The identification of compounds from apples that regulate adult hippocampal neurogenesis

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To the pearls of my life :

Mama, Nisa and Athifa

„The capacity to learn is a gift; the ability to learn is a skill;
the willingness to learn is a choice" – Brian Herbert

Zusamenfassung

Eine Ernährung die täglich reich an Obst und Gemüse ist, hat insbesondere bei älteren Menschen einen positiven Einfluss auf kognitive Fähigkeiten. Pflanzeninhaltsstoffe wirken als natürliche Antioxidantien, indem sie oxidative Stressoren neutralisieren. Weiterhin beeinflussen pflanzliche Nährstoffe molekulare Signalwege welche beim Überleben von Neuronen eine Rolle spielen. Die adulte hippocampale Neurogenese ist ein dynamischer, lebenslanger Prozess, bei dem aus Vorläuferzellen funktionelle neue Neuronen in der Körnerzellschicht des Gyrus dentatus gebildet werden. Dieser Prozess trägt zur Plastizität des Gehirns bei und spielt eine bedeutende Rolle beim Lernen und für das Gedächtnis. Externe Stimuli wie zum Beispiel eine reizreiche Umgebung und körperliche Aktivität wirken als positive Regulatoren und begünstigen die adulte hippocampale Neurogenese. Welche Rolle die Ernährung dabei spielt und ob Nahrungsbestandteile einen pro-neurogenen Effekt auf adulte hippocampale Vorläuferzellen haben ist kaum bekannt.

In diesem Projekt habe ich den Effekt von Nahrungsbestandteilen aus Äpfeln, welche eine bedeutende Quelle von pflanzlichen Nährstoffen in unserer Ernährung darstellen, auf die adulte hippocampale Neurogenese untersucht. Ich habe gezeigt, dass Querzetin, das am reichlichsten in Äpfeln enthaltende Polyphenol, in der Monolayer-Zellkultur den Austritt aus dem Zellzyklus induziert und die Differenzierung von adulten hippocampalen Vorläuferzellen fördert. Des Weiteren steigert Querzetin nach der Differenzierung in vitro die Anzahl an überlebenden Zellen. Dies geschieht durch die Aktivierung von endogenen Antioxidantien des Nrf2-Keap1-Signalweges und des für das Überleben von Zellen förderlichen Akt-Signalweges. Die Verabreichung von Querzetin in vivo als Nahrungsergänzungsmittel führte ebenfalls zu einem signifikanten Anstieg der Anzahl an überlebenden Zellen und neu gebildeten Nervenzellen im Gyrus dentatus. Um weitere potentiell aktive Wirkstoffe von Äpfeln zu bestimmen, habe ich eine Bioassay-ausgerichtete Fraktionierung durchgeführt, wobei der Fruchtfleischextrakt von Äpfeln der Sorte Pinova einer Fest-/ Flüssig-Separation unterzogen wurde. Die aktive Fraktion wurde anhand der primären Neurosphäre-Assay-Methode mit Zellen aus dem Gyrus dentatus adulter Mäuse ermittelt. Mittels spektrometrischer Analyse habe ich gezeigt, dass die aktiven Wirkstoffe im Fruchtfleischextrakt von Äpfeln zur Gruppe der Dihydroxybenzol-Glykosiden gehören, welche den nicht-flavonoiden Benzoesäure-Derivaten zuzuordnen sind.

Im in vitro Neurosphäre-Assay habe ich zudem gezeigt, dass die Isomere dieser Wirkstoffe, die 2,3- und die 3,5-Dihydroxybenzoesäuren, die Anzahl der Neurosphären signifikant erhöhen. Interessanterweise ist die 3,5-Dihydroxybenzoesäure ein Agonist des Laktatrezeptors Hydroxycarboxylic acid receptor 1 (HCAR1) und weist sogar eine noch höhere Affinität als Laktat auf. Es wird suggeriert, dass dieser Rezeptor neurotrophische Wirkungen vermittelt, wie zum Beispiel eine erhöhte Produktion von BDNF und dessen Ausschüttung. Zudem habe ich das Vorkommen dieses Reporters erstmalig bei adulten hippocampalen Vorläuferzellen nachgewiesen.

Um zu untersuchen, ob der Konsum handelsüblicher Obstprodukte die adulte hippocampale Neurogenese beeinflusst, habe ich Mäusen Apfelsaft ad libitum verabreicht. Nach der Gabe von Apfelsaft sah ich keinen signifikanten Anstieg der Gesamtneurogenese und keine Verbesserung der Leistungsfähigkeit im Morris-Wasserlabyrinth-Test. Dies ist bedingt durch eine zu geringe Konzentration der aktiven Wirkstoffe im Apfelsaft wodurch die wirksame Konzentration im Körper nicht erreicht wird.

Ich schlussfolgere, dass in Äpfeln potentielle pro-neurogene Inhaltsstoffe enthalten sind, welche die adulte hippocampale Neurogenese beeinflussen. Dies wird insbesondere durch die Aktivierung endogener antioxidativer Mechanismen und molekularer Signalwege vermittelt, die für das Überleben von Zellen von Bedeutung sind. Weitere Studien sind nötig, um zu bestimmen wie sich die Aktivierung von HCAR1 auf die adulte hippocampale Neurogenese auswirkt. Dies stellt einen potentiellen neuen Wirkmechanismus dar, welcher die gesundheitlichen Vorteile von Obst- und Gemüsekonsum belegt.

Summary

The high composition of fruits and vegetables in the daily diet is associated with cognitive well-being, especially in the elderly population. The phytonutrients are shown to have effects as antioxidants that neutralize oxidative stressors and can interact with molecular pathways to signal neuron survival. Adult hippocampal neurogenesis is a dynamic lifelong process of generating functional newborn neurons in the granular layer of the dentate gyrus from adult precursor cells. This process contributes to brain plasticity and plays a role in learning and memory. External stimuli such as environmental enrichment and physical activity are known to positively regulate this process. However, the role of nutrition and whether nutritional compounds have pro-neurogenic effects on adult hippocampal precursor cells are still elusive.

In this study, I investigated the impact of dietary compounds in apples, a significant source of phytonutrients in our food, on adult hippocampal neurogenesis. I demonstrated that quercetin, the most abundant polyphenol in apple, induces cell cycle exit and differentiation of adult hippocampal precursor cells in monolayer culture. Furthermore, this compound also increases the number of surviving cells upon differentiation *in vitro*, through the activation of endogenous antioxidants in the Nrf2-Keap1 pathway and the prosurvival Akt pathway. Quercetin supplementation *in vivo* is also shown to significantly increase the number of surviving cells and new neurons in the dentate gyrus. To search for other potential active compounds in apple, I performed bioassay-guided fractionation whereby the flesh extract from apples of the Pinova cultivar was subjected to liquid- and solid phase separation and the active fraction was determined using primary neurosphere assays using cells derived from adult mouse dentate gyrus. Using mass spectometry, we revealed that the active compounds in the apple flesh extract are dihydroxybenzoate glycosides, which are non-flavonoid benzoic acid derivatives. I also confirmed that the isomers of these compounds; 2,3- and 3,5 dihydroxybenzoic acids significantly increase the number of neurospheres. Interestingly, 3,5 dihdroxybenzoic acid is an agonist of lactate receptor hydroxycarboxylic acid receptor 1 (HCAR1), with an even higher affinity than lactate. This receptor is suggested to mediate neurotrophic actions such as increasing production and release of BDNF. I also demonstrated for the first time that this receptor is presence in adult hippocampal precursor cells.

To observe whether customary fruits or fruit-related products consumption affects adult hippocampal neurogenesis, I performed an experiment giving apple juice supplementation *ad libitum* to mice. I did not find a significant increase in net neurogenesis or the performance in the Morris water maze after apple juice supplementation. This is likely due to the low concentration of active compounds in apple juice failing to reach an effective concentration in the body.

I conclude that apples provide potential proneurogenic compounds that can influence adult hippocampal neurogenesis through the activation of endogenous antioxidant mechanisms and molecular pathways for cell survival. Further studies are necessary to investigate the role of HCAR1 activation on adult hippocampal neurogenesis, which is a potential new mechanism to explain the health benefits of fruit and vegetable consumption.

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Alhamdulillah, it is with my deepest gratitude to Allah the All-Praiseworthy for the lifetime, opportunities, health and other uncountable blessings that enable me to accomplish this challenging and long journey.

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Life as a PhD student for me is like walking in a labyrinth. Sometimes you cheer up with your exiting finding, but a minute later you figure out that you are in the same circle. In other time you stuck at the dead-end and don't know where to proceed. During this frustrating time, it was very helpful to share and talk with colleagues, accompanied by a cup of delightful coffee. I will be for sure missing the relaxing, funny but inspirational and enlightening discussion with Julia, Vijay, Rupert, Zeina, Tara and the others during the coffee break.

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Abbreviations

7-AAD	7-amino-actinomycin D
ACN	acetonitrile
AHN	adult hippocampal neurogenesis
$AlCl_3$	aluminium chloride
ANOVA	analysis of variance
APS	ammonium persulfate
BDNF	brain-derived neurotrophic factor
bFGF	basic fibroblast growth factor
BLBP	brain-lipid-binding protein
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
BuOH	butanol
CA	cornu ammonis
CldU	chlorodeoxyuridine
CPS	cryoprotection solution
CREB	cAMP-response element-binding protein
DAB	3,3'-Diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DCX	doublecortin
DG	dentate gyrus
DHBA	dihydroxybenzoic acid
DMSO	dimethylsulfoxide
EC	entorhinal cortex
ECM	extracellular matrix
EDTA	ethylene diamine tetraacetic acid
EGF	epidermal growth factor
eNOS	endothelial nitric oxide synthase
ERK	mitogenic extracellular signal-regulated protein kinase
ESI	electron spray ionization
EtOAc	ethylacetate
EtOH	ethanol
FACS	fluorescence-activated cell sorting
FDR	false discovery rate
GFAP	glial fibrillary acidic protein

Gpr81	G-protein-coupled receptor 81
Gsta3	glutathione S-transferase, alpha 3
HBSS	Hank´s balanced salt solution
HCAR 1	hydroxycarboxylic acid receptor 1
HCl	hydrochloric acid
HPLC	high-performance liquid chromatography
IdU	iododeoxyuridine
IGF-1	insulin-like growth factor-1
JNK	c-Jun N-terminal kinase
LTP	long term potentiation
MAPK	mitogen activated protein kinase
MeOH	methanol
MS	mass spectrometry
MWM	Morris water maze
$NaBH_4$	sodium borohydride
NaCl	sodium chloride
NGF	nerve growth factor
NiCl	nickel chloride
NP-40	nonident P40
OB	olfactory bulb
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDA	photodiode array detection
PDL	poly-D-lysin
PFA	paraformaldehyde
PI3K	phosphoinositide-3-kinase
PSA-NCAM	polysialylated-neural cell adhesion molecule
qTOF	quadrupole time-of-flight
RGC	radial glia-like cells
RMS	rostral migratory stream
SDS	sodium dodecyl sulfate
SGZ	subgranular zone
SVZ	ssubventricular zone
TBS	tris buffered saline
TEMED	tetramethyl ethylene diamine
THF	tetrahydrofurane
UPLC	ultra performance liquid chromatography

Figures and Tables

Publications

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Contents

1. Introduction

1.1. Food for thought: the effect of dietary intervention on cognition

Improvements in health care, hygiene, food sustainability and political stability over the past decades have increased life expectancy of human population globally, with the number of population aged 80 or over is predicted to reach 379 million people worldwide in 2050. Consequently, we are facing an increase in ageing-related health problems such as cognitive impairment, that impacts not only the quality of life of the elderly subjects and caregivers, but also the global economic conditions (Beaglehole *et al.*, 2003). Extensive studies are being conducted to understand and identify remedies for ageing-related diseases, however, currently available pharmacological interventions for cognitive impairment still lack the desired efficacy and only afford modest symptomatic improvements. Moreover, the progressive underlying neurodegeneration processes remain untreated. Thus, search for other modalities that have prevention and therapeutic potential for this condition is requisite (Solfrizzi *et al.*, 2008).

Apart from being the source of energy and building materials for the body, food is also believed to influence the individuals well being and protect against diseases (Gomez-Pinilla, 2008). The concept and belief that diet is an important precondition to achieve physical and intellectual fitness has been developed since ancient human civilization across various cultures. Hippocrates of Kos (5th century BC), the father of western medicine, described splendidly the role of diet for disease treatment in ancient Greek civilization. The same principle was also taught by Plato in his Platonic dialogues. The concept encouraged a balance in food composition and moderation of food intake as the essential elements to support optimal health and illness prevention. Particular foodstuffs and the dietary pattern depicted in Plato's works share many similarities with the recently so-called the Mediterranean diet (Skiadas and Lascaratos, 2001). The term "Mediterranean diet" is used to describe the food pattern typical of Crete and southern Italy in 1960s that mainly consists of minimally processed plant foods such as cereals, legumes, seeds, fruit, vegetables and olives. The main source of fat is from olive oil that is high in unsaturated fatty acids, while the consumption of meat products is low (Willett *et al.*, 1995). In Asia, ancient Indian culture established the Ayurvedic diet (Lele, 2012) and traditional Chinese culture follows the Yin/Yang concept in their diet to maintain health and promote the healing process (Lee and Shen, 2008). One of the

most studied dietary patterns in Asia is the Okinawan diet. The inhabitants of Okinawa, Ryukyu island have a high average life expectancy and an unusually high number of centenarians (the people with age 100 years old). The Okinawans food culture in the past was strongly influenced by Chinese concept of herbal medicine whereby the food was considered as "medicine for life". The daily calorie intake was low, yet nutritionally dense especially with plant-derived chemicals (phytonutrients or phytochemicals) such as from sweet potatoes (rich source of anthocyanin), leafy vegetables, seaweed, tofu and herbs such as fennel and curcumin (reviewed by Sho, 2001; Miyagi *et al.*, 2003).

Meta-analysis of published epidemiology studies suggests that adherence to Mediterranean diet is associated with slower cognitive decline and lower risk of Alzheimer's disease (Lourida *et al.*, 2013). Similarly, a population-based study reported that the centenarians in Okinawa were not only high in their number, but also their high physical and cognitive function until the very late of lifetime (Willcox *et al.*, 2008). In agreement with these studies, most of the recently popular healthy dietary patterns and guidelines recommend the daily intake of vegetables and fruits as significant ingredients to achieve higher life expectancy and better cognitive health (reviewed by Kiefte-de Jong *et al.*, 2014; Lamport *et al.*, 2014).

A growing number of studies are investigating the health benefits of the plant-specific constituents in our food, herein referred to as phytochemicals or phytonutrients, to establish the scientific rational to apply them as nutritionally active ingredients and further applications in disease treatment and prevention. Phytochemicals have been shown to provide health benefits through their roles such as the substrates for biochemical reactions, cofactors or inhibitors of enzymatic reactions, ligands of membrane or intracellular receptors, scavenger of reactive chemicals, and modulation of gut microbes (for review, see Dillard and German, 2000).

1.2. Diet, neuroprotection and brain plasticity

The term cognition refers to all processes by which the sensory input is transformed, reduced, elaborated, stored, recovered and used (Neisser, 1967). It is associated with individuals' learning and memory ability, which encompasses complex processes in the brain (Pessoa, 2008).

One of the processes underlying cognitive function is brain plasticity. It refers to the ability of the brain to perform structural and functional modification as the response to the internal and external changes that are associated with the functional changes of the individuals (eg. memory, addiction and recovery of function) in

order to react and adapt to those stimuli. The modifications can be the remodelling of dendritic length, spine density, synapse formation and neurogenesis (reviewed by Kolb and Whishaw, 1998; Bruel-Jungerman *et al.*, 2007). Although these processes occur throughout lifetime, this capacity decreases with age (Burke and Barnes, 2006).

The high content of unsaturated fatty acids, large oxygen utilization and weak antioxidant defence system are among several risk factors that make the brain highly vulnerable to oxidative damage (Floyd and Carney, 1992). Moreover, the accumulation of reactive oxygen species as the by-product of normal metabolism is reaching deleterious levels in senescent cells and is followed by cellular damage (such as mitochondrial decay) and cell death. As the individuals age, this process leads to progressive decline of neuronal connectivity and functions, leading to deterioration of cognition and memory (Mahncke *et al.*, 2006; Terman and Brunk, 2006). Supplementation with antioxidants provides neuroprotective effect by inhibition of oxidative damage, thus delaying mitochondrial decay and improving the age-associated memory decline in rats (Liu *et al.*, 2002).

A large body of *in vitro* and *in vivo* studies demonstrated active dietary compounds in foodstuffs that can promote brain plasticity and neuroprotection. Macro and micronutrients in food can modulate cognitive performance by directly influencing cellular and molecular functions in the brain including regulating neurotransmitter pathways, synaptic transmission, membrane fluidity and signal-transduction pathways (Table 1.1). Particular nutrients are classified as "good for brain" such as unsaturated fatty acids, polyphenols, vitamins and minerals. In contrast, a diet rich in saturated fat and refined carbohydrates are associated with the impairment of neural plasticity and increased risk of cognitive impairment in animals and human studies (Kalmijn *et al.*, 1997; Molteni *et al.*, 2002; Gomez-Pinilla, 2008). Compiling studies showed that flavonoids, the abundant phytonutrients in fruits and vegetables, are related to the improvement of cognitive performance. It is unlikely that the effect is exerted through their capacity to quench oxidative stress per se. Increasing evidence shows that flavonoids interact and modulate molecular signaling pathways (figure 1.1) to affect cellular functions that are involved in learning and memory (Williams *et al.*, 2004; Spencer, 2009).

Adult hippocampal neurogenesis is a unique form of brain plasticity whereby functional newborn neurons are generated throughout life and integrated into the existing neuronal circuitry that introduces a tremendous cellular and synaptic plasticity in the hippocampus (Altman and Das, 1965; van Praag *et al.*, 2002). The hippocampus is one of the brain structures that is essential in memory

consolidation, as well as contextual and spatial learning processes (Squire, 1992). Studies in rodents showed that increased adult neurogenesis improved spatial learning (van Praag *et al.*, 2005) and pattern separation (Sahay *et al.*, 2011). The ablation of adult hippocampal neurogenesis is shown to impair cognitive performance such as the impairment of long-term memory formation (Jessberger *et al.*, 2009) or attenuation of "re-learning" capability and learning strategy flexibility (Garthe *et al.*, 2009). Physical activity and environment enrichment are among the various factors that are robustly demonstrated to retain or induce adult neurogenesis resulting in the improvement in hippocampus-related behaviour (Brown *et al.*, 2003). Small molecules in plants (phytochemicals) are also discussed in the literature for their effect in the modulation of the adult hippocampal neurogenesis. This issue will be revisited on the later section of this chapter.

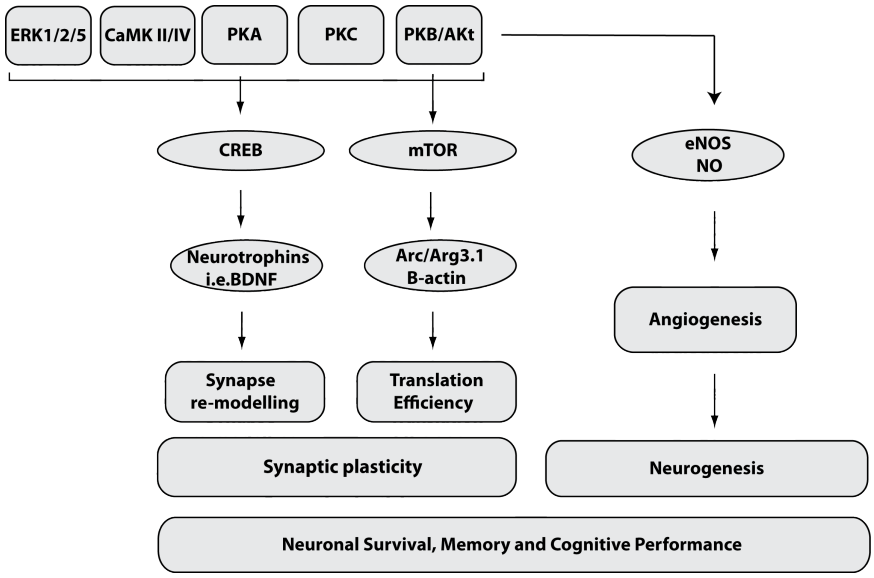

Figure 1.1. Signaling pathways involved in memory formation and cognitive performance through modulation of synaptic and cellular plasticity (neurogenesis). Figure is reproduced from Spencer (2009) with permission of The Royal Society of Chemistry.

Table 1.1. Several nutrients that affect cognitive performance (modified from Gomez-Pinilla (2008).

Nutrient	Effects on cognition	Food source
• Unsaturated fat (eg. Oleic acid, docoxahexanoic acid)	Restored spatial memory deficit in aged mice (Labrousse *et al.*, 2012) Increased hippocampal neurogenesis and dendritic arborization of new born neurons in aged mice (Cutuli *et al.*, 2014)	Olive, fish, walnut
• Polyphenols	Long term intake preserved verbal memory in elderly (Kesse-Guyot *et al.*, 2012) Improved memory performance and hippocampal functional connectivity (Witte *et al.*, 2014)	Fruits (apples, berries, grapes, etc), vegetables
• Micronutrients (vitamins & minerals)	Vitamin E reversed the impaired cognitive function due to high fat diet through normalization of BDNF, synapsin I and CREB level in hippocampus (Wu *et al.*, 2004) Vitamin B-6, B-12 or folate supplementation was associated with improved memory performance in various ages of woman subjects (Bryan *et al.*, 2002) Decreased selenium level correlated with lower cognitive score in elderly (Gao *et al.*, 2007).	wheatgerm, avocado, nuts. grains, nuts, asparagus, yeast, meat. cereals, fish, meat, nuts.
• Refined carbohydrates	Impaired place recognition memory, increased inflammation and oxidative stress in rat hippocampus (Beilharz *et al.*, 2014).	White bread, polished rice, table sugar, honey, corn syrup.

• Saturated fat	Decreased hippocampal BDNF level and spatial learning in rat (Molteni *et al.*, 2002) Associated with increased risk of cognitive decline in human (Greenwood and Winocur, 2005)	Coconut oil, palm oil, dairy products, lard, fatty meat

1.3. Adult hippocampal neurogenesis: a unique process that significantly contributes to learning and memory

Adult neurogenesis is the process of new functional neuron production in the adult brain. It is interpreted as the developmental process of the brain in the adult condition that is dedicated to an adaptive mechanism. Although it is clear that most of the developmental processes have been accomplished and cease in the adult brain, there are two regions in the mammalian brain where neuronal precursor cells reside in a permissive microenvironment and undergo the continuous development: the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of dentate gyrus in the hippocampus (figure 1.2-A) (Cameron *et al.*, 1993; Corotto *et al.*, 1993). In the subventricular zone, the precursor cells proliferate and migrate over relatively long distances along the rostral migratory stream toward the granular and glomerular cell layers of the olfactory bulb and differentiate into interneurons (Luskin, 1993). On the other hand, the precursor cells residing at the subgranular zone proliferate and only small numbers of survivors subsequently migrate, differentiate into neurons and ultimately integrate in the existing circuitry in the granule cell layer of dentate gyrus (van Praag *et al.*, 2002; Kempermann *et al.*, 2003). In this thesis, I use the terminology "precursor cells" to refer the stem and progenitor cells.

1.3.1. Hippocampus: Biological characteristics and function

The term hippocampus (derived from Latin word for "sea horse") was initially coined by the Bolognese anatomist Giulio Cesare Aranzi (ca. 1564) to name the bilateral structures, resembling this sea creature, that are found in the temporal lobes of the human brain. In most literature, the hippocampus is implicitly defined as one of several related brain regions comprising a functional system called the hippocampal formation. The hippocampal formation itself consists of the hippocampus proper (CA1, CA2 and CA3), dentate gyrus, subiculum,

presubiculum, parasubiculum and entorhinal cortex (Amaral and Lavenex, 2007). Phylogenetic studies considered the hippocampal formation to be a form of "old" cortex, however the dentate gyrus is "younger". In higher vertebrates, this structure is translocated into the inferior part of the lateral ventricle due to the expansion of the cerebral cortex. While in rodents, most parts of the hippocampus are located dorsally and lack the "sea horse" resemblance (Frotscher and Laszlo, 2006).

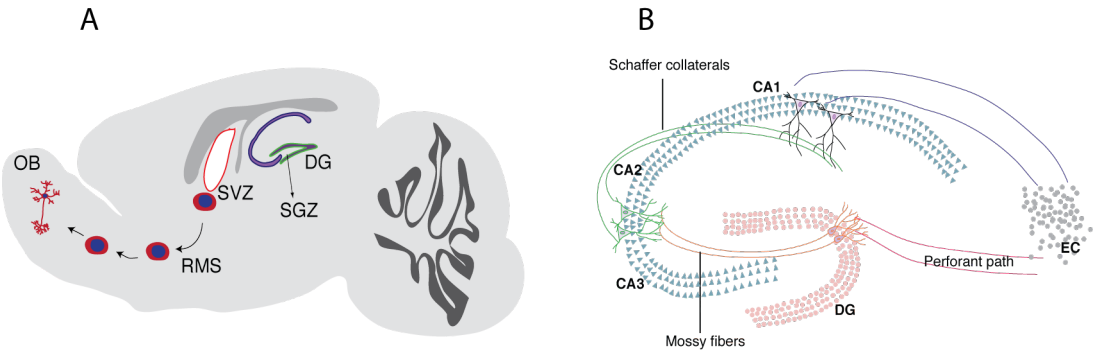

Figure 1.2. Schematic image of neurogenic regions in rodent brain (A) and the trisynaptic circuits in the hippocampus (B). Figures are adapted from Taupin and Gage (2002) and Scharfman (2007)

The hippocampus is the major component in processing long-term storage (consolidation) and retrieval of "episodic memory". It contributes to creating a spatio-temporal link to a certain event. Therefore spatial navigation and learning are suggested as hippocampal-dependent tasks (Fortin *et al.*, 2002; Morris, 2007). Furthermore, interaction between the hippocampus and amygdala in the limbic system associate the emotional content with the memorized events (Phelps, 2004).

Despite the difference in size, shape and complexity across species, the mammalian hippocampi share similarities in their basic architecture and pattern of connectivity, the so called "trisynaptic circuit" (figure 1.3-B). The first synapse is formed between the afferents from the entorhinal cortex and the dendrites of the granule cells in the dentate gyrus. These afferents bring the major input from the entorhinal cortex, which contain cortical sensory information, to the dentate gyrus via the fibers called perforant path to be further processed to ultimately form episodic memories. The axons of granule cells build the mossy fiber tract that reach pyramidal neurons in CA3 and establish the second synapse. The CA3 pyramidal neurons give rise to Schaffer collateral that innervate CA1 pyramidal neurons, where the third synapse takes place. The axons of CA1 pyramidal neurons project to subiculum and from the subiculum project back to entorhinal cortex (Andersen *et*

al., 1971; Amaral *et al.*, 2007). Albeit important and useful, this "trisynaptic circuit" is just a simplification of the functional circuitry in the hippocampal formation. Existing shortcuts and side paths increase the complexity of the whole system. For instance, CA3 and CA1 receive direct projections from the entorhinal cortex in addition to relay projections from granular cells. Furthermore, the entorhinal cortex receives feed back projections directly from CA1 and CA3 in addition to the one through subiculum (Amaral *et al.*, 2007; Scharfman, 2007).

Based on its anatomical and physiological characteristics, the dentate gyrus is suggested to be the gate of excitatory input from the entorhinal cortex that is also involved in filtering and compressing the incoming information (Hsu, 2007). It consists of three layers, i.e. the hilus, the granular cell layer and the molecular layer. The narrow band about three-cell-body wide from the most basal granule cell layer into the hilus, defined as subgranular zone (SGZ), provides a permissive microenvironment or "neurogenic niche" for life-long neuronal development (Kuhn *et al.*, 1996).

1.3.2. Adult hippocampal neurogenesis occurs in the neurogenic niche in the dentate gyrus

Adult neurogenesis occurs in a permissive microenvironment, namely the neurogenic niche, which comprises cellular and non-cellular components. Neural precursor cells (NPCs), mature neurons, astrocytes, microglia and vasculature are among the cellular components (figure 1.3), while the extracellular matrices are the non-cellular components.

Astrocytes provide significant contribution to the neurogenic niche of the dentate gyrus through both the secretion of soluble factors and cell-cell contact (Song *et al.*, 2002). Ephrins, membrane bound proteins expressed in astrocytes, activate β-catenin signaling via cell-cell interaction to induce neuronal differentiation of neural precursor cells (Ashton *et al.*, 2012). Other supportive factors such as Wnt3a, FGF-2, IL-1β and IL-6 are also secreted by neurogenesis-promoting astrocytes (Lie *et al.*, 2005; Shetty *et al.*, 2005; Barkho *et al.*, 2006).

Microglia are part of the cellular immune system in the brain that in the unreactive state have highly ramified morphology, dynamically wander in the brain parenchyma to phagocyte the dying cells and cellular debris without causing any inflammation (Nimmerjahn *et al.*, 2005). In the SGZ, most of the newborn cells fail to thrive due to apoptotic cell death, which is mediated by microglia (Sierra *et al.*, 2010). Not only involved in cell death, evidence from in vitro and in vivo studies demonstrated that microglia also positively affect proliferation, neuronal or glial

differentiation, and survival of newborn neurons via direct contact or secretion of neurotrophic factors such as IGF-1 and BDNF (for further review see Gemma and Bachstetter, 2013).

The close proximity of NPCs to blood vessels indicates that they may receive substantial factors from the vasculature for their development (Palmer *et al.*, 2000). The blood vessels not only play a role in transferring nutrients and other systemic factors, but also secrete the "endothelial factors" such as BDNF that maintain symmetric divisions of neural precursor cells, support neuronal recruitment and survival (Leventhal *et al.*, 1999; Shen *et al.*, 2004).

In addition to the contact with other cells, NPCs are surrounded by various extracellular matrices (ECM) such as laminins, glycoprotein tenascin-C, glycosaminoglycan (eg. hyaluronan) and proteoglycans. The ECM complexes contribute to NPC proliferation, differentiation and migration through modulation of growth factors signaling and cytoskeletal function. Hyaluronan is a major constituent of ECM in most tissues. It is suggested to decrease proliferative speed of NPCs and provide guidance cues for newborn cells and glial migration. Disruption of this matrix leads to lamination defects and inappropriate entorhinal fiber growth in the hippocampus. Furthermore, interaction of high molecular weight hyaluronan with CD44 at cell membranes regulates mitotic spindle orientation preferable for asymmetric cell division (Winkler *et al.*, 2012).

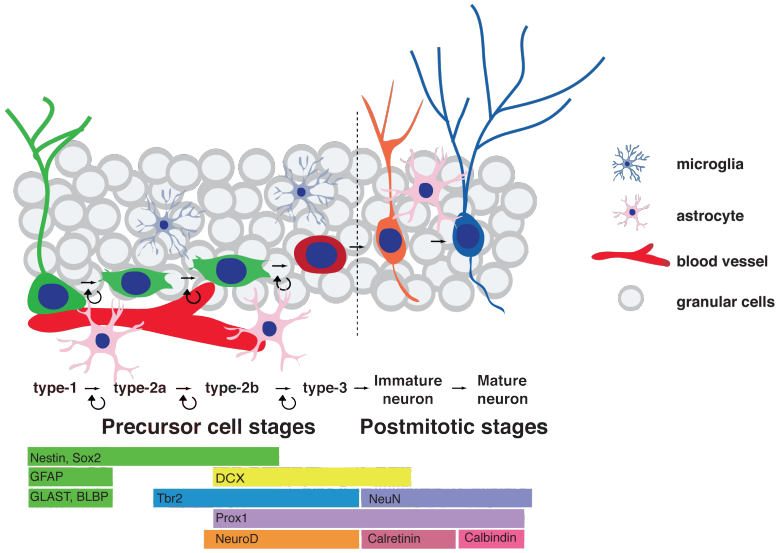

Figure 1.3. Schematic figure of neurogenic niche in dentate gyrus and the stages of cell development in adult hippocampal neurogenesis from precursor to mature cells. The expression of particular proteins are used as specific markers for each stage. Modified with kind permission from Donegá *et al.* (2013), Kempermann *et al.* (2004) and Nicola *et al.* (2015)

Adult hippocampal neurogenesis originates from the neural precursor cells that reside in the subgranular zone of the dentate gyrus. Based on the morphology, proliferation characteristic and cellular markers expression, these cells are categorized as type-1, type-2a, type-2b and type-3 cells (figure 1.3; Kempermann *et al.*, 2004).

Type-1 cells are considered as the putative stem cells of the adult SGZ that give rise to daughter progenitor cells through supposedly asymmetric division. They are called radial glia-like cells (RGCs) due to their morphology with long and strong apical process reaching the granular cell layer resembling radial glia and expression of the radial glia marker BLBP (Filippov *et al.*, 2003; Steiner *et al.*, 2006). The astrocytic properties are shown by the expression of the astrocyte marker such as glial fibrillary acidic protein (GFAP) expression and the existence of vascular endfeet (Filippov *et al.*, 2003). However RGCs lack S100β expression, another astrocyte marker (Steiner *et al.*, 2006). Other markers used to identify type-1 cells are nestin, Sox2, Id1 and Pax6 (Filippov *et al.*, 2003; Steiner *et al.*, 2006; Nam and Benezra, 2009).

Type-2 cells are the daughter cells of type-1 cells. They are characterized as highly proliferative cells, expressing nestin, lacking GFAP expression and with an irregular shape and horizontal short processes. Type-2 cells are subclassified into early type-2 cells (type-2a) and late type-2 cells (type-2b) based on the marker expression. In addition to BLBP and Sox2 expression, albeit less than in type-2a cells, type-2b cells start expressing doublecortin (DCX) and NeuroD, indicating the initiatiation of neuronal determination and migration. Type-2 cells have horizontal shorts processes, therefore are suggestively capable of tangential migration (Kuhn *et al.*, 1996; Kronenberg *et al.*, 2003; Steiner *et al.*, 2006; Roybon *et al.*, 2009).

Type-3 cells, also called neuroblasts, are characterized by rounded nuclei, showing a first apical dendrite, longer vertical processes, expressing neuronal markers such as DCX and PSA-NCAM, and an absence of nestin expression (Seri *et al.*, 2004; Plumpe *et al.*, 2006). Type-3 cells mostly stay in SGZ or migrate radially to the inner third of granular cell layer (Kempermann *et al.*, 2003).

On the third day after initial division, the majority of the precursor cells exit the cell cycle and enter the postmitotic state. In this stage, most of newborn cells are eliminated through apoptotic cell death. The surviving cells express the post mitotic neuronal marker NeuN and the immature neuronal marker calretinin. Morphologically, the cells in this stage have a rounded or slightly triangular nucleus and a prominent apical dendrite. The cells continue the maturation process and synaptic development, marked by switching the expression of calretinin to

calbindin. The final maturation stage is reached when the newborn neurons are integrated in the hippocampal circuitry, establishing the connection within the network. Approximately, it takes 4-7 weeks for the new cells to reach this stage (Hastings and Gould, 1999; van Praag *et al.*, 2002; Brandt *et al.*, 2003; Kempermann *et al.*, 2003). To establish the connection of the newborn neurons with the existing network, it is hypothesized that glutamate spillover from the existing synapses may be the chemoattractant and inducer of filopodia growth from newborn cells towards the active synapses (Toni and Sultan, 2011).

1.3.3. Benefit of adult hippocampal neurogenesis in learning and memory

The new functional neurons are added cumulatively into the granule cell layer during the adult hippocampal neurogenesis process and lead to functional alteration of the hippocampus (Bayer, 1985). Based on behavioral studies using ablation methods and computational modeling, the following hypotheses have been proposed to describe the functions of new neurons in learning and memory.

Memory acquisition and consolidation

Referred to as the "gateway to memory", the hippocampus processes the incoming information to be stored as long-term memory in another part of the brain (neocortex). Using irradiation ablation technique, Suarez-Pereira *et al.* (2015) demonstrated that newborn dentate gyrus granule cells are required in memory acquisition (learning) and consolidation of hippocampal-dependent task. This finding supports the previous results by Kitamura *et al.* (2009) that suggested more new neurons would decrease the hippocampal-dependent period of memory. The decay of hippocampal-dependency of memory is associated with memory consolidation in which the gradual increase in dependency upon extra-hippocampal region, such as the neocortex, occurs.

Efficiency in pattern separation

Everyday we experience several events or contexts that probably consist of high similarities and configurations. Somehow it is important to remember the differences between these contexts to be able to react efficiently with the environment (pattern separation) without losing the previously stored information (catastrophic interference). Animals with ablated hippocampal neurogenesis showed memory impairment in distinguishing similar locations in the radial arm maze (Clelland *et al.*, 2009). On the other hand, animals with increased hippocampal

neurogenesis improved the performance in differentiating two similar contexts (Sahay *et al.*, 2011). The problem of catastrophic interference can be avoided by adding new neurons in dentate gyrus through modifying the existing synaptic weights, whereby the old neurons maintain their synaptic weights and the new neurons take care of the novel features. Therefore the old codes remain undisturbed (Wiskott *et al.*, 2006).

Facilitation of temporospatial association

In episodic memory, the precise association among context, time and space is prerequisite. The task is even more challenging when more than just a single memory is involved. In this case both pattern separation and properly linked temporospatial association are necessary. The presence of new neurons might provide the solution to handle this challenge (Becker *et al.*, 2009).

Enable flexible and advance hippocampal-dependent learning strategy

Memory acquisition in spatial learning can be hippocampus-dependent or -independent. The allocentric navigation strategy, the one that encodes the location of particular object with respect to other objects, is hippocampus-dependent and involves advanced searching strategies. Analysis of search strategies used by the animals during navigation can distinguish the subtle differences in performance quality to identify between hippocampus-dependent and –independent aspects (Wolfer *et al.*, 2001).

The Morris water maze task (MWM) is one of the mostly used assays in behavioral neuroscience. Firstly introduced by Richard Morris in 1982 to study spatial learning and memory in rats, and later is also applied in mice (Morris *et al.*, 1982). In this task, the animals have to use the surrounding visual cues to build an allocentric cognitive map in order to locate a certain position. The instruments consist of a circular pool, filled with water that has been mixed with white pigment or milk powder. A small platform is submerged at one quadrant of the pool. The water temperature is set around 13 ºC below body temperature (around 25 ºC) to create a mild stressful condition in order to motivate the animals to find the platform as an escape. Stable visual cues are provided around the pool that can be used by the animal to navigate and determine the platform position. A camera connected to a computer with specialized tracking software is installed above the pool to record the swim path over time. Latency time to find the platform, path

length, time in quadrants, number of goal crossings, velocity and floating times are the common parameters used as the read outs of the task (Morris, 2006).

Using a particular test paradigm in the Morris water maze task and pharmacologic depletion of neurogenesis, Garthe *et al.* (2009) demonstrated that mice with impaired hippocampal neurogenesis needed a longer time to learn the precise goal location and rather relied primarily on the directed search strategy than the more precise place specific strategies. Furthermore, when the platform was moved to the opposite quadrant these mice showed inability to flexibly adapt their learning capacity with this change in order to find the new location. Therefore, it is suggested that the presence of newly generated adult granule cells play role in the development of flexible and efficient allocentric spatial navigation, especially to be able to adjust the coping strategy to spatial changes.

The dentate gyrus plays a role in preprocessing the information from the entorhinal cortex before it is stored at CA3. The existence of young new neurons in the dentate gyrus is beneficial to reduce the overlapping encoding of information in CA3, in particular regarding unrelated events that occur close in time. Therefore the newborn neurons promote pattern separation and prevent "catastrophic interference (Treves *et al.*, 2008). Considering this hypothesis, the "reversal learning" protocol of the Morris water maze appears to be the appropriate scheme to test the functional relevance of adult neurogenesis. In this protocol the animals initially learn the spatial relationship between cues and the platform position. Upon change of platform position the animals have to relearn the new spatial relationship within the same spatial cues. To have a successful navigation, effective pattern separation is necessary. In this case, the contribution of adult hippocampal neurogenesis to highly specific functional aspects of spatial learning is apparent. Nevertheless, the classical parameter in water maze performance such as the latency to reach the platform and swim path are not sufficient to assess the hippocampus-specific aspects of spatial learning. Counting the number of goal crossing during the probe trial session provides more information on spatial accuracy with which the platform position has been encoded and therefore reflecting the efficient pattern separation. Another useful approach i.e. analyzing search patterns or strategies can be used to depict and distinguish hippocampus-dependent (allocentric) and hippocampus-independent (egocentric) search strategies (for review, see Garthe and Kempermann, 2013).

1.3.4. The role of phytochemicals in the regulation of adult hippocampal neurogenesis

The term "regulation" refers to factors that mediate the deviation of neurogenesis from a baseline level. Since the baseline is mostly controlled intrinsically, the regulation depends on the behavioral activity and external stimuli. Any identifiable steps in neuronal development theoretically can be modulated by the regulatory factors, resulting in the change of quantity and/or quality of new neurons. The read out of this process is usually the number of bromodeoxyuridine (BrdU)-labelled cells that can be interpreted, depending on the experimental setting, as either cell proliferation or survival. Addressing the change in neurogenesis by means of using electrophysiology approaches, gene or protein expression analysis and systems biology accelerate our understanding of adult neurogenesis regulation (Kempermann, 2011a).

An increasing body of literature continually extends the growing list of potential regulators; such as exercise, environment enrichment, nutrition, nicotin, etc., showing that adult neurogenesis is extremely responsive to external stimuli. These regulators involve mechanisms from behavioral to molecular that form large and complex network of interdependencies, and which ultimately enable the fine-tuning of new neuron production as the response of the individual to environmental challenges (Kempermann, 2011b).

The age-related decline in adult hippocampal neurogenesis has been shown significantly in mice and rats, even already very obvious in young adulthood (Kuhn *et al.*, 1996; Ben Abdallah *et al.*, 2010). Physiological stimuli such as environmental enrichment and physical activity show a robust increase in the number of newborn neurons in aged mice dentate gyrus (Kempermann *et al.*, 1998; van Praag *et al.*, 2005). A study demonstrated that blueberry extract supplementation increased the number of surviving newborn cells in the dentate gyrus of aged rats through the activation of insulin-like growth factor 1 (IGF-1) signaling (Casadesus *et al.*, 2004). However, this study gave the impression that the surviving cells were mostly located in the hilus and did not clearly show whether these cells differentiated into neurons.

Some of the chemical substances in plants, known as phytochemicals, are produced as the plant's defense mechanism against environment insults such as insects and herbivores. They are able to modulate biological processes in mammals, therefore can be toxic at high concentrations. However, in the concentration below the toxic level, these compounds can induce "adaptive stress response" pathways

that result in increased capability to protect the organism against diseases for example through increased the expression of heat shock proteins, antioxidant enzymes and anti-apoptotic proteins (Mattson, 2008b; Kennedy and Wightman, 2011).

In the following table 1.2, I summarized the information from literature, showing a number of plants and phytochemicals that positively- or negatively-affect adult hippocampal neurogenesis. This effect can be either direct effect or indirectly through rescuing the adult hippocampal neurogenesis from detrimental effect of other health conditions.

1.4. Apple as brain food

"An apple a day keeps the doctor away". This adage is very well known worldwide and used to encourage fruit consumption and healthy lifestyle. Even though the phrase was firstly recorded in 1904, the apple's story in human kind has dated back to ancient times. As one of the first domesticated fruits, apples have been entangled in the culture and history of civilizations across the globe and associated with love, beauty, health, comfort, pleasure, temptation, sensuality and fertility (Janik, 2011). Apples have been used not only as food or beverages. Hippocratic doctors of ancient Greece classified apples as "cool and moist" food and should be consumed as a counterbalance of the "hot" food such as red meat. During medieval times, physicians promoted apples as treatment of bowel, lung and nervous system disturbances. In the renaissance, during the European exploration missions, apples helped to overcome the detrimental effect of scurvy on voyages (Janik, 2011). Studies in aged animals showed that apple supplementation prevented oxidative brain damage through increased endogenous antioxidative capacity, and also restored long-term potentiation and acetylcholine level, concurrently with improvement in behavioral tasks (Tchantchou *et al.*, 2005; Chan *et al.*, 2006; Viggiano *et al.*, 2006). A pilot study in human showed that consumption of apple juice improved behavioral symptoms in patients with Alzheimer's disease (Remington *et al.*, 2010). However, no significant cognitive change was observed after short time apple supplementation in healthy volunteers (Bondonno *et al.*, 2014).

As one of the most consumed fruits in Germany, available throughout the whole year at an affordable price, apples provide a significant source of phytochemicals (table 1.3) in our diet (BMELV, 2013). These phytochemicals, especially one group of phytonutrients known as flavonoids, have been widely studied and suggested to be actively involved in physiological processes, including in the modulation of memory formation and retrieval (Spencer, 2009).

Table 1.2. Phytochemicals that affect adult hippocampal neurogenesis

Compounds	Source	Effects on adult hippocampal neurogenesis
(-)epicathecin	Green tea Cocoa	(-) epicatechin supplementation in food pellet for 2 weeks led to no change in survival of dentate gyrus newborn cells. Nevertheless, the treated-group showed improved spatial memory in water maze task that was associated with the increased of angiogenesis and neuronal spine density in the dentate gyrus (van Praag *et al.*, 2007). Reduction of anxiety-like behaviour was also observed in the treated-group, accompanied by increased hippocampal BDNF and tyrosine hydroxylase level. The performance in pattern separation test and the survival of dentate gyrus newborn cells were not affected (Stringer *et al.*, 2015).
(-) epigallo-catechin gallate	Green tea	Dietary supplementation with (-) epigallocatechin gallate for 39 days showed no effect in the survival of newborn cells in dentate gyrus and contextual/cued fear conditioning test (Bhattacharya *et al.*, 2015).
Cannabidiol & Tetrahydrocannabinol	Cannabis	Despite decreased cell proliferation, cannabidiol supplementation for 6 weeks in food pellet increased survival of newborn dentate gyrus cells in mice. The performance in water maze task was not affected. However tetrahydrocannabinol supplementation decreased the number of proliferating cells wihtout affecting cell survival. Water maze performance was impaired (Wolf *et al.*, 2010).

Curcumin	Curcuma	In this experiment, ethanol extract of Curcuma longa was administered orally for 3 weeks to adult and D-galactose-induced aged mice. Curcuma extract supplementation increased cell proliferation and the number of DCX(+) cells in adult mice dentate gyrus, while rescuing the decrease of cell proliferation in aged-mice. Morris water maze performance was not affected in normal adult mice, however the performance in aged mice was significantly improved (Nam *et al.*, 2014).
Diallyl disulfide	Garlic	Diallyl disulfide administration per oral (10 mg/kg) for 3 weeks in 6-week old mice resulted in the decrease of dentate gyrus NPC proliferation without affecting the survival of newborn cells. Impaired memory retention was also observed in passive avoidance test. Protein level of BDNF, phospho-CREB and phospho-ERK in hippocampus was significantly decreased in treated mice (Ji *et al.*, 2013).
Ferulic acid	Grains, fruits, vegetables	Oral administration of 100 mg/kg ferulic acid prevented the decreased of hippocampal cell proliferation and ameliorated the stress-induced depression-like behaviour in corticosteroid-treated mice. The beneficial effects were brought about the increased in CREB and BDNF level in the hippocampus (Yabe *et al.*, 2010).
Fuzi polysaccharides (FPS)	Chinese-aconite	Single dose intraperitoneal administration of FPS extract significantly increased the number of proliferative cells in adult mice dentate gyrus. Longer period of extract supplementation (7 days) showed an increased in cell survival and neuron proportion. Hippocampal BDNF was increased after 6 hour FPS extract administration. Coadministration with BDNF receptor antagonist eliminated the effect of FPS extract (Yan *et al.*, 2010).

Gingko biloba extract (Egb 761)	Gingko biloba (leaf)	Increased hippocampal NPCs proliferation was observed in wild type aged mice and TgAPP/PS1 mice after 1 month oral supplementation with Egb 761 that may be mediated through activation of CREB (Tchantchou *et al.*, 2007).
Ginsenoside	Panax ginseng (root)	Intraperitoneal injection of ginsenoside (Rd) for 7 days increased proliferation of hippocampal NPCs in rats. However the percentage of newborn neurons was not changed (Lin *et al.*, 2012). Oral administration of Rb1 ginsenoside for 30 days in rats improved spatial cognitive performance in Morris water maze task. Increased cell survival in dentate gyrus and CA3 was observed with no effect on cell proliferation (Liu *et al.*, 2011).
Hypericum perforatum extract	St. John's wort	Long term intraperitoneal administration of Hypericum perforatum extract (3 weeks) prevented the decrease in hippocampal cell proliferation and ameliorated anxiety/depressive-like behaviour in chronic corticosterone-treated mice (Crupi *et al.*, 2011).
Oroxylin A	Scutellaria baicalensis (root)	Subchronic oral administration (14 days, 5mg/kg) of Oroxylin A increased both cell proliferation and new born cell survival in mice dentate gyrus (Lee *et al.*, 2010).
Swainsonine	Locoweed (Astragalus)	Oral administration of swainsonine alkaloid (10 μg/ml drinking water) for 4 weeks reduced proliferation and survival of adult mice hippocampal NPCs. Protein level of doublecortin and synaptophysin in the hippocampus were downregulated while caspase-3 and glial fibrillary acidic protein were upregulated. Hippocampus-dependent spatial learning and memory in Morris water maze task was impaired (Wang *et al.*, 2014).

Flavonoids are ubiquitously found in fruits and vegetables, comprising the most common group of polyphenols and the most studied phytonutrients. Flavonoids consist of two aromatic compounds as depicted in figure 1.4; benzopyrane (A and C rings) and benzene (B ring), and based on the hydroxylation pattern, conjugation between aromatic rings, glycosidic moieties, and methoxy groups are divided in six subgroups: flavanol (catechin, epicatechin, epigallocatechin gallate), flavone (apigenin, rutin, luteolin), flavonol (kaempferol, quercetin, myricetin, tamarixetin), flavanone (naringin, naringenin, taxifolin, hesperidin), isoflavone (genistin, genistein, daidzin, daidzein), anthocyanidin (apigenidin, cyanidin).

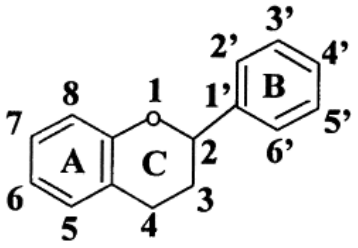

Table 1.3. Phytochemicals in apple (Kahle *et al.*, 2005; He and Liu, 2008)

Class	Compounds name
• Dihydrochalcone	Phloretin, Phloridzin
• Flavan-3-ols	Procyanidin B1 and B2 (+)-Catechin, (-)-Epicatechin
• Flavonols	Quercetin (aglycone and glycosides)
• Hydroxycinnamic acids	Chlorogenic acid, Caffeic acid, 4-p-Cumaroylquinic acid, Ferrulic acid
• Organic acids	Malonic acid, Maleic acid, Quinic acid
• Plant sterols	β-sitosterol, 1-hexadecanol, 2-hexadecanol, Tetradecan-7-ol
• Triterpenoids	β-amyrin and ursane-type triterpenoids

In accordance with the chemical structures, flavonoids are a potent hydrogen donor. Therefore their neuroprotective actions have been attributed to antioxidant properties. This antioxidant potency can be clearly observed in vitro (reviewed by Heim *et al.*, 2002; Manach *et al.*, 2004). Nevertheless, this effect unlikely occurs *in vivo* because their redox potential is significantly altered due to extensive metabolism before they reach the target site and low bioavailability (Spencer, 2007). Instead, accumulating evidence suggests that the effects are exerted through their ability to modulate intracellular signaling processes and gene expression and improve mitochondrial bioenergetics (Williams *et al.*, 2004; Jones *et al.*, 2012).

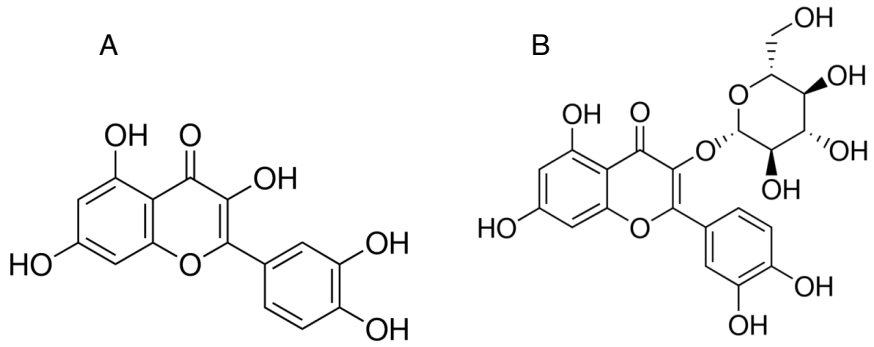

Figure 1.5. Chemical structure of quercetin aglycon (A) and quercetin-3-O-β-D-glucopyranoside (B).

Quercetin is the most abundant flavonol in plants, and its glycoside, quercetin-3-*O*-β-D-glucopyranoside, is the major flavonoid in apples (He and Liu, 2008). Chemical structures of these compounds are depicted in figure 1.5. The significant sources of quercetin are capers, red onion, apples, berries, broccoli and tea Average daily intake, depending on our food composition, ranges between 10 – 100 mg. Purified extract of quercetin is also commercially available as a food supplement (nutraceuticals) with daily dose 500-1000 mg, which equals to the concentration in 3-5 kg of apples (Bischoff, 2008). A growing body of literature reports the effect of flavonoids in learning and memory formation through the interaction with signaling pathways in the brain as depicted in figure 1.1. Several *in vitro* and *in vivo* studies that reported the influence of flavonoids, particularly quercetin, in modulation of cognitive function through these pathways are summarized in table1.4 (reviewed by Spencer, 2009).

Table 1.4. Interaction of flavonoids with molecular pathways in modulation of cognitive function

Pathways	Interaction	Reference
ERK/CREB	• Flavonoid fisetin facilitates long term potentiation (LTP) in rat hippocampal slice and improves object recognition task in mice through activation of ERK and induction of CREB phosphorylation • Quercetin and bilobalide enhance CREB phosphorylation and elevate BDNF in mouse hippocampus, resulting in increased dendritic processes and synaptogenesis in hippocampal neurons.	Maher *et al.* (2006) Tchantchou *et al.* (2009)
PI3K/Akt	• Flavonoid hesperitin increased survival of cortical neurons through Akt/PKB induction and pro-apoptotic proteins inhibition • Anthocyanin-rich blueberry consumption led to activation of mTOR and increased Arc expression in hippocampus, and associated with improved spatial memory in aged rats.	Vauzour *et al.* (2007) Williams *et al.* (2008)
eNOS	• Quercetin induces vasodilation and improves vascular funtion by inducing endothelial nitric oxide synthase (eNOS) activity. • Epicathecin increases angiogenesis in mouse dentate gyrus, associated with improved spatial memory.	Li *et al.* (2012), Shen *et al.* (2012) van Praag *et al.* (2007)

1.5. *In vitro* methods to study adult hippocampal neurogenesis

Although earlier breaking ground studies in adult hippocampal neurogenesis were done *in vivo*, this field will not gain ground without the supporting evidence from *in vitro* experiments. The understanding of neural stem cells biology and molecular mechanisms of neurogenesis mostly come from cell culture studies. Isolation of neural precursor cells and growing them in cell culture system enable the study of the properties, developmental potential, and molecular mechanisms of the precursor cells that is arduously done *in vivo* (Gage *et al.*, 1995). Moreover, the advance techniques in cell culture open the possibility to perform ex vivo manipulation of neural precursor cells by pharmacological compounds in high throughput manner in order to search for future medicine.

To study neurogenesis is tightly related with studying neural stem cells. Neural stem cells are loosely defined as the cells that have self renewal capacity, can generate or are derived from neural tissue, and can produce the progeny other than themselves (Gage, 2000). Neural cell lines have been established to fulfil these criteria and are widely used as the *in vitro* model of neural stem cells. The cell lines can be classified as tumor-derived cell lines such as neuroblastomas, glioblastomas and rat pheochromocytoma PC12 cells; and oncogene-expressing cell lines such as HiB5, C17, RN33B, ST14A cells. These cell lines are capable to undergo indefinite replication in culture and differentiate to neural cells upon stimulation of certain cues such as nerve growth factor (NGF) induces differentiation in PC12 cells (Greene and Tischler, 1976) and increase of temperature stimulates differentiation in HiB5 cells (Renfranz *et al.*, 1991). Nevertheless, in the natural condition, the stem cell division is tightly regulated whereas the tumor-derived cell lines lose this mechanism. Moreover, the oncogenetically immortalized cells are probably subjected to genetic alteration and additional mutation that make them tumorigenic or incompatible for studying the effect of normal genes (Gage, 2000; Gottlieb, 2002). The drawback of using tumor or immortalized cell lines can be overcome by the alternative method whereby the neural cell lines are obtained from the naturally existing adult neural progenitor cells that are located in the neurogenic niche i.e. subventricular zone or subgranular zone of the dentate gyrus (Gottlieb, 2002).

Precursor cells form different brain region are shown to have distinct characteristics, even in the identical *in vitro* condition (Ostenfeld *et al.*, 2002; Parmar

et al., 2003). Therefore, microdissection technique of the particular brain region of interest must be applied to obtain a high regional specificity of the isolated cell in culture. The position of hippocampus in rodent that is located adjacent to the wall of lateral ventricle increases the risk of contamination from SVZ-origin precursor cells if the hippocampal precursor cells are obtained from whole hippocampus homogenate. The microdissection technique as described by Babu *et al.* (2007) or Hagihara *et al.* (2009) can be used to improve the dentate-gyrus regional specificity of the obtained cells. Upon dissociation and homogenization steps, the precursor cells are disconnected with their physiological environment. The optimum *in vitro* conditions including specific nutrients, growth factors, pH and osmolarity have to be determined for the survival, proliferation and differentiation of the precursor cells. Using serum-free media is essential in order to have a tight control on the composition in the growth media and enables the investigation of regulatory extrinsic cues (Bottenstein and Sato, 1979; Brewer *et al.*, 1993). The growth condition for *in vitro* culture of adult mouse hippocampal progenitor cells has been established in our laboratory and is also adopted by others (Babu *et al.*, 2011; Walker and Kempermann, 2014).

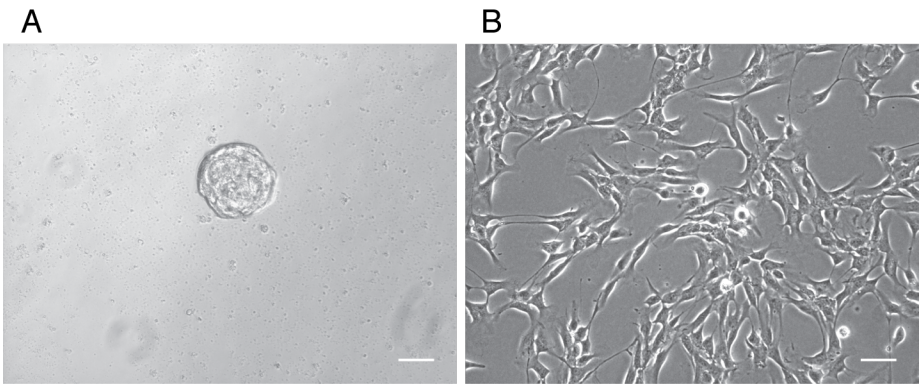

Figure 1.6. Photomicrograph of adult hippocampal neuronal precursor cell in neurosphere (A) and monolayer culture (B). Scale bar is 50 μm.

Two main *in vitro* models are widely used to isolate and propagate neural precursor cells in studying adult hippocampal neurogenesis; the neurosphere culture and the adherent monolayer culture (figure 1.6). These *in vitro* culture systems provide valuable tools to study intrinsic and extrinsic regulators of adult hippocampal neurogenesis. However, the completely different environment from *in vivo* condition makes *in vitro* methods do not adequately represent the *in vivo* situation. Therefore the interpretation of *in vitro* findings cannot be directly implied

to *in vivo* situation, and *vice versa*. Considering the advantages and the pitfalls of each system, one can decide which method fits the best as the model to answer the particular research questions.

1.5.1. Neurosphere culture

The neurosphere culture system from adult mammalian brain was initially reported by Reynolds and Weiss in 1992 to demonstrate the presence of cells with stem cell characteristics in adult brain. The isolated cells are suspended in media containing growth factors; epidermal growth factor (EGF) and/or basic fibroblast growth factor bFGF), and further plated in the vessels without adherent substrates. The vast majority of plated cells do not survive; only the ones that are responsive to the growth factors, which include stem and progenitor cells, can continue to expand and then form floating clusters, called neurospheres (Reynolds and Weiss, 1992). The neurospheres can be dissociated and form subsequent generations of spheres, or can be induced to differentiate under certain conditions into three major cell types of the central nervous system; neurons, astrocytes and oligodendrocyte. Therefore this culture system is a useful tool to analyze proliferation, self renewal capacity and multipotency of neural stem and progenitor cells (Jensen and Parmar, 2006).

Despite being a great *in vitro* tool in neural stem cell studies, the results that are obtained from neurosphere assay must be interpreted carefully. The detection of stem cell characteristics through serial passages and clonal analysis often leads to the wrong conclusion that every sphere is generated from a stem cell. In fact, the neurospheres can be formed by both stem and non-stem cell populations. To assure that the *bona fide* stem cells are isolated, the self renewal ability in an extended period of time, together with the generation of large number of progeny must be demonstrated (Reynolds and Rietze, 2005). Moreover, the dense stucture of cells in the neurospheres creates an uneven diffusion of nutrition and growth factor between the central and outer part of the spheres. Therefore the neurospheres consist of heterogenous cell types whereby more differentiated cells and even dead cells are found in the central part while the undifferentiated cells are located in the outer part of the spheres. This is why the neurospheres contain only a small number of true stem cells and are not suitable for studying individual cells (Mokry *et al.*, 1995; Lobo *et al.*, 2003).

Another important issue in neurosphere assay is the seeding density. Due to the high motility in the culture media, the cells or spheres have been shown to have a high tendency to fuse and create a "false-positive" neurosphere number and size (Singec *et al.*, 2006; Jessberger *et al.*, 2007). To minimize this drawback, it is very crucial to ascertain that the culture is started with single cell or at least an extremely low density (10 cells/μl for primary cells or 1 cell/μl for passaged spheres; Coles-Takabe *et al.*, 2008).

Overall, the neurosphere assay is the most widely used *in vitro* technique for isolation and understanding of neural stem cells biology. Due to its sensitivity to the the extrinsic factors in the growth media, the neurosphere assay become an assay of choice to study the effect of extrinsic cues such as pharmacological compounds on neural precursor cells (Irvin *et al.*, 2003; Walker *et al.*, 2008).

1.5.2. Adherent monolayer culture

The monolayer culture system for precursor cells from adult rat hippocampus was initially reported by Palmer and colleagues in 1995 (Palmer *et al.*, 1995). About a decade later, the protocol for isolation and monolayer culture of precursor cells from adult mice dentate gyrus was also established (Babu *et al.*, 2007).

In contrast to neurosphere culture, the isolated cell suspension is plated in the presence of mitogens on a surface that has been coated with adherence substrates such as poly-d-lysin or polyornithine and laminin. Within 1 week in culture, small proliferative colonies containing relatively homogenous population of fibroblasts, oligodendrocytes, astrocytes and progenitor cells are formed (Palmer *et al.*, 1995). A centrifugation in Percoll gradient could be done to selectively enrich the precursor cells in the cell suspension (Babu *et al.*, 2011). Nonetheless, the non-precursor cells are more adherent than the precursor cells, hence will not be harvested during passaging. After several passages, the culture is sufficiently enriched with precursor cells and can be further passaged for extended period of time (~25 passages) without loss of multipotency and other precursor cell characteristics (Palmer *et al.*, 1995; Babu *et al.*, 2011). Therefore, the heterogeneity issue that occurs in neurosphere culture can be overcome using adherent monolayer culture. Each cell will be maintained at the highest level of homogeneity during proliferation while the spontaneous differentiation can be minimized due to the uniform exposure of growth factors. Upon growth factor withdrawal or addition of

particular factors, the cells in monolayer culture can be differentiated into mature neurons, astocytes or oligodendrocytes (Babu *et al.*, 2007).

Due to the significant reduction of cell-cell contacts in monolayer culture, the individual cell development or function such as cell migration, differentiation, maturation and electrophysiology can be easily studied even in real-time resolution. However, this characteristic can also be the disadvantage of this culture system because the cells lose the "niche-like" environment that can influence survival and differentiation (Kempermann, 2011a).

Due to the homogenous cell population, cell multipotency and defined growth media constituents, this culture system is beneficial to study the effect of extrinsic compounds on cell proliferation, cell fate determination, survival and also the analysis of underlying mechanisms such as signaling pathway analysis. Furthermore, once the culture is established, the cells can be propagated extensively to provide vast amount of hippocampal precursor cells *ex vivo* (Babu *et al.*, 2007). This enables the researchers to perform experiments that need a big number of cell materials such as genes/siRNA transfection using nucleofection and protein analysis.

1.6. Bioassay-guided fractionation and structure elucidation as the tool for active compounds discovery

The function of flowering plants is not merely as a source of nutrition but also as a library of phytochemicals that bear a great potency as new drugs for treating diseases of human beings and other mammals. Many methods have been developed in order to perform extractions and test their bioactivities scientifically. The extraction methods aim to isolate the putative biological active substances from plants. Extraction using liquid solvents is the most common method. Other methods such as steam distillation, supercritical fluid extraction or pressurized gas extraction can also be applied. The selection of extraction methods can be based on the chemical properties of the target phytochemicals (eg. flavonoids, alkaloids, carotenoids, terpenoids, etc.). In the case when the compounds of interest are not predetermined (such as in screening program), the extraction methods that enable to extract out the widest possible range of chemical types should be applied (Houghton and Raman, 1998).

These extraction methods will produce the so called "crude extracts" which are relatively complex and would need to be followed up by the "cleaning up" process such as fractionation procedures to separate the mixture of compounds into several groups. In fractionation procedures, the mixture of phytochemicals will be separated based on their solubility, size, shape, electrical charge and several other properties. The aim of this separation is to obtain the fractions that contain one or two compounds. This step will mitigate the structure elucidation step of the active compounds (Houghton and Raman, 1998). These extracts or fractions are subsequently tested to reveal the biological activities. The testing methods range from cell-free enzyme system and receptor-ligand binding assay to clinical testing to human volunteers. The active fractions are then analyzed using mass spectrometry or nuclear magnetic resonance spectroscopy chemical structure identification (Weller, 2012).

Since the aim of bioassay guided fractionation is for novel compound discovery, the common problem that may occur is that the active compounds that are found in the screening probably have been identified previously. To avoid wasting resources, a system that can prevent replication should be applied. This system relies on the good database of existing identified compounds that enables the identification of known compounds in the screened-extracts before elaborate fractionation steps are performed (Liu, 2008). Despite the the fact that the active compounds *per se* are not novel, the bioassay guided fractionation method can also be as an unbiased approach to determine the novel biological activities of the known compounds.

1.7. Aim

In our laboratory, we have shown that lifestyle factors such as physical activity and environmental enrichment are strong positive regulator of adult hippocampal neurogenesis. We are also interested in studying the effect of diet, which is also part of the lifestyle, and to identify the particular components in the diet in the regulation of adult hippocampal neurogenesis. We decided to study the effect of apple due to its wide availability, affordability and the reputation as a healthy food, especially in Germany.

Therefore the aim of this study is to elucidate the phytochemicals in apple fruit that have proneurogenic activity in adult hippocampal precursor cells.
In order to achieve the aim, this study includes the following objectives:

1. The vast majority of recent studies investigated flavonoids as the active compounds in fruits that exert the beneficial effect on learning and memory, including their effect on adult neurogenesis. Therefore initially I studied the effect of quercetin, the most abundant flavonoid in apples and its molecular mechanisms on adult hippocampal precursor cells *in vitro* and *in vivo.*
2. To investigate other possible active compounds in apple, apart from flavonoids, I used the unbiased approach i.e. bioassay-guided fractionation whereby the sequential fractionation steps are applied to the extract and tested in the bioassay to screen the bioactivity. The active fraction is further analyzed to identify the chemical structure.
3. To observe whether the customary apple consumption shows the acute effect on adult hippocampal neurogenesis, an *in vivo* experiment was done by giving apple juice supplementation to the mice. The mice were subjected to a behavioral task to assess the spatial learning and memory performance, and the number of newborn neurons was assessed histologically.

2. Materials and Methods

2.1. Mice for in vitro experiment

For *in vitro* experiments, male and female wild-type mice of strain C57BL/6JRj were purchased from Janvier and subsequently housed at the animal facility of Medizinisch- Theoretisches Zentrum at Technische Universität Dresden, Germany. Animal handling in these experiments were conducted in accordance with the applicable European (86/609/EEC) and National (Tierschutzgesetz) regulations and approved by the local ethical committee. The mice were anesthesized with isoflurane prior to decapitation and brain isolation.

2.2. Quercetin experiment *in vivo*

In vivo experiments to study the effect of quercetin was performed in National Institute of Psychiatry Ramón de la Fuente Muñiz, Mexico City. The C57BL/6NHsd mice were purchased from Harlan Laboratories, Mexico City. All institutional and legal regulations regarding to animal ethics and handling were followed in accordance to the regulations established in the Mexican official norm (NOM-062-ZOO-1999) and had the approval of the Ethics Committee on Experimentation of the "Instituto Nacional de Psiquiatría Ramón de la Fuente Muñiz" (CEI/C/009/2013).

Chlordeoxyuridine (CldU) 42.5 mg/kg bw and Iododeoxyuridine (IdU) 57.5 mg/kg bw was injected at the beginning of the experiment and 2 hours before the animals were perfused with 4% paraformaldehyde (PFA), respectively. Quercetin 50 mg/kg bw was prepared fresh in propylene glycol and administered daily through oral gavage (total volume 200 μl). The control group received similar route and volume of propylene glycol. Following brain processing, IdU- and CldU-positive cells were visualized with immunofluorescence staining and subsequently counted exhaustively using fluorescence microscope (Apotome, 40x objective) throughout rostro-caudal axis of the granular cell layer.

2.3. Apple juice experiment *in vivo*

In vivo experiments with apple juice supplementation and the water maze task was performed in CRTD, TU Dresden following the protocol that is approved

by Animal Experiment Committee of Freestate Saxony (DD24-5131/207/27). The Pinova apples were purchased from Obsthof Schlage Dresden-Pillnitz, harvested in autumn 2014. The juice was prepared simply using a kitchen juicer. Eight liters of apple juice was obtained from 13 kg fresh apples, aliquoted and stored in -80ºC.

After 1-week acclimatization period, the mice were kept in a group of five in each cage and then divided into 3 groups that received water, sugar water or apple juice, respectively. The sugar water and apple juice was replaced with the new aliquote daily. The amount of food and liquid intake was measured by measuring the weight of the chow and the drinking bottle everyday and substracted from the weight in the previous day the food and the bottle weight was substracted from the weight of the previous day. The body weight was measured weekly. The single injection of BrdU was given at the first day of experiment. The mice were perfused one day after the water maze task accomplished

2.4. Instruments

	Type	Vendor
ApoTome microscope	Axioimager Z1	Carl Zeiss
Confocal microscope	Zeiss LSM 780	Carl Zeiss
Fluorescence microscope	DM5500B	Leica
Inverted light-microscope	CKX41	Olympus
Stereomicroscope	SZ61	Olympus
Absorbance plate-reader	NanoQuant Infinite® M200	Tecan
Fluorescence plate-reader	NanoQuant Infinite® F200	Tecan
Centrifuge	5810 R 5415 R	Eppendorf
Cell culture hood	LFV 12	Schulz Lufttechnik
Cell incubator	APT.line™ CB	Binder
Incubating shaker	Mini shaker	VWR
Orbital shaker	KM-2	Edmund Bühler
Rocker shaker	WT 16 Wipptisch	Biometra
Spektralphotometer	Nanodrop ND-1000	Peqlab
Real-time PCR	DNA Engine Opticon2	MJ Research
Vortex mixer	D-6012	Neolab
Magnetic stirrer	C-Mag HS 7	IKA
Analytic Balance	AC 210 S	Sartorius

Balance	Practum1102-1S	Sartorius
Computer scanner	hp scanjet 5590	Hewlett-Packard
Fluocytometer	BD LSR II	Becton Dickinson

2.5. Chemicals and Consumables

Item	Company	Cat. Nr.
2,3 dihydroxybenzoic acid	Aldrich	126209
3,4 dihydroxybenzoic acid	Sigma	P5630
3,4 dihydroxybenzoic acid	Aldrich	D110000-100G
3,3'-Diaminobenzidine	Sigma	D5905
7-amino-actinomycin D	BD Pharmingen	559925
Accutase	PAA	L11-007
Acetic acid	Roth	6755.1
Acetonitrile (HPLC grade)	Fisher Scientific	A998-212
Acrylamide	Roth	A124.1
Albumin Standard (2mg/ml)	Thermo Scientific	23209
Aluminium chloride	Sigma-Aldrich	237078
Ammonium persulfate	Merck	1.01201.0100
Aqua Poly/Mount	Polysciences	18606
B27 Supplemement	Life Technologies	17504-044
basic Fibroblast Growth Factor	Peprotech	100-18B
bisBenzimide trihydrochloride	Sigma	14533
Bovine Serum Albumin	Sigma	A8412
BrdU	Sigma	B5002
Bromophenol blue	Sigma	B8026
Butanol	Fisher Scientific	A399
Chloranil	Fluka	45374
Nylon mesh cell strainer (40μm)	BD Falcon	C3977-6
Citric acid	Roth	5110.3
CldU	MP Biomedical	105478
Cytotoxicity Detection Kit	Roche	11 644 793 001
D-(+)-Glucose	Carl Roth	X997.2
Dc Protein assay kit	Bio-Rad	500-0114
DMEM/F-12 Medium	Life Technologies	21331-020
DMEM/F-12, powder	Life Technologies	32500
DMSO	Sigma	D8418

dNTP mix	Life Technologies	18427-013
Donkey Serum	Jackson Immuno Research	017000121
E-plate 96 for xCELLigence	ACEA Bioscience	05232368001
Encore Biotin Module	NuGen	4200-12
Epidermal Growth Factor	Peprotech	AF-100-15
Ethanol absolute	VWR	20821.320
Ethyl acetate	Fisher Scientific	E145
Ethylene diamine tetraacetic	Sigma	E5134
Fructose	Sigma	F0127
Gelatin	Carl Roth	4308.1
Glutamax supplement	Life Technologies	35050-061
Glycerol	VWR	24.388.295
Hank´s balanced salt solution	PAA	H15-009
HCl 37%	Merck	1.00317.1000
HEPES	Sigma	H3375
Heparin	Sigma	H3393
Hydrochloric acid	Merck	1.00317.1000
Hydrogen Peroxide 30%	Merck Millipore	107298
Hyperfilm ECL	Amersham	28906836
IdU	MP Biomedicals	100357
Isoflurane	Baxter	HDG9623
Ketamin	Pharma Partner	12G038
Laminin	Roche	11243217001
Malic acid	Aldrich	240176
Methanol (HPLC grade)	Fisher Scientific	A452
Microscope glass-slide	Marienfeld	0800000
$Na_2HPO_4 . 2H_2O$	Merck	1.06580.1000
$NaH_2PO_4 . 2H_2O$	Merck	1.06346.1000
$NaHCO_3$	Amresco	144558
NEO-CLEAR®	Merck Millipore	1.09843.5000
NEO-MOUNT®	Merck Millipore	1.09016.0100
Neural Tissue Dissociation Kit	Miltenyi Biotec	130-092-628
Neurobasal Medium	Life Technologies	21103-049
NeuroCult™ Proliferation	Stemcell Technologies	05701
Nickel Chloride	Sigma Aldrich	31462
Nitrocellulose membrane	Amersham	10600010
Nonident P40	Applichem	A22390025

oligo (dT) primer	Life Technologies	18418-012
Ovation Pico WTA System V2	NuGen	3302-12
Paraformaldehyde	Sigma	P6148
Penicillin-Streptomycin	Life Technologies	15140-122
Pierce® ECL Western Blotting	Thermo Scientific	32106
Poly-D-Lysin	Sigma	P7405
Ponceau S Solution 0.1%	Sigma-Aldrich	P7170
Precision Plus Protein™	Bio-Rad	1610374
Propidium iodide	Sigma-Aldrich	P4170
Propylene glycol	SIGMA	P1009
Protease inhibitor	Sigma	P8340
Quercetin	Sigma-Aldrich	Q4951
Quercetin hydrate	Sigma-Aldrich	337951
Quinic Acid	Sigma-Aldrich	138622
Resazurin	Sigma	R7017
RNase A	Invitrogen	12091-039
RNeasy mini kit	Qiagen	74104
RNeasy micro kit	Qiagen	74004
Sodium borohydride	Aldrich	452882
Sodium citrate	Sigma	S-4641
Sodium chloride	Merck	106400
Sodium deoxycholate	Sigma	D6750
Sodium dodecyl sulfate	Roth	2326.1
Sodium tetraborate	Sigma	B9876
Sucrose	Sigma	S1888
Superscript II RTase	Life Technologies	18064-014
SuperSignal® West Femto	Thermo Scientific	34094
SYBR green	Qiagen	204243
Tetrahydrofuran	Sigma	34865
Tetramethylethylenediamine	Bio-Rad	161-0800
Tris Hcl	Roth	9090.3
Triton X 100	Sigma	T9284
Trizma Base	Sigma	T1503
Tween 20	Merck	8221840500
Tween 80	Sigma	P8074
Vanillin	Sigma-Aldrich	V1104
Xylazine	Pharma Partner	106500/1

Xylene	Sigma Aldrich	534056

2.6. Antibodies

2.6.1. Primary antibodies

	Company	Cat.Nr
Goat anti Doublecortin (1:250)	Santa Cruz Biotechnology	sc-8067
Mouse anti Idu/BrdU (1:500)	BD Bioscience	347580
Mouse anti Map2(ab) (1:1000)	Sigma	M-1406
Mouse anti Nestin (1:400)	BD Bioscience	611658
Mouse anti S100β (1:1000)	Sigma	S 2532
Mouse anti β-Actin (1:1000)	Santa Cruz Biotechnology	sc-81178
Mouse anti β-III-Tubulin (1:1000)	Promega	G712A
Rabbit anti Vinculin (1:1000)	ThermoFisher Scientific	700062
Rabbit anti Cdk4 (1:1000)	Abcam	ab7955
Rabbit anti HCAR-1 (1:100)	Sigma	SAB1300090
Rabbit anti cleaved Caspase-3 (1:1000)	Cell Signaling Technology	9661
Rabbit anti Cyclin D1 (1:1000)	Abcam	ab16663
Rabbit anti FOX3/NeuN (1:500)	Abcam	Ab104225
Rabbit anti GFAP (1:2000)	Dako	Z0334
Rabbit anti GSTA3 (1:1000)	Proteintech	16703-1-AP
Rabbit anti Ki67p (NCL) (1:500)	Novocastra	NCL-Ki67p
Rabbit anti p27 (C-19) (1:1000)	Santa Cruz Biotechnology	sc-528
Rabbit anti phospho-Akt (1:2000)	Cell Signaling Technology	4060S
Rabbit anti SOX2 (1:500)	Merck Millipore	AB5603
Rabbit anti-BCL2L1 (1:400)	Sigma	AV30475
Rat anti-BrdU (1:500)	AbD Serotec	OBT0030

2.6.2. Secondary antibodies

Donkey anti goat Biotin	Jackson ImmunoResearch	705-065-147
Donkey anti goat HRP	Jackson ImmunoResearch	705-035-003
Donkey anti mouse Cy3	Jackson ImmunoResearch	715-505-151
Donkey anti mouse Cy5	Jackson ImmunoResearch	715-175-151

Donkey anti mouse DyLight 488	Jackson ImmunoResearch	715-485-150
Donkey anti rabbit Biotin	Jackson ImmunoResearch	711-065-152
Donkey anti rabbit Cy3	Jackson ImmunoResearch	711-165-152
Donkey anti rabbit	Dianova	711-185-152
Donkey anti rat Biotin	Jackson ImmunoResearch	712-065-153
Donkey anti rat Cy 3	Dianova	712-225-153
Goat anti mouse HRP	Jackson ImmunoResearch	115-035-003
Goat anti rabbit horseradish peroxidase (HRP)	Jackson ImmunoResearch	111-035-003
Goat anti rat HRP	Jackson ImmunoResearch	112-035-003

2.7. Primers

Bcl2l1 Forward: 5′-GCGTGGAAAGCGTAGACAAG-3′
Reverse : 5′-GCTGCATTGTTCCCGTAGAG-3′
Bcl2l11 Forward: 5′-GCCAGGCCTTCAACCACTAT-3′
Reverse : 5′-GCTCCTGTGCAATCCGTATC-3′
β-actin Forward: 5′-TGACCCAGATCATGTTTGAGA-3′
Reverse : 5′-GGAGAGCATAGCCCTCGTAG-3′

2.8. Reagents and Buffers preparation

10x Phosphate Buffer Saline (PBS)

NaCl	80	g
KCl	2	g
$Na_2HPO_4.2H_2O$	7.64	g
KH_2PO_4	2	g

ddH_2O to make the solution up to 1000 ml. Adjust the pH to 7.3

10x Tris Buffer Saline (TBS)

NaCl	87.66	g
Trizma base	12.11	g

ddH_2O to make the solution up to 1000 ml. Adjust the pH to 8.0

0.2 M Phosphate buffer pH 7.4

0.2 M Na_2HPO_4	243	ml
0.2 M NaH_2PO_4	57	ml

adjust the pH to 7.4

Coating Solution

Poly-d-lysine (PDL) solution

5 mg of poly-D-lysine (lyophillized powder) was dissolved in cold 5 ml ddH_2O. This stock solution (1 mg/ml) was alliquoted in eppendorf tubes and stored in -20°C. The working concentration was 10 µg/ml PDL in ddH_2O.

Laminin solution

Laminin (1mg/2ml) was diluted in ice-cold DMEM media into working concentration (5 µg/ml).

Preparation of PDL and Laminin should be done on ice.

Monolayer culture media

Neurobasal media	96 ml
B27 supplement (50x)	2 ml
Glutamax	1 ml
Penicillin-Streptomycin (10000 U/ml)	1 ml

Proliferation media

Monolayer culture media + EGF 20 ng/ml + bFGF 20 ng/ml

Differentiation media

Monolayer culture media without growth factors

Neurosphere media

DMEM : F12 powder	3 g
Glucose	0.93 g
Sodium bicarbonate	0.28 g
Hepes	0.29 g

ddH_2O to make the solution up to 225 ml, pH adjusted to 7.2 , sterile filtered

Added freshly before cell seeding: NeuroCult™ proliferation supplement (1 ml in 9 ml media)

EGF	20 ng/ml
bFGF	10 ng/ml
Heparin	2 µg/ml
BSA	2 %
PenStrep	100U

BrdU 10 mM (*in vitro*)

3.7 mg BrdU was dissolved in 1 ml NaCl 0.9 %, heated in 40°C for 15 minutes and mixed rigorously.

BrdU (*in vivo*)

To prepare 10 mg/ml BrdU solution:

50 mg BrdU was dissolved in 5 ml NaCl 0.9%, heated in 50°C for 10-15 minutes and mixed rigorously every 5 minutes. Sterile filtered before use. The dose was 50 mg/kg body weight.

CldU (*in vivo*)

To prepare 10 mg/ml CldU solution:

50 mg CldU was dissolved in 5 ml NaCl 0.9%, heated in 50°C for 10-15 minutes and mixed rigorously every 5 minutes. Sterile filtered before use. The dose was 42.5 mg/kg body weight.

IdU (*in vivo*)

To prepare 10 mg/ml IdU solution:

50 mg IdU was dissolved in 4 ml NaCl 0.9% + 1 ml 0.2 M NaOH, heated in 50°C for 10-15 minutes and mixed rigorously every 5 minutes. Sterile filtered before use. The dose was 57.5 mg/kg body weight.

SDS PAGE 12% (resolving gel)

30% Acrylamide mix	4	ml
1.5M Tris (pH 8.8)	2.5	ml
10% SDS solution	0.1	ml
10% APS solution	0.1	ml
ddH_2O	3.3	ml
TEMED	0.004	ml

SDS PAGE (stacking gel)

30% Acrylamide mix	0.83	ml
0.5M Tris (pH 6.8)	0.63	ml
10% SDS solution	0.05	ml
10% APS solution	0.05	ml

ddH_2O	3.4	ml
TEMED	0.005	ml

Lysis buffer for protein extraction

1M Tris HCl, pH 7.5	0.5	ml
0.5 M EDTA, pH 8.0	20	µl
1M NaCl	1.5	ml
10% Sodium deoxycholate	0.25	ml
10% SDS	0.1	ml
10% NP-40	1	ml
ddH_2O to make the solution up to	10	ml

10x Laemmli buffer

Tris HCl 1M, pH 6.8	2.5	ml
SDS	1	g
Glycerol	5	ml
Bromophenol blue	5	mg
ddH_2O to make the solution up to	10	ml

The buffer should be stored in 4°C.

10x Running buffer

Trizma base	30.2	g
Glycin	144	g
SDS	10	g
ddH_2O to make the solution up to	1000	ml

1x Transfer buffer

Trizma base	3.02	g
Glycin	14.4	g
Methanol	200	ml
ddH_2O to make the solution up to	1000	ml

5% milk solution

5 gr non-fat milk was dissolved in 100 ml TBS-T solution

1x TBS-T

10x TBS	100	ml
Tween 20	1	ml

Citrate buffer (0.1 M)

Sodium citrate 14.61 g in 500 ml ddH_2O

Adjust the pH to 6.0 with citric acid (2M). Filter or autoclave for long storage.

PFA 4%

heat up 300 ml ddH_2O + 2 pcs of NaOH pellets (until ≤ 60ºC)

add parafolmadehyde powder 40 g to the hot water and stir until PFA powder completely dissolved

add 0.2 M Phosphate buffer 500 ml to the PFA solution

add ddH_2O to bring final volume until 1000 ml.

Adjust pH to 7.4

Cryoprotection solution (CPS)

Ethylene glycol	500	ml
Glycerol	500	ml
0.2M Phosphate buffer	1000	ml

Blocking solution (immunostaining)

Donkey serum	500	µl
10 % Triton X	100	µl

PBS to make the solution up to 5 ml

Blocking solution with Saponin

Donkey serum	500	µl
2 % Saponin	500	µl

PBS to make the solution up to 5 ml

PBS-plus solution

Donkey serum	150	µl
10 % Triton X	100	µl

PBS to make the solution up to 5 ml

PBS-plus solution (with saponin)

Donkey serum	150	µl
2% Saponin	500	µl

PBS to make the solution up to 5 ml

2.9. Cell culture

2.9.1. Isolation of adult hippocampal neural precursor cells

The isolation procedure of adult hippocampal neural precursor cells was adapted from Babu *et al.* (2011). Under isofluorane anesthesia, the mice were killed by neck dislocation. After desinfection the head and neck of the mice with 70% ethanol, the brains were collected by cutting the head skin and the skull along sagital suture. The brains were placed in ice-cold PBS and immediately subjected to dissection step.

Dentate gyrus part was dissected following the procedure as described by Hagihara *et al.* (2009). The brains were cut along the longitudinal fissure using surgical scalpel to separate both hemispheres. Under stereomicroscope the diencephalon was removed using fine-tip forceps to expose the medial side of hippocampus. Dentate gyrus was dissected out from hippocampus by inserting sharp needle-tip (#27-G) into both side of dentate gyrus (the border of dentate gyrus and Ammon's horn) and sliding it along septo-temporal axis of hippocampus. The dentate gyrus was then picked up using forceps and transferred into sterile dish containing a small amount of HBSS media and continued with dissociation step.

The tissue was dissociated using Neural Dissociation Kit-P (Miltenyi Biotec) following the manufacturer's instructions. After being minced into smaller pieces using surgical scalpel, the tissue was then transferred into a 15-ml tube containing 2 ml Enzyme Mix and incubated for 15 minutes at 37°C. Subsequently, the tissue was triturated by pipetting up and down gently for 10 times using medium-bore, fire-polished glass pipette. After another 10 minutes incubation at 37°C, the trituration step was repeated using a small-bore, fire-polished glass pipette. HBSS buffer was added into the mixture to add up the volume until 10 ml and then centrifuged at 300g for 5 minutes, room temperature. The supernatant was removed by suctioning and the cell pellet was resuspended in 1 ml growth media with growth factor. The cells were then plated either into one well of PDL- and laminin-coated 12-well plate and incubated in 37°C, 5% CO_2 for monolayer culture or into uncoated 96-well plate for neurosphere culture.

The tested compounds/extracts were dissolved in water or DMSO, depending on the solubility. When DMSO is used as vehicle, the final concentration in culture media does not exceed 0,1 %.

2.9.2. Adherent monolayer cell culture

Five to six male and female wild-type mice of strain C57BL/6JRj were used to establish a monolayer culture of precursor cells. After tissue dissociation the precursor cells were plated on the PDL-/laminin-coated plate and incubated in 37°C with 5% CO_2. To prepare the coating, adequate volume of ice-cold PDL working solution was dispensed on culture vessels and left overnight at room temperature in the laminar flow hood (avoid UV light). After 3 times washing with ddH_2O, the surface was left until dry in laminar flow hood. Subsequently, the same volume of ice-cold Laminin solution was added into the vessels and incubated in 37°C overnight. The coated vessels can be stored at -20°C for up to 6 months. The laminin solution was removed prior to cell seeding.

The table below shows the volume of PDL and Laminin used in each type of vessel:

Type	Volume (ml)
T25 Flask	3
T75 Flask	7
Ø 10 cm Dish	5
Ø 6 cm Dish	3
6-well plate	1 (per well)
12-well plate	0.5 (per well)
24-well plate	0.4 (per well)
96-well plate	0.1 (per well)

Every two days, half of media was replaced with fresh proliferation media with the full amount of growth factors. After reaching 80% confluency, the cells were then ready to be passaged. The cells were washed quickly with PBS (without Mg^{++} and Ca^{++}) and then incubated with 500 µl Accutase for 3 minutes at 37°C. After all cells detached, 10 ml PBS was added to collect cells from the flask, followed by 3 minutes centrifugation at 300 g. Cell pellet was resuspended in 1 ml proliferation medium and subsequently the cell number was determined using a Neubauer improved chamber. Replating was done at a constant seeding density 10.000 cells/cm^2 growth surface. The cells can be stored by proliferation media containing 10% DMSO with cell density 10^6 cells/ml/cryovial in -80ºC. To bring the frozen cells back to culture, the cryovial was put in a water bath and then the cells

was washed with 9 ml PBS to remove the residue of DMSO. After 3 minutes centrifugation at 300 g, the cell pellet was resuspended in 5 ml proliferation media and plated in a coated T25 flask In order to let the cells adapt to the culture condition after the freezing step, at least 2 passages were performed prior to plating the cells for the experiments. The cells used for experiments did not exceed 15 passages.

2.9.3. Neurosphere assay

Freshly isolated precursor cells from dentate gyrus were resuspended in complete neurosphere media to reach the approximate density of 2 hippocampi in 10 ml media and subsequently filtered through 40 μm nylon mesh cell-strainer to remove the clumped cells. This cell suspension was then dispensed in falcon tubes, 10 ml each per group. After adding the tested compounds or vehicle, cells were plated in a 96-well plate and incubated at 37°C, 5% CO_2 for 12 days. Neurospheres with the size ≥ 40 μm were counted under inverted light microscope.

2.9.4. Cell impedance assay

Cell impedance, linearly correlated with cell number, was measured using xCELLigence system (Roche Applied Science and ACEA Bioscience). The system consists of the RTCA analyzer, RTCA SP station, computer installed with RTCA software and disposable E-plate 96 (Urcan *et al.*, 2010).

Cells were plated on PDL/Laminin-coated E-plate 96 with a seeding density of 8000 cells/well. After 30 minutes equilibrium time at room temperature, the E-plate was installed on RTCA SP station that stood in the incubator. Prior to cell seeding, 50 μl growth media was dispensed in each well and the background signal was measured. The impedance was measured at 30 second intervals during the first 30 minutes to observe cell attachment followed by 30 minute interval measurements to monitor cell proliferation.

After 24 hours cell seeding, the media was refreshed and test compounds at various concentration were added. The cell impedance at 48 hours after treatment was divided by corresponding cell impedance at the time when treatment was given and then analyzed.

2.10. Cell cycle analysis with Propidium Iodide staining

Cells were harvested and fixed with ice-cold ethanol 70%. Following 2 washing steps with PBS, the cells pellet was treated with 50 μl RNase A (100 μg/ml solution). Subsequently, 400 μl propidium iodide solution (50 μg/ml solution) was added into the cell solution and incubated for 1 hour in the dark prior to fluorescence-activated cell sorting (FACS) analysis.

2.11. Cell death assay with 7-aminoactinomycin D (7-AAD) staining

Cells were harvested into 15-ml falcon tubes and washed 2 times with PBS. Cell pellet was resuspended with 400 μl PBS and treated with 20 μl (1 μg) of 7-AAD solution. As the positive control, 0.2% Triton X (final concentration) was added to the cell solution. FACS analysis was performed after 10 minutes incubation.

2.12. Immunostaining

2.12.1. Brain preparation

Mice were deeply anesthesized by intraperitoneal injection of xylazin+ketamin and subsequently perfused with 0.9% NaCl and PFA 4%. The brains were collected and immersed in PFA 4% solution for 24 hours and subsequently equilibrated in 30% sucrose solution. After the brains completely sank in the solution, the slicing step was performed.

Serial coronal cryosections were performed using a microtome (Leica) in 40 μm thickness and then stored in cryoprotection solution at -20°C. Every sixth section of each brain were pooled in one series for immunostaining.

2.12.2. In vitro BrdU labelling and immunostaining

BrdU labelling technique followed by immuno-fluorescence staining was used to measure proliferation rate of AHPCs. Cells were plated on coated glass-coverslips in 24-well plates with a seeding density 10000 cells/cm^2 and incubated in proliferation media for 48 hours. The media was replaced with fresh proliferation media containing test compounds or vehicle (DMSO) and further incubated for another 24 hours. BrdU (10 µM) was applied to each well for 2 hours prior to the end of the experiment. The cells were then fixed with ice-cold PFA 4% for 10 minutes and subsequently washed with PBS solution three times.

Prior to BrdU immunofluorescence staining, the fixed cells on coverslips were washed twice with NaCl 0.9% and then incubated in 1N HCL at 37°C for 30 minutes followed by once wash with 0.1M Borate Buffer pH 8 and two washes with PBS. The blocking step was performed afterward by incubating the cells in blocking solution (see "Reagents & Buffers preparation"section) containing 10% Donkey serum and 0.2% Triton X in PBS at room temperature for 1 hour. Subsequently, anti-BrdU antibody (1:500) in PBS-plus solution (containing 3 % Donkey serum and 0.1 % Triton X) was dispensed on each coverslip and incubated either for 2 hours in room temperature or overnight at 4°C. After 3 times washing with PBS, the cells were incubated in fluorescent secondary antibody (1:500, in PBS-plus solution) for 90 minutes at room temperature. Next, two washes with PBS was followed by 10 minutes incubation with Hoechst 33342 (1:3000 in PBS). After a final two washes with PBS, the coverslips were mounted on glass slides using Aqua-Poly/Mount for fluorescence microscopy.

Cell counting was performed using a Leica DM5500B fluorescence microscope with StereoInvestigator (Microbrightfield) software. The number of BrdU-positive cells in a given population of cells was quantified from several visual fields and the average was presented as the cell percentage.

2.12.3. Immunofluorescence staining of cells on coverslips

Standard protocol for staining cells on coverslips was performed by blocking the unspecific binding with blocking solution for 1 hour at room temperature. Over night incubation at 4°C with primary antibodies in PBS plus solution was performed immeditely after removing the blocking solution, without washing step. After 3 times washing with PBS, the cells were incubated in fluorescent secondary antibody (1:500, in PBS-plus solution) for 90 minutes at room temperature followed by twice washing with PBS. Counter staining was done by 10 minutes incubation with Hoechst 33342 (1:3000 in PBS) followed by another two washes with PBS. Finally, coverslips were mounted on glass slides using Aqua-Poly/Mount for fluorescence microscopy.

In HCAR-1 staining (membrane antigen), 0.2% Triton X blocking solution and PBS plus solution was replaced with 0.2 % Saponin. Antigen retrieval step was not necessary for staining the monolayer NPC on coverslips.

2.12.4. Fluorescence & DAB immunostaining for brain tissue

For immunofluorescence staining, brain sections were blocked in blocking solution for 1 hour at room temperature prior to overnight incubation of primary antibodies in 4ºC. After a thorough washing step with PBS, incubation with fluorescence secondary antibody was done for 3-4 hours at room temperature. The tissues were subsequently mounted on glass coverslip with Aqua Polymount anti-fading solution.

In DAB staining, the sections were initially incubated with 0.6% H_2O_2 for 30 minutes to quench endogenous peroxidase in the tissue. After thorough washing with TBS, incubation with 1 hour blocking solution followed by overnight incubation with primary antibodies was performed.

The sections were incubated with biotinylated secondary antibodies for 4 hours at room temperature followed by the incubation with avidin/biotinylated enzyme complex solution (ABC elite) for 1 hour at room temperature. Thorough washing steps were performed between incubation steps. The chromogen 3,3'-diaminobenzidine was used as the enzyme substrate to develop a dark brown color for visualization of the labeled antigens.

The sections were mounted on gelatin-coated glass slide, dehydrated in Neoclear® solution and then covered with glass coverslip in Neo-mount® solution before microscopic analysis.

For HCAR-1 immunofluorescence staining, antigen retrieval process using citrate buffer was performed prior to standard immunostaining protocol (using Saponin 0.2% for cells permeabilization). The sections were washed with TBS and then incubated in 10 mM citrate buffer for 5 minutes in 95ºC (water bath). The process was repeated twice with 15-20 minutes interval by leaving the sections in room temperature to cool down. The standard immunofluorence protocol was followed afterwards.

2.13. Confocal microscopy and image analysis

On the LSM 780, images were acquired using a Plan-Apochromat 20x/0.8 air objective. DAPI, GFP, Cy3 and Cy5 were excited using the laser lines 405 nm, 488 nm, 561 nm and 633 nm, respectively. For emission detection the following wavelength areas were used: DAPI: 415–450 nm, GFP: 499–534 nm, CY3: 588–623 nm and CY5: 649–690 nm.

Images were processed offline using Fiji (National Institute of Health) and

Adobe Photoshop CS5®(Adobe Systems Incorporated). The image composites and the figures were assembled using Adobe Illustrator CS4.

2.14. Cells quantification in brain sections (BrdU-, CldU-, or IdU-labeled cells)

The number of labeled cells was determined in series of every 6^{th} sections Positive cells were counted exhaustively throughout rostro-caudal axis of the granule cell layer using 40x objectives. The resulting numbers were multiplied by six to obtain the estimated total number of labeled cells per animal.

For phenotyping, the number of colocalization between $NeuN^+$ or $S100\beta^+$ cells was determined in randomly selected 100 cells for each dentate gyrus and displayed as cell percentage.

2.15. Resazurin survival assay

Stock solution of 0.5 mM was prepared by dissolving Resazurin dye (7-hydroxy-3H-phenoxazin-3-one 10-oxide) in water and subsequently sterile filtered. This stock solution should be kept in 4°C and avoided from light. The final working concentration (0.05 mM) was prepared by diluting the 1-volume of stock solution in 9- volume of growth media. The cells were incubated in this working solution for 2 hours in 37°C and the fluorescence intensity was subsequently measured in a plate reader using 560 nm Ex/590 nm Em filter setting. As the background, the solution was incubated in the wells without cells. After substraction all the fluorescence values with background value, the relative values were obtained by normalized the fluorescence values of treated group to control group.

2.16. Western Blot

For western blot analysis, the cells were collected in lysis buffer with protease inhibitor cocktail and disrupted by trituration using syringe with 20G needle. The proteins were separated by SDS-PAGE gels and transferred to nitrocellulose membranes. The membranes were blocked and then probed with primary antibodies and HRP-labeled secondary antibodies. Protein bands were detected using a horseradish peroxidase/chemiluminescence system (ECL Western Blotting Substrate, Pierce) and visualized on Hyperfilm ECL (Amersham).

The films were scanned and band intensities were determined by using ImageJ (http://imagej.nih.gov/ij/).

2.17. RNA collection and amplification

The total RNA from monolayer cell culture treated with quercetin was prepared using Qiagen RNeasy mini kit, following the manufacturer's protocol.
The total RNA from primary cells treated with apple extract was prepared using Qiagen RNeasy micro kit, followed by amplification step using Ovation® Pico and PicoSL WTA Systems V2 and Encore® Biotin Module as described by manufacturer. Prior to microarray analysis, the quality and integrity of RNA was assessed by using Bioanalyzer.

2.18. RNA microarray

RNA samples were amplified using the TotalPrep™ RNA Amplification Kit (Illumina) and hybridized to MouseWG-6 v2.0 Expression BeadChips (Illumina). Raw data were preprocessed with quantile normalisation in R/Bioconductor using the package *beadarray*. The array was reannotated by querying each probe sequence against the mm9 mouse genome using the BLAT algorithm (kindly supplied by Dr. Jim Kent; http://hgwdev.cse.ucsc.edu/~kent/exe/linux). The genomic position of probes returning a single hit was then used to assign the probe to an NCBI Entrez GeneID. Probes targeting the same GeneID were collapsed as means to yield data for 21155 unique genes.

Using Benjamini-Hochberg method the gene expression result was corrected with the adjusted p value < 0.05 as the significance cut off and then visualized in volcano plot. The gene expression was presented as "expression relative to control" which was the result of log2 of fold change. The value "1" indicates 2 fold change of differentially upregulated expression and "-1" indicates 2 fold change of differentially downregulated expression. The list of differentially expressed genes was enriched into KEGG pathways (Kanehisa and Goto, 2000) and Wikipathways (Kelder *et al.*, 2009) using WebGestalt, a web-based gene enrichment analysis tool.

2.19. Real time PCR

Total RNA was prepared using the Qiagen RNeasy mini kit. The adherent cell cultures were washed once with PBS and then harvested using a cell scraper and RLT buffer. Subsequently, RNA was extracted following the manufacturer's

protocol, including the optional Dnase step. The concentration and the purity of RNA were analyzed using a NanoDrop 1000 spectrophotometer. For reverse transcription, 1 μg of RNA was used. The cDNA was prepared using Superscript II kit using oligo(dT) primers following the manufacturer's protocol. A cDNA negative reaction was also set up without addition of reverse transcriptase enzyme to ensure no genomic DNA contamination.

The quantitative real-time RT-PCR (qPCR) analysis for Bcl2l1 and Bcl2l11 was performed using a SYBR Green PCR Mix (Qiagen) following the manufacturer's protocol. The template cDNA was prepared as above and each reaction contained cDNA from 0.1 μg total RNA. Thermal cycling and fluorescence detection were carried on an Opticon 2 DNA Engine (MJ Research). The quantification was done using the ΔΔCt method after normalizing to the β-actin housekeeping gene. The ΔΔCt shows the relative change of gene expression in the treated group compared to the control group.

2.20. Apple extraction and fractionation

The extract was prepared from Pinova apple cultivar that was purchased from local apple orchard (Schlage Obsthof) at Pillnitz, Dresden in autumn 2011. The peel was separated from the flesh using an apple peeler. The apple peel (100 g fresh weight) and flesh (200 g fresh weight) were separately mixed in 200 ml 80% methanol using a kitchen blender. The apple sludge was filtered through Whatman filter paper Grade 1. The process was repeated 3 times.

The solution was then centrifuged for 10 minutes, 6000g at 4ºC to remove the small particles from the extract. The supernatant was collected and evaporated in rotary evaporator at 40ºC until reaching maximum dryness. The extracts were tested for the activity in neurosphere assay and then stored in -20ºC for further fractionation steps.

The first fractionation steps were performed using solvent fractionation method. The extracts were dissolved in ddH_2O and 1 volume of ethyl acetate was added. Using separation funnel, water and ethyl acetate phase was separated and collected. The solvent was evaporated and the water fraction was further separated in the mixture of water and butanol (1:1). From these steps, I obtain 3 fractions each from apple peel and the solvent in all fractions was evaporated until maximum dryness

The active water fraction from butanol separation was further fractionated by column separation method using Reverse phase C8 column (Vydac). The fraction was dissolved in 20% methanol solution before being injected into the column and then eluted with 20%-, 40%-, 60%- and 100% methanol consecutively for 30 minutes each. The elutes were then dried using centrifugal evaporator prior to be tested in the neurosphere assay.

The active fraction (the one collected in 40% methanol) was further fractionated using Eurosphere C18 column using a gradient system with 1% aqueous acetic acid and 100 % acetonitril (ACN) as solvent. The gradient started with 80% ACN, then after 20 minutes decreased to 50% ACN, further decreased to 30% ACN within 20 minutes and finally within 20 minutes to 5%, followed by an equilibration step to the initial run condition. In this separation technique, 4 fractions were collected from different time range, i.e. minute 0-10, 10-20, 20-30 and 30-60, respectively. The solvent was evaporated in centrifugal evaporator prior to the neurosphere assay. The active fraction from this step was then analyzed using UPLC-MS system for structure elucidation.

2.21. Compound elucidation by UPLC/ESI-QTOFMS

Chromatographic separations were performed at 40°C on an Acquity UPLC system (Waters) equipped with a HSS T3 column (100 × 1.0 mm, particle size 1.8 μm, Waters)applying the following binary gradient at a flow rate of 150 μL min^{-1}: 0-1 min, isocratic 95% A (water/formic acid, 99.9/0.1 v/v), 5% B (acetonitrile/formic acid, 99.9/0.1 v/v); 1-16 min, linear from 5 to 50% B; 16-18 min, isocratic 95% B; 18-20 min, isocratic 5% B. The injection volume was 2.6 μL (full loop injection). Eluting compounds were detected from 190-500 nm using a photodiode array detector (Waters) and from *m/z* 100-1000 using a MicrOTOF-Q hybrid quadrupole time-of-flight mass spectrometer (Bruker Daltonics) equipped with an Apollo II electrospray ion source in negative ion mode. The following instrument settings were applied: nebulizer gas, nitrogen, 1.6 bar; dry gas, nitrogen, 6 l/min, 190°C; capillary, +4000 V; end plate offset, -500 V; funnel 1 RF, 200 V; funnel 2 RF, 200 V; in-source CID energy, 0 V; hexapole RF, 100 V; quadrupole ion energy, 3 eV; collision gas, argon; collision energy, 10 eV; collision RF 200/400 V (timing 50/50); transfer time, 70 μs; pre pulse storage, 5 μs; pulser frequency, 10 kHz; spectra rate, 3 Hz.

Mass spectra were acquired in centroid mode. Calibration of *m/z* was performed for individual raw data files on lithium formate cluster ions obtained by automatic infusion of 20 μl lithium hydroxide (10 mM) in the mixture of isopropanol/water/formic acid, 49.9/49.9/0.2 (v/v/v) respectively at the end of the gradient using a diverter valve.

For the acquisition of collision-induced dissociation mass spectra appropriate precursor ions were isolated using the first quadrupole (isolation width *m/z* 4) and fragmented inside the collision cell applying collision energies in the range of 15-30 eV.

2.22. Total flavonoid assay

Total flavonoids in apple cultivars were measured using sodium borohydride/chloranil-based assay as describe by He *et al.* (2008). Dried samples and quercetin standards were reconstituted in 1 ml of THF/EtOH (1:1,v/v) in a glass test tube. Each test tube was added with 0.5 ml of 50 mM $NaBH_4$ and 0.5 ml of 74.56 mM $AlCl_3$ solution, followed by 30 minute mixing step in an orbital shaker at room temperature. An additional 0.5 ml of $NaBH_4$ solution was added with continuing shaking for another 30 minutes at room temperature. Subsequently, 2 ml of 0.8 M of cold acetic acid solution was added into the test tube, thoroughly mixed and then incubated in the dark for 15 minutes. The tubes were then heated at 100ºC with shaking for 60 minutes after adding 1ml of 20 mM chloranil into each tube. The tubes were cooled down using tap water, and the final volume was brought to 4 ml with methanol. Then, 1 ml of 1052 mM vanillin was added into each tube and mixed thoroughly. Finally, 2 ml of concentrated HCl was added into each tube and kept in the dark for 15 minutes after a thorough mix. For detection, 200 μl of final reaction solutions were added into each well of a 96-well plate and the absorbances were measured in microplate reader at 490 nm.

2.23. Morris water maze task

Three weeks after apple juice supplementation, mice were trained to locate a submerged escape platform in a circular pool (2 m diameter). Water was made opaque with non-toxic titan dioxide and kept at temperature of 19-20ºC. Each mouse received 6 trials a day for 5 consecutive days. The position of the platform was changed at day 4 to the opposite quadrant (reversal). Dropping position was changed every day and remained constant for the whole trials during each day.

Mice were allowed to search up to 120 seconds for the platform. At the end of each trial, mice were guided to the platform and allowed to remain there for 15 seconds. The first 30 second time frame on first trial of day 4 was used as probe trial. Swim paths were recorded using Ethovision (Noldus) and further analyzed using Matlab (the Mathworks, USA).

2.24. Statistical analysis

Statistical analysis was performed using Graphpad Prism software (version 5.0, GraphPad Software Inc.). Depending on the number of groups, statistical analysis was done using t-test, one-way or two-way ANOVA. One sample t-test was performed to analyze 2 groups, whereby each replicate in the treatment group is presented as normalized to control. Therefore the control group has only one value, which was then set as the hypothethical value. The Dunnett's test was used as the post hoc test in one-way ANOVA.

Microarray data analysis was done using R for Mac OS X (3.1.1). False discovery rate (FDR) method was applied to perform multiple comparison tests on the microarray data.

All data are shown as means with standard error (SEM) unless stated otherwise.

3. Results

3.1. The effect of Quercetin on adult hippocampal neural precursor cells *in vitro* and *in vivo*

Quercetin and its glycosides are the most abundant flavonoid in apples, especially in the peels, and have been studied extensively for their antiproliferative effects on cancer cells. Additionally, this class of phytochemicals has a prominent ability to exert an antioxidant effect due to their capacity in scavenging reactive oxygen species. The biological activities of quercetin not only depend on its antioxidant properties, but also the potency in modulation of molecular pathways. An increasing number of studies demonstrate the beneficial effects of the quercetin and other flavonoids consumption in maintaining brain health and preventing the detrimental effects of aging and neurodegenerative diseases. However, the role of quercetin in adult hippocampal neurogenesis, a process that provides cellular plasticity in the brain and is susceptible to the effect of neurodegenerative diseases, is still elusive. Therefore I studied the effect of this compound in adult hippocampal neurogenesis that is possibly responsible, in part, for the beneficial effect of eating apples.

3.1.1. Quercetin (25 μM) inhibits cell cycle without inducing cell death of neural precursor cells *in vitro*

The adherent monolayer culture system was used as the *in vitro* model to investigate the effect of quercetin on NPCs proliferation and differentiation. One of the routine methods to assess cell proliferation is by using BrdU assay. BrdU is an analog to thymidine that competes with endogenous thymidine base during DNA synthesis in the cell cycle. Therefore, incubation with BrdU for a given time labels the cohort of proliferating cells during that time range.

To test the effect of quercetin on cell proliferation, the NPCs were cultured in the presence of growth factors (EGF and bFGF) for 48 hours. Following complete media change, the cultures were continued for subsequent 24 hours with or without quercetin and then labeled with BrdU (10 μM) for 2 hours prior to the end of the experiment (figure 3.1-A). A dose response assay was performed to find the optimal quercetin concentration.

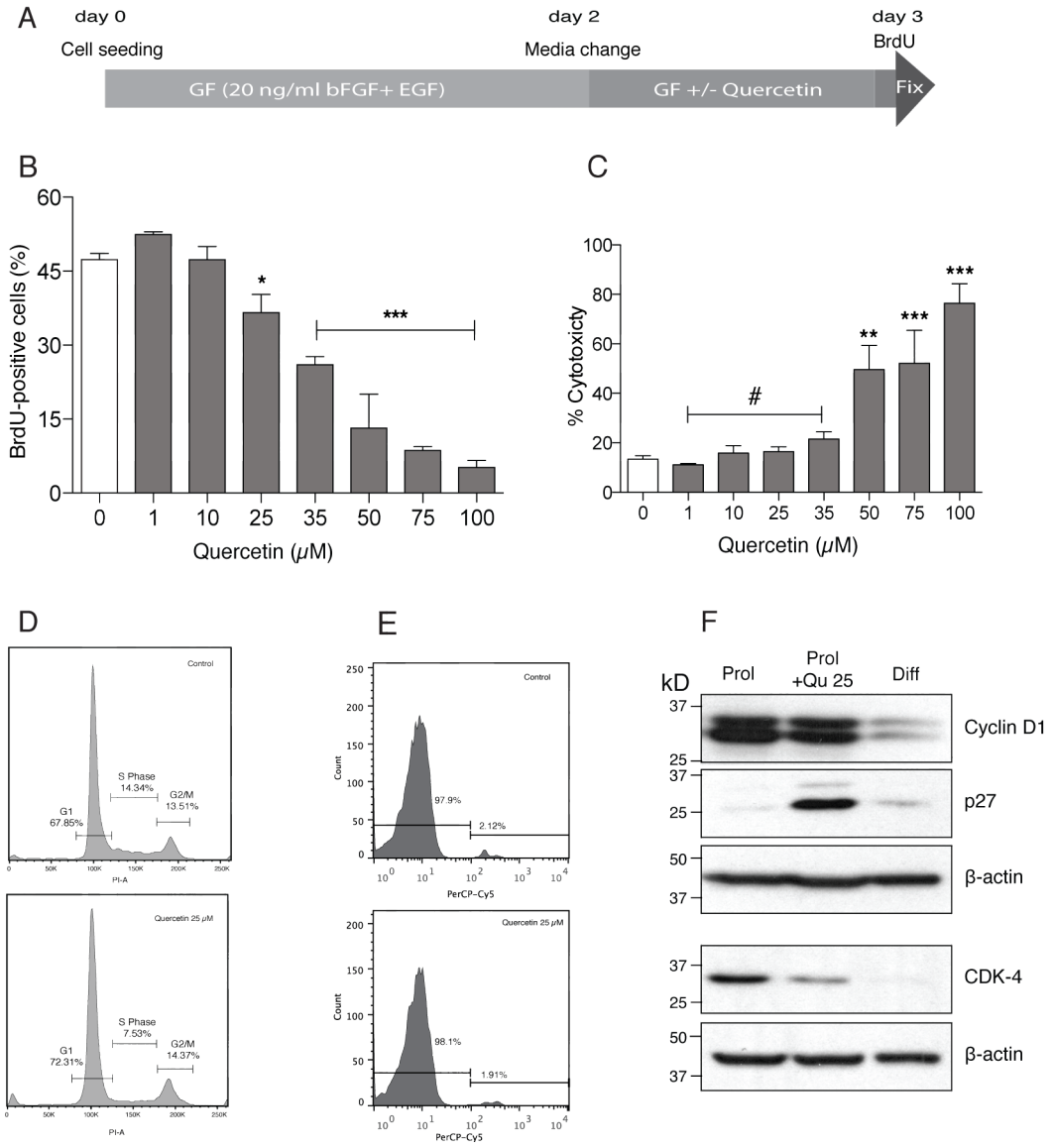

Figure 3.1. Quercetin (25 μM) inhibits cell cycle without inducing cell death. (A) Experimental scheme of monolayer NPCs culture with 24 hours quercetin treatment. (B) Quercetin decreases BrdU-labeled cells dose dependently (C) Cytotoxicity was determined by measuring the LDH release into media by colorimetric assay. Error bars represent SEM. (D) Cell cycle analysis using propidium iodide staining detected by FACS showing the decreased number of cells entering S-phase after 24 hour incubation with 25 μM quercetin treatment. (E) Cells stained with 7-AAD detected by FACS confirming that quercetin (25 μM) do not induce cell death. (F) Western blot of cell cycle-related proteins. Cells cultured in 24 hours without growth factor (diff) were used as a control for cell cycle arrest.

(# $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by posthoc Dunnett's test).

I observed that 25 μM quercetin is the lowest tested concentration with a significant decrease in the number of BrdU-positive cells compared to the DMSO-treated group (33.53 ± 2.12 % vs. 52.23 ± 2.42 %. n = 4, $p < 0.001$. Figure 3.1-B). In the higher concentrations of quercetin, the number of BrdU-positive cells was much lower but a lot of cells showed morphological changes such as cell shrinkage and fragmentation, indicating cell death.

To determine the cytotoxic concentration of quercetin, the lactate dehydrogenase (LDH) assay was performed. I found that higher quercetin concentrations, ≥ 50 μM, significantly increased the number of dead cells compared to control (49.59 ± 9.818 % vs. 13.46 ± 1.32 %. n = 4, $p < 0.01$; figure 3.1-C), while at 25 μM there was no significant difference to control. To confirm this finding, 7-AAD staining analyzed by FACS was performed as cell death assay. There was also no significant difference in the number of 7-AAD stained cells between control and 25 μM quercetin, confirming that this concentration is not cytotoxic in NPC (figure 2.1-E). Considering the result from the BrdU and LDH experiments I concluded that 25 μM is the most promising concentration to be applied for further investigation of the effect of quercetin in NPCs.

In line with the BrdU counts, cell cycle analysis using propidium iodide staining detected by FACS also showed that a significant proportion of cells were accumulating in G1 phase (72.31 ± 0.54 % vs. 67.85 ± 0.66 %, n = 3, $p < 0.001$), whereas fewer cells were entering the S phase (7.53 ± 0.3 % vs. 14.34 ± 0.28 %, n = 3, $p < 0.001$) in the quercetin-treated group compared to the control group, respectively (figure 3.1-D). This finding indicated that quercetin inhibits cell cycle progression of NPCs from G1 to S phase.

Western blot analysis was applied to observe the expression of several proteins that are involved in cell cycle regulation. In the quercetin-treated group, cyclin-dependent kinase 4 (cdk4) was downregulated, while cyclinD1 remained unchanged. On the contrary, cell cycle inhibitor p27 was upregulated, to an even greater extent than by withdrawal of the growth factors (figure 3.1-E).

Altogether, these data indicate that quercetin upregulates the “brake” signal in cell cycle to counteract the mitogenic effect of growth factors.

3.1.2. Quercetin (25 μM) induces NPCs differentiation despite the presence of growth factor

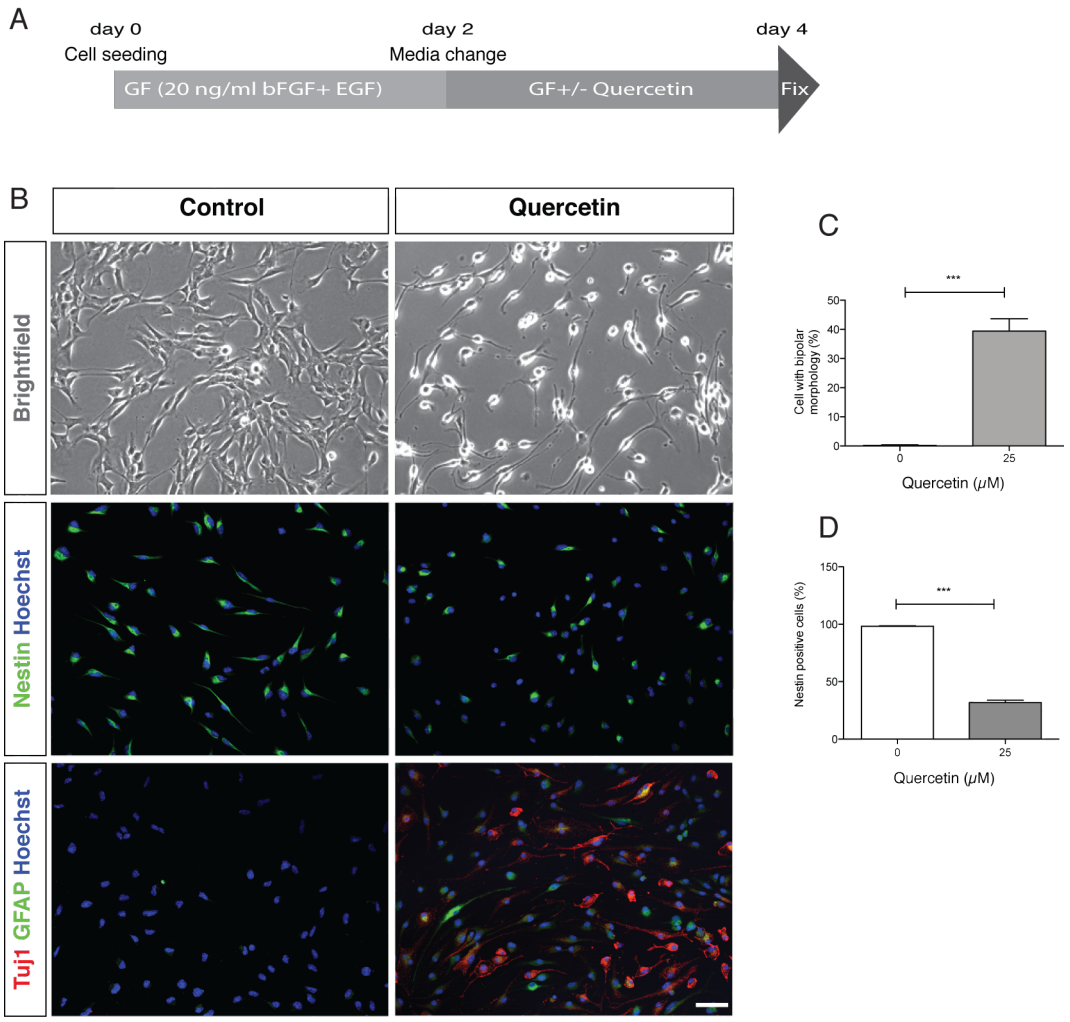

Figure 3.2. Quercetin (25 μM) induces NPCs differentiation. (A) Experimental scheme (B) The morphological change of NPCs into "neuron-like" bipolar morphology, (upper panels), downregulation of nestin expression (middle panels) and expression of neuronal marker β-III-Tubulin (Tuj1) and the astrocyte marker GFAP (lower panels). Scale bar 50 μm. (C) Quantification of the cells with bipolar morphology (D) Percentage of nestin-positive cells
Error bars represent SEM. ***p<0.001 by Student's *t*-test.

Under proliferation conditions, NPCs are phase bright with a typical morphology (polygonal) as shown in figure 1.6-B, with two or three short processes and expressing nestin. After 48 hours incubation with 25 μM quercetin (experimental scheme depicted in figure 3.2-A), a significant number of NPCs changed into neuron-like morphology, characterized with spindle-shaped body

with bipolar processes (39.45 ± 4.19 % vs. 0.002 % for quercetin-treated and control group, respectively. n = 5, $p < 0.001$, Student's *t*-test. Figure 3.2-B upper panel and 3.2-C). In parallel, a significant decrease in nestin expression was also observed after 48 hours of quercetin treatment (31.91 ± 1.99 %) compared to control (98.2 ± 0.46 %). n = 6, $p < 0.001$, Student's *t*-test, figure 2.2-B middle panel and 3.2-D.

These findings led me to the question whether the NPCs exit the cell cycle and proceed to cell differentiation after quercetin treatment. To answer this question, the cells were stained with β-III-tubulin (Tuj1) and GFAP, the markers for neurons and astrocytes, respectively. The NPCs treated with quercetin showed the population of cells that expressed these differentiated cells markers (figure 3.2-B lower panel), supporting the hypothesis that quercetin induces cell cycle exit and differentiation.

3.1.3. Quercetin promotes cell survival during differentiation by inducing endogenous antioxidants and the Akt pathway

The NPCs in monolayer culture can be induced to differentiate into neurons or astrocytes by culturing them in the growth medium without EGF and bFGF. Growth factor withdrawal is a stressful condition for the cells that leads to massive cell death (figure 3.3-B). As polyphenols have been shown to exert neuroprotective effect, I next tested whether quercetin supports cell survival *in vitro* during differentiation condition.

Prior to growth factor withdrawal, the NPCs were initially cultured for 48 hours in proliferation condition to acclimatize them in the culture condition. Quercetin was added to the differentiation media during the next 96 hours (figure 3.3-A). At the end of the experiment (day 6), the number of surviving cells was measured using the resazurin viability assay. Resazurin is a non-toxic and non-fluorescent compound that will be metabolized by healthy cells into fluorescent compound (resorufin). The fluorescence signal intensity is associated with the number of healthy cells in the sample.

I observed that quercetin (25 μM) increased the survival rate of differentiated NPCs 182.6 ± 19.06 % higher than in the control group (n = 5, $p < 0.001$ by Dunnett's test. Figure 3.3-C).

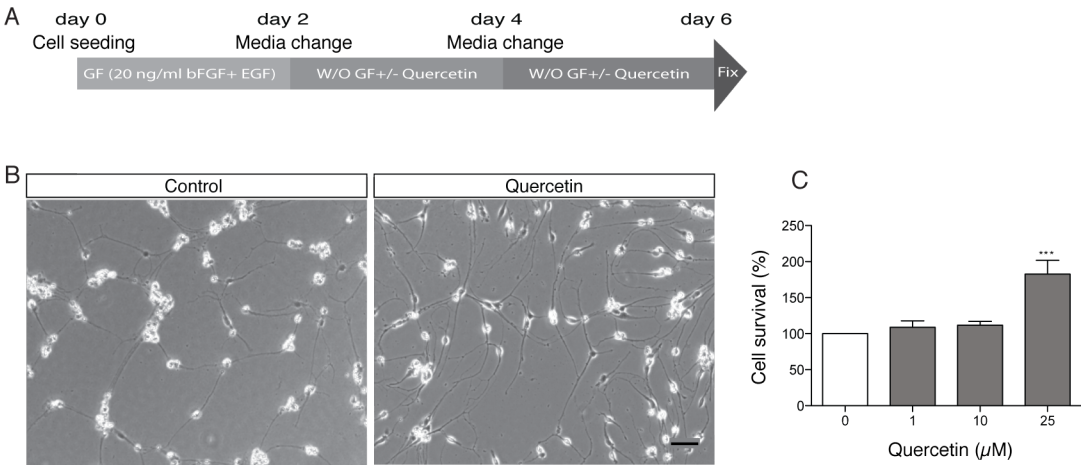

Figure 3.3. Quercetin (25 μM) promotes NPCs survival during differentiation (A) Experimental scheme of monolayer NPCs culture in differentiation condition. (B) Brightfield images of NPCs after 96 hour growth factor withdrawal. Scale bar 50 μm. (C) Quercetin (25 μM) increases cell survival during differentiation. Bar graph shows the percentage of resorufin fluorescence intensity compared to control ($p < 0.001$ by Dunnett's test. Error bars represent SEM).

To follow up the underlying mechanisms of quercetin on cell survival, I performed RNA microarray analysis. The RNA samples were collected from NPCs 24 hours after growth factor withdrawal, in the presence or absence of quercetin (25 μM). The raw data were processed through the workflow (see material and methods section) and then visualized as a volcano plot. FDR adjustment for $p < 0.05$ was regarded as statistically significant (figure 3.4-A). The differentially expressed genes were presented as relative expression to control, which was calculated as the result of log2 fold change. The value "1" indicates 2 fold increase of gene expression while "-1" indicates 2 fold decrease of gene expression relative to the gene expression in the control group.

The analysis revealed the expression of 3900 genes that were significantly regulated upon quercetin treatment. The top 10 highest up- and down-regulated genes are listed in table 3.1. To interprete the gene expression data as biological functions, I used a web-based enrichment analysis tool; namely Web-based Gene Set Anaysis Toolkit (WebGestalt; Wang *et al.*, 2013). The differentially expressed genes were then enriched into pathways from the KEGG (Kanehisa and Goto, 2000) and WikiPathways databases (Kelder *et al.*, 2009). Following hypergeometric testing, the top 10 of the most significantly enriched pathways in each database

were displayed. Next, I sorted the pathways based on the ratio of enrichment, which shows the number of differentially expressed genes in the samples compared to the total genes expected in each pathway. Therefore the higher the ratio, the more enriched the pathway is (figure 3.4-B & C).

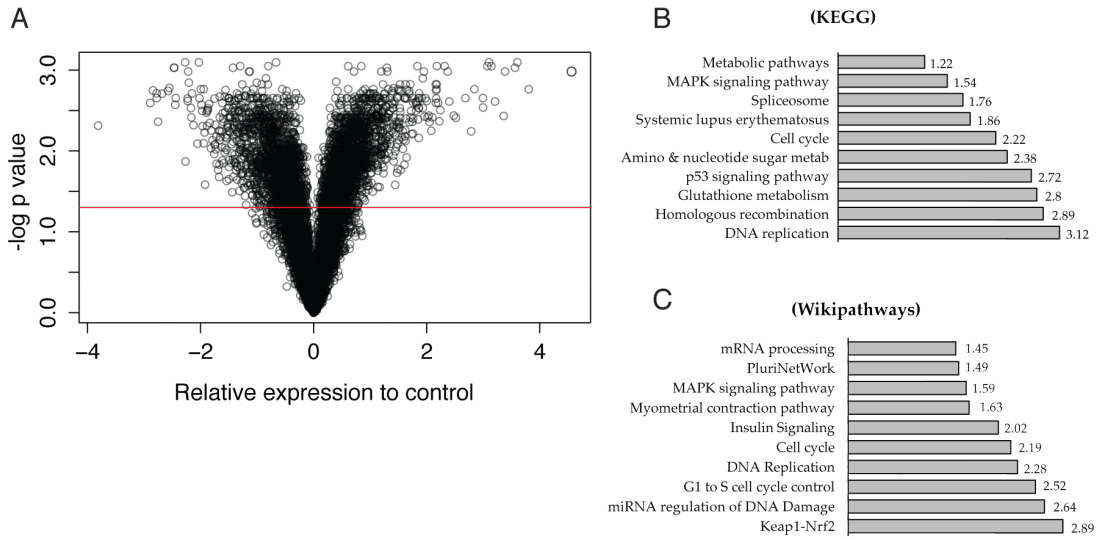

Figure 3.4. RNA microarray analysis of NPCs after 24 hour growth factor withdrawal, treated with 25 μM quercetin. (A) Volcano plot of changes in gene expression. (B) The top 10 most significantly enriched pathways from KEGG and (C) WikiPathways databases, sorted based on the ratio of enrichment.

Table 3.1 List of top 10 upregulated and downregulated genes after quercetin treatment in differentiation condition

Gene ID	Gene name	Description	Relative expression	Adjusted p value
14859	Gsta3	glutathione S-transferase, alpha 3	4.56	0.001
228775	Trib3	tribbles homolog 3 (Drosophila)	3.8	0.002
76650	Srxn1	sulfiredoxin 1 homolog (S. cerevisiae)	3.6	0.001
71839	Osgin1	oxidative stress induced growth inhibitor 1	3.56	0.001
15220	Foxq1	forkhead box Q1	3.38	0.001
23886	Gdf15	growth differentiation factor 15	3.36	0.004
19773	Rln1	relaxin 1	3.2	0.002

14630	Gclm	glutamate-cysteine ligase, modifier subunit	3.14	0.001
53945	Slc40a1	solute carrier family 40 (iron-regulated transporter), member 1	3.1	0.001
12705	Cited1	Cbp/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain 1	3.07	0.001
64075	Smoc1	SPARC related modular calcium binding 1	-3.81	0.005
16979	Lrrn1	leucine rich repeat protein 1, neuronal	-2.89	0.003
330166	Miat	myocardial infarction associated transcript (non-protein coding)	-2.84	0.002
70433	Draxin	dorsal inhibitory axon guidance protein	-2.78	0.002
18761	Prkcq	protein kinase C, theta	-2.77	0.002
12799	Cnp	2',3'-cyclic nucleotide 3' phosphodiesterase	-2.75	0.004
18377	Omg	oligodendrocyte myelin glycoprotein	-2.68	0.002
70784	Rasl12	RAS-like, family 12	-2.58	0.002
18481	Pak3	p21 protein (Cdc42/Rac)-activated kinase 3	-2.55	0.002
101488	Slco2b1	solute carrier organic anion transporter family, member 2b1	-2.47	0.001

In both databases, the pathways involved in cell cycle, endogenous antioxidant system, and cell survival are significantly enriched. The flavonoids were reported to exert their effect on cell survival through modulation of mitogen activated protein kinase (MAPK) and phosphoinositide-3-kinase (PI3K-Akt) pathways. Depending on the chemical structures, flavonoids can either inhibit or activate these pathways (reviewed by Spencer, 2009).

The MAPK pathways consist of mitogenic extracellular signal-regulated protein kinase (ERK) that is associated with pro-survival signalling and the stress activated c-Jun N-terminal kinase (JNK) and p38 cascades that induce apoptotic signalling. The sustained activation of JNKs subsequently induces the transcription of proapoptotic genes or modulates mitochondrial function that leads to cell death. However the acute and transient activation of JNK due to the parallel activation of pro survival pathways can rescue cell death (reviewed by Dhanasekaran and Reddy, 2008).

The enrichment analysis of MAPK pathway showed that ERK was not differentially expressed upon quercetin treatment. In contrast, I observed the upregulation of JNK2 and JNK3 while JNK1 was downregulated. The transcripts of JNKs-related proapoptotic genes namely Bid, Bax and PUMA was also upregulated in quercetin-treated group (table 3.2). To observe whether the apoptotic signals occur at the protein level, I checked the expression of cleaved caspase-3 which is the important protein in the execution phase of apoptosis. The western blot analysis showed that the cleaved caspase-3 protein was downregulated after 48 hour quercetin treatment (75.08 ± 18.08 % compared to control, n = 4, p = 0.07, one sample *t*-test (figure 3.8-E). This finding indicates the existence of rescue mechanisms that counteract the proapoptotic signals and protect the cells from apoptosis.

Table 3.2 The differential expression of JNKs and related pro-apoptotic genes in the MAPK pathway after quercetin treatment in differentiation condition

Gene ID	Gene name	Description	Relative expression	Adjusted p value
26419	Mapk8	mitogen activated protein kinase-8 (JNK1)	-0.57	0.02
26420	Mapk9	mitogen activated protein kinase-9 (JNK2)	0.49	0.04
26414	Mapk10	mitogen activated protein kinase-10 (JNK3)	0.95	0.01
12028	Bax	BCL2-associated X protein	0.9	0.01
170770	PUMA	BCL2 binding component 3 (Bbc3)	0.53	0.01
12122	Bid	BH3 interacting domain death agonist	1.05	0.002

To observe whether the pro-survival and anti-apoptotic signals were simultaneously upregulated, I next analyzed the enrichment of the differentially expressed genes in the PI3K/Akt pathway from the KEGG database (figure 3.5) and the Nrf2-Keap1 pathway from the WikiPathways database (figure 3.7).

In the PI3K/Akt pathway I observed the significant upregulation of pro-survival genes such as Akt, CREB, MDM2 and Bcl xL (Bcl2l1). On the contrary, the apoptosis-inducing genes such as p53 and Bim (Bcl2l11) are downregulated (table 3.3). To confirm the transcriptomic data, I did qPCR to quantify the expression level of Bcl xL (Bcl2l1) and Bim (Bcl2l11). In line with the microarray data, I found that

the Bcl2l1 was upregulated over time in the quercetin-treated group (ΔΔCt 1.85 ± 0.2 and 2.55 ± 0.67 after 24 and 48 hours of treatment, respectively. Figure 3.6-A). On the contrary, the expression of pro-apoptotic gene Bcl2l11 was downregulated over time (ΔΔCt -1.55 ± 3.6 and -3.5 ± 1.5 after 24 and 48 hours treatment, respectively. Figure 3.6-B).

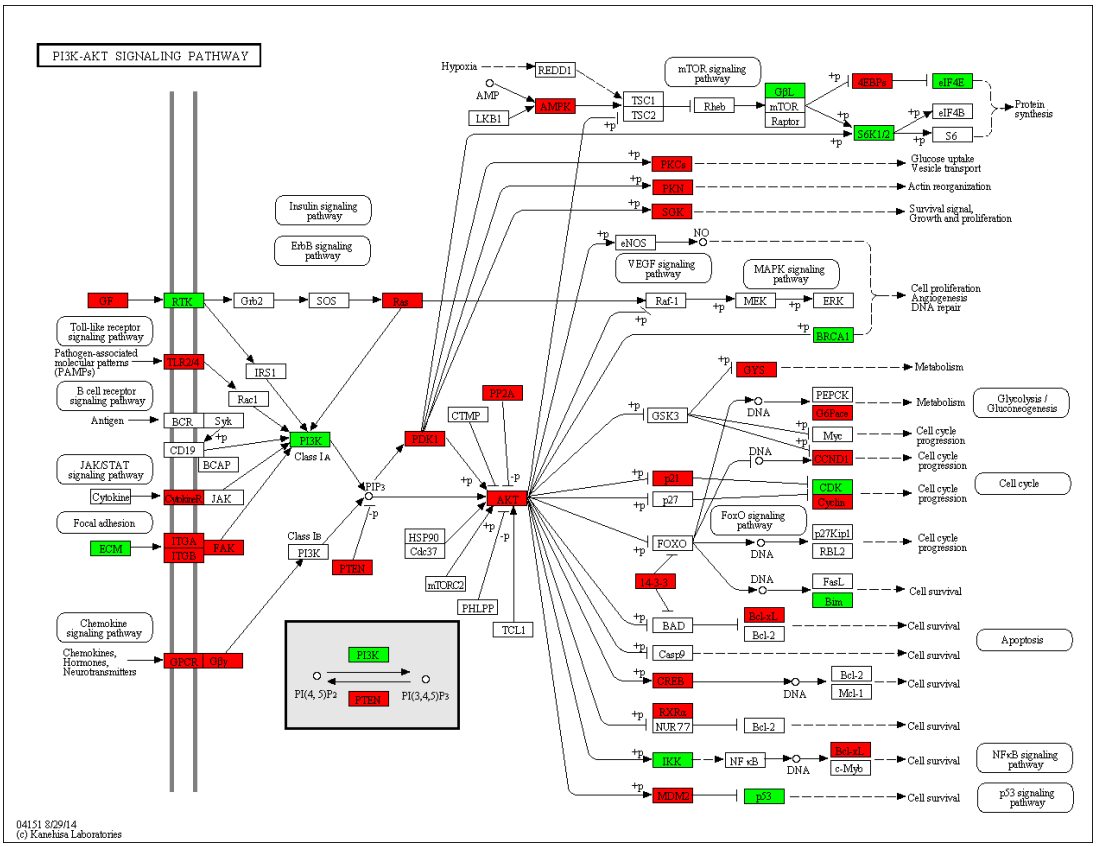

Figure 3.5. The differentially expressed genes from quercetin-treated NPCs in differentiation conditions are mapped to the PI3K-Akt pathway in KEGG pathways map04151. Upregulated and downregulated genes are higlighted as red and green, respectively.

The increased expression of phospho-Akt (pAkt) and Bcl2l1 was also observed in protein level (figure 3.8-A). After 48 hour incubation with 25 μM quercetin, the pAkt was significantly upregulated (202 ± 23.29 % compared to control group, n = 4, p = 0.02. Figure 3.8-B). A similar pattern was also observed in Bcl2l1 protein expression (427.2 ± 145.6 % compared to control group, n = 3, p = 0.15. Figure 3.8-C. The p values were calculated using one-sample t-test).

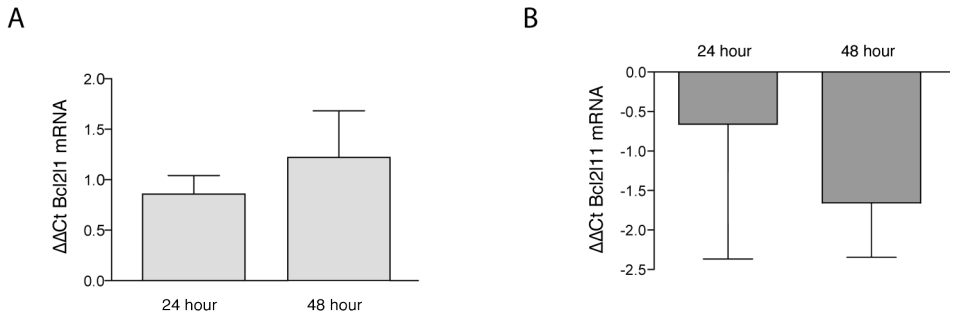

Figure 3.6. Relative expression of Bcl2l1 gene (A) and Bcl2l11 (B) in differentiating NPCs after 24- and 48 hour quercetin (25 μM) treatment by qPCR. (n = 3, error bars represent SEM).

Table 3.3 The list of differentially expressed genes, mapped in PI3K-Akt pathway (KEGG pathways mmu04151) after quercetin treatment in differentiation condition

Gene ID	Gene name	Description	Relative expression	Adjusted p value
22339	Vegfa	vascular endothelial growth factor A	2.17	0.002
20393	Sgk1	serum/glucocorticoid regulated kinase 1	1.87	0.002
13685	Eif4ebp1	eukaryotic translation initiation factor 4E binding protein 1	1.48	0.008
14164	Fgf1	fibroblast growth factor 1	1.47	0.005
11911	Atf4	activating transcription factor 4	1.41	0.013
108079	Prkaa2	protein kinase, AMP-activated, alpha 2 catalytic subunit	1.41	0.012
17246	Mdm2	transformed mouse 3T3 cell double minute 2	1.3	0.005
14696	Gnb4	guanine nucleotide binding protein (G protein), beta 4	1.26	0.004
16590	Kit	kit oncogene	1.23	0.016
12048	Bcl2l1	BCL2-like 1	1.11	0.004
12575	Cdkn1a	cyclin-dependent kinase inhibitor 1A (P21)	1.02	0.025
241226	Itga8	integrin alpha 8	0.98	0.011
14268	Fn1	fibronectin 1	0.96	0.025
16410	Itgav	integrin alpha V	0.92	0.01

22628	Ywhag	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma polypeptide	0.91	0.024
21898	Tlr4	toll-like receptor 4	0.9	0.005
22341	Vegfc	vascular endothelial growth factor C	0.9	0.012
74769	Pik3cb	phosphatidylinositol 3-kinase, catalytic, beta polypeptide	0.88	0.017
12444	Ccnd2	cyclin D2	0.88	0.024
20181	Rxra	retinoid X receptor alpha	0.87	0.004
18750	Prkca	protein kinase C, alpha	0.86	0.01
14083	Ptk2	PTK2 protein tyrosine kinase 2	0.84	0.004
13640	Efna5	ephrin A5	0.84	0.017
208647	Creb3l2	cAMP responsive element binding protein 3-like 2	0.84	0.02
104099	Itga9	integrin alpha 9	0.8	0.017
12443	Ccnd1	cyclin D1	0.77	0.031
192897	Itgb4	integrin beta 4	0.75	0.002
14205	Figf	c-fos induced growth factor	0.75	0.017
26427	Creb3l1	cAMP responsive element binding protein 3-like 1	0.72	0.003
109333	Pkn2	protein kinase N2	0.71	0.002
226849	Ppp2r5a	protein phosphatase 2, regulatory subunit B (B56), alpha isoform	0.7	0.013
16337	Insr	insulin receptor	0.68	0.03
16194	Il6ra	interleukin 6 receptor, alpha	0.65	0.036
15461	Hras	Harvey rat sarcoma virus oncogene 1	0.63	0.036
18607	Pdpk1	3-phosphoinositide dependent protein kinase 1	0.63	0.012
12977	Csf1	colony stimulating factor 1 (macrophage)	0.6	0.02
23797	Akt3	thymoma viral proto-oncogene 3	0.6	0.004
14183	Fgfr2	fibroblast growth factor receptor 2	0.56	0.012
12913	Creb3	cAMP responsive element binding protein 3	0.55	0.009
14182	Fgfr1	fibroblast growth factor receptor 1	0.51	0.03
12831	Col5a1	collagen, type V, alpha 1	0.5	0.008

16402	Itga5	integrin alpha 5 (fibronectin receptor alpha)	0.5	0.029
14706	Gng4	guanine nucleotide binding protein (G protein), gamma 4	0.49	0.002
269643	Ppp2r2c	protein phosphatase 2 (formerly 2A), regulatory subunit B (PR 52), gamma isoform	0.48	0.026
16151	Ikbkg	inhibitor of kappaB kinase gamma	0.43	0.015
19053	Ppp2cb	protein phosphatase 2 (formerly 2A), catalytic subunit, beta isoform	0.43	0.017
19211	Pten	phosphatase and tensin homolog	0.38	0.034
54635	Pdgfc	platelet-derived growth factor, C polypeptide	0.38	0.036
19052	Ppp2ca	protein phosphatase 2 (formerly 2A), catalytic subunit, alpha isoform	0.37	0.014
14936	Gys1	glycogen synthase 1, muscle	0.36	0.044
14745	Lpar1	lysophosphatidic acid receptor 1	0.33	0.026
14171	Fgf17	fibroblast growth factor 17	0.3	0.02
14600	Ghr	growth hormone receptor	0.19	0.018
52432	Ppp2r2d	protein phosphatase 2, regulatory subunit B, delta isoform	0.18	0.026
14377	G6pc	glucose-6-phosphatase, catalytic	0.13	0.04
14170	Fgf15	fibroblast growth factor 15	-0.13	0.032
12189	Brca1	breast cancer 1	-0.27	0.016
72508	Rps6kb1	ribosomal protein S6 kinase, polypeptide 1	-0.34	0.041
78134	Lpar4	lysophosphatidic acid receptor 4	-0.35	0.041
22370	Vtn	vitronectin	-0.37	0.006
12445	Ccnd3	cyclin D3	-0.42	0.02
21827	Thbs3	thrombospondin 3	-0.48	0.036
22059	Trp53	transformation related protein 53	-0.50	0.014
16398	Itga2	integrin alpha 2	-0.52	0.031
13684	Eif4e	eukaryotic translation initiation factor 4E	-0.56	0.022
12830	Col4a5	collagen, type IV, alpha 5	-0.66	0.006
12447	Ccne1	cyclin E1	-0.69	0.047
12832	Col5a2	collagen, type V, alpha 2	-0.69	0.012

56716	Mlst8	MTOR associated protein, LST8 homolog (S. cerevisiae)	-0.71	0.013
373864	Col27a1	collagen, type XXVII, alpha 1	-0.71	0.029
12125	Bcl2l11	BCL2-like 11 (apoptosis facilitator)	-0.72	0.005
13649	Egfr	epidermal growth factor receptor	-0.72	0.045
17311	Kitl	kit ligand	-0.79	0.025
12566	Cdk2	cyclin-dependent kinase 2	-0.84	0.023
18709	Pik3r2	phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 2 (p85 beta)	-0.84	0.009
12675	Chuk	conserved helix-loop-helix ubiquitous kinase	-0.9	0.006
21826	Thbs2	thrombospondin 2	-0.95	0.004
12567	Cdk4	cyclin-dependent kinase 4	-0.97	0.005
14168	Fgf13	fibroblast growth factor 13	-1.39	0.014
18595	Pdgfra	platelet derived growth factor receptor, alpha polypeptide	-1.43	0.008
18710	Pik3r3	phosphatidylinositol 3 kinase, regulatory subunit, polypeptide 3 (p55)	-1.45	0.003
14708	Gng7	guanine nucleotide binding protein (G protein), gamma 7	-1.49	0.002

Another highly enriched pathway that explains the mechanism of quercetin in promoting cell survival of differentiating NPCs is the Nrf2-Keap1 pathway (figure 3.7). During stress conditions, there is an activation and accumulation of Nrf2 in the nucleus (Nguyen *et al.*, 2009). The heterodimer of Nrf2 with small Maf (musculoaponeurotic fibrosarcoma) has been shown to increase the binding affinity with ARE, that leads to the transcription of phase II enzymes (Itoh *et al.*, 1997). These enzymes play important roles in endogenous protection systems by neutralizing the harmful by-products of oxidative stress (Nguyen *et al.*, 2003).

One of the downstream genes in this pathway, namely the Gsta3 (glutathione S-transferase alpha 3) is the most highly expressed gene in differentiating NPCs upon 25 μM quercetin treatment (table 3.1). The increased was also detected in protein expression (427.2 ± 145.6 % compared to control group, n = 3, p = 0.15 by one-sample t-test. Figure 3.8-D). The other genes in NRF2-Keap1 pathway that were differentially expressed during hippocampal precursor cells differentiation after quercetin treatment, were listed in table 3.4.

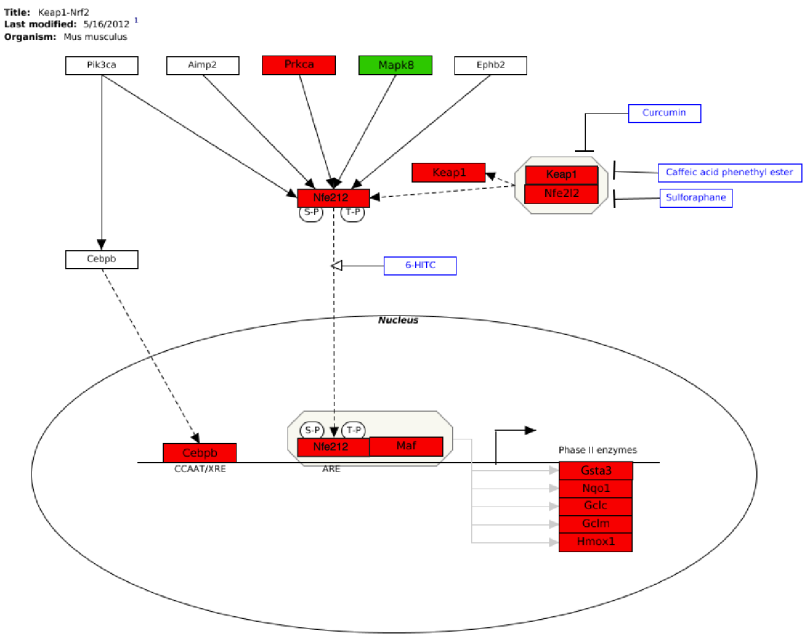

Figure 3.7. RNA microarray data from quercetin-treated NPCs in differentiation conditions are mapped to Nrf2-Keap1 pathway curated by Wikipathways. Up- and down-regulated genes are highlighted as red and green, respectively. (www.wikipathways.org/index.php/Pathway:WP1245).

Table 3.4 List of regulated genes after quercetin treatment in differentiation condition mapped in Nrf2-Keap1 pathway (Wikipathways WP1245)

Gene ID	Gene name	Description	Relative expression	Adjusted p value
14859	Gsta3	glutathione S-transferase, alpha 3	4.56	0.001
12608	Cebpb	CCAAT/enhancer binding protein (C/EBP), beta	1.96	0.004
15368	Hmox1	heme oxygenase (decycling) 1	2.24	0.003
17132	Maf	avian musculoaponeurotic fibrosarcoma (v-maf) AS42 oncogene homolog	0.36	0.033
18104	Nqo1	NAD(P)H dehydrogenase, quinone 1	1.91	0.002
18750	Prkca	protein kinase C, alpha	0.86	0.01
14630	Gclm	glutamate-cysteine ligase, modifier subunit	3.14	0.001
50868	Keap1	kelch-like ECH-associated protein 1	0.53	0.01
14629	Gclc	glutamate-cysteine ligase, catalytic subunit	0.87	0.006
18024	Nfe2l2	nuclear factor, erythroid derived 2, like 2	0.34	0.03
26419	Mapk8	mitogen-activated protein kinase 8	-0.57	0.021

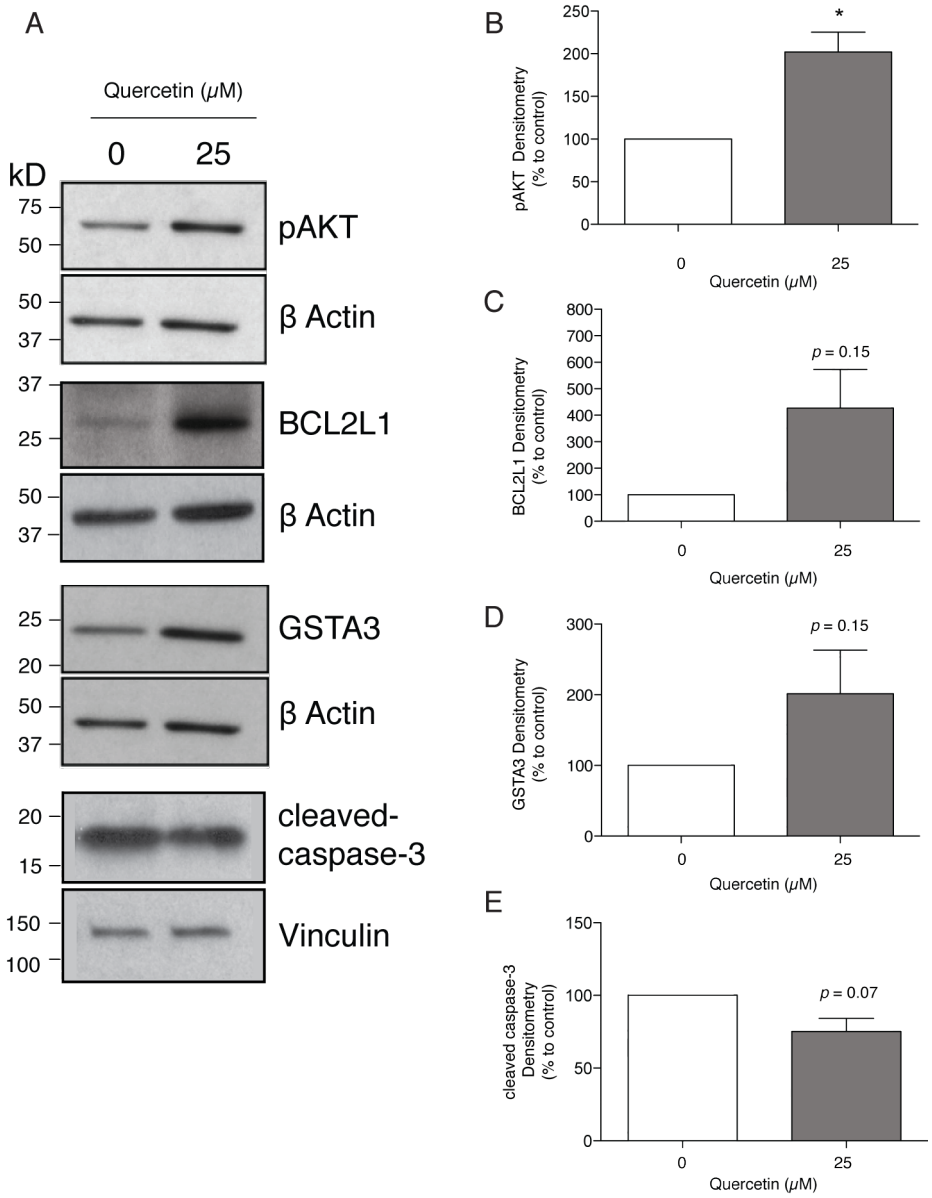

Figure 3.8. The expression of proteins in PI3K-Akt and Nrf2-Keap1 pathways after quercetin treatment were determined by western blot (A) and the semi-quantitative analysis normalized by beta-actin or vinculin (B,C,D,E). *$p < 0.05$, one sample *t*-test. Error bars represent SEM.

In addition to Nrf2 upregulation, the Keap1 (kelch-like ECH-associated protein 1) was also upregulated in the quercetin-treated group. This protein is the opponent of Nrf2 through facilitating the nucleo-cytoplasmic shuttling mechanism of Nrf2 and subsequently promoting its ubiquitinylation and degradation (Nguyen *et al.*, 2005). Bell *et al.* (2015) demonstrated that the overexpression of Nrf2 inhibits neurite outgrowth and neuronal maturation. Therefore it is likely that the upregulation of Keap1 gene expression, and possibly its protein level upon

quercetin treatment, is beneficial to prevent the detrimental effect of excessive accumulation of Nrf2.

Considering the *in vitro* data altogether, I conclude that quercetin (25 μM) induces NPC differentiation through cell cycle inhibition and activates the endogenous antioxidant system to promote cell survival during differentiation.

3.1.4. Quercetin increases survival of dentate gyrus newborn neurons *in vivo*

The next step, I proceed to perform an *in vivo* experiment to observe whether the effect *in vitro* also occurs *in vivo.* I hypothesize that quercetin affects adult hippocampal neurogenesis through inducing NPC neuronal differentiation and promoting the survival of newborn granule cells.

To reflect the situation that quercetin is part of the diet, I administered the quercetin through the oral route. When administered orally, quercetin undergoes extensive metabolism starting in the digestive tract by gut microflora and then in the liver before ultimately being excreted from the body through the kidney. Only trace amounts of quercetin and/or its metabolites can reach the brain and cross the blood brain barrier (reviewed by Hollman, 2004). A previous study by Ishisaka *et al.* (2011) showed that quercetin metabolites accumulate in the rat brain following oral administration of 50 mg/kg body weight (bw) quercetin dissolved in propylene glycol. Therefore I applied the same concentration of quercetin in the following experiment (figure 3.9-A). To ensure equal amount of intake, quercetin solution was administered daily by oral gavage.

In this experiment, I took advantage of a double-labeling protocol using the different halogenated thymidine analogs CldU and IdU to observe the effect of quercetin on the survival and fate determination of newborn cells in the dentate gyrus, and also the effect of quercetin on hippocampal precursor cell proliferation. Using the experimental scheme as depicted in figure 3.9-A, CldU labeled the cells that were "born" at day 0 of the experiment. This labelling enabled me to trace the phenotype of these cells and also to determine the number of surviving newborn cells at the end of the experiment. On the other hand, IdU was incorporated into the cells that were in S phase on the last day of the experiment. Therefore, the number of IdU-positive cells in this case represented the cell proliferation rate. The availability of antibodies that can recognize these molecules distinctly allows us to

detect and quantify these two cell-populations by immunofluorescence staining (Vega and Peterson, 2005).

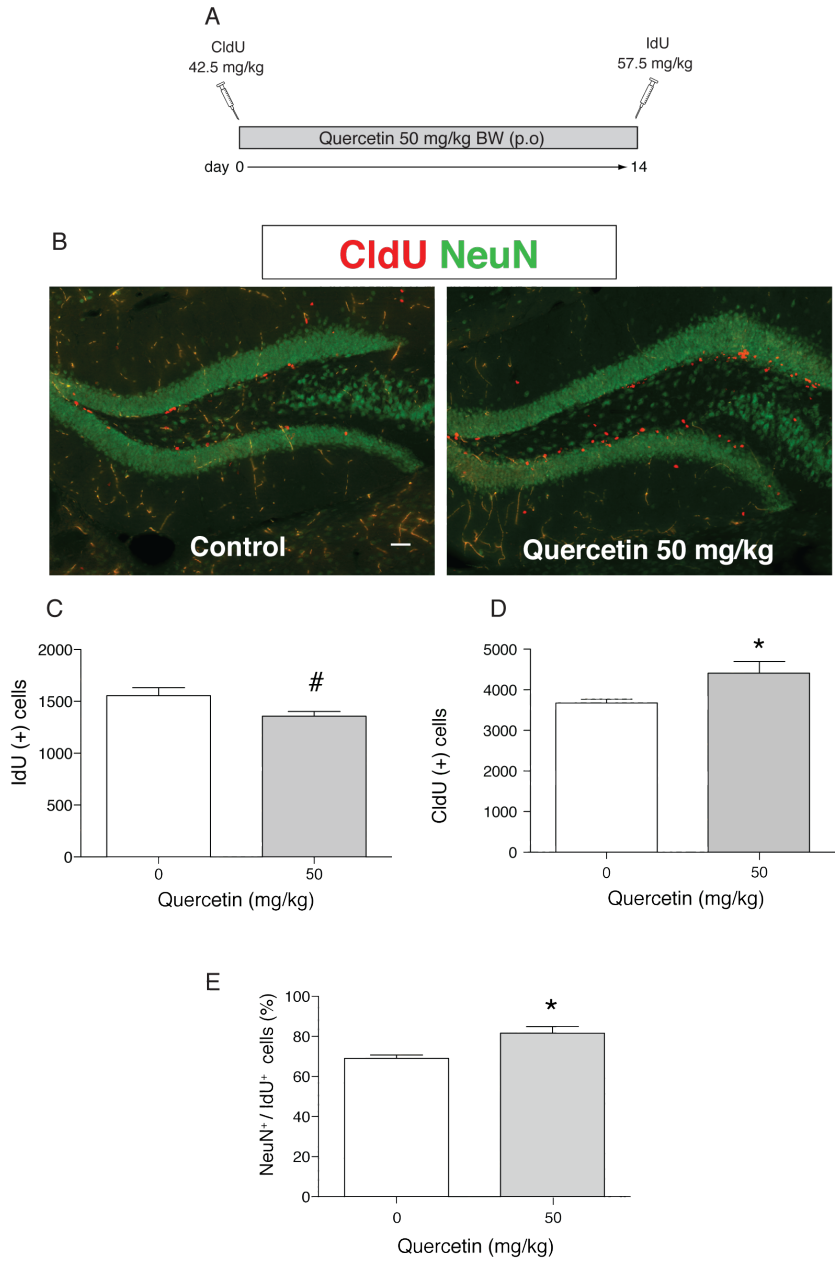

Figure 3.9. Quercetin increases cell survival and neuronal differentiation of newborn granule cells *in vivo*. (A) Experimental scheme. (B) Immunofluorescence staining shows the cohort of cells that CldU (red) and NeuN positive (green). Bar graphs show the number of IdU-positive cells (C), CldU-positive cells (D) and CldU/NeuN double positive cells (E). Error bars represent SEM, *p<0.05, # p:0.068, Student's *t*-test. Scale bar 50 μm.

After 2 weeks of quercetin treatment, I observed a trend towards a decrease in IdU-positive cells in the quercetin-treated group compared to control (1358 ± 45.52 cells vs. 1556 ± 76.77 cells, n = 4, p = 0.068. Figure 3.9-C). Interestingly, the number of CldU-positive cells was significantly increased after quercetin treatment (4410 ± 286.1 vs. 3672 ± 94.58 cells n = 4, p = 0.049. Figure 3.9-D). These results indicate the increase in numbers of surviving newborn cells and decrease of cell proliferation after quercetin treatment.

To determine the phenotype of CldU-positive cells, I did co-staining with NeuN (neuronal marker) and S100β (astrocyte marker) and quantified the percentage of CldU cells that colocalized with the neuronal or astrocyte marker. The result shows that the quercetin-treated group has a significantly higher percentage of NeuN/CldU-positive cells compared to the control group (81.67 ± 3.18 % vs. 69 ± 1.73 %, respectively. n = 3, p = 0.025. Figure 3.9-B; 3.9-E). Nevertheless only a small portion of CldU-positive cells showed the astrocytic phenotype with no significant statistical difference between two groups (0.98 ± 0.008 % vs. 2.33 ± 0.88 % for control and quercetin-treated group, respectively. n = 3, p = 0.2. All p values in this experiment were calculated using Student's t-test).

Altogether, the *in vivo* results correspond to the *in vitro* data whereby quercetin plays role in the regulation of adult hippocampal neurogenesis by affecting cell proliferation, promoting cell survival and cell fate determination into neuron.

3.2. Apple under scrutiny: searching for dietary compounds in Pinova apples that affect hippocampal precursor cells

Apples are a rich source of flavonoids and many other phytochemicals. The previous results have demonstrated that quercetin, the major flavonoid in apples, influences the proliferation and survival of hippocampal precursor cells. In this study I am also interested in searching for other active compounds in apples with the potential to exert an effect on hippocampal precursor cells.

To do this, I prepared extracts from Pinova apples and further tested them on hippocampal precursor cells in monolayer and neurosphere culture. The Pinova cultivar was selected in this study due to its abundant availability during the time of experiment and the favourable taste. Moreover, the Pinova cultivar is indigenously a Dresden apple cultivar because it was initially bred and cultivated in the Dresden-Pillnitz area by the breeders from the former Fruit Research

Institute Dresden Pillnitz (Fischer *et al.*, 2000). The high production yield and well-acceptable taste support this cultivar as a sustainable source of dietary phytochemicals.

3.2.1. Total flavonoid content of Pinova apple

Flavonoids are one of the major phytochemicals in apples that have been reported in many studies for their health benefits including anti-oxidative activity and anticancer properties. Therefore I performed an assay to quantify and compare the flavonoid content in Pinova apples and another cultivars.

Several varieties of apples were purchased from "Obsthof Schlage" in the Hosterwitz/Pillnitz district of Dresden, which were grown under a controlled-cultivation system. That means the use of fertilizers and pesticides are strictly controlled and minimized. The apple extracts were prepared and subsequently tested for their total flavonoid content using the sodium borohydride-chloranil assay.

The result from this assay showed that the flavonoid content in the peel is much higher than in the flesh for all tested apple cultivars. The total flavonoid content in the flesh is insignificantly different among all tested cultivars. A bigger variation is seen in the peel, whereby the flavonoid content in the Jonagold cultivar is significantly lower than in the Pinova cultivar. Pinova apples that were used in the experiment contained relatively high amount of total flavonoids, albeit insignificantly lower than the Elstar cultivar (table 3.5 and figure 3.10-A).

Table 3.5. Total flavonoid content in apple varieties.

Cultivar	Total flavonoid content (equivalent to mg quercetin/100 g fresh weight)	
	Peel	Flesh
Pinova	379 ± 93.95	42.45 ± 17.63
Elstar	428.9 ± 103.7	96.74 ± 32.61
Pilot	344.3 ± 74.19	67.36 ± 25.43
Rebella	186.6 ± 44.4	48.82 ± 15.10
Roter Berlepsch	148.1 ± 60.55	18.74 ± 7.86
Jonagold	87.85 ± 28.45	36.98 ± 15.53

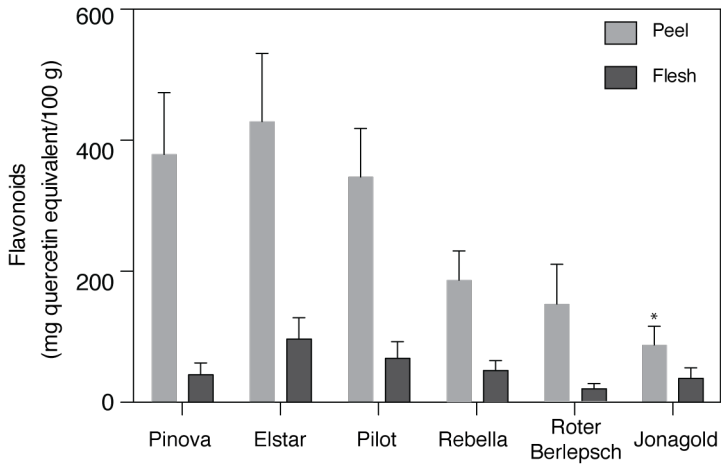

Figure 3.10. Total flavonoid content in the peel and flesh of several apple varieties from Pillnitz. The total amount of flavonoids is presented as equivalent of mg quercetin/100 gram fresh weight. Error bars represent SEM. *$p < 0.05$ by Dunnett's test to Pinova.

3.2.2. The neurosphere assay is a sensitive *in vitro* model to study the effect of apple extracts on neural precursor cells

Apple peel and flesh were extracted separately using 80% methanol/water. The solvent was removed using a rotary- and centrifugal evaporator until the extract reached maximum dryness. The extracts were dissolved in DMSO and then tested on the monolayer NPC culture. The cells were cultured for 48 hours in the presence of the extracts and then incubated with BrdU for 2 hours before the end of the experiment. The number of BrdU-positive cells was recorded after immunofluorescence staining to observe the effect of the extracts on cell proliferation.

The number of BrdU-positive cells was not significantly different to the control group after Pinova peel extract. There was a trend towards an increase at a lower concentration and a trend towards a decrease at a higher concentration (figure 3.11-A). A significant increase in the number of BrdU-positive cells was observed after treatment with a higher concentration of Pinova flesh extract compared to the control group (50.02 ± 1.55 % vs. 42.2 ± 0.63 %. n = 3, $p = 0.02$ by Dunnett's test. Figure 3.11-B).

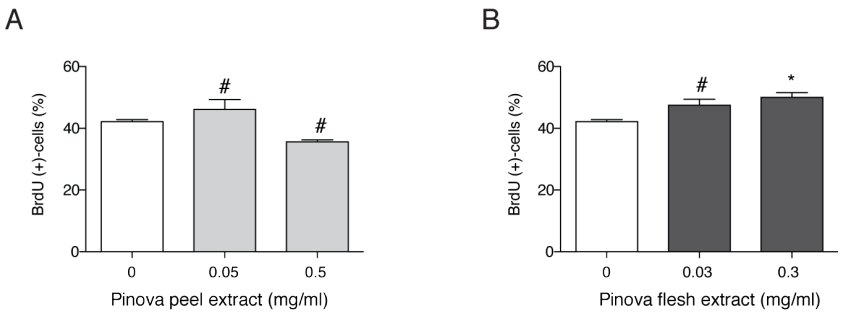

Figure 3.11. The effect of Pinova extracts on cell proliferation in monolayer culture based on BrdU-labelling method. Error bars represent SEM. # $p > 0.05$, *$p < 0.05$ by Dunnett's test.

As well as the monolayer culture, the neurosphere assay is also used as an *in vitro* model to study the effect of certain compounds on neural precursor cells (Walker *et al.*, 2008; Fritsche *et al.*, 2011). The primary cells from the dentate gyrus were dissociated and then incubated with the peel and flesh extracts. At the end of the experiments (day 12), the number of neurospheres in each group was counted as the read-out. Since this assay uses primary cells that are taken directly from living brain tissue, I did the replicates only for the promising concentration.

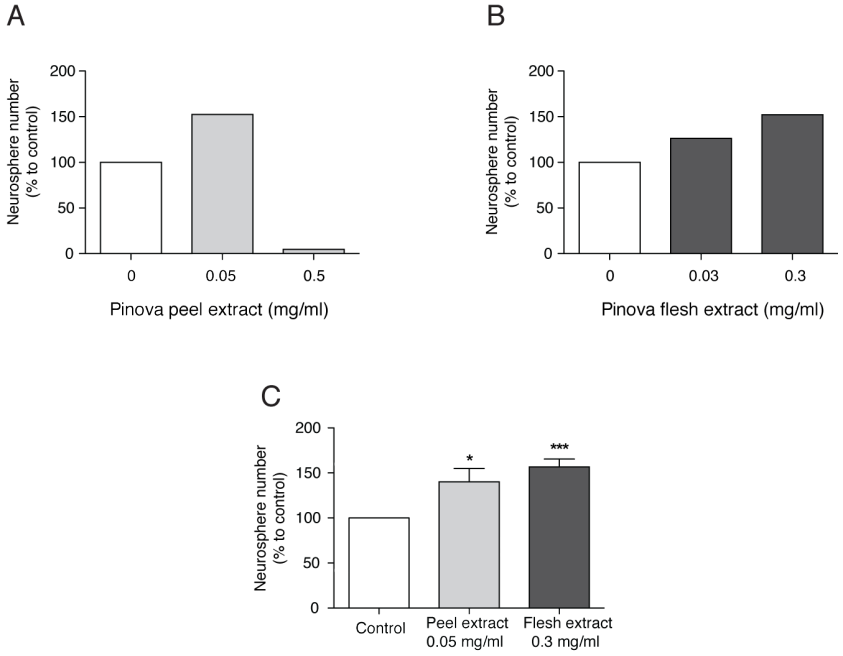

Figure 3.12. The effect of Pinova extracts on neurosphere assay. The replicates were performed on the concentrations with the highest effect in increasing neurosphere number (C). * $p < 0.05$, *** $p < 0.001$, n = 3, Dunnett's test. Error bars represent SEM.

The neurosphere assay shows that the peel extract increases the neurosphere number at the concentration 0.05 mg/ml while the flesh extract exerts the highest effect at 0.3 mg/ml (Figure 3.12-A & B). I did three replicates for 0.05 mg/ml peel extract and 0.3 mg/ml flesh extract. There was a significant increase of neurosphere number in the group treated with the peel extract (140.1 ± 14.73 % to control, n = 3, $p < 0.05$) and the flesh extract (156.8 ± 8.67 % to control, n = 3, $p < 0.001$. Figure 3.12-C).

In both the neurosphere assay and BrdU-labelling method in monolayer culture, I observed a similar pattern of the the peel extract effect, whereby the lower concentration increased $BrdU^+$ cells or neurosphere number while the higher concentration showed the opposite effect. In the group treated with the flesh extract, the neurosphere number increases dose dependently.

To determine which assay is more sensitive to observe the effect of apple extracs and the fractions , I calculated the discriminability index (d') of both assays. This index is derived from the equal–variance Gaussian signal-detection model that indicates the separation between the noise (control group) and signal (treated group) distribution (Wickens, 2002; Gale and Perkel, 2010). A larger d' indicates that the signal is more readily detected. The formula to calculate d' is as follows :

$$d' = \frac{\mu_S - \mu_N}{\sqrt{\frac{1}{2}(\sigma_S^2 + \sigma_N^2)}}$$

μ_S = mean of signal (treated group)

μ_N = mean of noise (control group)

σ_S = standard deviation of signal (treated group)

σ_N = standard deviation of noise (control group)

Table 3.6. The discriminability index (*d'*) of the monolayer culture and neurosphere assay in the detection of apple extracts' effect on neural precursor cells.

	Peel extract (0.05 mg/ml)		Flesh extract (0.3 mg/ml)	
	Monolayer	Neurosphere	Monolayer	Neurosphere
d'	1.003	1.925	3.82	4.63

By using this calculation I found that the neurosphere assay has higher discriminability index than the BrdU-labelling method (table 3.6). Therefore the neurosphere assay was selected to test the activity of the following fractions.

3.2.3. Apple juice supplementation does not affect adult hippocampal neurogenesis in mice

Apple juice is one of the most widely consumed fruit beverages, including in Germany. The high flavonoid and other phytonutrient content in apple juice has been reported to aleviate oxidative damage and cognitive decline in aged mice (Tchantchou *et al.*, 2005). To follow up the finding from the neurosphere assay and as a proof of principle for the notion that fruit consumption may improve cognitive performance, an *in vivo* experiment using 10-week old female C57BL/6 mice was designed in which the mice had free access to Pinova apple juice (AJ) *ad libitum* for 4 weeks. The Morris water maze task was performed at the end of the experiment to assess hippocampal-dependent learning and memory performance. A BrdU injection was given at the beginning of the experiment to label the newborn cells and then follow up the survival and fate determination (Figure 3.13-A). To exclude the effect of fruit sugar as a possible confounding factor, a group that received equicaloric sugar water (SW) was provided in addition to the control group with normal drinking water (W). The analysis of sugar content in apple juice sample detected sucrose (49.8 g/l), glucose (12.3 g/l) and fructose (70.2 g/l).

At the beginning of the experiment the weight of mice (mean ± SD) was 19.8 ± 1.08 g in the W group; 19.8 ± 1.08 g in the SW group; and 19.7 ± 1.23 g in the AJ group. At the end of experiment the weights of mice were increased to 21.6 ± 0.9 g in the W group; 21.73 ± 1.44 g in the SW group; and 20.87 ± 1.96 g in the AJ group. The data showed a significant increased in body weight of the mice at the end of experiment compared to the initial weight in all groups [$F(4,168) = 24.95$, $p < 0.001$]. However there was no significant difference in body weight between groups [$F(2,42) = 0.4$, $p = 0.67$. Figure 3.13-B].

The amount of food consumption was significantly different between groups [$F(2,6) = 29.7$, $p < 0.001$. Figure 3.13-C]. The daily food consumption (mean ± SD) of each mouse was 3.17 ± 0.48 g in (W) group; 2.04 ± 0.47 g in (SW) group; and 2.41 ± 0.53 g in (AJ) group.

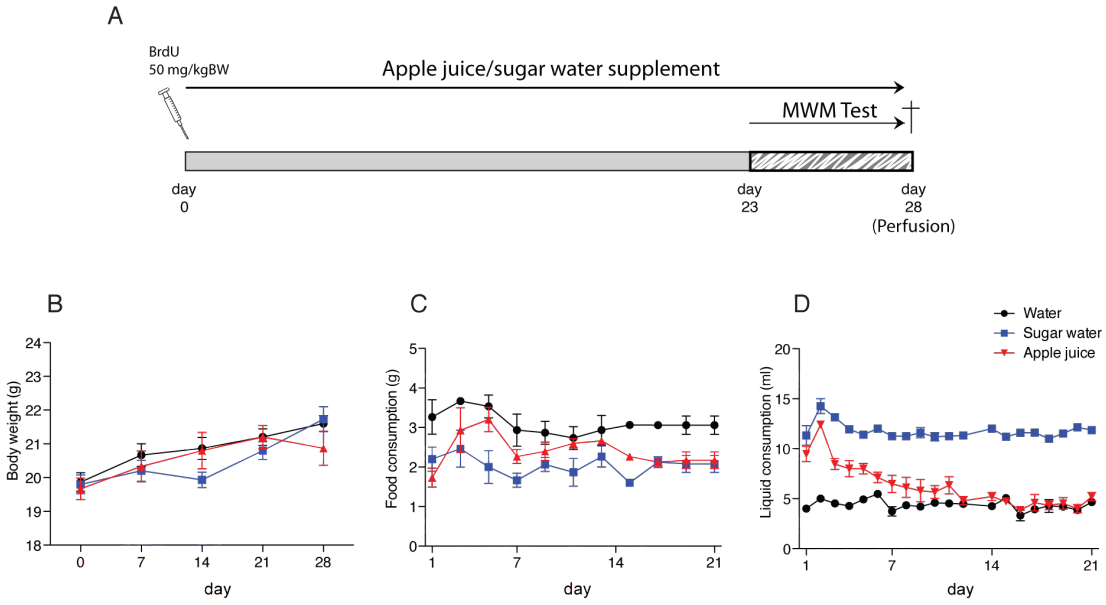

Figure 3.13. I*n vivo* experiment with apple juice. (A) Experiment scheme. (B) The change of body weight across the experiment. (C) The amount of food consumption across experiment. (D) The amount of liquid consumption across the experiment.

The pleasant taste of sugar water and apple juice is very attractive to the mice. It was expected that a significant difference in the amount of liquid consumption between groups would be observed. The amount of daily liquid consumption (mean ± SD) of each mouse was 11.75 ± 0.9 ml in the SW group; and 6.26 ± 2.3 ml in the AJ group, while the mice in the W group consumed 4.4 ± 0.65 ml [$F(2,6) = 211.12$, $p < 0.001$. Figure 3.13-D].

To observe the effect of apple juice supplement on hippocampus-dependent learning, the reversal learning protocol of the Morris water maze task as described in Garthe *et al.* (2009) was performed. In this protocol, the mice are trained with 6 trials per day for 5 days to find the hidden platform in the pool. The mice are provided with the visual cues outside the pool for the spatial navigation. On the beginning of day 4, the position of platform was reversed to the opposite quadrant, while the outside cues remain unchanged (figure 3.14-A). This platform reversal forces the mice to re-learn the new position of the platform by rearranging the encoding of previously obtained spatial cues to the new configuration in order to succesfully master the task. In that study (Garthe *et al.*, 2009), the absence of newborn hippocampal neurons resulted in a reduced ability to cope with this environmental change. Therefore this version of MWM task is a sensitive tool to test the presence and functional role of adult hippocampal newborn neurons.

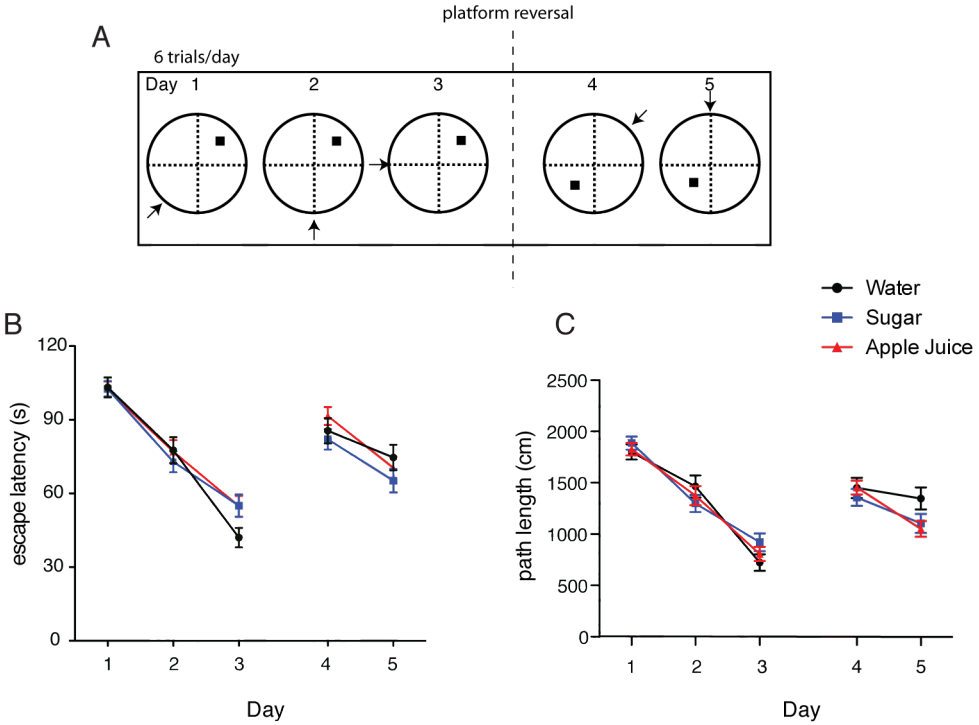

Figure 3.14. The performance in the Morris water maze task after apple juice supplementation. (A) Experimental scheme. The mice were trained for 5 days, 6 trials each day to find the submerged platform (black square). The position of the platform was reversed on day 4 and 5. The dropping position (arrow line) was changed every day. (B) The escape latency (in seconds) indicates the time needed to find the platform. (C) Path length (cm) is the swim distance to reach the platform

The escape latency time (s) and path length (cm) are among the parameters that are recorded to assess the performance in MWM. In the present study, I observed significant changes in latency time [$F(2,711) = 111.17$, $p < 0.001$, figure 3.14-B] and path length [$F(2,711) = 116.7$, $p < 0.001$, figure 3.14-C] over the first three aquisition days. This means that the mice in all groups were able to learn and solve the task. There was no significant difference observed between groups.

When the platform was moved to another quadrant on day 4, the mice took more time to find the new platform position. However the performance of all mice in all groups was significantly increased on the next day [latency time $F(1,454) = 18.73$, $p < 0.001$; path length $F(1,473) = 12.39$, $p < 0.001$], indicating that the mice were able to relearn and cope with the environmental change. No significant difference was observed between groups.

At the end of experiment, the mice were perfused and the brains were processed for immunostaining. The number of BrdU-positive cells in this

experiment indicates the number of surviving newborn cells in the dentate gyrus. We observed no significant difference in the number of BrdU-positive cells in the dentate gyrus after apple juice supplementation (figure 3.15).

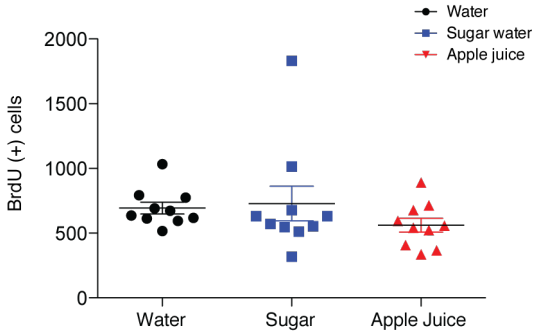

Figure 3.15. The number of BrdU-positive cells after apple juice supplementation. (p = 0.375, one way ANOVA, n = 10).

The low amount, instability and poor bioavailability of the active compounds in the apple juice are the rational explanations of why the effect of apple juice supplementation was not detected *in vivo*. The measurement of quercetin in the apple juice (done by GfL Gesellschaft für Lebensmittel-Forschung mbH in Berlin) detected a small amount of quercetin (< 2 mg/L). Based on this data I estimated the quercetin intake of each mouse in the AJ group was < 12.5 μg/day or equal to 0.6 mg/kg bw. This amount of intake is very low to reach the effective concentration in the brain. Furthermore, some active compounds are unstable and easily degraded or oxidized during the administration.

From this experiment, I conclude that the consumption of apple juice cannot provide a sufficient quantity of active phytochemicals to modulate the process of adult hippocampal neurogenesis.

3.2.4. Using bioassay-guided fractionation approach to search for other active compounds in apple extracts

The first *in vitro* study of apple extracts showed that Pinova flesh extract increased the number of BrdU-positive cells and the neurosphere number, more significantly than the Pinova peel (figure 3.11 & 3.12). This effect was the opposite of what I observed after quercetin treatment. Therefore I asked the question whether there are other active compounds beside quercetin in Pinova flesh extract that exert the effect, most likely in inducing cell proliferation. To answer this question, I used a bioassay-guided fractionation approach, whereby the Pinova

flesh extract was separated into fractions using liquid- and solid phase separation methods and then the activity was tested using the neurosphere assay (figure 3.16). I selected to use neurosphere assay due to its higher discriminability index than the BrdU-counting method in monolayer culture.

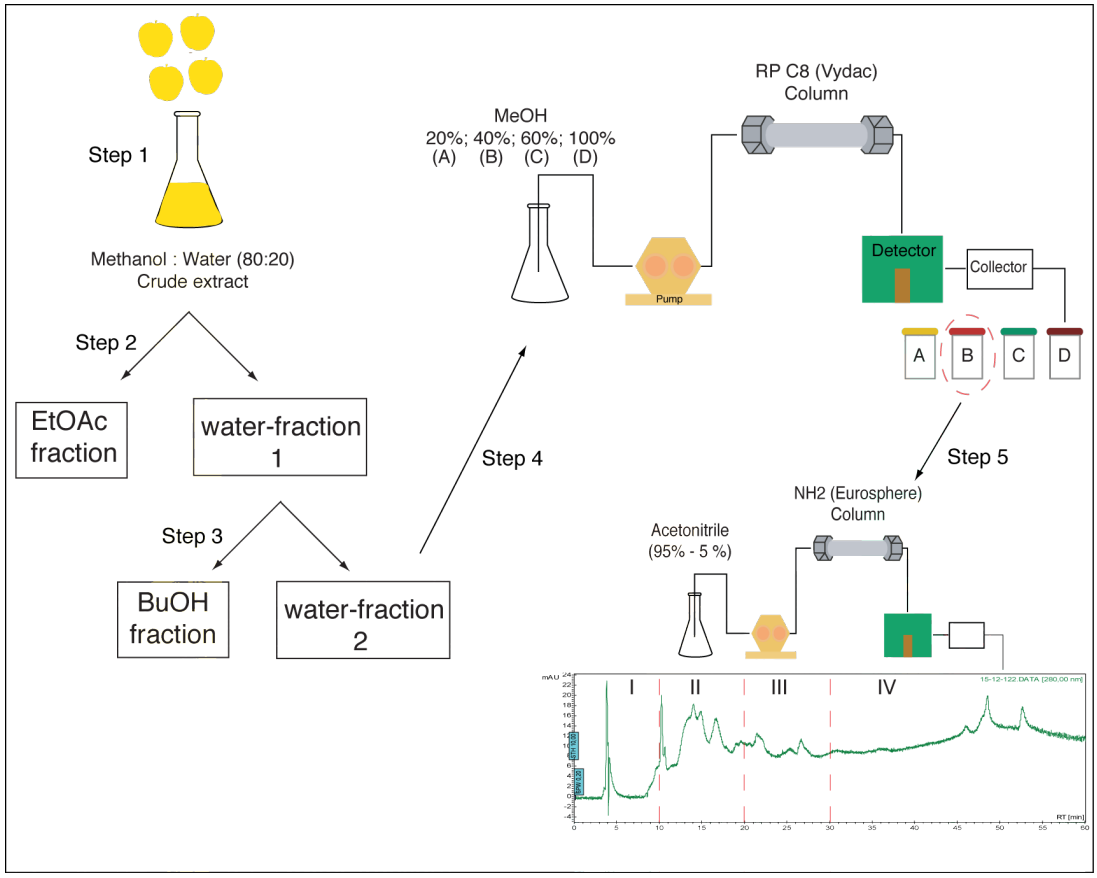

Figure 3.16. Extraction and fractionation scheme of apple extract. Apple flesh was extracted with methanol 80% in water (step 1). Nonpolar compounds are enriched in EtOAc (step 2). Less polar compounds are enriched in BuOH (step 3). The polar compounds in water fraction are further fractionated by column fractionation using C8 column in reverse phase HPLC system (step 4). The active fraction from step 4 (fraction B, eluted in MeOH 40%) is subsequently fractionated using NH2

The Pinova flesh extract was dissolved in an equal mixture of water and ethyl acetate (EtOAc) to separate the polar and nonpolar compounds. The water-fraction 1 was further fractionated by adding an equal volume of butanol (BuOH), generating water-fraction 2 and butanol fraction. Three fractions were obtained at this step, i.e.: water-, EtOAc- and BuOH fractions. To avoid confusion, the water-fraction 2 will be assigned as "water fraction" for the rest of this thesis. The solvent

was completely removed by centrifugal evaporator and then the dried fractions were dissolved in water (for water fraction) or DMSO (for EtOAc and BuOH fractions) prior to testing.

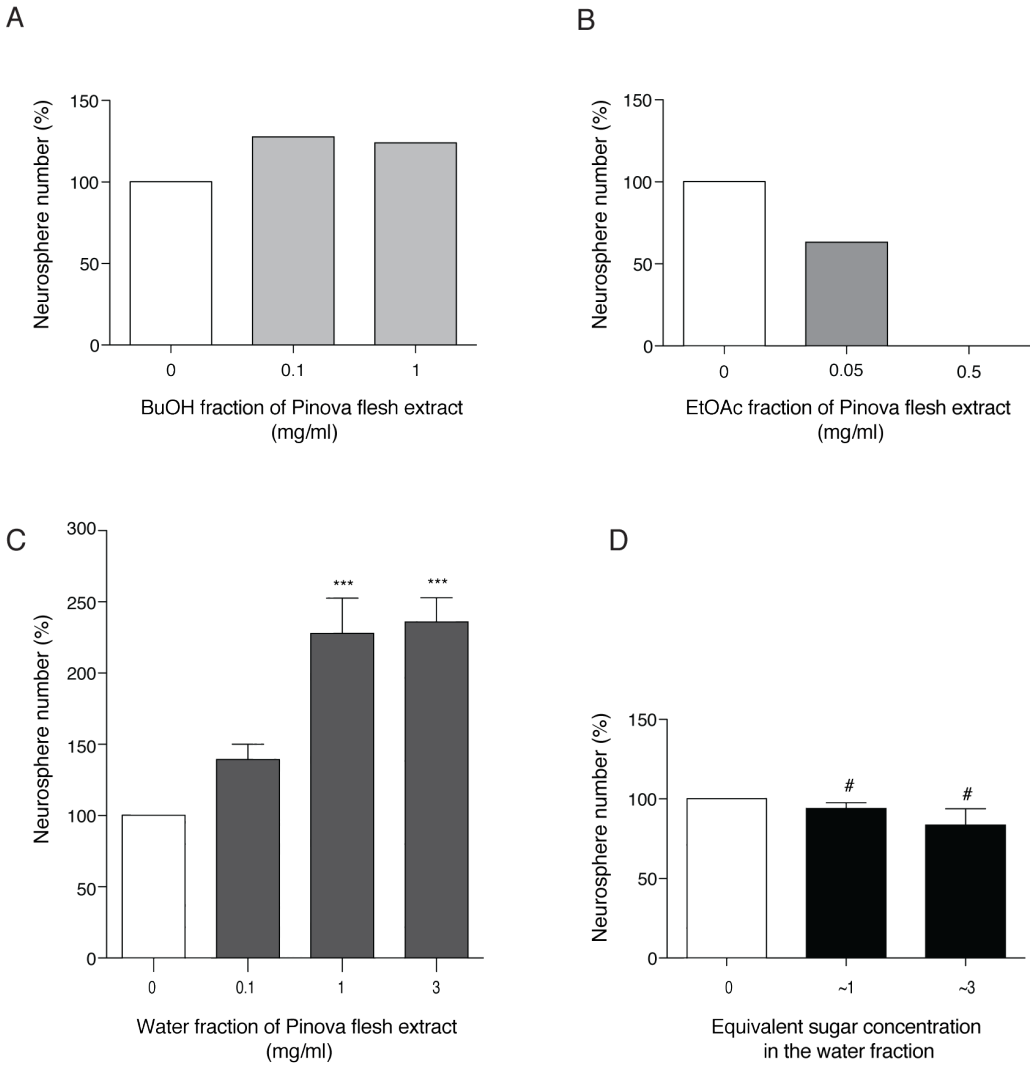

Figure 3.17. Neurosphere assay of Pinova flesh extract and fractions from solvent separation steps. (A) BuOH fraction. (B) EtOAc fraction. (C) Water fraction (D) Sugar (equivalent concentration to the water fraction). *$p < 0.05$; ***$p < 0.001$; #$p > 0.05$ by Dunnett's test. Error bars represent SEM.

Among these 3 fractions, I found that the water fraction showed a dose-dependent significant increase in neurosphere number (139.4 ± 10.8 %; 227.8 ± 24.86 %, n = 5, $p < 0.001$ and 235.8 ± 17.12 %, n = 5, $p < 0.001$ by Dunnett's test compared to control for the 0.1, 1 and 3 mg/ml concentrations, respectively. Figure 3.17-C).

The BuOH fraction increased the neurosphere number at 0.1 mg/ml (128 % of control, figure 3.17-A). Although this fraction was quite promising to be followed up, the yield was not enough to do sufficient replicates and for further separation steps. On the other hand, the EtOAc fraction showed a decrease of neurosphere number dose dependently. No sphere was found after treatment with 0.5 mg/ml EtOAc fraction (figure 3.17-B).

The water fraction of Pinova extract contained fruit sugar that can affect cell growth. To exclude this confounding factor, I tested sugar in the concentrations equivalent to the sugar content (sucrose, glucose and fructose) in the fraction. There was no significant effect of sugar on neurosphere number (figure 3.17-D).

To continue the fractionation step, I did solid phase separation using reverse phase C8 column in an HPLC machine. The water fraction were eluted and collected every 30 minutes using different polarity of solvent, starting with higher polarity solvent followed by the lower polarity solvent (20 %, 40 %, 60 % and 100 % of methanol (MeOH) in water, consecutively). In this system the hydrophilic (more polar) compounds will be eluted first, while the hydrophobic (less polar) compounds will be bound (adsorbed) to the column and eluted at a later time point. After solvent evaporation, dried fractions were dissolved in water and tested using the neurosphere assay.

The fraction from 40% MeOH eluent increased neurosphere number dose dependently compared to control (85.09 ± 7.3 %; 147.2 ± 5.88 %; and 184.4 ± 4.18 % for 0.01, 0.1, and 1 mg/ml, respectively; n = 4, $p < 0.001$ by Dunnett's test; figure 3.18-A). However 10 mg/ml concentration decreased neurosphere number drastically (1.3% of control). Fractions eluted in 20% and 60% MeOH did not show any increase in neurosphere number (figure 3.18-B) and no fraction was obtained during elution with 100 % methanol.

The promising fraction from 40% methanol-water eluent was further fractionated using an NH_2 column, which has properties as a "normal phase" column; meaning that the hydrophilic compounds will be adsorbed by the column and the less hydrophilic ones will be eluted first. The medium polarity solvent acetonitril mixed with H_20/acetic acid 1% was used as eluent. The gradient elution from 80% to 5% acetonitril was applied for 60 minutes and the fraction was collected in different time frame (figure 3.19-A). The solvent was evaporated using a Genevac centrifugal evaporator until maximum dryness and then dissolved in water for testing.

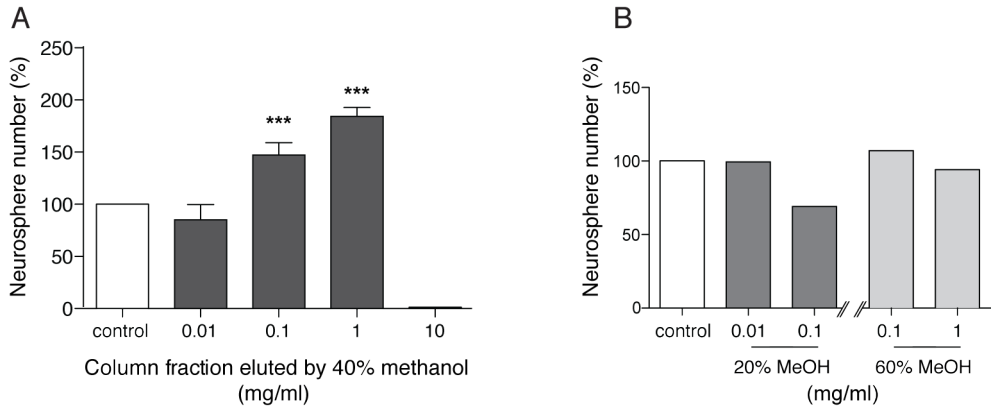

Figure 3.18. Neurosphere assay of the water-fraction after RP C8 column separation. (A) fraction eluted in 40% methanol. (B) fractions eluted in 20% and 60% methanol. (***$p < 0.001$. Error bars represent SEM).

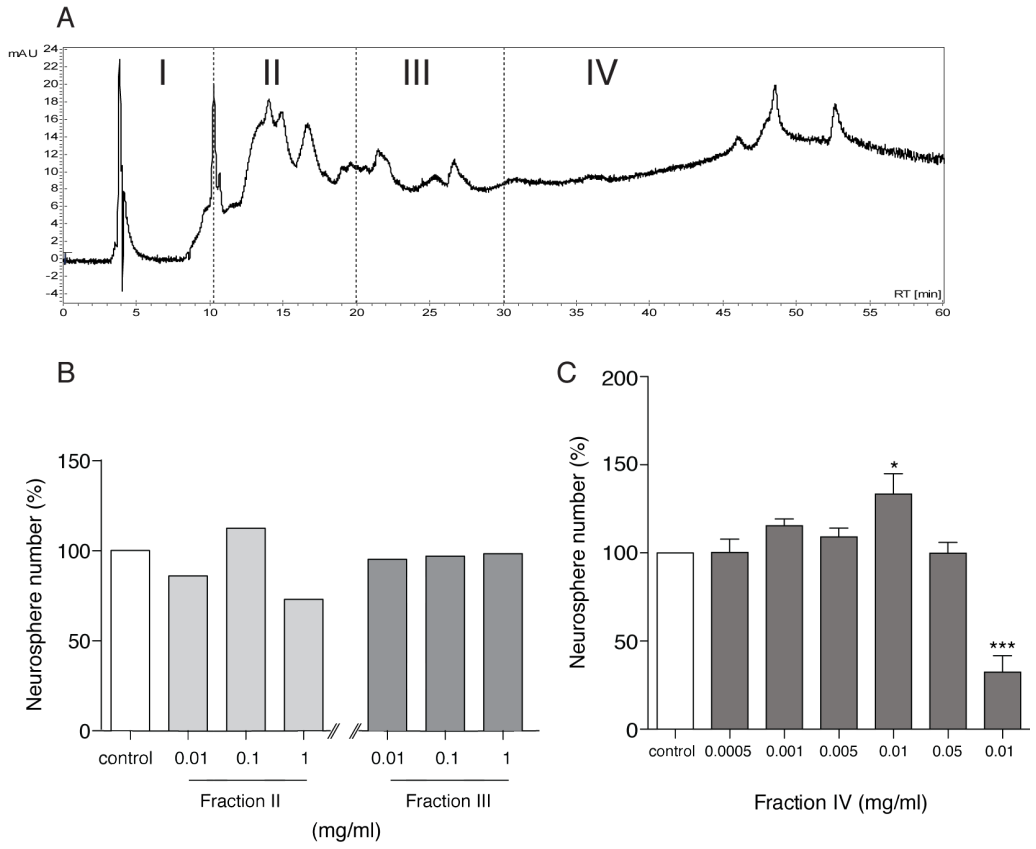

Figure 3.19. Neurosphere assay of the 40% MeOH fraction separated in NH2-column. (A) HPLC spectra of the fractions and the time frame for fractions collection. (B) Neurosphere assay of fraction collected from time frame II and III. (C) Neurosphere assay of fraction collected in time frame IV. *$p < 0.05$, ***$p < 0.001$. Error bars represent SEM.

The neurosphere assay revealed that fraction IV increased the neurosphere number dose dependently. The maximum effect was observed at 0.01 mg/ml concentration (133.5 ± 11.46 % of control, n = 6, $p < 0.05$ by Dunnett's test. Figure 3.19-C). The higher concentration (0.01 mg/ml) significantly decreased the neurosphere number (32.43 ± 9.32 % of control, n = 4, $p < 0.001$ by Dunnett's test). The fraction III showed no increase in neurosphere number while a slight increase was observed in 0.1 mg/ml of fraction II (figure 3.19-B). Fraction I could not be tested as only minuscule amount of dry weight was obtained.

3.2.5. Mass spectrometry analysis revealed dihydroxybenzoic acids as the active compounds in Pinova apple flesh

Due to the limited amount of sample, the fraction IV could not be fractionated further. In collaboration with Dr. Christoph Böttcher at the Leibniz-Institut für Pflanzenbiochemie in Halle, we performed ultra-performance liquid chromatography coupled with photodiode-array detector and electrospray ionization quadrupole time-of-flight mass spectrometry (UPLC/PDA/ESI-qTOF-MS) analysis in positive- and negative ion mode to identify and elucidate the compounds in fraction IV.

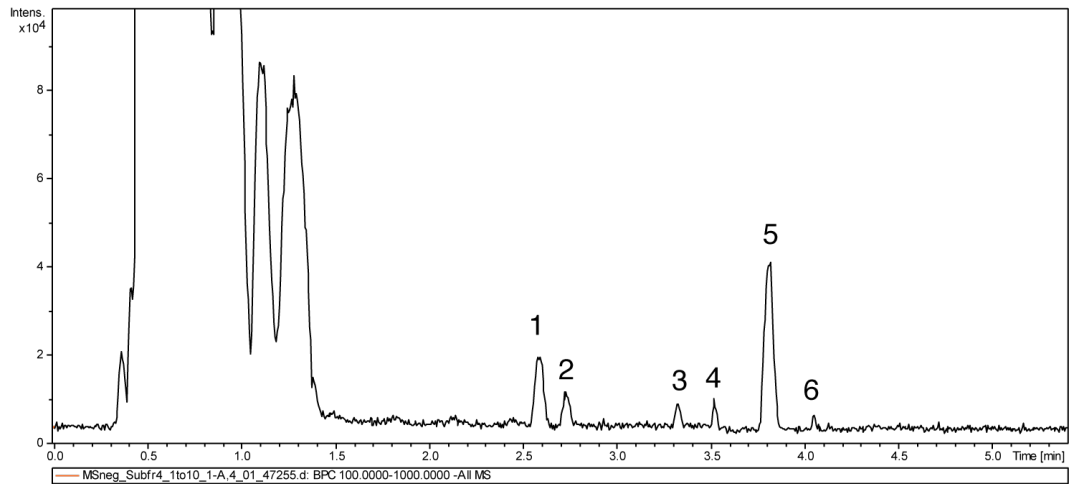

Figure 3.20. Base peak chromatogram m/z 100-1000, gradient elution, ESI(-) of fraction IV. Six peaks were annotated as described in table 2.7.

The compounds eluted in the first 1.5 minutes (shown in figure 3.20) are very difficult to be further separated and therefore could not be identified. The subsequent peaks (peak number 1 - 6) were subjected to TOF-MS and collision

induced dissociation (CID). Interestingly, 2 of the peaks were identified as the glycosides of dihydroxybenzoate (table 3.7). The spectra of these compounds are shown in figure 3.21 A-B. Dihydroxybenzoate is the conjugate base of dihydroxybenzoic acid, which belongs to the non-flavonoid phenolic compounds (reviewed by Liu, 2004).

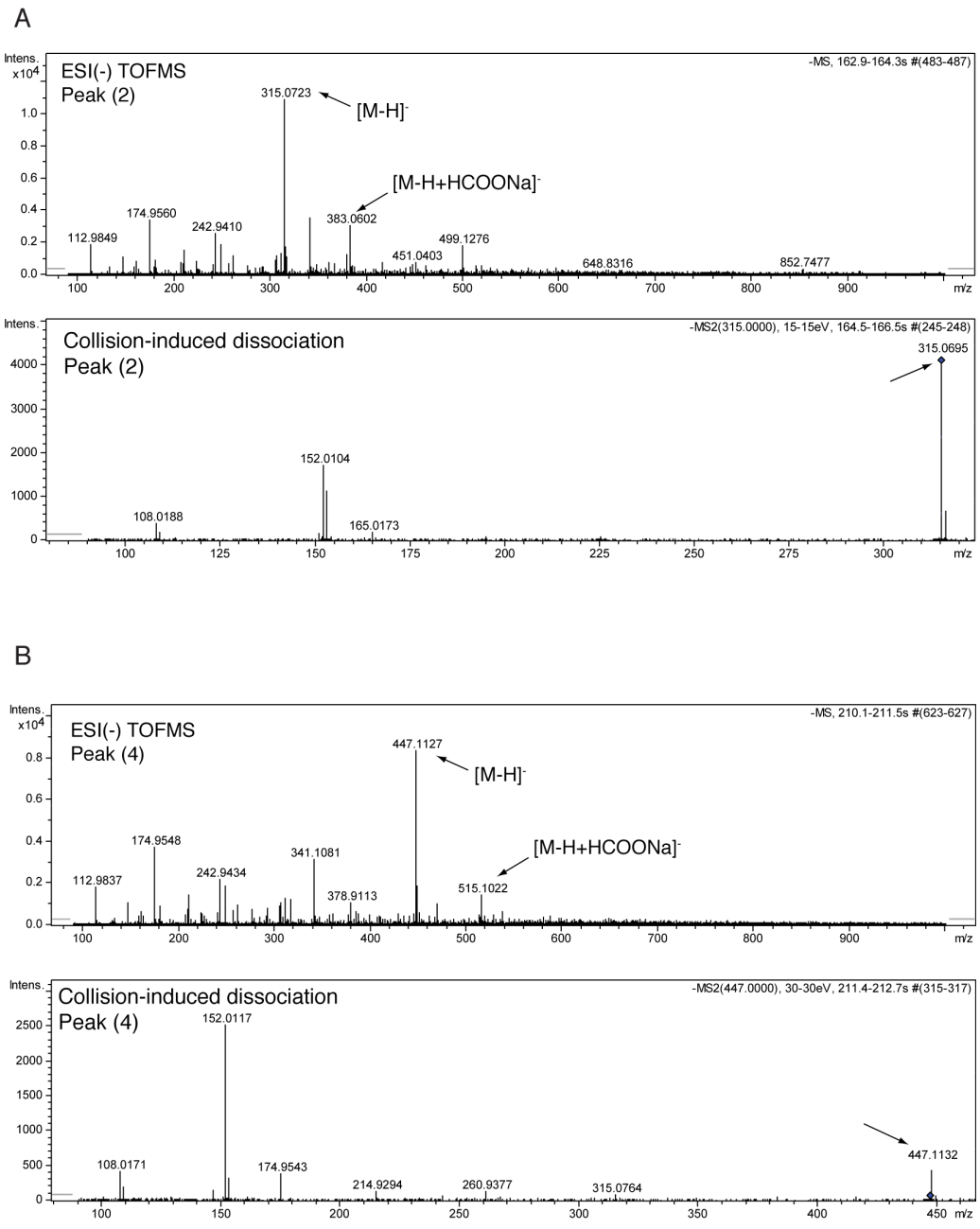

Figure 3.21. UPLC/PDA/ESI-QTOFMS analysis of fraction IV. (A) Mass spectra of peak 2 (B) Mass spectra of peak 4 in ESI (-) TOFMS and collision induced dissociation.

Table 3.7 Peak annotation of UPLC/PDA/ESI-QTOFMS analysis of fraction IV based on accurate mass measurement and high-resolution CID mass spectra.

Peak no	*m/z*, ESI(+)	*m/z*, ESI(-)	elemental composition	comments
1	347.0942, $[M+Na]^+$	323.0979, $[M-H]^-$	$C_{12}H_{20}O_{10}$	Hexoside
2	339.0668, $[M+Na]^+$	315.0713, $[M-H]^-$	$C_{13}H_{16}O_9$	Dihydroxybenzoate *O*-Hexoside
3	361.1099, $[M+Na]^+$	337.1133, $[M-H]^-$	$C_{13}H_{22}O_{10}$	Hexoside, Homolog of **1**
4	471.1082, $[M+Na]^+$	447.1132, $[M-H]^-$	$C_{18}H_{24}O_{13}$	Dihydroxybenzoate *O*-(Hexoside-Pentoside)
5	361.1091, $[M+Na]^+$	337.1140, $[M-H]^-$	$C_{13}H_{22}O_{10}$	Hexoside, Isomer of **3**
6	660.1686, $[M+H]^+$	658.1546, $[M-H]^-$	???	???

CID = collision-induced dissociation

3.2.6. Isomers of dihydroxybenzoic acids increase neurosphere number and cell proliferation in monolayer cultures of adult hippocampal precursor cells

The discovery of dihydroxybenzoate derivatives in the active fraction is very intriguing. However, dihydroxybenzoate glycosides are not available as isolated pure compounds. Therefore, to confirm the activity of the identified compound, I purchased several commercially available isomers of dihydroxybenzoic acids (DHBA) to be tested in the neurosphere assay.

A significant increase in neurosphere number was observed in 2,3-DHBA (115.9 ± 4.69 % of control in 1 μM concentration, n = 5, $p < 0.05$ by one sample *t*-test) and 3,5-DHBA (161.12 ± 14.97 % of control in 10 μM, n = 5, $p < 0.001$ by one sample *t*-test), as shown in figure 3.22-A&C. Another isomer (3,4-DHBA) showed a trend to increase neurosphere number (figure 3.22-B). Nevertheless, higher concentration of these compounds decreases the neurosphere number.

In addition to the neurosphere assay, I tested this compound on monolayer culture by using an impedance-based assay namely xCELLigence system. This system is equipped with microelectrode at the bottom of the well plates that can continuously detect the impedance changes caused by the presence of the attached cells, indicated as a Cell Index (CI). The more surface area are occupied by the cells e.g due to increased cell number, the higher the Cell Index. This system can be used to monitor the cell dynamics from the begining until the end of the culture period in real time. Therefore by comparing the Cell Index at the end and at the beginning of experiment, the estimation of the increase in cell number is more accurate than just labelling the S phase at the end of experiment such as in the classic BrdU-

labelling method. Moreover, the results are quickly obtained without laborious immunostaining and counting steps.

The result of cell impedance measurement showed that the normalized Cell Index (CI at the end of experiment divided to CI at the compound administration) was significantly increased at 100 and 250 μM 3,5-DHBA in a dose dependent manner. The increase of cell impedance can be interpreted as the increase cell number.

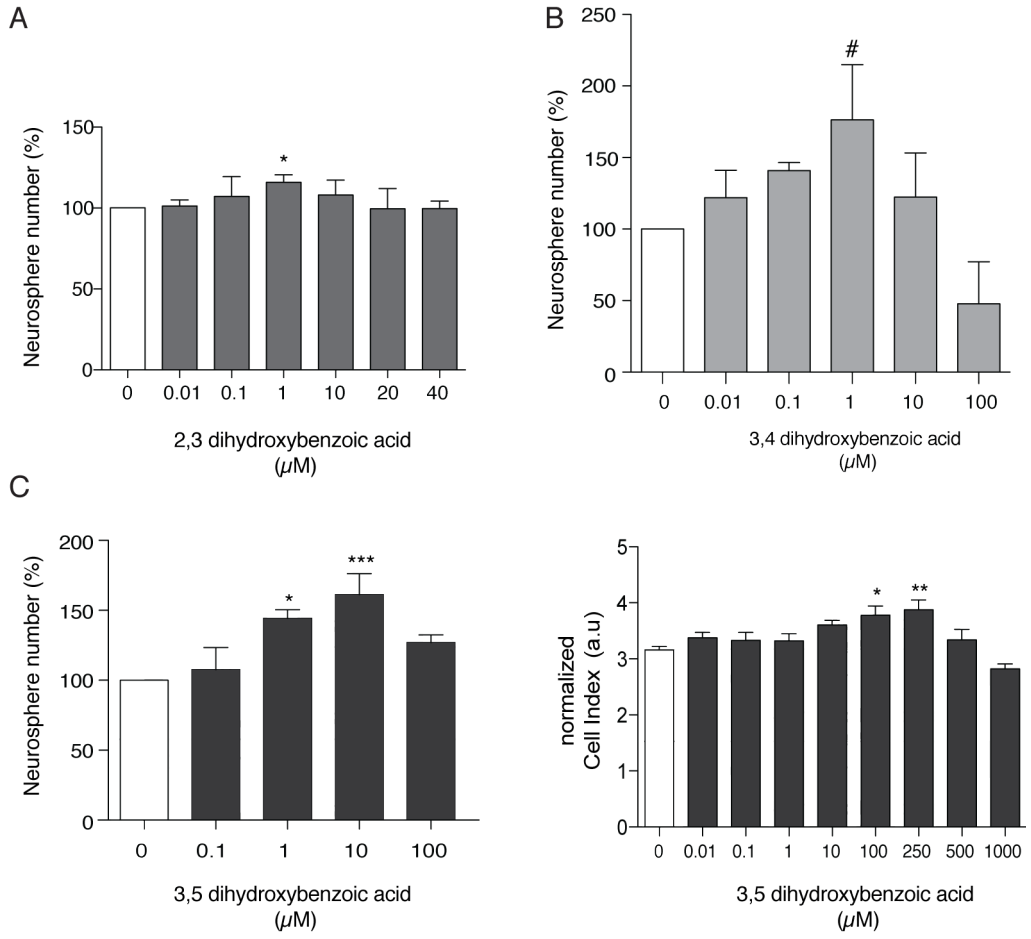

Figure 3.22. Neurosphere assay of dihydroxybenzoic acid isomers (A) 2,3-dihydroxybenzoic acid; (B) 3,4-dihydroxybenzoic acid and (C) 3,5-dihydroxybenzoic acid. (D) Normalized Cell Index in monolayer culture of adult hippocampal precursor cells after 3.5 DHBA treatment (n=8).
*$p < 0.05$,**$p < 0.01$, ***$p < 0.001$, # p = 0.1861 by Dunnett's post hoc test. Error bars represent SEM.

Compared with the neurosphere assay, a higher concentration of 3,5-DHBA is required to elicit the effect on monolayer cultures. This could be explained because

the cells in monolayer cultures have been exposed for many passages with supraphysiologic concentration of growth factors, resulting in the high basal proliferation rates and changes in cell physiology (Hebert *et al.*, 2009). Meanwhile, the cells in neurosphere assay are from primary cells that resemble more the *in vivo* condition and consist of a more heterogenous cell population that shows different responses and sensitivity to the treatment.

3.3. The hydroxycarboxylic acid receptor-1 (HCAR-1) is detected in adult hippocampal precursor cells *in vitro* and *in vivo*

HCAR-1 was initially thought of as orphan receptor until Liu *et al.* (2009) and Liu *et al.* (2012) demonstrated that lactate and 3,5-DHBA activate this receptor at adipocytes to inhibit lipolysis. A year later, Lauritzen *et.al.* reported the existence of this receptor in the brain, particularly in the hippocampus and cerebellum. Moreover, Castillo *et al.* (2015) specifically showed the existence of HCAR-1 in dentate gyrus.

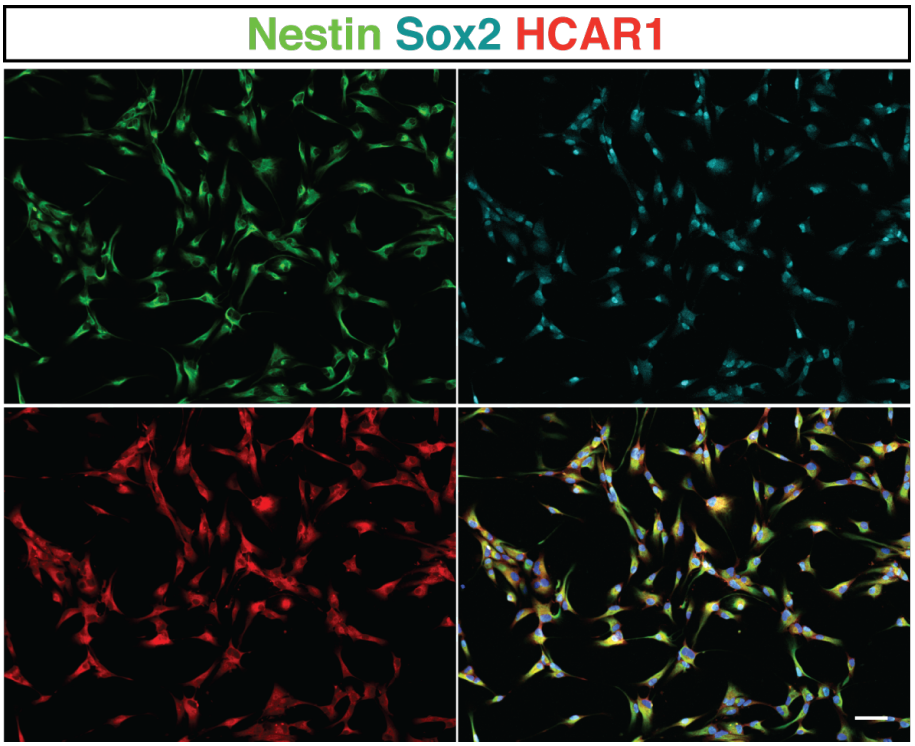

Figure 3.23. Immunofluorescence staining of proliferating adult hippocampal precursor cells in monolayer culture. HCAR1 (red) ,Nestin (green) and Sox2 (cyan). Scale bar: 50 μm.

In this study I demonstrated that this receptor is also present in adult hippocampal precursor cells. Immunofluorescence staining of proliferating hippocampal precursor cells in monolayer cultures showed membrane specific localization of the HCAR-1 in Nestin^{+}/Sox2^{+} cells (figure 3.23).

Immunofluorescence staining of brain sections showed HCAR1 present in the granular cells of the dentate gyrus, in line with the result as reported previously by Castillo *et al.* (2015). Interestingly, HCAR1 is also colocalized with Sox2^{+} and Nestin^{+} cells in the subgranular zone (figure 3.24). Considering the morphology of Nestin^{+} cells, this indicates that HCAR1 is present in type 1 and type 2 cells.

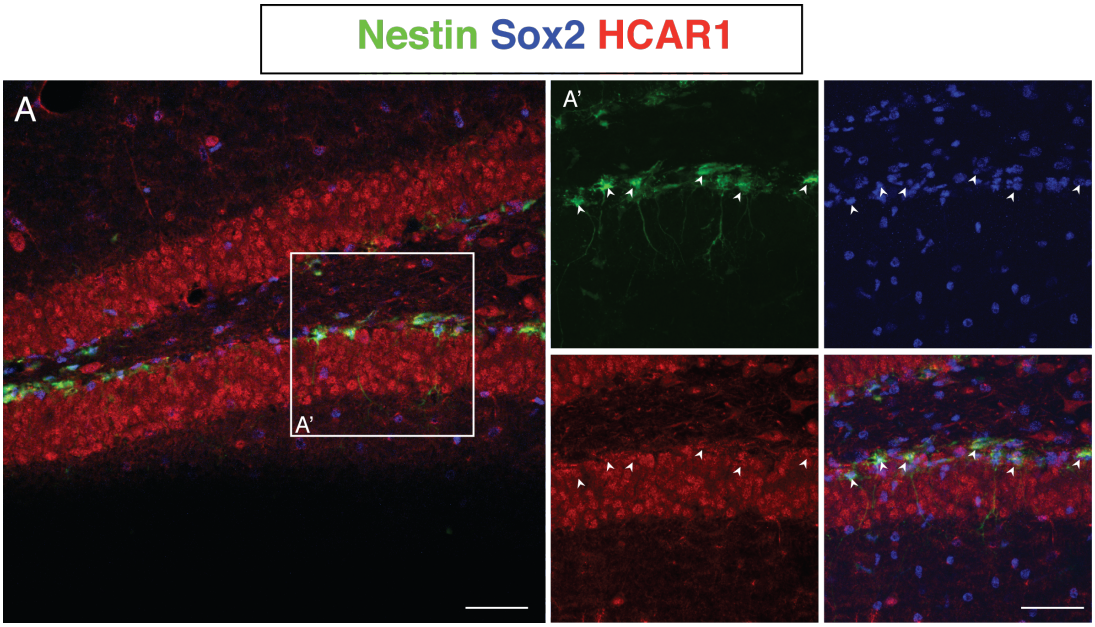

Figure 3.24. The expression of HCAR1 in adult hippocampal precursor cells. Confocal images of brain sections from Nestin-GFP mice with 20x objective magnification (A) and 40x objective magnification (A'). HCAR1 (red), Nestin (green) and SOX2 (dark blue). Arrowheads show the cells with HCAR1, Nestin and Sox2 colocalization. Scale bar: 50 μm.

4. Discussion

4.1. Quercetin effects on kinases are underlying its effect on precursor cell proliferation, survival and apoptosis

Quercetin is one of the most abundant flavonoids in our foodstuffs, such as in capers, red onion, apples, berries, broccoli and tea, whereby it mostly presents as glycoside forms. It is also the most extensively studied flavonoid (Bischoff, 2008). Due to the presence of two antioxidant pharmacophores within its molecule, i.e. the catechol group in the B-ring and OH group, quercetin has optimal activity in scavenging free radicals *in vitro*. Therefore it is the most potent antioxidant within the flavonoid family (Hanasaki *et al.*, 1994; Heijnen *et al.*, 2002). However, this antioxidant capacity is not the only explanation of the quercetin effect. Many studies have shown that quercetin exerts its effect through inhibitory or stimulatory actions at different components of protein and lipid kinase signaling cascades, resulting in alteration of phosphorylation sites of these molecules and/or modulation of gene expression that involved in cell survival (reviewed by Williams *et al.* (2004)).

A growing number of studies have shown the effect of quercetin in rescuing adult hippocampal neurogenesis in pathological conditions such as Alzheimer's disease, stress, aging, depression and anxiety (Tchantchou *et al.*, 2009; Tongjaroenbuangam *et al.*, 2011; Dias *et al.*, 2012). It is also not very clear whether quercetin directly modulate the molecular mechanism in the precursor cells or acts simply by neutralizing the detrimental levels of oxidative stress that are produced during the course of these pathologic conditions. Therefore in this study I investigated the effect of quercetin on adult hippocampal precursor cells in or derived from healthy, young adult mice.

Using BrdU labeling and cell cycle analysis of the precursor cells monolayer culture, I demonstrated that quercetin in 25 μM concentration arrests the cell cycle in the G0/G1 phase. By staining the cells with markers of precursor cells, neurons and astrocytes, I provided the evidence that these cells exit the cell cycle and further commit to differentiate into neurons or astrocytes even in the presence of growth factors. As the molecular explanation of this effect, I showed the downregulation of cyclin-dependent kinase 4 (Cdk4) in the quercetin-treated cells. In parallel, I also provided evidence that the cyclin-dependent kinase inhibitor

$p27^{kip1}$ is upregulated. Activity of the Cdk family of kinases is important for cell cycle progression from G0/G1 to S phase (Harbour and Dean, 2000). Moreover, Cdks are required for the phosphorylation of Neurogenin 2 (Ngn2), in order to prevent the expression of proneurogenic genes expression in precursor cells and maintain cell stemness (Ali *et al.*, 2011). In contrast, beside its role as a negative regulator of cell cycle progression through inhibition of cyclin/cdk complexes, $p27^{kip1}$ stabilizes the differentiation factor Ngn2 and therefore promotes gene expression for neuronal differentiation in neural precursor cells (Sherr and Roberts, 1999; Nguyen *et al.*, 2006).

It has been shown that the antiproliferative effect of quercetin is mediated through the inhibition of epidermal growth factor receptor (EGFR) phosphorylation, resulting in the downregulation of cyclins and CDKs complexes (Huang *et al.*, 1999). In present work, I demonstrated that the protein expression of Cdk4 is downregulated while the cyclin D1 is unchanged. It has been reported that the cyclin D1, can exert the effect in promoting cell survival, independent of the Cdk4, through DNA repair mechanism together with DNA repair proteins, such as RAD51 (Jirawatnotai *et al.*, 2011). This can be one explanation why quercetin at this concentration inhibits precursor cell proliferation without inducing cell death.

Upon growth factor withdrawal I observed the differentiation of precursor cells simultaneously with the evidence of massive cell death. I demonstrated for the first time that quercetin (25 μM) increases the number of surviving cells during precursor cell differentiation. I also verified that the activation of the Akt pathway is one of the underlying mechanisms of the prosurvival effect of quercetin. Quercetin (25 μM) significantly increases the protein level of phosphorylated Akt, together with the upregulation of the downstream antiapoptotic protein Bcl2l1. The significant downregulation of cleaved caspase-3 after 48 hour quercetin treatment is confirming the prosurvival effect of quercetin.

It has been reported in another study that quercetin inhibits Akt phosphorylation to induce apoptosis in cancer cells. However it was shown that this effect occurs at a higher concentration of quercetin (> 50 μM) while the lower concentration showed the activation of Akt (Granado-Serrano *et al.*, 2006).

4.2. Quercetin increases cell survival during differentiation through induction of the adaptive response of neural precursor cells

Reactive oxygen species (ROS) level is increased during precursor cells differentiation, in paralel with the decrease of endogenous antioxidant capacity (Bigarella *et al.*, 2014). Moreover, upon growth factor withdrawal the intracellular superoxide anion (O_2^-) and caspase activity is increased and ultimately causes cell death. Substances like ROS scavengers or antioxidants and caspase inhibitors could be beneficial in reducing cell death (Lieberthal *et al.*, 1998).

As mentioned earlier, quercetin has the capability to scavenge the radical oxygen species and has been shown as the most effective inhibitor of superoxide anion (O_2^-) formation (Robak and Gryglewski, 1988). Uniquely, in the reaction to stabilize O_2^-, quercetin is oxidized and transiently forms the pro-oxidant metabolite o-semiquinone and hydroxy radicals. The generation of pro-oxidant compounds is dependent on the given concentration of quercetin, whereby 100 μM has been shown to significantly enhanced the production of radical compounds and cell death (Laughton *et al.*, 1989; Metodiewa *et al.*, 1999).

Using RNA microarray analysis I demonstrated the differential expression of genes that are involved in cell survival after 24 hour quercetin treatment. Interestingly, I also found the upregulation of genes in MAPK pathway involved in inducing apoptosis such as JNKs, PUMA, Bax and Bid. At the same time, the genes in the Nrf2-Keap1 pathway were also significantly upregulated. During stress conditions, Nrf2 is activated, accumulated in the nucleus and dimerizes with Maf to increase the binding affinity with the antioxidant response element (ARE), resulting in the transcription of phase II enzymes such as Gst, Nqo1, Gcl and Hmox1 in order to increase cell capacity in quenching the toxin or oxidative stressors (Rushmore *et al.*, 1991; Venugopal and Jaiswal, 1996; Itoh *et al.*, 1997; Nguyen *et al.*, 2009; Kansanen *et al.*, 2013). Interestingly, the upregulation of Gsta3 (one member of the Gst family) at the protein level was still observed after 48 hours treatment, indicating that the cells are initially triggered in the stressful condition and then activate the adaptation mechanism by increasing the endogenous antioxidant capacity. This process provides a capability of the cells to survive in a higher level of stress, such as growth factor withdrawal.

This adaptive phenomenon is also known as *hormesis.* It is defined as the response of cells or organisms to the low dose of environmental insults such as

toxins, caloric restriction, exercise, hypoxia and other stressors, resulting in the increase of resistance to the more severe level of stress (Calabrese *et al.*, 2007). The molecular mechanisms in the hormetic response include proteins such as ion channels, kinases, deacetylases and transcription factors that regulate the expression of genes for cell protection and survival (reviewed by Mattson, 2008a).

In contrast to the in vitro findings, the published data on the neuroprotective effect of quercetin *in vivo* are still elusive. The extensive metabolism and the limitation of quercetin to cross the blood brain barrier were suggested as the factors underlying these inconsistent findings. Most of the reported neuroprotective effect was found in the ischemic brain or aging, where the blood brain barrier is compromised and therefore increases the access of quercetin into the brain tissue (reviewed by Ossola *et al.*, 2009). However, Ishisaka (2011) detected the accumulated concentration of quercetin and its methylated metabolites that reached the plateau after 1 week oral administration of 50 mg/kg bw quercetin in healthy adult rats. Assuming 1 gram of brain tissue lysate is equal to 1 ml, the concentration of quercetin and methylquercetins was 40 nM and 48 nM respectively. Furthermore they also demonstrated the attenuation of oxidative stress in the hippocampus and striatum after chronic forced swimming with this concentration of quercetin. In the present study, I demonstrated that the same concentration of quercetin (50 mg/kg bw) increased the number of surviving newborn neurons in the adult dentate gyrus. Further study is needed to confirm the molecular pathways that are involved in causing this effect and to test the effect on the behavior such as spatial learning and memory.

4.3. Apple juice consumption on adult hippocampal neurogenesis : pharmacologic dose matters?

To the best of my knowledge, this study is the first to show the effect of apple juice consumption on hippocampal neural precursor cells in healthy young adult mice. Other studies have shown the positive effect of apple juice supplementation in alleviating oxidative damage and cognitive decline in aged mice, folate- and vitamin E deficient diet, high iron diet or transgenic murine model with compromised endogenous antioxidant potential (Tchantchou *et al.*, 2004; Tchantchou *et al.*, 2005; Chan *et al.*, 2006).

In the present study, I did not find any difference in the number of newborn neurons or performance in MWM between the apple juice-treated group and

control. The low amount of flavonoids in the apple juice sample is suggested as one reason for the absence of the expected effect. Using quercetin as the reference active compound in apple, the pharmacologic dose of apple juice was not reached in this study. On average, each mouse only took 1 % of the effective quercetin concentration.

The amount of flavonoids and other polyphenols in apple juice depends on the processing method. The flavonoids are stored in the intracellular vacuoles, therefore the liquefaction method by using intensive enzyme treatment to solubilize the cell wall results in the higher release of these substances in the juice than with the pressing method (Schols *et al.*, 1991). Moreover, the cultivar, ripening stage, storage condition and geographic factors influence the composition and amount of phytochemicals in the apples (Guo *et al.*, 2013). To date at least 7500 apple cultivars, that are suited for fresh consumption, or processed (for cooking or cider), or for both purposes (Elzebroek, 2008) are recorded. Using a sodium borohydride/chloranil-based assay I showed that the Pinova apple cultivar used in this study has a relatively high total flavonoid content. Nevertheless, the tested Pinova apple extract was processed by sonication followed by solvent (methanol-water mixture) extraction, therefore the cell wall was massively disrupted and the maximal amount of flavonoids and other phenolic compounds could be obtained.

In addition to the low content of polyphenols in the apple juice sample, the BrdU injection paradigm and the duration of apple juice supplementation are also suggested as a limitation in this study. Considering the low amount of active compounds in the apple juice sample, the longer duration of supplementation could increase the accumulation of active compounds in the body. Furthermore, using multiple BrdU injections allows the labelling of more proliferating cells at the beginning of the experiment and therefore increases the chance to observe subtle changes in the number of surviving cells at the end of the experiment.

4.4. Dihydroxybenzoic acids as active compounds in apple

As noted earlier, apples are the source of phytochemicals, not only quercetin, that provide a library of potential chemical compounds for medical uses. Extractions followed by testing the activity in bioassays are the classical approach in drug discovery from natural products. Further isolation steps are the separation of chemical mixtures based on their physico-chemical properties, such as polarity and adsorption. Ultimately, the active compounds and the chemical structures can

be identified and elucidated using spectrometry methods such as nuclear magnetic resonance (NMR) and mass spectrometry (MS). This approach is unbiased because there is no preconception about the chemical structures and properties of the active compounds that are being sought (Houghton and Raman, 1998). I demonstrated for the first time the use of the primary neurosphere assay from adult mouse dentate gyrus precursor cells as an effective method to find the active compound in apple flesh extract.

The neurosphere assay has been widely used in many studies to observe the activity of stem cells after treatments and also characterization of factors that influence stem cell properties and potentialities (Reynolds and Rietze, 2005). During the initial days of culture, the death of the majority of the cell population was observed while only a small population (<0.1%) of EGF-and bFGF-responsive cells survived and going to proliferate (Reynolds and Weiss, 1992). Additionally, this assay has been used to demonstrate the activity of KCl in the activation of the latent progenitor cell population (Walker *et al.*, 2008). Therefore, the number of neurospheres, as the read-out of the assay in this present study, can be interpreted as the effect of the tested fractions or compounds on cell proliferation, survival, or precursor cell activation.

Initially, I show the effect of apple peel and apple flesh crude methanolic extracts in increasing the neurosphere number. Higher concentration of apple peel extract showed the decrease of neurosphere number. However, the effect of lower concentration of apple peel is almost equivalent to the higher concentration of apple flesh extract. This is due to the higher concentration of antiproliferative compounds such as triterpenoids and quercetin in the peel than in the flesh (Wolfe *et al.*, 2003; He and Liu, 2007). For further fractionation steps, the apple flesh extract was selected.

The separation process was continued through subsequent liquid phase extraction with ethyl acetate and butanol, consecutively. In these steps, the organic solvent and water form 2 separate layers and the compounds in the extract will be fractionated based on their polarity and affinity to the solvents. In a previous study, ethyl acetate fraction of apple extract was reported to contain triterpenoids, flavonoids (quercetin glycosides and aglycone), plant sterols and organic acids while in the butanol fraction more polar flavonoids (catechin and epigallocatechin) together with organic acids (caffeic acid, ferulic acid, chlorogenic acid and quinic acid) were identified (He and Liu, 2008). The ethyl acetate and butanol fractions are

less soluble in water, therefore the fractions are firstly dissolved in DMSO to ensure the solubility of the fractions in the growth media. The remaining water fraction from the liquid phase extraction steps contains the more polar compounds such as sugar. Besides being the main source of energy, high glucose concentration can induce cell proliferation (Wolf *et al.*, 1992). I demonstrated in this study that the increased neurosphere number is not caused by sugar, suggesting the existence of other active compounds in the water fraction.

To continue the separation steps, I performed solid phase separation by using HPLC column. The active fraction from this steps was subjected to mass spectrometry analysis and detected as dihydroxybenzoic acid (DHBA) derivatives. To confirm this finding, I tested several commercially available isomers. Interestingly, I found 2 isomers that significantly increased neurosphere number, i.e. 2,3 DHBA and 3,5 DHBA. Although these are not novel compounds, their effect on adult hippocampal progenitor cells has never been reported in the literature.

Dihydroxybenzoic acids are classified as non-flavonoid phenolic acids from the benzoic acid derivatives subgroup. These compounds have widespread occurence in fruits and vegetables and are known for their antioxidant activity (reviewed by Khadem and Marles, 2010). Interestingly, flavonoids such as quercetin are degraded by gut microflora to form dihydroxybenzoic acids (Kim *et al.*, 1998). It is also shown that 2,3- and 2,5-dihydroxybenzoic acid are present in the body as the product of acetyl salicylic acid (aspirin) metabolism (Grootveld and Halliwell, 1988).

2,3 DHBA (pyrocatechuic acid) is suggested to induce the expression of Sirt1, peroxisome proliferator-activated receptor-gamma co-activator-1α (PGC-1α), and NAD(P)H quinone oxidoreductase 1 (Nqo1) genes through its oxidation/reduction reactions which involve the production of reactive oxygen species (Grootveld and Halliwell, 1988; Kamble *et al.*, 2013). The protein encoded by these genes are involved in the adaptive mechanisms against stressful conditions (reviewed by Liang and Ward, 2006; Li, 2013). Iron and precipitated hemoglobin are involved in the formation of free radicals leading to cell membrane peroxidation. 2,3 DHBA has the capacity as an iron-chelating agent to increase urinary iron excretion and also to scavenge the formed free radicals (Graziano *et al.*, 1974; Graziano *et al.*, 1976).

3,5 DHBA (α-resorcylic acid) is reported to bear moderate antioxidant and hydrogen peroxyde scavenging activity (Sroka and Cisowski, 2003). Only a trace amount of 3,5 DHBA is detected in green tea, however, the significant presence of

this compound in the body is suggested from the metabolism of alkylresorcinols, the phenolic lipids that are abundant in wholegrain cereals (Ross *et al.*, 2004; Gruz *et al.*, 2008). In addition to its potent activity to quench lipid peroxidation, earlier studies showed its anti-lipolytic activity upon binding to the hydroxycarboxylic acid receptor-1 (HCAR1) in adipocytes (Liu *et al.*, 2012). There is no study, to the best of my knowledge, that investigates the effect of 3,5 DHBA on brain function.

In line with the finding in the neurosphere assay, 3,5 DHBA is also shown to increase the cell number in monolayer precursor cell culture, confirming the effect of 3,5 DHBA in increasing cell proliferation. Altogether, the effect of 3,5 dihydroxybenzoic acid in precursor cells, and its high affinity to bind with the lactate receptor are very intriguing because physical activity, during which the level of lactate in the blood is increased dramatically, robustly increases proliferation of neuronal precursor cells in the dentate gyrus (Ide *et al.*, 2000; Kempermann, 2011b).

4.5. HCAR1 activation is speculated as a novel mechanism to mediate the proneurogenic effect of dietary compounds

HCAR1, also known as G-protein-coupled receptor-81 (GPR81) belongs to the same subfamily as nicotinic acid receptor GPR109A and is highly expressed in adipose tissue (Lee *et al.*, 2001; Wise *et al.*, 2003). However, its affinity for nicotinic acid is very low (Wise *et al.*, 2003). Initially, no endogenous ligand was identified to specifically bind this receptor. In 2009, lactate was first reported as the endogenous ligand of this receptor and interestingly, 3,5-DHBA was subsequently demonstrated to specifically bind this receptor with higher binding affinity than lactate (Liu *et al.*, 2009; Liu *et al.*, 2012).

Aside from being present in adipose tissue, *in situ* hybridization revealed the distribution of HCAR1 mRNA in the astrocytes and neurons, predominantly in cortical, hippocampal and cerebellar neurons (Bergersen and Gjedde, 2012). In 2014, Lauritzen *et al.* initially demonstrated the distribution of this receptor by immunohistochemistry in endothelial cell membranes at the blood-brain barrier, cerebellar Purkinje neurons, hippocampal pyramidal cells, the hilus of the dentate gyrus and cerebral neocortex. More specifically, HCAR1 was observed at the highest density at synaptic membranes of excitatory-type synapses in

hippocampus. However, the expression of HCAR1 in hippocampal precursor cells has never been reported.

In this study, using immunostaining techniques on brain sections from Nestin-GFP C57BL/6 mice and monolayer culture of adult hippocampal precursor cells, I demonstrated for the first time the expression of HCAR1 in adult hippocampal precursor cells. There is still a lack of studies elucidating the role of HCAR1 activation in the brain. It has been shown that upon binding to HCAR1, the physiological concentration range of lactate (0.1 - 30 mM) and 3,5 DHBA (IC 50 ~1.4 mM) elicit the inhibition of forskolin-induced cAMP synthesis in hippocampal slice lysates. This finding indicates that the HCAR1 in central nervous system is responsive to the ligands in the same way as in the peripheral adipose tissue (Lauritzen *et al.*, 2014).

The decrease of cAMP levels upon receptor activation hardly explains the effect of 3,5 DHBA in increasing precursor cell proliferation in the neurosphere assay and monolayer culture. It is known that the activation of the cAMP cascade is related to the activation of its downstream transcription factor cAMP response element-binding protein (CREB), leading to the increase of precursor cell proliferation in the adult mouse hippocampus (Nakagawa *et al.*, 2002). In this study, I found the effective concentration of dihydroxybenzoic acids in the neurosphere assay to be ~10 μM and ~100 μM in monolayer culture, which are much lower than the previously reported concentration for cAMP inhibition. This indicates another alternative mechanism that may be involved to elicit the effect. As often observed in other G-coupled-protein receptors, HCAR1 may activate the intracellular signaling pathway through a non-canonical mechanism that is cAMP independent, such as through β-arrestin. With this mechanism, HCAR1 activation may mediate neurotrophic actions such as facilitating the production and release of BDNF (Reiter *et al.*, 2012; Bergersen, 2015). This hypothesis is very intriguing to be followed up for further understanding of the downstream mechanism and the effect of this receptor activation at the behavioral level.

Altogether, the identification of dihydroxybenzoic acid in apple, the activity in neural precursor cells, and the presence of HCAR1 in hippocampal precursor cells provide a novel hypothetical mechanism for the proneurogenic effect of dietary compounds on adult hippocampal precursor cells.

5. Conclusion

The findings in the present study show that quercetin, the major flavonoid in apples, influences the proliferation, differentiation and survival of adult hippocampal precursor cells *in vitro* and *in vivo*. The effects are exerted through the interaction of quercetin with the cell cycle regulator proteins and molecular pathways that are involved in controling these processes. Moreover, quercetin is also shown to activate the endogenous antioxidant system, the Nrf2-Keap1 pathway, to overcome the stressful condition during the differentiation process.

The major finding of this work is the identification of the active compounds in apple flesh other than flavonoids that exert potential proneurogenic effect. Using bioassay-guided fractionation followed by mass spectrometry analysis, these active compounds were identified as dihydroxybenzoate glycosides, which belong to the class of benzoic acid derivatives. In addition to its antioxidative properties, an isomer of these compounds, namely 3,5 dihydroxybenzoic acid, was shown to bind and activate the hydroxycarboxylic acid receptor-1 (HCAR1). I demonstrated the presence of this receptor in the adult hippocampal precursor cells, both *in vivo* and *in vitro,* and from there emerged the possibility that the activation of HCAR1 might underlie the proneurogenic effect of 3,5 dihydroxybenzoic acid. Further study is required to confirm the hypothesis that activation of HCAR1 enhances the production of neurotrophic factors.

As the amount of apple juice consumption is quite significant in our society, I performed the experiment to test whether apple juice consumption has a beneficial effect in hippocampal precursor cells and spatial cognition in mice. Unexpectedly, there is no significant effect in the number of newborn cells in the dentate gyrus and the performance in the Morris water maze task between apple-juice treated mice and the control group. The insufficient concentration of active compounds in the apple juice is proposed as the explanation of the absence of the effect.

The pharmacologic concentration of quercetin that is shown in the *in vitro* and *in vivo* experiments is hardly achieved by normal physiological daily food and beverages intake. Supplementation or fortification of food and beverages with quercetin should be done to increase the bioavailability of these compounds, especially in the brain. However, the bioavailability of dihydroxybenzoic acids is

higher than the flavonoids because the flavonoids and phenolic lipid alkylrecorcinols are metabolized to form dihydroxybenzoic acids in the body.

Eventually, this work provides more evidence about the health benefit of fruit and vegetable consumption. Although an apple a day is not enough to reach effective concentration of active phytochemicals, but it taught us a lesson that the unexpected simpler compounds like benzoic acid derivatives are apparently the potential proneurogenic compound in apples. The good news for the ones who don't like or allergic to apples is that these compounds are also available in other plant-derived foodstuffs and even can be generated in the body as the product of polyphenols metabolism.

6. References

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