain research Brain research reviews 37, 133-140.

Rajnicek, A.M., Foubister, L.E., and McCaig, C.D. (2007). Prioritising guidance cues: directional migration induced by substratum contours and electrical gradients is

controlled by a rho/cdc42 switch. Developmental biology 312, 448-460.

Rakic, P. (1972). Mode of cell migration to the superficial layers of fetal monkey neocortex. The Journal of comparative neurology 145, 61-83.

Rasika, S., Nottebohm, F., and Alvarez-Buylla, A. (1994). Testosterone increases the recruitment and/or survival of new high vocal center neurons in adult female canaries. Proceedings of the National Academy of Sciences of the United States of America 91, 7854-7858.

Recknor, J.B., Sakaguchi, D.S., and Mallapragada, S.K. (2006). Directed growth and selective differentiation of neural progenitor cells on micropatterned polymer substrates. Biomaterials 27, 4098-4108.

Richardson, J.A., Rementer, C.W., Bruder, J.M., and Hoffman-Kim, D. (2011). Guidance of dorsal root ganglion neurites and Schwann cells by isolated Schwann cell topography on poly(dimethyl siloxane) conduits and films. Journal of neural engineering 8, 046015.

Rodriguez, J.J., Jones, V.C., Tabuchi, M., Allan, S.M., Knight, E.M., LaFerla, F.M., Oddo, S., and Verkhratsky, A. (2008). Impaired adult neurogenesis in the dentate gyrus of a triple transgenic mouse model of Alzheimer's disease. PloS one 3, e2935.

Rodriguez, J.J., Montaron, M.F., Petry, K.G., Aurousseau, C., Marinelli, M., Premier, S., Rougon, G., Le Moal, M., and Abrous, D.N. (1998). Complex regulation of the expression of the polysialylated form of the neuronal cell adhesion molecule by glucocorticoids in the rat hippocampus. The European journal of neuroscience 10, 2994-3006.

Rossi, C., Angelucci, A., Costantin, L., Braschi, C., Mazzantini, M., Babbini, F., Fabbri, M.E., Tessarollo, L., Maffei, L., Berardi, N., et al. (2006). Brain-derived neurotrophic factor (BDNF) is required for the enhancement of hippocampal neurogenesis following environmental enrichment. The European journal of neuroscience 24, 1850-1856.

Ruiz, C.R., Shi, J., and Meffert, M.K. (2014). Transcript specificity in BDNF-regulated protein synthesis. Neuropharmacology 76 Pt C, 657-663.

Saha, K., Keung, A.J., Irwin, E.F., Li, Y., Little, L., Schaffer, D.V., and Healy, K.E. (2008). Substrate modulus directs neural stem cell behavior. Biophysical journal 95, 4426-4438.

Sahay, A., Scobie, K.N., Hill, A.S., O'Carroll, C.M., Kheirbek, M.A., Burghardt, N.S., Fenton, A.A., Dranovsky, A., and Hen, R. (2011). Increasing adult hippocampal neurogenesis is sufficient to improve pattern separation. Nature 472, 466-470.

Sanchez, T., and Hla, T. (2004). Structural and functional characteristics of S1P receptors. Journal of cellular biochemistry 92, 913-922.

Sapolsky, R.M. (2004). Is impaired neurogenesis relevant to the affective symptoms of depression? Biological psychiatry 56, 137-139.

Scardigli, R., Capelli, P., Vignone, D., Brandi, R., Ceci, M., La Regina, F., Piras, E., Cintoli, S., Berardi, N., Capsoni, S., et al. (2014). Neutralization of nerve growth factor impairs proliferation and differentiation of adult neural progenitors in the subventricular zone. Stem cells 32, 2516-2528.

Scharfman, H., Goodman, J., Macleod, A., Phani, S., Antonelli, C., and Croll, S.

(2005). Increased neurogenesis and the ectopic granule cells after intrahippocampal BDNF infusion in adult rats. Experimental neurology 192, 348-356.

Schloesser, R.J., Lehmann, M., Martinowich, K., Manji, H.K., and Herkenham, M. (2010). Environmental enrichment requires adult neurogenesis to facilitate the recovery from psychosocial stress. Molecular psychiatry 15, 1152-1163.

Schonemann, M.D., Muench, M.O., Tee, M.K., Miller, W.L., and Mellon, S.H. (2012). Expression of P450c17 in the human fetal nervous system. Endocrinology 153, 2494-2505.

Schubiger, M., and Palka, J. (1985). Genetic suppression of putative guidepost cells: effect on establishment of nerve pathways in Drosophila wings. Developmental biology 108, 399-410.

Schumacher, M., Guennoun, R., Robel, P., and Baulieu, E.E. (1997). Neurosteroids in the Hippocampus: Neuronal Plasticity and Memory. Stress 2, 65-78.

Seri, B., Garcia-Verdugo, J.M., Collado-Morente, L., McEwen, B.S., and Alvarez-Buylla, A. (2004). Cell types, lineage, and architecture of the germinal zone in the adult dentate gyrus. The Journal of comparative neurology 478, 359-378.

Shi, J., Longo, F.M., and Massa, S.M. (2013). A small molecule p75(NTR) ligand protects neurogenesis after traumatic brain injury. Stem cells 31, 2561-2574.

Shichita, T., Sugiyama, Y., Ooboshi, H., Sugimori, H., Nakagawa, R., Takada, I., Iwaki, T., Okada, Y., Iida, M., Cua, D.J., et al. (2009). Pivotal role of cerebral interleukin-17-producing gammadeltaT cells in the delayed phase of ischemic brain injury. Nature medicine 15, 946-950.

Shimizu, E., Hashimoto, K., Watanabe, H., Komatsu, N., Okamura, N., Koike, K., Shinoda, N., Nakazato, M., Kumakiri, C., Okada, S., et al. (2003). Serum brain-derived neurotrophic factor (BDNF) levels in schizophrenia are indistinguishable from controls. Neuroscience letters 351, 111-114.

Shors, T.J., Townsend, D.A., Zhao, M., Kozorovitskiy, Y., and Gould, E. (2002). Neurogenesis may relate to some but not all types of hippocampal-dependent learning. Hippocampus 12, 578-584.

Sierra, A., Encinas, J.M., Deudero, J.J., Chancey, J.H., Enikolopov, G., Overstreet-Wadiche, L.S., Tsirka, S.E., and Maletic-Savatic, M. (2010). Microglia shape adult hippocampal neurogenesis through apoptosis-coupled phagocytosis. Cell stem cell 7, 483-495.

Simitzi, C., Stratakis, E., Fotakis, C., Athanassakis, I., and Ranella, A. (2015). Microconical silicon structures influence NGF-induced PC12 cell morphology. Journal of tissue engineering and regenerative medicine 9, 424-434.

Singh, C., Liu, L., Wang, J.M., Irwin, R.W., Yao, J., Chen, S., Henry, S., Thompson, R.F., and Brinton, R.D. (2012). Allopregnanolone restores hippocampal-dependent learning and memory and neural progenitor survival in aging 3xTgAD and nonTg mice. Neurobiology of aging 33, 1493-1506.

Singh, M. (2006). Progesterone-induced neuroprotection. Endocrine 29, 271-274.

Smith, R., Petersen, A., Bates, G.P., Brundin, P., and Li, J.Y. (2005). Depletion of rabphilin 3A in a transgenic mouse model (R6/1) of Huntington's disease, a possible culprit in synaptic dysfunction. Neurobiology of disease 20, 673-684.

Snyder, J.S., Hong, N.S., McDonald, R.J., and Wojtowicz, J.M. (2005). A role for adult neurogenesis in spatial long-term memory. Neuroscience 130, 843-852.

Snyder, J.S., Soumier, A., Brewer, M., Pickel, J., and Cameron, H.A. (2011). Adult hippocampal neurogenesis buffers stress responses and depressive behaviour. Nature 476, 458-461.

Spritzer, M.D., and Galea, L.A. (2007). Testosterone and dihydrotestosterone, but not estradiol, enhance survival of new hippocampal neurons in adult male rats. Developmental neurobiology 67, 1321-1333.

Stein-Behrens, B.A., Lin, W.J., and Sapolsky, R.M. (1994). Physiological elevations of glucocorticoids potentiate glutamate accumulation in the hippocampus. Journal of neurochemistry 63, 596-602.

Stemple, D.L., and Anderson, D.J. (1992). Isolation of a stem cell for neurons and glia from the mammalian neural crest. Cell 71, 973-985.

Stratakis, E., Eda, G., Yamaguchi, H., Kymakis, E., Fotakis, C., and Chhowalla, M. (2012). Free-standing graphene on microstructured silicon vertices for enhanced field emission properties. Nanoscale 4, 3069-3074.

Sun, D., McGinn, M., Hankins, J.E., Mays, K.M., Rolfe, A., and Colello, R.J. (2013). Aging- and injury-related differential apoptotic response in the dentate gyrus of the hippocampus in rats following brain trauma. Frontiers in aging neuroscience 5, 95.

Sun, X., Sun, X., Liu, T., Zhao, M., Zhao, S., Xiao, T., Jolkkonen, J., and Zhao, C. (2015). Fluoxetine enhanced neurogenesis is not translated to functional outcome in stroke rats. Neuroscience letters 603, 31-36.

Surget, A., Tanti, A., Leonardo, E.D., Laugeray, A., Rainer, Q., Touma, C., Palme, R., Griebel, G., Ibarguen-Vargas, Y., Hen, R., et al. (2011). Antidepressants recruit new neurons to improve stress response regulation. Molecular psychiatry 16, 1177-1188.

Suter, D.M., and Miller, K.E. (2011). The emerging role of forces in axonal elongation. Progress in neurobiology 94, 91-101.

Suzuki, M., Wright, L.S., Marwah, P., Lardy, H.A., and Svendsen, C.N. (2004). Mitotic and neurogenic effects of dehydroepiandrosterone (DHEA) on human neural stem cell cultures derived from the fetal cortex. Proceedings of the National Academy of Sciences of the United States of America 101, 3202-3207.

Svensson, P., Wang, K., Arendt-Nielsen, L., and Cairns, B.E. (2008). Effects of NGF-induced muscle sensitization on proprioception and nociception. Experimental brain research 189, 1-10.

Takahashi, H., Belser, P.H., Atkinson, B.F., Sela, B.A., Ross, A.H., Biegel, J., Emanuel, B., Sutton, L., Koprowski, H., and Herlyn, D. (1990). Monoclonal antibody-dependent, cell-mediated cytotoxicity against human malignant gliomas. Neurosurgery 27, 97-102.

Takasugi, N., Sasaki, T., Ebinuma, I., Osawa, S., Isshiki, H., Takeo, K., Tomita, T., and Iwatsubo, T. (2013). FTY720/fingolimod, a sphingosine analogue, reduces amyloid-beta production in neurons. PloS one 8, e64050.

Tamplenizza, M., Lenardi, C., Maffioli, E., Nonnis, S., Negri, A., Forti, S., Sogne, E.,

De Astis, S., Matteoli, M., Schulte, C., et al. (2013). Nitric oxide synthase mediates PC12 differentiation induced by the surface topography of nanostructured TiO2. Journal of nanobiotechnology 11, 35.

Tanapat, P., Hastings, N.B., and Gould, E. (2005). Ovarian steroids influence cell proliferation in the dentate gyrus of the adult female rat in a dose- and time-dependent manner. The Journal of comparative neurology 481, 252-265.

Tanapat, P., Hastings, N.B., Reeves, A.J., and Gould, E. (1999). Estrogen stimulates a transient increase in the number of new neurons in the dentate gyrus of the adult female rat. The Journal of neuroscience : the official journal of the Society for Neuroscience 19, 5792-5801.

Tashiro, A., Sandler, V.M., Toni, N., Zhao, C., and Gage, F.H. (2006). NMDA-receptor-mediated, cell-specific integration of new neurons in adult dentate gyrus. Nature 442, 929-933.

Taylor, A.M., Blurton-Jones, M., Rhee, S.W., Cribbs, D.H., Cotman, C.W., and Jeon, N.L. (2005). A microfluidic culture platform for CNS axonal injury, regeneration and transport. Nature methods 2, 599-605.

Terry, A.V., Jr., and Buccafusco, J.J. (2003). The cholinergic hypothesis of age and Alzheimer's disease-related cognitive deficits: recent challenges and their implications for novel drug development. The Journal of pharmacology and experimental therapeutics 306, 821-827.

Tervonen, T.A., Ajamian, F., De Wit, J., Verhaagen, J., Castren, E., and Castren, M. (2006). Overexpression of a truncated TrkB isoform increases the proliferation of neural progenitors. The European journal of neuroscience 24, 1277-1285.

Thompson, D.M., and Buettner, H.M. (2006). Neurite outgrowth is directed by schwann cell alignment in the absence of other guidance cues. Annals of biomedical engineering 34, 161-168.

Torigoe, K., Tanaka, H.F., Takahashi, A., Awaya, A., and Hashimoto, K. (1996). Basic behavior of migratory Schwann cells in peripheral nerve regeneration. Experimental neurology 137, 301-308.

Tronel, S., Belnoue, L., Grosjean, N., Revest, J.M., Piazza, P.V., Koehl, M., and Abrous, D.N. (2012). Adult-born neurons are necessary for extended contextual discrimination. Hippocampus 22, 292-298.

Tuszynski, M.H., U, H.S., Amaral, D.G., and Gage, F.H. (1990). Nerve growth factor infusion in the primate brain reduces lesion-induced cholinergic neuronal degeneration. The Journal of neuroscience : the official journal of the Society for Neuroscience 10, 3604-3614.

Vallee, M., Mayo, W., and Le Moal, M. (2001). Role of pregnenolone, dehydroepiandrosterone and their sulfate esters on learning and memory in cognitive aging. Brain research Brain research reviews 37, 301-312.

van der Borght, K., and Brundin, P. (2007). Reduced expression of PSA-NCAM in the hippocampus and piriform cortex of the R6/1 and R6/2 mouse models of Huntington's disease. Experimental neurology 204, 473-478.

Van der Borght, K., Mulder, J., Keijser, J.N., Eggen, B.J., Luiten, P.G., and Van der Zee, E.A. (2005). Input from the medial septum regulates adult hippocampal neurogenesis. Brain research bulletin 67, 117-125.

Van Doorn, R., Van Horssen, J., Verzijl, D., Witte, M., Ronken, E., Van Het Hof, B., Lakeman, K., Dijkstra, C.D., Van Der Valk, P., Reijerkerk, A., et al. (2010). Sphingosine 1-phosphate receptor 1 and 3 are upregulated in multiple sclerosis lesions. Glia 58, 1465-1476.

Vithlani, M., Hines, R.M., Zhong, P., Terunuma, M., Hines, D.J., Revilla-Sanchez, R., Jurd, R., Haydon, P., Rios, M., Brandon, N., et al. (2013). The ability of BDNF to modify neurogenesis and depressive-like behaviors is dependent upon phosphorylation of tyrosine residues 365/367 in the GABA(A)-receptor gamma2 subunit. The Journal of neuroscience : the official journal of the Society for Neuroscience 33, 15567-15577.

Voelker, M., and Fromherz, P. (2005). Signal transmission from individual mammalian nerve cell to field-effect transistor. Small 1, 206-210.

Vollmayr, B., Simonis, C., Weber, S., Gass, P., and Henn, F. (2003). Reduced cell proliferation in the dentate gyrus is not correlated with the development of learned helplessness. Biological psychiatry 54, 1035-1040.

Wade, K.C., Guttentag, S.H., Gonzales, L.W., Maschhoff, K.L., Gonzales, J., Kolla, V., Singhal, S., and Ballard, P.L. (2006). Gene induction during differentiation of human pulmonary type II cells in vitro. American journal of respiratory cell and molecular biology 34, 727-737.

Waeber, C., and Chiu, M.L. (1999). In vitro autoradiographic visualization of guanosine-5'-O-(3-[35S]thio)triphosphate binding stimulated by sphingosine 1-phosphate and lysophosphatidic acid. Journal of neurochemistry 73, 1212-1221.

Walker, T.L., and Kempermann, G. (2014). One mouse, two cultures: isolation and culture of adult neural stem cells from the two neurogenic zones of individual mice. Journal of visualized experiments : JoVE, e51225.

Walsh, J.F., Manwaring, M.E., and Tresco, P.A. (2005). Directional neurite outgrowth is enhanced by engineered meningeal cell-coated substrates. Tissue engineering 11, 1085-1094.

Wang, L.P., Kempermann, G., and Kettenmann, H. (2005). A subpopulation of precursor cells in the mouse dentate gyrus receives synaptic GABAergic input. Molecular and cellular neurosciences 29, 181-189.

Wei, Y., Yemisci, M., Kim, H.H., Yung, L.M., Shin, H.K., Hwang, S.K., Guo, S., Qin, T., Alsharif, N., Brinkmann, V., et al. (2011). Fingolimod provides long-term protection in rodent models of cerebral ischemia. Annals of neurology 69, 119-129.

Weiss, P. (1947). The problem of specificity in growth and development. The Yale journal of biology and medicine 19, 235-278.

Wen, X., and Tresco, P.A. (2006). Effect of filament diameter and extracellular matrix molecule precoating on neurite outgrowth and Schwann cell behavior on multifilament entubulation bridging device in vitro. Journal of biomedical materials research Part A 76, 626-637.

Wheeler, D., Bandaru, V.V., Calabresi, P.A., Nath, A., and Haughey, N.J. (2008). A defect of sphingolipid metabolism modifies the properties of normal appearing white matter in multiple sclerosis. Brain : a journal of neurology 131, 3092-3102.

White, F.A., Silos-Santiago, I., Molliver, D.C., Nishimura, M., Phillips, H., Barbacid,

M., and Snider, W.D. (1996). Synchronous onset of NGF and TrkA survival dependence in developing dorsal root ganglia. The Journal of neuroscience : the official journal of the Society for Neuroscience 16, 4662-4672.

Winner, B., Geyer, M., Couillard-Despres, S., Aigner, R., Bogdahn, U., Aigner, L., Kuhn, G., and Winkler, J. (2006). Striatal deafferentation increases dopaminergic neurogenesis in the adult olfactory bulb. Experimental neurology 197, 113-121.

Winner, B., Lie, D.C., Rockenstein, E., Aigner, R., Aigner, L., Masliah, E., Kuhn, H.G., and Winkler, J. (2004). Human wild-type alpha-synuclein impairs neurogenesis. Journal of neuropathology and experimental neurology 63, 1155-1166.

Xu, B., Yang, R., Chang, F., Chen, L., Xie, G., Sokabe, M., and Chen, L. (2012). Neurosteroid PREGS protects neurite growth and survival of newborn neurons in the hippocampal dentate gyrus of APPswe/PS1dE9 mice. Current Alzheimer research 9, 361-372.

Yamada, M., Onodera, M., Mizuno, Y., and Mochizuki, H. (2004). Neurogenesis in olfactory bulb identified by retroviral labeling in normal and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated adult mice. Neuroscience 124, 173-181.

Yoshimi, K., Ren, Y.R., Seki, T., Yamada, M., Ooizumi, H., Onodera, M., Saito, Y., Murayama, S., Okano, H., Mizuno, Y., et al. (2005). Possibility for neurogenesis in substantia nigra of parkinsonian brain. Annals of neurology 58, 31-40.

Yu, T.T., and Shoichet, M.S. (2005). Guided cell adhesion and outgrowth in peptide-modified channels for neural tissue engineering. Biomaterials 26, 1507-1514.

Zeidan, Y.H., Jenkins, R.W., and Hannun, Y.A. (2008). Remodeling of cellular cytoskeleton by the acid sphingomyelinase/ceramide pathway. The Journal of cell biology 181, 335-350.

Zhang, H., Petit, G.H., Gaughwin, P.M., Hansen, C., Ranganathan, S., Zuo, X., Smith, R., Roybon, L., Brundin, P., Mobley, W.C., et al. (2013). NGF rescues hippocampal cholinergic neuronal markers, restores neurogenesis, and improves the spatial working memory in a mouse model of Huntington's Disease. Journal of Huntington's disease 2, 69-82.

Zhao, C., Teng, E.M., Summers, R.G., Jr., Ming, G.L., and Gage, F.H. (2006). Distinct morphological stages of dentate granule neuron maturation in the adult mouse hippocampus. The Journal of neuroscience : the official journal of the Society for Neuroscience 26, 3-11.

Zhao, M., Momma, S., Delfani, K., Carlen, M., Cassidy, R.M., Johansson, C.B., Brismar, H., Shupliakov, O., Frisen, J., and Janson, A.M. (2003). Evidence for neurogenesis in the adult mammalian substantia nigra. Proceedings of the National Academy of Sciences of the United States of America 100, 7925-7930.

Zhu, Y., Jin, K., Mao, X.O., and Greenberg, D.A. (2003). Vascular endothelial growth factor promotes proliferation of cortical neuron precursors by regulating E2F expression. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 17, 186-193.