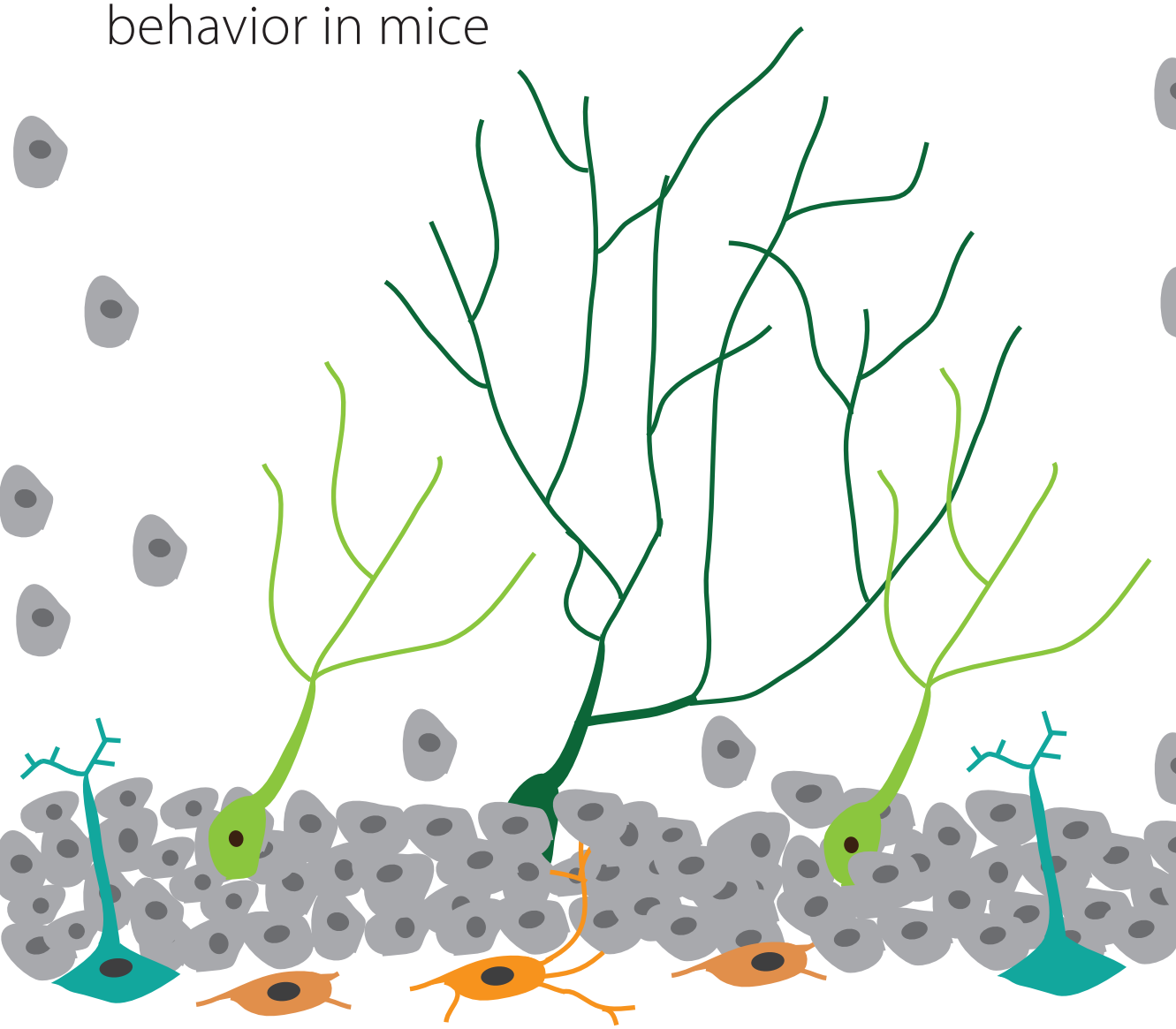
Hasan Mohammad

Åbo Akademi University

# JNK regulation of neurogenesis, neuroplasticity and anxiety-related behavior in mice



# JNK regulation of neurogenesis, neuroplasticity and anxiety-related behavior in mice

Hasan Mohammad

Department of Biosciences, Åbo Akademi University
Turku Centre for Biotechnology,
Doctoral Network in Molecular Biosciences (MolBio)
University of Turku and Åbo Akademi University
Åbo, Finland, 2016

From the Turku Centre for Biotechnology, University of Turku and Åbo Akademi University, Faculty of Science and Engineering, Åbo Akademi University, and Turku Doctoral Network in Molecular Biosciences.

**Supervised by**
Docent Eleanor Coffey, Ph.D.
Research Director
Åbo Akademi University
Vice-Director Turku Centre for Biotechnology
Turku, Finland

**Reviewed by**
Professor Esa R. Korpi
Professor of Pharmacology
Group Leader
University of Helsinki
Helsinki, Finland

and

Docent Riikka Lund, Ph.D.
Adjunct Professor
Turku Centre for Biotechnology
University of Turku and Åbo Akademi University
Turku, Finland

**Opponent**
Professor Nuno Sousa
Full Professor at the School of Health Science
University of Minho
Minho, Portugal

**Author's Address**
Neuronal Signaling Lab
Turku Centre for Biotechnology
Tykistokatu 6B,
20520, Turku
Finland
email:hasan.mohammad@btk.fi

ISBN 978-952-12-3360-9 (Print)
ISBN 978-052-12-3361-6 (Digital)
Painosalama Oy – Turku, Finland 2016

*Dedicated to my Father*

# Table of Contents

# List of Publications

This thesis is based on the following original publications and manuscripts. The original articles are reprinted in the appendix with the permission of copyright holders.

I. Björkblom B*, Padzik A*, **Mohammad H**, Westerlund N, Parvianen L, Papageorgiou T, Iljin K, Kallioniemi O, Kallajoki M, Courtney M, and Coffey ET. c-Jun N-terminal kinase phosphorylates MARCKSL1 determines actin stability and migration in neurons and cancer cells. Mol. Cell. Biol. 2012; 32:17 (* equal contribution)

II. Komulainen E, Zdrojewska J, Freemantle E, **Mohammad H**, Kulesskaya N, Deshpande P, Marchisella F, Mysore R, Hollos P, Michelsen K A, Mågard M, Rauvala H, James P and Coffey E T. JNK1 controls dendritic field size in L2/3 and L5 of the motor cortex, constrains soma size, and influences fine motor coordination. Frontiers in Cellular Neuroscience 2014; 8: 272

III. **Mohammad H**, Marchisella F, Eerola K, Komulainen E, Freemantle E, Savontaus E, Kulesskaya N, Rauvala H, and Coffey ET. JNK signaling regulates adult hippocampal neurogenesis and anxiety behavior in mice. (Manuscript 1)

IV. **Mohammad H**, Komulainen E, Varidaki A, Coffey ET. Novel role of JNK and its potential substrates in regulating dendritic spines architecture. (Manuscript 2)

# Abbreviations

| | |
|---|---|
| 5HT | 5-Hydroxytryptamine |
| AD | Alzheimer's disease |
| ADP | adenosine diphosphate |
| AHN | adult hippocampal neurogenesis |
| ALS | amyotrophic lateral sclerosis |
| AMPAR | α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor |
| ATP | adenosine triphosphate |
| BDNF | brain derived neurotrophic factor |
| BLA | basolateral amygdala |
| BNST | bed nucleus stria terminalis |
| BSA | bovine serum albumin |
| BrdU | bromodeoxyuridine |
| CA1 | cornus ammonis 1 |
| CA3 | cornus ammonis 3 |
| CBT | cognitive behavioral therapy |
| CNS | central nervous system |
| CRF | corticotrophic releasing hormone |
| DCX | doublecortin |
| DIV | days *in vitro* |
| DJNKI-1 | D-*retroinverso* peptide inhibitor of JNK kinase |
| E15 | embryonic day 15 |
| ED | effector domain |
| ERK | extracellular signal regulated protein kinase |
| GABA | gamma-aminobutyric acid |
| $GABA_A$ | gamma-aminobutyric acid receptor A |
| GAD | general anxiety disorder |
| GFAP | glial fibrillary acidic protein |
| GFAP-TK | herpes simplex virus thymidine kinase expressed under GFAP promoter |
| GluA | glutamate receptor subunit of the AMPAR |
| GR | glucocorticoid receptor |
| GTP | guanidine triphosphate |
| HMW | high molecular weight |
| HPA | hypothalamus-pituitary-adrenal axis |
| HRP | horseradish peroxidase |
| HSF | heat shock factor |
| IFN | interferon |
| IL-1β | interleukin- 1β |
| JBD | JNK-binding domain |
| JIP | JNK-interacting protein |
| JNK | c-Jun-N-terminal kinase |

| | |
|---|---|
| KSR | kinase suppressor of Ras1 |
| LAB | low anxiety behavior |
| LTD | long-term depression |
| LTP | long-term potentiation |
| MAM | methylazoxymethanol acetate |
| MAOIs | monoamine oxidase inhibitors |
| MAP2 | microtubule associated protein |
| MAPK | mitogen activated protein kinase |
| MAP2K | mitogen activated protein kinase kinase |
| MAP3K | mitogen activated protein kinase kinase kinase |
| MARCKSL1 | mysritoylated alanine rich C-kinase substrate like protein |
| MCAO | middle cerebral artery occlusion |
| MEK | MAP/ERK kinase |
| MLK | mixed lineage kinase |
| mPFC | medial prefrontal cortex |
| MR | mineralocorticoid receptor |
| MRP | MARCKS-related protein |
| MTOC | microtubule organizing centre |
| NGF | nerve growth factor |
| NIH | novelty induced hypophagia |
| NMDA | N-methyl-D-aspartate |
| NMDAR | N-methyl-D-aspartate receptor |
| NSF | novelty suppressed feeding |
| NT-3 | neurotrophic factor-3 |
| OB | olfactory bulb |
| OCD | obsessive compulsive disorder |
| PD | Parkinson's disease |
| PFA | paraformaldehyde |
| PIP2 | phosphatidylinositol 4, 5-bisphosphate |
| p-JNK | phosphorylated JNK |
| PKC | protein kinase C |
| PRD | proline-rich domain |
| PSA-NCAM | polysialated neural cell adhesion molecule |
| PSD95 | postsynaptic density 95 |
| PTSD | posttraumatic stress disorder |
| PVN | paraventricular nucleus |
| RTK | receptor tyrosine kinase |
| SSRI | selective serotonin reuptake inhibitors |
| SNRI | selective norepinephrine reuptake inhibitors |
| SGZ | subgranular zone |
| SVZ | subventricular zone |
| SAPK | stress activated protein kinase |
| SCG10 | superior cervical ganglion 10 |
| shRNA | short hairpin RNA |
| siRNA | small interfering RNA |

| | |
|---|---|
| TCA | tricyclic antidepressant |
| TNF | tumor necrotic factor |
| VEGF | vascular endothelial growth factor |

# Abstract

Anxiety disorders are dramatically increasing and are therefore affecting the well-being of modern society. The available treatment alternatives are currently limited because the molecular mechanisms of anxiety are not well understood. However, with the advance of imaging and genetic manipulation techniques, various structural changes have been associated with anxiety disorders. Neuroanatomical studies have revealed impairments of the dendrite architecture, spines and adult hippocampal neurogenesis in anxiety disorders. Adult hippocampal neurogenesis has gained much more attention in this context. It has been suggested that adult hippocampal neurogenesis plays an important role in the pathophysiology of anxiety disorders and it has also been implicated in the behavioral efficacy of some antidepressants.

In recent years, MAP kinases have been linked to the development of mood disorders as well as adult hippocampal neurogenesis. The JNK subfamily of MAPKs are stress-activated kinases that are activated by various external stresses. Basal JNK activity is low in normal cells, but cellular stress induces elevated JNK activity, which results in apoptosis. JNK kinases have been considered as potential therapeutic targets in neurodegenerative disorders, but their role in mood disorders has not yet been addressed.

The work presented in this thesis addresses the role of JNK signaling in regulating anxiety behavior in mice. Using *Jnk1* knockout mice and pharmacological inhibition of JNK signaling, JNK was found to regulate adult neurogenesis, which underlies anxiety behavior in mice. The *Jnk1-/-* mice displayed reduced anxiety and increased adult hippocampal neurogenesis. The inhibition of JNK signaling in mouse brain also reduced anxiety and enhanced adult hippocampal neurogenesis, as observed with some antidepressant treatments. Inhibition of JNK activity not only increased adult hippocampal neurogenesis, but also facilitated the maturation of newborn neurons in the adult brain of mice. Additionally, dendrite architecture and spine distribution of CA3 neurons in the hippocampus of *Jnk1-/-*mice were found to be altered. In a subsequent screen for JNK substrates, two JNK effectors, MARCKSL1 (an actin-associated protein) and MAP2, (a microtubule-associated protein), were found to regulate neuronal architecture. Taken together this implies that JNK signaling might control the dendrite and spine changes associated with anxiety disorders.

In summary, the work presented in this thesis provides a deeper understanding of the molecular events that are putatively involved in regulating the neurogenic and dendritic changes implicated in anxiety disorders. Moreover, these findings imply that the targeting of JNK signaling might be a potential therapeutic strategy for combating anxiety disorders.

# SAMMANFATTNING (Swedish Abstract)

I vår moderna värld har förekomsten av ångeststörningar drastiskt ökat, vilket påverkar välfärden i våra samhällen. Eftersom de molekylära mekanismerna bakom ångest är relativt okända, är möjligheterna till behandling av ångeststörningar begränsade. I och med utvecklingen av genetiska manipuleringmetoder och avbildningstekniker har strukturella förändringar associerade med ångeststörningar kunnat konstateras. Neuroanatomiska studier har påvisat störningar i dendritarkitektur, dendrittaggar och i neurogenesen hos vuxna individer. Särskilt neurogenesen i hippocampus anses viktig i detta sammanhang. Neurogenes i hippocampus har föreslagits spela en viktig roll i ångeststörningarnas patofysiologi och för hur vissa antidepressiva läkemedel förmedlar sin effekt.

Under senare år har MAP-kinaser (MAPK) föreslagits vara involverade både i uppkomsten av affektiva störningar och i neurogenes i hippocampus. JNK är en grupp kinaser inom MAPK-familjen som aktiveras av olika externa stressfaktorer. I normala celler är aktiviteten hos JNK låg. Cell-stress ökar aktiviteten hos JNK vilket leder till bl.a. apoptos. JNK kinaser anses vara potentiella terapeutiska mål för behandling av neurodegenerativa sjukdomar men deras potential som mål för behandling av affektiva sjukdomar har tillsvidare inte utretts.

Den här avhandlingen behandlar betydelsen av JNK för ångestrelaterat beteende hos möss. Med hjälp av *Jnk1*-knockout möss och farmakologisk inhibering av JNK-signalering, demonstreras att JNK reglerar neurogenes i hippocampus, vilket i sin tur ligger bakom mössens ångestrelaterade beteende. *Jnk1*-knockout möss var mindre ängsliga och uppvisade ökad neurogenes i hippcampus jämfört med kontrollmöss. Inhibering av JNK-signalering i hjärnan hos möss gjorde dem också mindre ängsliga och ökade neurogenesen i hippocampus på samma sätt som vissa antidepressiva läkemedel. Inhibering av JNK-aktivitet ledde inte bara till ökad neurogenes i hippocampus, utan främjade också mognandet av nybildade nervceller hos vuxna möss.

Resultaten visar också att dendritarkitekturen och fördelningen av dendrittaggar hos CA3 neuroner i hippocampus är förändrad hos *Jnk1*-knockout möss. Genom screening av substratmolekyler för JNK hittades två JNK-effektormolekyler, MARCKSL1 (ett aktin-associerat protein) och MAP2 (ett mikrotubulus-associerat protein), som reglerade neuronernas sttruktur. Det här tyder på att JNK-signalering kan kontrollera ångeststörningsrelaterade förändringar hor dendriter och dendrittaggar.

Sammanfattningsvis ger resultaten som presenteras i avhandlingen en ökad insikt i molekylära mekanismer som kan leda till ångeststörningsrelaterade förändringar i neurogenes och dendritstruktur. Därtill föreslås att JNK-signalbanan har potential som terapeutiskt mål för behandling av ångeststörningar.

# Review of Literature

## 1. Introduction to Anxiety disorders

### 1.1. Anxiety disorders

Anxiety disorders are currently the most prevalent psychiatric disorders and add a great strain upon the health care resources (Alonso *et al.* 2004; Kessler *et al.* 2009). These comprise a group of psychiatric diseases that include acute disorders, such as generalized anxiety disorder (GAD), social anxiety disorder, phobias, and more devastating pathologies, such as panic disorder, obsessive-compulsive disorder (OCD) and post-traumatic stress disorder (PTSD) (Fig.1). GAD patients are constantly worried about everyday life tasks. This unreasonable persistent worry develops into a permanent fear of failing in every task. Patients with social anxiety disorder suffer from fear of being judged and of being embarrassed. Patients suffering from this condition feel extremely fearful and uncomfortable around others. Phobias are very common anxiety disorders, in which a person displays persistent and highly disproportionate fear of a particular situation or an object. In the more devastating diseases, such as panic disorder, a person suffers from sudden and repeated panic attacks that can last for several minutes or longer. During the attack, patients lose control because of the fear of disaster, even in the absence of real danger. OCD patients have a huge problem coping with their daily lives, they always check and re-check things repeatedly, for example, washing their hands over and over again. They find themselves lost in an attempt to gain control of their over obsessions. PTSD is the other most disconcerting anxiety disorder, and is observed in war veterans, survivors of sexual abuse, or any other serious life event. The death of a loved one can also leave individuals with PTSD. It usually results in bad dreams, flashbacks, guilt feelings, depression, and hyper-arousal in the patients. There is a high degree of comorbidity between mood and anxiety disorders. Fourteen percent of the European population is affected with one or both disorders in their lifetime (Williamson *et al.* 2005). As these pathologies can last for months or even longer, medication is needed to lessen or prevent their severity. Although a range of treatments are available, they are ineffective in a large number of patients or result in side effects (von Moltke & Greenblatt 2003). These disorders also differ in their prevalence and response to medication among genders. In many cases, there is a relapse of the condition after the treatment. Due to this diversity and ineffective drugs, there is the incessant demand for new therapeutic approaches.

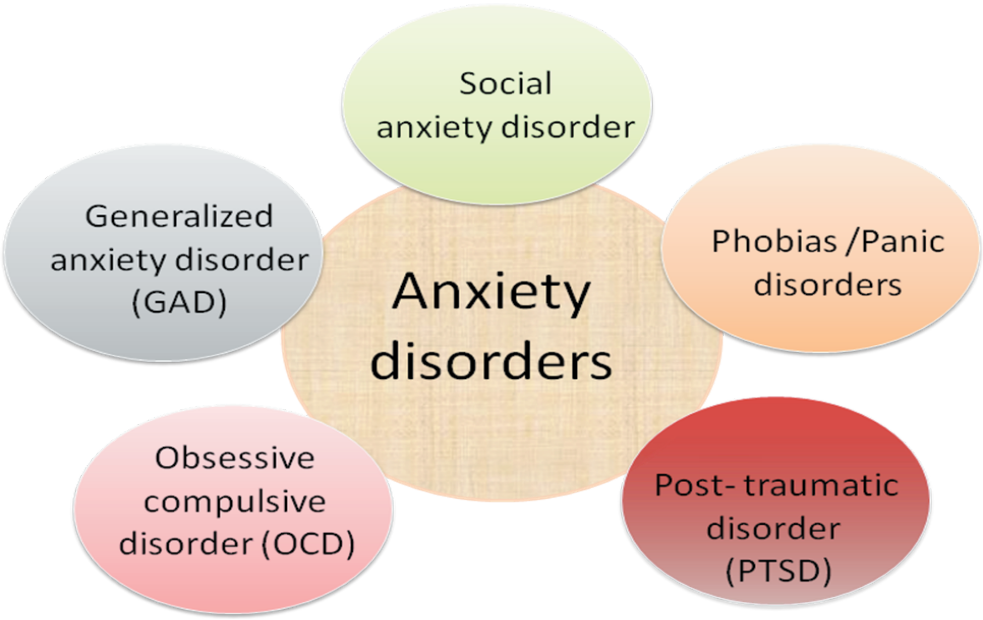



**Figure 1.** Different types of anxiety disorders

## 1.2. Stress and anxiety disorders

In the past two decades, there has been an immense increase in epidemiological studies on humans, as well as research with animal models, to know how stress affects emotions and cognition. Stress is a physiological response to a threatening or hostile environment. It prepares the body for a quick and energetic reaction, which is referred to as a "flight or fight" response. However, sustained stress results in severe pathological states, which influence brain function and behavior, such as anxiety disorders. The stress response accompanies a plethora of changes, including alteration in autonomic function, and release of multiple hormones, which eventually affect the behavior. Neuroendocrine responses to stress include secretion of chemical mediators, such as corticotrophin-releasing factor, vasopressin, serotonin (5-hydroxytryptamine, 5-HT) and catecholamines. Additionally, neuropeptides, such as substance P, neuropeptide Y and cholecystokinin are released in stress responses (Carrasco & Van de Kar 2003). The hormones produced in response to stress lead to maladaptive changes in the brain. Regions of the brain, including the brain stem, hypothalamus, prefrontal cortex, and hippocampus, are involved in stress responses and are altered in stress-related disorders, such as anxiety. These regions represent highly interconnected circuits, which influence each other directly or indirectly by modifying neural activity (Bremner 2006). There are various predisposing factors that make individuals more vulnerable, for instance, early life experiences have sustained physiologi-

cal and behavioral effects. There are several genetic or allelic differences that are critical in determining an individual's response to stressful life experiences, for example, the short form of the serotonin receptor gene predisposes to chronic stress and can subsequently lead to depressive behavior (Luddington *et al.* 2009). Similarly, the genetic variant (Val66Met allele) of brain derived neurotrophic factor polymorphism (BDNF) has been associated with anxiety related behavior (Chen *et al.* 2006b). The latter polymorphism also affects neuronal plasticity by reducing dendritic branching as well as mRNA trafficking, which might underlie these psychiatric disorders (Baj *et al.* 2013).

Hypothalamus-pituitary-adrenal (HPA) axis activation is a hallmark of the stress response and acts as a normal physiological feedback process that maintains homeostasis. The HPA axis consists of the hypothalamus, the anterior lobe of the pituitary gland, and the adrenal cortex. Corticotrophin–releasing factor (CRF), synthesized and released by neurons in the paraventricular nucleus (PVN) of the hypothalamus, is a principal regulator of the HPA axis. In response to stress, CRF initiates a cascade of events that leads to the production of glucocorticoids from the adrenal cortex. Glucocorticoids normally mediate adaptive effects through the intracellular receptors to increase the cell survival. However, an excessive or inadequate HPA axis activation results in the development of stress related disorders, such as PTSD (Smith & Vale 2006). CRF deficient mice display reduced anxiety and a disrupted HPA axis, which suggests that CRF plays an important role in regulating anxiety behavior by maintaining HPA homeostasis (Smith *et al.* 1998). Glucocorticoids are the primary stress hormones released in response to stress induced activation of the HPA axis. Glucocorticoids (cortisol in humans and corticosterone in rodents) regulate various physiological and behavioral responses (Carrasco & Van de Kar 2003; Charmandari *et al.* 2005). Their effects are mediated via glucocorticoid and mineralocorticoid receptors (GRs and MRs respectively). These receptors are distributed throughout the limbic structures and in the peripheral tissues (Herman *et al.* 2003). Polymorphisms of the glucocorticoid receptors alter sensitivity towards stress hormones, and when encountered with environmental stressful events make individuals more susceptible to chronic anxiety disorders (Lian *et al.* 2014). Genetic manipulations and pharmacological studies using agonists and antagonists have provided evidence that both GRs and MRs modulate anxiety responses (Calvo & Volosin 2001; Gass *et al.* 2001). Based on such evidence, HPA signaling has been considered as a potential target for stress related disorders (Anacker *et al.* 2011).

## 1.3. Neurobiology of Anxiety

From an evolutionary perspective, it has been postulated that the set of interrelated limbic structures, i.e. the septo-hippocampal system, areas of the hypothalamus, central nuclei of the amygdaloid complex, and the peri-aqueductal gray matter of the mid brain, represent the anatomical core of anxiety (Charney & Redmond 1983). This anatomical core evaluates threatening situations and selects an appropriate defense response. The hippocampus, for instance, is thought to process context-related information and express anxiety responses. There is consistent literature suggesting the crucial role of the amygdaloid complex of the limbic system in producing patterns of behavior resembling responses after fearful stimuli (Davis 1997; Davidson 2002). For example, the lesions in the amygdala blocked the response to fearful stimuli (Maren 1996). The amygdaloid complex consists of functionally and morphologically varying subnuclei with multifaceted neural links. The basolateral amygdala (BLA) is glutamatergic, and the central amygdala mostly composed of GABAergic neurons (Tye *et al.* 2011). The BLA is a locus for fear memory and provides information about the environment to central amygdala through robust projections (LeDoux 2000). The central amygdala makes projections to the hypothalamus and peri-aqueductal gray matter to mediate anxiety response, for example the avoidance of open areas by rodents (Adhikari 2014). The bed nucleus of stria terminalis (BNST), also suggested to be a part of "extended amygdala" (Alheid *et al.* 1998), modulates anxiety by processing inputs from the amygdala. Furthermore, the interplay of the BLA with the medial prefrontal cortex (mPFC) and the ventral hippocampus provides another level of regulation of anxiety. It has been suggested that BLA integrates the contextual and sensory input from the mPFC and ventral hippocampus to regulate central amygdala and BNST, leading to activation of downstream regions to control anxiety-like behavior (Adhikari 2014).

Anxiety, as a survival mechanism in threatening situations, is intertwined with the emotional processes as well as cognitive functions. Therefore, many neurotransmitter pathways are involved, including γ-aminobutyric acid (GABA), glutamate, serotonin, and norepinephrine. Several studies have indicated the critical role of these neurotransmitter pathways in anxiety behavior. GABA is a primary inhibitory neurotransmitter in the central nervous system and its down regulation has been linked to the pathophysiology of anxiety disorders. The pharmacological targeting of the GABA receptors has been used in anxiolytics (Lydiard 2003).

In contrast, glutamate is the major excitatory neurotransmitter in the nervous system. The modulation of excitatory glutamate transmission has been shown to induce anxiolytic effects (Bergink *et al.* 2004). Glutamate transmission occurs via glutamate gated ions channels, known as N-

methyl-D-aspartate (NMDA) receptors. Administration of NMDA and non-NMDA receptor antagonists to the basolateral amygdala reduced anxiety in animal models (Kim & McGaugh 1992). Alternatively, the neurotransmission of GABA can be increased to achieve suppression of excitatory glutamate output from the amygdala. Thus, an imbalance between glutamate receptor mediated excitation and GABA receptor mediated inhibition is associated with anxiety (Sajdyk & Shekhar 1997). Both of these neurotransmitters are abundant in the amygdala and other limbic structures, and have been targeted for the treatment of anxiety disorders (Lydiard 2003; Bergink *et al.* 2004).

Far more attention, however, has been dedicated to the role of serotonin (Jones & Blackburn 2002). Preclinical and clinical studies in the last 50 years have shown the therapeutic efficacy of drugs targeting serotonin (5-HT, 5-hydroxytryptamine) receptors in various anxiety disorders. In particular, 5-HT1A knockout mice showed increased stress sensitivity and anxiety (Heisler *et al.* 1998). 5-HT1A is an auto-receptor and maintains a negative feedback loop by inhibiting the release of serotonin. The partial agonist of 5-HT1A buspirone has been effective in the treatment of generalized anxiety disorder (GAD). Compounds with antagonistic properties against 5-HT2A and 5-HT3 receptors have also reduced anxiety in preclinical studies (Katz *et al.* 1993; Olivier *et al.* 2000). Currently, the most extensively prescribed drugs for treating anxiety-depressive disorders are aimed at the modulation of serotonin transmission, and are discussed in the next section.

Noradrenergic systems have also been implicated in stress and anxiety, e.g. the noradrenergic neurons of the locus coeruleus play a critical role in fear and stress responses (Bremner *et al.* 1996). Norepinephrine effects are mediated via presynaptic α2 receptors and postsynaptic α1 and β1 receptors. The α2 receptor is also an auto-receptor that inhibits the release of norepinephrine from the presynaptic terminals. Therefore, α2 receptor agonists show reduced anxiety-like behavior in mice (Bremner *et al.* 1996).

## 1.4. Treatments of anxiety disorders

Both pharmacotherapy and psychological therapies have been used for treating anxiety disorders. There are different classes of psychotropic medications that have been used to treat anxiety disorders, with the most frequently investigated class being antidepressants. Tricyclic antidepressants (TCAs) have been used in psychiatry since the 1950s. TCAs are reported to damp down the anticipatory anxiety and also reduce the number of panic attacks (Andersch *et al.* 1991). Despite notable effects, they have unwanted side effects, such as sedation, sexual dysfunction, consti-

pation and the likelihood of toxic overdose (Ravindran & Stein 2010). Due to these side effects, alternative drugs have been sought after. The monoamine oxidase enzyme inhibitors (MAOIs), selective serotonin reuptake inhibitors (SSRIs) and serotonin norepinephrine reuptake inhibitors (SNRIs) are the other class of drugs that has been used frequently. MAOIs, SSRIs, and SNRIs have shown improvements in the efficacy of treatment, and have fewer side effects than TCAs. Therefore, SSRIs and SNRIs are now considered as the front line of pharmacotherapy agents for anxiety disorders, due to their overall efficacy, safety, and tolerability (Koen & Stein 2011). However, despite being more effective, they increase the risk of suicidal behavior in adolescents. On account of this risk, these drugs are marked with black-box labeling by the U.S. Food and Drug Administration (US Food and Drug Administration, 2007).

Another line of drugs used for the treatment of psychiatric disorders are anticonvulsants. Benzodiazepines and tiagabine are most commonly used because of their rapid onset and efficacy (Stevens & Pollack 2005). They both have anxiolytic and anticonvulsive effects at lower doses, but higher doses produce significant adverse effects such as amnesia, sedation and even unconsciousness (Saari *et al.* 2011). In addition, uncomfortable withdrawal symptoms have been reported in patients who discontinued benzodiazepine use. Consequently, this therapy becomes more inappropriate for individuals having a history of substance abuse (Ravindran & Stein 2010). Therefore, non-benzodiazepines anticonvulsants are preferred for the treatment of psychiatric illnesses. Pregabalin is one example which is prescribed for GAD, however, it has also been reported to have adverse effects and has led to deaths due to drug abuse (Baldwin *et al.* 2013).

Most experts recommend that psychotherapy should be combined with pharmaceutical treatment. Currently, there are two different psychotherapeutic approaches that have been used: 1) Behavioral therapy and 2) Cognitive therapy. In such treatments, the therapists strive to help the patients to combat undesirable behavior or adapt their disturbing thoughts into healthy ones. Both treatment methods are overlapping in the sense of educating the mind of the patient, and are broadly classified as cognitive-behavioral therapy (CBT). Psychotherapy is used to treat all forms of anxiety disorders (American Psychological Association 2004). Due to the diverse risks and side-effects of the established anxiolytics, there is a need for the development of new therapeutic approaches to treat anxiety disorders. Some novel strategies have been suggested recently, such as the potential use of endogenous modulators of glutamate or neuropeptide signaling, as well as the HPA axis. Currently, the glutamate antagonists, neuropeptides, such as oxytocin, neuropeptide Y, vasopressin cholecystokinin (CKK), and CRF receptor antagonists, are under investigation (Mathew *et al.* 2008; Clynen *et al.* 2014; Farb & Ratner 2014).

## 1.5. Modeling anxiety

Animal models have excelled in the research on psychiatric disorders. *In vivo* models are critical for evaluating novel treatment approaches. With the advent of genetically modified mice, a substantial degree of research has been focused on developing different knockout models with extreme anxiolytic and anxiogenic phenotypes. To develop appropriate models for testing new anxiolytics, researchers have concluded that more than one behavioral test is required. Also, when considering the heterogeneity of these disorders, it is believed that no animal model will be able to combine all the aspects of human anxiety disorders (Cryan & Holmes 2005; Rotzinger *et al.* 2010). For decades, the research in non-humans, primarily rodents, has been central for investigating the neural system mediating emotions. Rats have been used extensively for preclinical research, because of their size and vigor which facilitates invasive manipulations, including cannula implantations and catheterizations for testing the efficacy of drugs. Moreover, rats also perform well in the cognitive and operant tasks, which are the foundation of behavioral pharmacology. Various selective inbreeding strategies have been used to develop high anxiety behavior (HAB) and low anxiety behavior (LAB) rats (Landgraf & Wigger 2002).

The development of gene targeting approaches has led to the widespread use of mice in neuropsychiatric research. Mice are more amenable to these genetic manipulations and most importantly offer an economic and practical advantage of breeding and housing in large numbers (Cryan & Holmes 2005). There are several different mouse lines that have been developed by targeting genes of the pathways hypothesized to be involved in anxiety disorder, these include $5HT1_A$, $GABA_A$ and CRH receptors (Holmes 2001). In addition to pharmacological manipulations, these gene mutant mice have provided valuable information in understanding the neural substrates of anxiety. These mouse models are validated for phenotypes using various tests. They are categorized as conditioned response tests or unconditioned response tests. Conditioned responses are Pavlovian in nature, involve a stressful event and mostly comprise conflict test, defensive burying and active/passive avoidance behavior. The most commonly used unconditioned tests are exploration based, startle responses or social tests (Belzung & Griebel 2001). Basically, these tests explore the innate behavior of rodents to avoid bright, novel, open and/or aversive stimuli. These tests are validated by various anxiolytic agents such as SSRIs and benzodiazepines (Bailey & Crawley 2009).

### 1.5.1. Exploratory-based approach-avoidance conflict tests

Exploratory-based avoidance tests are most widely used to assess anxiety behavior in rodents. The elevated plus maze (Pellow *et al.* 1985), the open

field test (Christmas & Maxwell 1970) and the light-dark box (Crawley & Goodwin 1980) are classical exploratory based anxiety tests. These tests provide an environment for rodents in which there is an option to explore an adverse area or remain in a less anxiety-provoking location. *The elevated plus maze* test takes advantage of the natural tendency of mice to explore a novel situation. The rodents are allowed to spend time in elevated open or closed arms. Normally, mice tend to avoid the unprotected open areas and spend time in enclosed spaces. This approach-avoidance conflict in the elevated plus maze results in behavior that are indicators of psychological stress. On the contrary, mice explore the open arms more willingly following anxiolytics treatment, without showing any effect on their general locomotion. The more sensitive measures of the effects of anxiolytic compounds are measured by analyzing additional behavioral indices including head dipping, grooming and stretched attend postures in elevated plus maze (Borsini *et al.* 2002).

The *open field* test has also been proven to be equally successful in assessing anxiety behavior in rodents. In addition to anxiety behavior, this test measures the novel environment exploration and general locomotor activity. In this test, subjects are allowed to explore in an open arena. There are two major factors that affect the anxiety behavior in this test, the social isolation and the brightly lit unprotected test environment. The test indicates anxiety levels by measuring variables such as time spent and distance travelled by rodents exploring brightly lit open spaces, as usually rodents show thigmotaxic behavior. *The Light-dark box* test is another exploration based test more similar to elevated plus maze. Here, rodents are subjected to a novel environment with light (unprotected area) and dark compartments (protected area). A similar inherent tendency of risk avoidance is explored in this test. Mostly rodents prefer to stay in dark and protected areas. Therefore, a change in the willingness to explore the unprotected illuminated area is a key measure of anxiety in this paradigm. This change is reflected in the time spent in each compartment and the number of transitions made between compartments during the test. The anxiolytics, such as benzodiazepines, show reduced anxiety in all three tests, as measured by increased exploration in the light compartment of light-dark box, the open arms of elevated plus maze, and the center of an open field. The advantage of these tests is that they show good correlation between the typical behavioral effects of commonly used anxiolytics. Additionally, the patterns of behavior in these tests are relatively replicable across the species and studies (Walf & Frye 2007; Griebel & Holmes 2013).

Even though these tests continue to remain the mainstay of assessing anxiety behavior, there are major cautions associated with their use. As they are based on the interplay of approach and avoidance, they are unable to separate decreased anxiety related avoidance from increased novelty seeking behavior (Cryan & Holmes 2005). The exploration based tests

are reliant on the sensory and motor functions. The assessment of locomotory, neurological and sensory functions must also be performed and taken into account to obtain trustworthy results.

### 1.5.2. Other anxiety related tests

In addition to the above tests, there are a number of others that have been used to investigate anxiety-like and/or repetitive-like behavior. The marble burying test is one that explores the most commonly referred to defensive burying behavior in response to the aversive stimuli in rodents (De Boer & Koolhaas 2003). It takes advantage of the innate burying behavior of rodents to hide novel, but harmless, objects. Early pharmacological studies have established marble burying as a potential model of anxiety because of its sensitivity to the anxiolytics such as benzodiazepines (Broekkamp *et al.* 1986; Nicolas *et al.* 2006). Hyponeophagia paradigms are also being used to assess anxiety, and have been designed on the basis of the "bait shyness" phenomenon in rodents. Bait shyness is an inherent approach evolved in rodents to avoid the potential toxicity of novel food. The novelty-induced hyponeophagia (NIH) and novelty-suppressed feeding (NSF) (Bodnoff *et al.* 1988; Dulawa *et al.* 2004) are two such paradigms used to evaluate anxiolytic drugs (Merali *et al.* 2003; Dulawa & Hen 2005).

## 1.6. Neuroplasticity changes associated with anxiety disorders

Existing treatments for psychiatric disorders provide symptomatic relief rather than reversing the underlying abnormalities. Recent reviews proposed that structural and neuroplastic changes might underlie anxiety disorders (Myers & Davis 2007; Pittenger & Duman 2008; Tovote *et al.* 2015). Neuroimaging has evolved as a new tool for understanding the pathophysiology of psychiatric disorders. For instance, functional neuroimaging of PTSD patients has revealed many insights into the structural and neuroplastic abnormalities involved in such pathological states. The most consistent finding is hyperactivity of the amygdala, which is involved in the assessment of threatening stimuli and necessary for processing of fear conditioning (LeDoux 2000; Davis & Whalen 2001). This heightened activity of the amygdala reflects an elevated fear response. In some brain regions, such as the hippocampus, cingulate cortex and medial frontal cortex, reduced activity has been reported (Shin *et al.* 2006; Hayes *et al.* 2011). The hippocampus and the medial frontal cortex are known to interact with the amygdala for encoding contextual fear memory (Dolcos *et al.* 2004; McGaugh 2004) and processing fear extinction (Morgan *et al.* 1993; Quirk *et al.* 2000; Milad & Quirk 2002), respectively. The altered activity in these brain regions reveals disturbed fear and fear extinction responses, as well as problems in the identification of safe contexts in

anxiety disorders (Deckersbach *et al.* 2006). Additionally, there are volumetric changes reported in the brain regions discussed above (Fig.2). The hippocampus volume is reduced in severe anxiety disorders, such as PTSD (Gilbertson *et al.* 2002; Felmingham *et al.* 2009) (Fig.2). Also, reduction in the volume and shape of cingulated cortex has been reported, but for the amygdala volumetric changes are inconsistent between studies (Shin *et al.* 2006). However, it is unclear whether these changes predispose or cause the pathological condition.

Due to ethical reasons, there are limitations on mechanistic investigations in humans, but the animal models have shed some more light on the intricate mechanisms that underlie these changes and their associations with anxiety disorders. Kalisch and colleagues suggested that a positive relationship exists between anxiety and hippocampal volume using HAB and LAB rats (Kalisch *et al.* 2005). It has long been apparent that a relationship exists between stress and the development of psychiatric disorders, such as anxiety and depression (Finlay-Jones & Brown 1981; Kendler *et al.* 1999; Kendler *et al.* 2001). Stress can have long-lasting effects on the brain structure and circuitry, which ultimately influence the behavior. The intimate association between the effects of stress, mechanisms of neuroplasticity, and the pathophysiology of mood disorders and the mechanism of antidepressant action has gained much appreciation in the last decades (McEwen 1999; Pittenger & Duman 2008). A sustained level of stress induces morphological changes in the brain regions involving hippocampus, prefrontal cortex and amygdala. Stress induced atrophy and retraction of dendritic arborization in the hippocampus (Watanabe *et al.* 1992; Magarinos *et al.* 1996; Sapolsky 2000) and the prefrontal cortex (Wellman 2001; Cook & Wellman 2004), whereas hypertrophy of dendritic arborization is reported in the amygdala (Vyas *et al.* 2002; Vyas *et al.* 2003) (Fig.2). Spine density is also altered in response to different stress paradigms in animal models. Dendritic spines are small protrusions on dendrites, which were first described by Santiago Ramón y Cajal in the 19th century. They make synapses, which are basic units of information transmission and provide another level of plasticity to the brain. Spine distribution and morphology is also perturbed in response to stress, as is dendritic architecture. The spine density is reduced in the prefrontal cortex (Radley *et al.* 2006; Goldwater *et al.* 2009) and increased in the amygdala (Mitra *et al.* 2005) in response to chronic stress. However, there are a number of discrepancies among studies in relation to spine density in the hippocampus, because some studies show a decrease in the density, whereas others suggest increased spine density in response to stress (Christoffel *et al.* 2011). Post-mortem studies in aged humans with anxiety and depressive symptoms but no history of psychiatric illnesses also, revealed neuroanatomic changes, including a reduction in the dendritic arborization and dendritic spines in the hippocampus (Soetanto *et al.* 2010) (Fig.2).

The studies in humans and in rodents have clearly shown that structural changes in brain areas, such as the prefrontal cortex, amygdala and hippocampus, are associated with anxiety and depressive behavior. The hippocampus has been one of the principal regions of interest in the context of anxiety disorders. A more recent structural change in hippocampus that impacts neuroplasticity and hence hippocampus function is neurogenesis in the adult hippocampus. It has gained considerable interest in the field of psychiatry and has been altered by different forms of stress (Dranovsky & Hen 2006; Mirescu & Gould 2006). Adult hippocampal neurogenesis has been suggested to be involved in anxiety behavior (Revest *et al.* 2009) (Fig.2) and it appears to be required for the behavioral effects of antidepressants (Santarelli *et al.* 2003), which is discussed in more detail in the next section. Overall, these studies suggest that chronic stress alters the structural morphology of the brain and thus affect neuroplasticity.

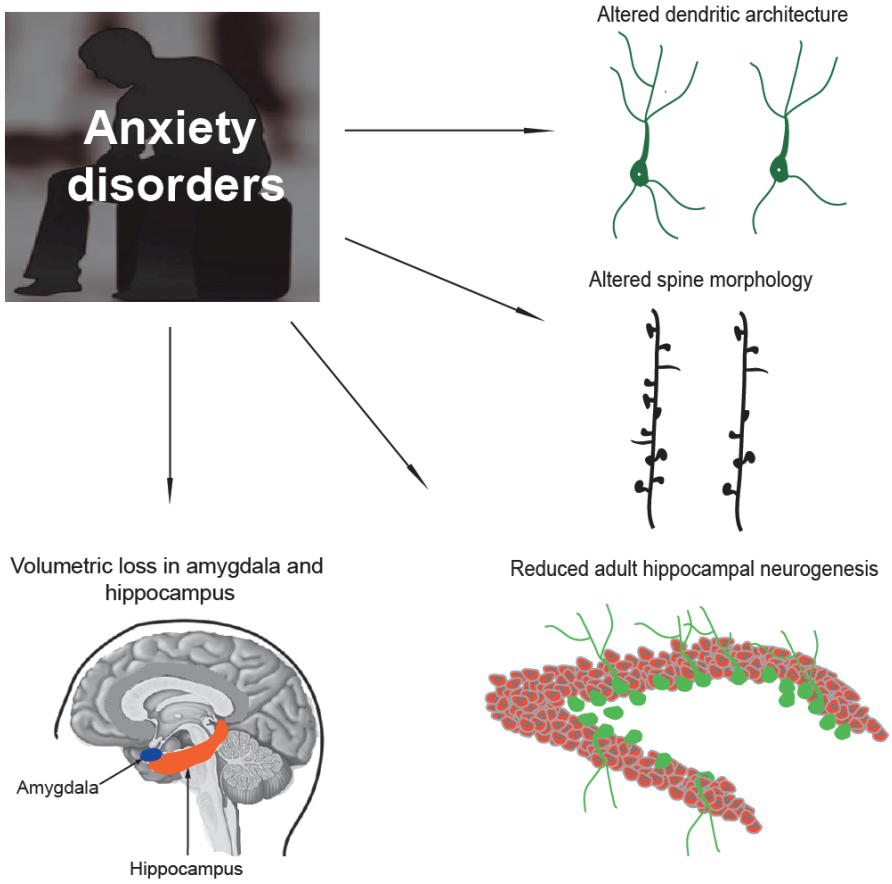



**Figure 2.** Neuroplastic changes associated with anxiety disorders. (The figure is adapted from Kalisch et al 2005 Neuropsychopharmacology 31 (5) 925-932, Revest et al 2009 Mol Psychiatry14 (10) 959-67, Soetanto et al 2010 Arch Gen Psychiatry 67 (5) 448-57.

## 2. Adult neurogenesis

Traditionally, it was believed that neurogenesis occurs only during embryonic development in the mammalian central nervous system. Only recently, decades after the discovery of newborn neurons in the adult brain, the phenomenon of adult neurogenesis is now generally accepted (Ming & Song 2005). Joseph Altman observed the presence of newborn neurons in the adult brain for the first time in the1960s (Altman & Das 1965). The acceptance by the scientific community that new neurons are also formed in the adult brain was a slow process, and numerous confirmatory studies were undertaken to overcome the earlier dogma. In the past few years, significant progress has been made in understanding the process of adult neurogenesis. Although it was initially difficult to trace the fate of these rare dividing cells and prove that they were neurons rather than glia, the use of $^{3}$H-thymidine, and more recently bromodeoxyuridine (BrdU a synthetic analog of thymidine), has revolutionized the field (Sidman *et al.* 1959; Gratzner 1982). These analogs were successfully incorporated into the replicating DNA and made the tracing of labeled cells easier. With these technical innovations this field has advanced considerably, and now more sophisticated methods, such as transgenic mice and retrovirus labeling of newborn cells, have further expanded the understanding of maturing neurons (Abrous *et al.* 2005; Ming & Song 2005). Several inducible and non-inducible transgenic mice have been produced to express or knockdown particular genes of interest (Dhaliwal & Lagace 2011). The retroviruses, most commonly Muloney murine leukemia viruses, which integrate into the host DNA during mitosis, thus making them good indicators of cell division (Lewis & Emerman 1994). The labeling of viruses with reporters, such as green or red fluorescent proteins, allowed visualization and analysis of newborn cells (Abrous *et al.* 2005; Ming & Song 2005). Further, the identification of specific markers that neurons express during maturation, for example, PSA-NCAM, DCX expressed in immature neurons and NeuN in mature neurons, enabled the fate specification of newly born neurons (Kempermann *et al.* 2004) (Fig.3). The discovery of stem cells in the brain has attracted a great deal of interest, because it changed the current concept of brain plasticity and raised possible strategy for brain repair.

Adult neurogenesis is a process whereby new functional and mature neurons are formed from neural stem cells residing in the discrete locations. These new neurons are generated throughout life in their niches where neural stem cells undergo such steps as proliferation, differentiation, migration, maturation and final integration into the established neuronal circuits. In mammals, the active neurogenesis occurs in 3 niches: the subventricular zone (SVZ) of the lateral ventricle, the subgranular zone (SGZ) of the dentate gyrus in the hippocampus and the olfactory bulbs (OB) (Fig.3). Adult neurogenesis has been reported in the SVZ and SGZ of

rodents (Kaplan & Hinds 1977; Alvarez-Buylla & Garcia-Verdugo 2002), in non-human primates (Yuan *et al.* 2014) as well as in humans (Eriksson *et al.* 1998). However, adult neurogenesis in the OB has been questioned in humans (Ernst & Frisen 2015).

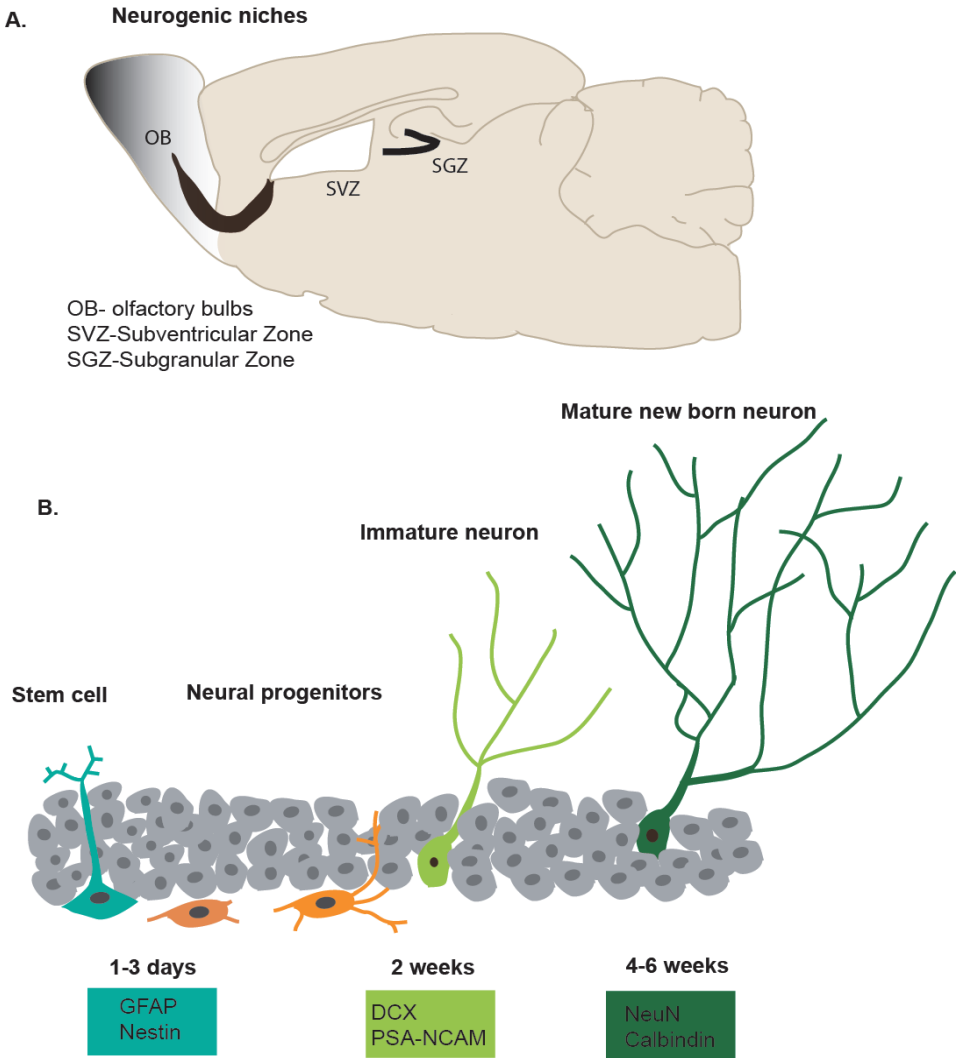



**Figure 3.** A) Neurogenic niches in the mouse brain Subgranular zone, Subventricular Zone and Olfactory bulbs. Figure modified from Frankland and Miller 2008 Nature Neuroscience **11**:1124-26). B) The developmental stages of adult born neurons in the dentate gyrus of hippocampus with examples of markers expressed at different stages. (Figure modified from Josef Bischofberger, 2007 Nature Neuroscience **10**:273-75)

Recently, adult-born neurons have also been detected in the striatum (Ernst *et al.* 2014) and hypothalamus (Rojczyk-Gołębiewska *et al.* 2014) regions adjacent to these neurogenic niches. The birth of new neurons in the SGZ of the dentate gyrus is termed as adult hippocampal neurogene-

sis (AHN), which has been extensively studied in the context of mood and anxiety disorders.

## 2.1. Role of adult hippocampal neurogenesis in brain

Within the dentate gyrus of the hippocampus, the newborn neurons are generated from the neural stem cells in the SGZ. From here they migrate to the inner granule cell layer and integrate into the hippocampal circuitry (Ming & Song 2005). The detailed analysis of neurogenesis suggests that there are approximately 9000 new cells formed per day in the rodent hippocampus (Curlik *et al.* 2014). Approximately fifty percent of them differentiate and express neuronal markers (Cameron & McKay 2001). These newly born neurons in the dentate gyrus make axonal projections with CA3 pyramidal cell layer. They extend their dendrites into the molecular layer and grow into a complex innervated structure in a month (Vivar & van Praag 2013). The functional integration of newly born neurons commences within the first week after proliferation and continues to form synaptic contacts in the following weeks. In comparison to mature granule neurons, the immature or young neurons display distinctive properties, such as enhanced synaptic plasticity and long-term potentiation (Song *et al.* 2005). These findings indicate that these new neurons with distinctive characteristics are sufficient to contribute in a significant manner to hippocampal function. The newborn neurons in the hippocampus have been implicated in hippocampal dependent functions, including learning, memory and pattern separation (Clelland *et al.* 2009; Deng *et al.* 2010; Sahay *et al.* 2011). The inhibition of adult neurogenesis in the hippocampus has been shown to alter the long term spatial memory as well as the ability to differentiate between highly similar stimuli (Snyder *et al.* 2005; Luu *et al.* 2012). On the other hand, recent reports also suggest that AHN regulates the process of forgetting in infants and adults (Akers *et al.* 2014). It appears that neurogenesis in the hippocampus has an anterograde impact in facilitating memory acquisition, whereas a retrograde impact while erasing old memories. The age-related decline of spatial memory has been associated with the decline of neurogenesis with age (Lazarov *et al.* 2010). It is anticipated that due to the finite storage capacity of the dentate gyrus, the reduced neurogenesis in aged animals is unable to clear the old memories and so the capability of the hippocampus to store new information is diminished.

Hippocampal neurogenesis has been extensively studied in the context of emotional and mood regulation. Recent reports support the significant role of adult hippocampal neurogenesis in mediating the effects of drugs in mood disorders such as anxiety and depression (Samuels & Hen 2011). Even though there is considerable evidence for the critical role of hippocampal neurogenesis, still there are many open questions, like how exact-

ly newborn neurons contribute to learning, memory, age- related cognitive decline and emotions.

## 2.3. Factors regulating the adult hippocampal neurogenesis

Adult neurogenesis is a highly dynamic process that is regulated at different stages by several exogenous factors, including antidepressants, age, exercise, early life stress and disease (Zhao *et al.* 2008; Lucassen *et al.* 2010; Kuipers *et al.* 2015). Research into hippocampal neurogenesis has also identified additional endogenous stimuli, numerous proteins, hormones, neurotrophins, neurotransmitters and epigenetic factors that regulate the multiple steps of neurogenesis (Balu & Lucki 2009; Hagg 2009; Yao & Jin 2014). These factors alter adult neurogenesis in different ways by acting at distinct steps, and are being investigated extensively.

Mood improving antidepressants (Malberg *et al.* 2000; Manev *et al.* 2001), exercise (Praag *et al.* 2005), environmental enrichment (Kempermann *et al.* 1997; Clemenson GD 2015) and learning (Gould *et al.* 1999) have been reported to increase adult hippocampal neurogenesis, whereas other factors, such as aging (Klempin & Kempermann 2007), stress (Warner-Schmidt & Duman 2006), and disease (Winner *et al.* 2011) reduce neurogenesis.

## 2.4. Stress and adult hippocampal neurogenesis

Stress is known to inhibit the adult neurogenesis in various mammalian species, including mouse, rat, tree shrew, and marmoset (Mirescu & Gould 2006). Different stress paradigms, such as psychosocial stress, resident intruder stress, foot-shock, predator odor and others, inhibit one or more phases of adult neurogenesis (Gould *et al.* 1997; Gould *et al.* 1998; Tanapat *et al.* 2001; Malberg & Duman 2003). Both acute and chronic stress show similar effects and alter all phases of neurogenesis, inhibiting both proliferation and survival (Westenbroek *et al.* 2004; Mirescu & Gould 2006). The stress-induced reduction in neurogenesis can be a result of increased apoptosis of progenitor cells or the cell cycle arrest (Heine *et al.* 2004a; Heine *et al.* 2004b). Therefore, there are many ways in which stress can influence neurogenesis. Substantial evidence suggests that the stress hormones play an important role by modulating cross talk with other stress-related mechanisms (Egeland *et al.* 2015). Glucocorticoids and their receptors GRs and MRs have been pointed out as regulators of hippocampal neurogenesis (Wong & Herbert 2004; Wong & Herbert 2005; Wong & Herbert 2006). The inhibition of the HPA axis by removing circulating adrenal steroids increased neurogenesis, whereas, in contrast, exogenous corticosterone administration reduced neurogenesis (Cameron &

Gould 1994). Persistent inhibition of neurogenesis has been observed despite restoration of normal glucocorticoid levels. Similarly, the persistent reduction in cell proliferation has been reported in adult rats (where basal levels of corticosterone were normal) that were subjected to early life maternal separation. This highlights the effect of early life stress on adult neurogenesis (Mirescu *et al.* 2004).

Studies also suggest that stress might affect neurogenesis by increasing glutamate excitatory neurotransmission in the hippocampus, which reduces proliferation (Abraham *et al.* 1998). Activation of NMDA receptors has been observed to inhibit cell proliferation, whereas the blockade had the opposite effect (Cameron *et al.* 1995; Nacher *et al.* 2003). Stress also increases pro-inflammatory cytokines in the hippocampus, for instance, interleukin-1β (IL-1β). The hippocampal progenitors express the IL-1β receptor, and administration of IL-1β has been seen to reduce hippocampal cell proliferation, whereas blockade of IL-1β receptors with an antagonist attenuates hippocampal neurogenesis after chronic unpredictable stress (Koo & Duman 2008). Moreover, the IL-1β receptor knockout mice and the IL-1β receptor antagonist over-expressing transgenic mice were protected against the reduction of neurogenesis after stress. Two other important cytokines, namely interferons (IFNs) and tumor necrosis factor (TNF), also regulate the processes integral to neurogenesis, including proliferation, differentiation (Borsini *et al.* 2015).

In addition to cytokines, a reduction of trophic factors might contribute to the deleterious effects of stress on hippocampal neurogenesis. Various stress paradigms, both acute and chronic, reduce the trophic support in the brain. For example, the expression of BDNF, the most well studied neurotrophin, is reduced in the hippocampus after stress. The reduction of BDNF levels might be due to elevated glucocorticoids in response to stress because adrenalectomy increased BDNF expression, whereas the chronic administration of corticosterone decreased BDNF levels (Duman & Monteggia 2006). Glucocorticoids can also regulate the pathways downstream of BDNF, including MAP kinase and phospholipase C pathways. Also, glucocorticoids activate the BDNF receptor TrkB, and promote cell survival of hippocampal neurons (Jeanneteau *et al.* 2008). These studies have indicated that stress negatively affects neurogenesis via the inhibitory crosstalk of glucocorticoids signaling on BDNF signaling. Stress induced reduction of other neurotrophic factors, such as nerve growth factor (NGF), NT-3 and vascular endothelial growth factor (VEGF) could also influence neurogenesis (Balu & Lucki 2009; Egeland *et al.* 2015).

Therefore, it is clear that stress reduces adult hippocampal neurogenesis, but the mechanism and functional consequences still require further investigation. Overall, stress influences diverse cellular processes, including neurotransmission, growth factors, dendritic architecture, so it is most

likely that inhibition of neurogenesis contributes to stress in concert with multiple other mechanisms (Balu & Lucki 2009).

## 2.5. Role of adult hippocampal neurogenesis in anxiety

Stress suppresses hippocampal neurogenesis, while the pharmacotherapies, such as antidepressants, elevate adult neurogenesis. Such evidence is the basis of the neurogenesis hypothesis of depression, which postulates that a decrease in newborn neurons in the dentate gyrus is related to the pathophysiology of depression. Interestingly, the ablation of adult hippocampal neurogenesis alone does not induce a depressed mood (Saxe *et al.* 2006; David *et al.* 2007; Levone BR 2015). Nevertheless, it can prevent the ability of some, but not all, antidepressants to protect from behavioral changes in response to stress (Santarelli *et al.* 2003; Surget *et al.* 2008; David *et al.* 2009). These studies do suggest that adult hippocampal neurogenesis influences and contributes to antidepressant induced recovery from stress, but negative findings have also been reported (Petrik *et al.* 2012). However, studies also indicate the involvement of neurogenesis-independent mechanisms in depressive behavior (David *et al.* 2007; Mendez-David *et al.* 2014). The neurogenesis-independent phenomena, including neuronal remodeling, dentate gyrus evoked activity and neuroplasticity, have been linked to onset and recovery of depressive behavior in rodents (Meltzer *et al.* 2005; Airan *et al.* 2007; Bessa *et al.* 2009). The high level of comorbidity and their similarities suggest that depression and anxiety may have shared etiology.

Hippocampal neurogenesis in the milieu of anxiety has shown inconsistent outcomes among the different studies. In the paradigms that enhance neurogenesis, such as exercise, both increased (Burghardt *et al.* 2004; Fuss *et al.* 2010) and decreased anxiety (Salam *et al.* 2009) is observed, which suggests a complex relationship. The inconsistency among studies might depend on variable rodent strains, housing conditions, exercise protocol, the timing of tests in relation to running, and the particular test used to assess anxiety behavior. On the other hand, the suppression of hippocampal neurogenesis in rodents is unable to clarify the relationship. In some studies anxiety was heightened (Bergami *et al.* 2009; Revest *et al.* 2009), while others observed reduced (Uchida *et al.* 2011) anxiety, or no link (Saxe *et al.* 2006) after suppression of hippocampal neurogenesis.

The hippocampus is functionally dissociated along the dorso-ventral axis. The dorsal pole regulates cognition related behavior, while the ventral pole mediates emotional responses (Ferbinteanu & McDonald 2001; Bannerman *et al.* 2002). A recent study suggested a similar functional dissociation of adult born neurons in the hippocampus, and demonstrat-

ed that ablation of ventral newborn neurons diminishes the anxiolytic/antidepressant effect of fluoxetine under chronic stress. Such studies advocate that the location of newborn neurons is vital (Satvat *et al.* 2012; Wu & Hen 2014). A more recent study suggested that enhancing adult hippocampal neurogenesis is sufficient to reduce both anxiety and depressive behavior in response to stress, but has no effect on the baseline anxiety and depression (Hill *et al.* 2015). Despite these inconsistent outcomes, the evidence is strong that adult hippocampal neurogenesis is required for some beneficial effects of antidepressants. The chronic administration of antidepressant targeting serotonin neurotransmitter system requires hippocampal neurogenesis to be effective in the hyponeophagia task (Santarelli *et al.* 2003) and to reverse behavioral effects of chronic mild stress (Surget *et al.* 2008). Moreover, it has also been demonstrated that antidepressants, such as fluoxetine, require hippocampal neurogenesis for mood improving action in some, but not in all behavioral tests, which suggests that drugs can have both neurogenesis-dependent and independent actions (David *et al.* 2009). The drugs that act via different mechanisms, however, such as the CRF1 antagonist and the vasopressin 1B antagonist, are effective in the absence of adult hippocampal neurogenesis (Surget *et al.* 2008). Environmental enrichment is also a mood improving stimulus that does not require adult hippocampal neurogenesis. It has been observed that environmental enrichment reduced anxiety in mice where adult hippocampal neurogenesis was blocked by X-ray irradiation (Meshi *et al.* 2006). Even though the X-ray irradiation technique robustly blocks the neurogenesis, it also influences the proliferation, differentiation and microglia activation (Tada *et al.* 2000; Mizumatsu *et al.* 2003). Therefore, this reduction in anxiety could be a consequence of the negative effects of X-ray irradiation in the hippocampus and other brain regions. The role of adult hippocampal neurogenesis in the treatment of mood and anxiety disorders is highly debatable and remains under intense research.

## 2.6. Manipulation of neurogenesis in rodents

To understand the functional relevance of adult neurogenesis, researchers have used a number of methods for suppressing or enhancing adult neurogenesis, including chemical treatments, modifications and genetic manipulations. Chemicals, such as methylazoxymethanol acetate (MAM) and cytarabine (cytosine arabinoside), have been used to inhibit the proliferation of cells. These drugs have been used to demonstrate the cell types involved in neurogenesis (Doetsch *et al.* 1999) and the role of adult born neurons in learning and memory (Shors *et al.* 2002; Bruel-Jungerman *et al.* 2005). Cytosine arabinoside, however, is neurotoxic (Courtney & Coffey 1999), rendering this approach problematic.

The focal X-ray irradiation has been a very popular technique for investigating the functional relevance of neural stem cells in the hippocampus. The X-rays suppress neurogenesis for up to 3 months and reduce around 80 percent of cells (Tada *et al.* 2000; McGinn *et al.* 2008). This has been shown to be a consistent and reproducible approach and has allowed the generation of a large group of neurogenesis deficient mice in a short time. The use of X-ray irradiation has some drawbacks, such as increased microglia activations, disrupted angiogenesis and altered differentiation (Tada *et al.* 2000; Mizumatsu *et al.* 2003). Nonetheless, the approach has been crucial in advancing the understanding of adult neurogenesis. Using this approach it has been established that neurogenesis is required for the behavioral effects of antidepressants (Santarelli *et al.* 2003).

Genetic approaches have been used extensively to develop transgenic mice with ablated or enhanced adult neurogenesis to complement the x-ray irradiation studies. One such transgenic approach is based on the use of GFAP-TK mice, where the herpes simplex virus thymidine kinase is expressed under the control of a GFAP promoter (Saxe *et al.* 2006). Administration of ganciclovir eliminates the proliferating GFAP and TK positive cells, and thus ablates neurogenesis. More recently, this model was modified to achieve increased specificity by expressing thymidine kinase under the nestin promoter to ablate adult neurogenesis. Another approach involved the doxycycline-induced overexpression of pro-apoptotic Bax protein in progenitor cells under the nestin promoter. This leads to a significant reduction in the adult neurogenesis in the hippocampus and alters anxiety-related behavior. On the other hand, the tamoxifen-induced knockdown of Bax protein under nestin promoter has been used to enhance adult neurogenesis, which improved the pattern separation (Sahay *et al.* 2011) in rodents and also reduced the anxiety-related behavior under chronic stress (Hill *et al.* 2015).

Many cell cycle proteins, including cyclin D2, Cdk5, and Bmi1, which are necessary for hippocampal neurogenesis, have been deleted to attenuate neurogenesis (Kowalczyk *et al.* 2004; Zencak *et al.* 2005; Lagace *et al.* 2008). A multitude of transgenic mouse lines have been used to alter adult neurogenesis by targeting different genes, such as HDAC2, Notch1, NeuroD1, Prox1, Smad4, Sox2 and Trkb (Dhaliwal & Lagace 2011). The development of constitutive, as well as inducible transgenic models, have provided additional clues in our understanding of the function and importance of adult born neurons in naive and disease state. Each model has its own strength and limitations in defining the role of adult born neurons. However, there is still a need for improvement in the techniques used to manipulate adult hippocampal neurogenesis in order to clarify the dependency of antidepressant drugs on neurogenesis.

# 3. Mitogen activated protein kinases (MAPKs)

MAPKs (mitogen activated protein kinases) are a network of serine-threonine kinases, which transmit extracellular signals into their intracellular targets and regulate numerous cellular activities, including cell proliferation, differentiation, transformation, survival, and cell death (Seger & Krebs 1995; Kim & Choi 2010). MAP kinases are evolutionarily conserved from yeast to humans and share a three-tiered signaling cascade (Kyriakis & Avruch 2012). Malfunctions in this cascade can result in severe disease states. MAPK signaling has been implicated in the pathogenesis of various neurodegenerative diseases, such as Parkinson's disease (PD), Alzheimer's disease (AD) and Amyotrophic lateral sclerosis (ALS) (Kim & Choi 2015).

## 3.1. Introduction to MAPK signaling pathways

In the mammalian family of MAPKs, there are three major kinases: c-Jun N-terminal kinase (JNK), p38MAPKs, and extracellular signal-regulated kinase (ERK). Each of these exists in several isoforms. JNK has three isoforms, JNK1, JNK2 and JNK3, which play critical functions in cellular physiology as well as in diseases (Johnson & Nakamura 2007; Yamasaki *et al.* 2012; Coffey 2014). p38 MAPK has four isoforms (p38α, p38β, p38γ, and p38δ), of which the extensively studied p38α and p38β are implicated in neurodegenerative disorders (Correa & Eales 2012; Kim & Choi 2015). ERK has eight isoforms, among which ERK1, ERK2, and ERK5 have been studied in the context of mood disorders (Einat *et al.* 2003). The ERK1and 2 isoforms are activated by different upstream MAPK/ERK kinases 1 or 2 (MEK1/2), whereas ERK5 is activated via MEK5.

Each MAPK signaling cascade is composed of three sequential kinase components: a MAPK kinase kinase (MAP3K), a MAPK kinase (MAP2K), and a MAPK (Fig 4). The hierarchical sequential phosphorylation of these components activates the MAPKs, which in turn phosphorylate diverse targets, such as c-Jun, c-Myc, ATF2, Bcl-2 and Bad. MAPK pathways are activated either via binary interaction between kinase components or by the formation of a multiple kinase signaling complex docked to a scaffold protein. Such scaffold proteins orchestrate the specificity and subcellular localization of MAPK signaling. JNK-interacting proteins (JIPs) 1-3 serve as a scaffold for the JNK pathway, whereas JIP4 is specific for the p38 MAPK pathway. Kinase suppressor of Ras1 (KSR) and MEK partner 1(MP1) act as a scaffold for the ERK signaling pathway. B-arrestin1/2 acts as scaffolding proteins for both the JNK and ERK signaling pathways (Brown & Sacks 2009). Both JNK and p38MAPK signaling are triggered in response to a variety of cellular stresses, such as oxidative stress, osmotic stress, and genotoxic stress. The pro-inflammatory cytokines, interleukin

1β (IL1- β) and tumor necrosis factor-α (TNF- α) as well as microbial component (eg. bacterial lipopolysaccharide), also activate these pathways (Arthur & Ley 2013). The activation of the JNK pathway is mediated via the interaction and sequential phosphorylation of its components JNK, a MAP2K, e.g. MKK4 or MKK7 and a MAP3K e.g. MEKK1, ASK3, mixed lineage kinase (MLK) or transforming growth factor-β-activating kinase 1 (TAK1) (Davis 2000). The same MAP3Ks (AK3, TAK1) phosphorylate the distinct MAP2Ks, MKK3 and MKK6 to activate p38 signaling. The ERK1/2 kinases are activated by the MEK1/2 upstream kinases in ERK signaling pathways. The MEK proteins are activated by three isoforms of Raf: A-Raf, B-Raf, and C-Raf, which in turn are activated by small GTPase Ras (K-Ras, H-Ras or N-Ras). The receptor tyrosine kinase (RTK)-Grb2-SOS signaling axis mediates activation of GTPase Ras family proteins, which play an important role in the transmission of the extracellular signal (Dhillon *et al.* 2007) (Fig.4).

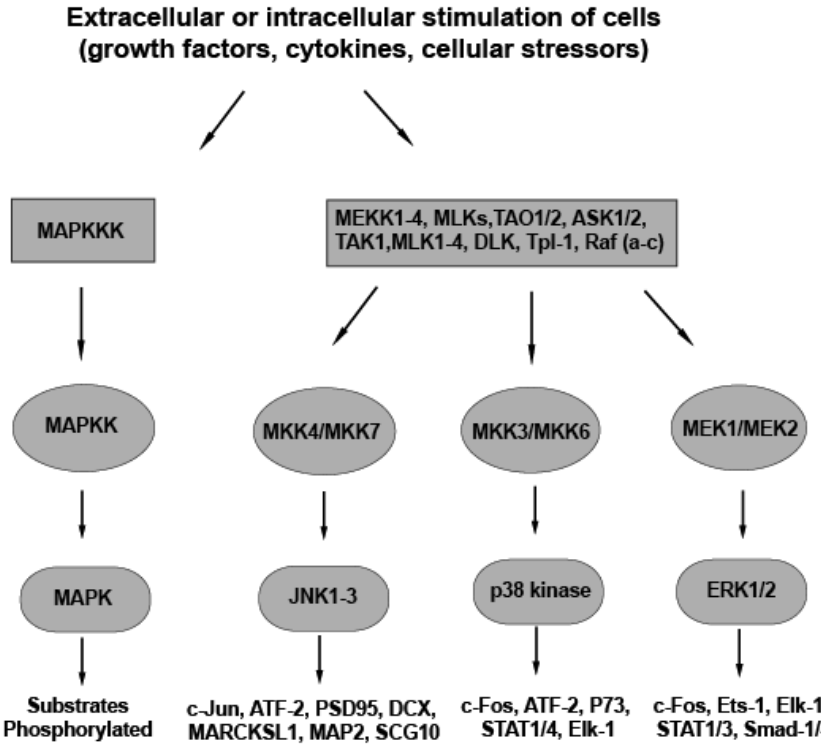



**Figure 4**. A simple schematic of the MAPK cascade. Various extra- and intracellular stimuli including growth factors, cytokines, cellular stressors activates the 3 tier cascade. (Figure modified from Sweeney and Firestein, 2007, Nature Reviews Rheumatology **3:** 651-660)

## 3.2. MAPK signaling in the central nervous system and implications in anxiety behavior

MAPK pathways play specialized roles in the central nervous system (CNS) by regulating cellular processes, including neuronal differentiation, neuronal migration, long-term potentiation (LTP), long-term depression (LTD), and synaptogenesis. The function of MAPK signaling in the CNS has been demonstrated using knockout and conditional mouse models, as well as inhibitor treatments and cell culture studies. A plethora of studies have demonstrated the role of ERK signaling in learning and memory. ERK1 knockout mouse model has shown that ERK signaling is critical in striatal mediated learning and memory (Mazzucchelli *et al.* 2002). The forebrain specific conditional deletion of MEK isoform B-raf kinase affects the hippocampal LTP and hippocampal dependent learning (Chen *et al.* 2006a). The intra-hippocampal administration of JNK inhibitor DJNKI-1 and knockout of JNK isoforms suggested a role for JNK signaling in associative learning (Sherrin *et al.* 2010). p38 MAP kinases have also been reported to contribute to associative learning and memory consolidation (Zhen *et al.* 2001; Rossato *et al.* 2006). The suppression of JNK activity is proposed to promote LTP indirectly by impairing depotentiation, which may serve to enhance synaptic plasticity (Yang *et al.* 2011). Synapse reorganization is an important cellular event in mediating LTP and LTD for maintaining synaptic plasticity. Strong evidence suggests the requirement of MAP kinases, including p38, JNKs and ERKs, in synaptic plasticity in the hippocampus (Thomas & Huganir 2004). The reorganization of dendritic morphology and dendritic spines underlies synaptic plasticity. Electrophysiological studies have implicated the ERK/MAPK pathway in activity dependent formation of dendritic filopodia (Thomas & Huganir 2004). Recent evidence suggested the requirement of JNK kinases in dendrite formation and axonal guidance by regulating microtubule and actin dynamics (Bjorkblom *et al.* 2005; Coffey 2014; Komulainen *et al.* 2014). p38 MAPK signaling has been postulated to be involved in actin remodeling in the spines, which are small protrusions where synapses are formed (Correa & Eales 2012).

In addition, JNK signaling is required for neuronal migration and axonal elongation during brain development and for the stress induced neuronal cell death in the adult brain (Yamasaki *et al.* 2012; Coffey 2014). ERK/MAPK signaling influences neuronal differentiation and maturation, as well as axonal growth, as has been demonstrated using conditional knockout models. The deletion of the ERK2 isoform specifically in the neural progenitors reduces the overall cortical neurogenesis by impairing the proliferation and differentiation during neural development (Samuels *et al.* 2008).

More recently, the ERK5 isoform that shares sequence homology with ERK1/2 has emerged as a regulator of adult neurogenesis. The inducible and conditional deletion in the neurogenic regions of adult mice attenuated adult neurogenesis in the hippocampus and disrupted the hippocampal dependent memory. In contrast, the gain of function knock-in mouse mutant used to specifically activate ERK5 in the neurogenic regions increased adult born neurons in the dentate gyrus by augmenting the survival, differentiation and dendritic complexity (Pan *et al.* 2013).

Besides its role in the aforementioned processes, in the last decade the ERK/MAPK signaling has been implicated in the anxiety and depressive behavior (Duman *et al.* 2007). ERK/MAPK signaling has been activated in the patients treated with mood stabilizers, such as lithium or valproic acid (Einat *et al.* 2003). Preclinical studies revealed an altered activity of ERK/MAPK signaling after administration of antidepressants like fluoxetine and imipramine, although the results are inconsistent among studies (Tiraboschi *et al.* 2004; Fumagalli *et al.* 2005). Moreover, reduced levels of pERK1/2 have been reported in the post-mortem brains of patients suffering from depression (Dwivedi *et al.* 2001). Interestingly, a recent study showed increased activity of ERK1/2, JNK and p38MAPK in the hippocampus of mice subjected to an acute session of forced swim test and tail suspension test. They also showed antidepressant-like effects after acute blockade of MAPK signaling using MEK, p38MAPK and JNK inhibitors (Galeotti & Ghelardini 2012). Very little is known about the role of MAP kinases in regulating emotional behavior, in particular, JNK family of MAPK has not been studied in this perspective. The work presented in this thesis has mainly addressed the role of JNK subfamily of MAPKs.

## 4. c-Jun- N-terminal kinase (JNK) pathway

The JNK subfamily of MAPKs was first purified as protein kinases from the rat liver after cyclohexamide treatment, and they were able to phosphorylate microtubule associated protein 2 (MAP2) on Ser/Thr residues (Kyriakis & Avruch 1990). Later, they were renamed as c-jun N-terminal kinases (JNKs) due to their strong phosphorylation of the c-jun transcription factor (Pulverer *et al.* 1991). JNKs are also known as stress activated protein kinases (SAPKs), as they are activated by many types of external stress, including heat shock, UV radiations, and inflammatory cytokines. There are three JNK genes encoded in the mammalian genome, namely *jnk1 (Mapk8)*, *jnk2 (Mapk9)* and *jnk3 (Mapk10)*. In humans, the *jnk1*, *jnk2* and *jnk3* genes are mapped to chromosome 10, 5 and 4, respectively. In rodents, they are present on chromosome 14, 11 and 5 in mouse, and chromosome 16, 10 and 14 in the rat (Haeusgen *et al.* 2011). There are 10 alternative transcripts for the *jnk* gene, *jnk1* and *jnk2* have four transcripts, whereas *jnk3* has two transcripts. All the isoforms arise from *jnk*

gene transcripts after alternative splicing in two subdomains (α and β JNK isoform) and the C-termini (JNK type 1 and 2), and fall in the weight categories of 46 and 54 kDa (Gupta *et al.* 1996). The expression pattern of JNKs differs significantly during development and adulthood; *jnk1* and *jnk2* expression begins early at embryonic day 8, and *jnk1* is detectable at embryonic stage 11. JNK1 and JNK2 are ubiquitously expressed in the mammals, whereas JNK3 is exclusive to the brain and testis (Kuan *et al.* 1999).

JNK kinases phosphorylate numerous cytoplasmic and nuclear substrates, including the c-jun, and AP-1 transcription factors, various apoptotic proteins and microtubule associated protein (MAPs) (Bogoyevitch & Kobe 2006). Through these substrates, JNKs regulate stress responses as well as physiological processes. The activation of JNK signaling follows the classical three-tiered-cascade, also known as a core-signaling module. JNKs are activated by two upstream kinases, mitogen activated protein kinase kinase 4 (MKK4) and MKK7. These are dual specificity Threonine (Thr) and Tyrosine (Tyr) kinases, but preferentially MKK4 phosphorylates Tyr residues, and MKK7 phosphorylates Thr residues in the activation loop of JNKs (Kishimoto *et al.* 2003). Both of these kinases are activated by various MAP3Ks, including mixed lineage protein kinases (MLKs), apoptosis signal-related kinases (ASKs) and dual leucine zipper kinases (DLK) (Cuevas *et al.* 2007). In addition, the high degree of specificity of JNK signaling is mediated by various scaffold proteins, including JNK interacting protein (JIP)1, JIP2 and JIP3 (JNK/SAP associated protein-1, JSAP1) (Yasuda *et al.* 1999). These scaffold proteins create a multienzyme complex and provide a physical conduit for the signal transduction. JNK activity is tightly regulated and employed at moderate levels in most of the mammalian tissues, yet it is constitutively active in the brain. Gene knockout studies of JNKs in mice have revealed diverse and essential roles of JNK signaling in the brain.

### 4.1. Physiological functions of JNK signaling in the brain

JNK kinases are multifunctional and are involved in many physiological processes. The JNK subfamily plays a crucial role in the fundamental developmental processes, such as organogenesis by regulating proliferation, cell survival, and apoptosis. Both *in vitro* and *in vivo* studies have emphasized the importance of JNK signaling in the nervous system. Indisputable evidence from studies with JNK-knockout mice has highlighted the requirement of JNK activity in brain development. All three JNK isoforms, as well as the upstream activators MKK4 and MKK7, have been knocked out to understand the importance of JNK signaling. Mice lacking only one of these genes, i.e. *jnk1*, *jnk2* or *jnk3*, survived normally without any developmental defects. Even the double mutant mice lacking *jnk1* and *jnk3*

or *jnk2* and *jnk3* survived, but the double mutant lacking *jnk1* and *jnk2* die between embryonic stages 11 (E11) and E12 and display the exencephaly phenotype due to deregulated apoptosis in the brain (Coffey 2014). Apoptosis was found to be increased in the forebrain of *Jnk1-/-*, *Jnk2-/-*mice with increased capase-3 activity, whereas cell death was reduced in the lateral edges of hindbrain before the neural tube closure (Johnson *et al.* 1993). Taken together, this suggests that JNK1 and JNK2 have both pro- and anti-apoptotic functions in brain development. The double knockout of *Jnk1* and *Jnk3* or *Jnk2* and *Jnk3* does not exhibit any neural tube defects (Kuan *et al.* 1999; Sabapathy *et al.* 1999). Interestingly, mice with genetic ablation of an upstream JNK activator, MKK4 or MKK7, does not display any defects in neural tube development. However, the single knockouts of *Mkk4* and *Mkk7* die between E11.5-E13.5 due to impaired liver formation (Asaoka & Nishina 2010). Among the JNK substrates, null mice of *c-jun*, *Atf2* do not phenocopy the neural tube defects of *Jnk1-/-*, *Jnk2-/-*mice. The mice lacking c-jun die because of hepatic abnormalities similar to *Mkk4-/-* or *Mkk7-/-* mice, whereas the *Atf2-/-* mice die at birth due to respiratory failure (Johnson *et al.* 1993; Maekawa *et al.* 1999). To date, only one JNK substrate, myristoylated alanine rich C-kinase substrate like protein (MARCKSL1, also known as MacMARCKS) has been identified (Björkblom *et al.* 2012), which causes neural tube defects after ablation (Chen *et al.* 1996a).

Studies using JNK inhibitor and *Jnk1-/-* mice established that JNK1 is required during the later stages of brain development. After neural tube formation, the neural progenitors proliferate and give rise to immature neurons. These further migrate to their final positions, where they extend neurites. In the developing cortex, immature neurons migrate to form cortical layers where they extend neurites for proper brain development. However, in *Jnk1-/-* mice the radial migration of cortical neurons is abnormally accelerated, which leads to thicker cortical plates and thinner ventricular zones. These results suggest that JNK1 regulates neuronal migration and is required for proper cortical layer formation during cortical development (Westerlund *et al.* 2011). Moreover, a large body of evidence from knockout mice and from isolated neurons shows that JNK1 regulates neurite formation and maintenance. Two types of neurites are extended from a developing neuron: dendrites and axons. In the cortex and the cerebellum of *Jnk1-/-* mice, the dendritic complexity was increased and the dendritic length was reduced in comparison to the wild type mice. The isolated cerebellar granule neurons from *Jnk1-/-* mice also show evidence of shorter dendrites and more primary dendrites (Coffey *et al.* 2000; Bjorkblom *et al.* 2005). In contrast, the genetic ablation of JNK1 does not affect the axon formation during initial embryonic development, the corpus callosum and anterior commissures appear normal. However, after postnatal day 6, the anterior commissure tract degenerates with progressive loss of microtubules in axons and dendrites (Chang *et al.*

2003). Moreover, the neurons lacking *jnk* isoforms also display signs of axonal hypertrophy. In addition, the genetic deletion of JNK pathway components creates axonal abnormalities (Xu *et al.* 2011). For example, JIP3-deficient mice display defects in axon guidance (Ha *et al.* 2005). Taken together, these studies demonstrate the role of JNK1 in maintaining the axonal trajectories. The mechanism by which JNK maintains the neuronal architecture has been attributed to both neuronal and cytoskeletal substrates. One mechanism is via the phosphorylation of MAPs (microtubule associated proteins), such as MAP1b, MAP2, SCG10 (superior cervical ganglion 10) and DCX (doublecortin) (Chang *et al.* 2003; Bjorkblom *et al.* 2005; Tararuk *et al.* 2006; Westerlund *et al.* 2011). MAPs are known to bind microtubules and modulate their stability. The phosphorylation of these MAPs by kinases such as JNK, regulates their binding to the microtubules. Phosphorylation of MAP1B, MAP2 and SCG10 is reduced in *Jnk1-/-* mice. Moreover, the JNK-site phosphomimetic mutants of MAP2 and SCG10 restore the normal phenotype in brains of *Jnk1-/-* embryos (Westerlund *et al.* 2011; Komulainen *et al.* 2014). Overall, JNK is an important kinase that regulates the critical events during brain development.

In the adult brain, JNK is reported to regulate synaptic plasticity. Synaptic plasticity forms the fundamental basis of learning and memory. It is a biological process that results in the change in synaptic strength in response to stimuli. The vital events that modulate synaptic plasticity involve NMDAR activation (NMDA glutamate receptors), calcium influx and AMPARs recruitment. NMDARs are known to activate JNKs (Mukherjee *et al.* 1999), and also the lack of JIPs has been suggested to affect NMDAR function (Kennedy *et al.* 2007). Moreover, JNKs phosphorylate PSD95 (Kim *et al.* 2007) and GluA2 (AMPAR subunit) (Ahn & Choe 2010) proteins, which are important regulators of synaptic plasticity. In addition, the absence of LTD has also been reported in *Jnk1-/-* mice and JNK inhibitor treated rats. Another study has shown JNK2 is required for late phase LTP (Chen *et al.* 2005). Indeed, the importance of JNK in maintaining synaptic plasticity is evident, but its targets need to be elucidated. To gain further understanding of the role of JNK in the adult brain the work presented in this thesis has addressed issues of the motor functions and emotional functions in *Jnk1-/-*mice (Study II and III).

## 4.2. Role of JNK in regulating neuronal cytoskeleton

The cytoskeleton is a structural element that defines eukaryotic cells, and neurons are very specialized eukaryotic cells that extend neural processes to form synaptic connections in the central nervous system. Like other eukaryotic cells, neurons comprise of three main polymers: microtubules, actin filaments, and intermediate filaments. Each polymer has a distinc-

tive composition, structure and organization that may be further specialized in the particular cell type (Kirkpatrick & Brady 1999).

Neuronal microtubules are quite similar in their basic structure to the ones found in eukaryotes. The core of microtubules is a polymer of tubulin subunits. The alpha and beta-tubulin heterodimers align on their ends to form protofilaments. Thirteen of these protofilaments join laterally to form a rod-like microtubule structure. These are quite dynamic, and are able to undergo repetitive cycles of growth and disassembly, which is controlled by the GTPase activity of tubulin dimers. The tubulin dimers are added at the 'plus' end where β-tubulin is exposed due to GTP hydrolysis. The opposite end, which grows more slowly, is called the 'minus' end. The balance between assembly and disassembly at both ends defines the net growth in free microtubules. In the case of non-neuronal cells, the minus end is tethered to a nucleation site, which is associated with the microtubule-organizing center (MTOC). The anchoring and nucleation help to define the polarity of microtubules.

However, in the brain the pool of microtubules is composed of a strikingly more diverse range of components. These include a variety of tubulin isotypes, for example, some neuron specific class III and IVa isotypes, many post-translational tubulin modifications, and various microtubule-associated proteins. The brain microtubules also differ in their organization according to their location, which enables them to perform designated tasks in a particular environment. For example, they differ between axons and dendrites by at least two aspects. The axonal microtubules exhibit uniform polarity, with their plus ends extending distal to the cell body, whereas dendrite microtubules display mixed polarity. In addition, they also differ in the composition of the microtubule-associated proteins, for example, MAP2 is primarily found in dendrites whereas tau is present in axons (Conde & Caceres 2009). The brain microtubules exhibit a variety of different post-translational modifications (e.g. phosphorylation, acetylation, palmitoylation, polyglutamylation, polyglycylation and detyrosination), which affect the stability of the microtubule structure (Fukushima *et al.* 2009). In addition, there are some small molecules, such as taxol, epothilones, discodermolide, that stabilize the microtubule assembly, while others, such as colchicine and vinblastine, destabilize it (Stanton *et al.* 2011).

The actin cytoskeleton is more diverse in composition and organization. Actin microfilaments are made of actin monomers, which get twisted around each other to appear like a string of pearls. Actin filaments are distributed throughout neurons and glia, and are particularly enriched in the presynaptic terminals, dendritic spines and growth cones. They are most apparent along the membrane and axonal microtubules as a meshwork of short oligomers. Actin filaments are also highly dynamic, under-

going continuous polymerization and depolymerization. The hydrolysis of ATP mediates the switch between the monomeric G-actin form and filamentous F-actin form. The F-actin polymers also display structural polarity with pointed (minus) and barbed (plus) ends. The ATP bound G-actin monomers are added at the barbed end to maintain the dynamic growth, whereas the ADP bound monomers detach from the pointed end. This treadmilling process of actin filament polymerization and depolymerization is the basis of filopodia and lamellipodia which are required for such processes as adhesion, motility, endocytosis and cell migration.

Another cytoskeletal element that is found throughout the cell body is the intermediate filament. This is a large family of proteins classified into five groups and expressed in a wide range of organisms from invertebrates to mammals. In neurons, this set of filaments is known as neurofilaments, which consist of three subunits, high, medium and low, according to their molecular weight. They are expressed by a separate set of genes and classified under group IV intermediate filaments along with nestin and synemin. Some of the brain related intermediate filaments also belong to class III, such as vimentin, periphrin, and GFAP. Neurofilaments play an important role in maintaining cellular and axonal volume and are suggested to provide mechanical strength and stabilize cytoskeletal framework to eukaryotic cells (Yuan *et al.* 2012).

### 4.2.1. JNK regulates microtubule dynamics via Microtubule associated protein 2 (MAP2)

A large body of evidence suggests that JNK1 is directly involved in the maintenance of microtubule integrity in axons and dendrites during development and aging of the nervous system. It has been suggested that JNK1 regulates microtubule dynamics by phosphorylating MAP2. MAP2 belongs to a family of microtubule stabilizing proteins that are efficiently phosphorylated by several protein kinases, including MAP kinases. MAP2 is directly phosphorylated by JNK1 *in vitro* (Kyriakis & Avruch 1990) and *in vivo* (Bjorkblom *et al.* 2005). Its isoform, JNK2, shares 83% sequence homology with JNK1, but does not phosphorylate MAP2 as efficiently as JNK1 (Chang *et al.* 2003). In comparison to other MAP kinases, JNK was more efficient than p38 or ERK in phosphorylating MAP2. JNK1 is expressed ubiquitously throughout the body. However, its microtubule assembly function appears to be specific to neurons, which might be because MAP2 is primarily enriched in dendritic compartments of the neuronal subpopulation. Moreover, these two proteins colocalize in the soma-to-dendritic compartments of neurons. Further, it has been demonstrated that the cytoplasmic pool of JNK is regulating neurite outgrowth by phosphorylating MAP2 (Bjorkblom *et al.* 2005). *Björkblom et al.* have also shown that activation of JNK increased the specific phosphorylation of the Thr-Pro motif in the C-terminal of MAP2 by many fold.

In *Jnk1-/-* mice, MAP2 phosphorylation is reduced on the proline-rich domain PRD residues, which have been suggested as the phosphorylation sites of JNK1 (Bjorkblom *et al.* 2005). These *Jnk1-/-* mice also displayed a significantly increased level of immunoreactivity to the antibody raised to detect dephosphorylated MAP2 in Thr 1620 and 1623 residues of the PRD region (Bjorkblom *et al.* 2005).

### 4.2.2. JNK regulates actin dynamics via Myristoylated alanine-rich C-kinase substrate like protein (MARCKSL1)

MARCKSL1, (also known as MacMARCKS and MRP) along with its homolog MARCKS, belongs to a distinct family of protein kinase C substrates (Aderem 1992). It has been implicated in such processes as cell spreading and exocytosis. MARCKSL1 and MARCKS are both known to interact with the plasma membrane and bind actin. Both proteins are similar in many other aspects, e.g., they are acidic, amphiphilic, have conserved N-terminal domains with a myristoylation recognition site responsible for membrane attachment, and have a central basic effector domain (ED) for facilitating actin and Ca+/calmodulin binding. However, the protein MARCKSL1 is 30 % smaller than MARCKS, and they differ in their affinity towards the plasma membrane. It has been suggested that MARCKSL1 is always bound to the plasma membrane after phosphorylation (Verghese *et al.* 1994; van den Bout *et al.* 2008). Their expression patterns also differ. MARCKSL1 is expressed prominently in the brain and reproductive tissues, whereas MARCKS is omnipresent (McNamara & Lenox 2000). MARCKSL1 plays a critical role during the development of the nervous system, as was illustrated in MARCKSL1 knockout animals, which presented a range of neural tube defects, including exencephaly and spina bifida, resembling *Jnk1-/- Jnk2-/-* mice phenotype. These studies suggest that MARCKSL1 knockouts might be a model to understand the underlying mechanism of neural tube defects. The neural tube closure is basically mediated by events that are highly dependent on the coordinated control of actin dynamics.

The MARCKS family proteins regulate actin bundling through the ED, which contains the actin binding site (Wohnsland *et al.* 2000). PKC phosphorylates MARCKS on serine 152, 156 and 163 and MARCKSL1 on ser93 and ser104 in the ED, and modulates their interaction with actin. MARCKS has been studied broadly for its role in actin bundling. The actin binding and polymerization is disrupted when the ED of MARCKS is phosphorylated by PKC or upon Ca+/calmodulin binding. The phosphorylation leads to conformational changes, which detach MARCKS from the plasma membrane and also inhibit cross-linking with the actin cytoskeleton. It has also been shown that a peptide containing the MARCKSL1 ED cross linked the actin cytoskeleton and increased polymerization and bundling *in vitro* while the full length intact MARCKSL1 did not affect the

actin polymerization (Wohnsland *et al.* 2000; Arbuzova *et al.* 2002). This discrepancy suggests that intact MARCKSL1 has to undergo another level of regulation, such as conformational change or proteolytic cleavage, to make the ED accessible.

There is still much to learn about the role of MARCKS family proteins in the regulation of actin bundling in cells. It has been proposed that the actin regulating potential of MARCKS and MARCKSL1 is a secondary effect of their capability of sequestering acidic phospholipids at the membrane. Basically, the hypothesis suggests that upon PKC phosphorylation MARCKS is translocated to the cytosol and exposes other actin regulating proteins to acidic phospholipids, such as PIP2 (Arbuzova *et al.* 2002). However, MARCKSL1 does not translocate to the cytosol upon PKC mediated phosphorylation (Chen *et al.* 1996a), so the proposed hypothesis seems to be incorrect for MARCKSL1. In the work presented as Study I of this thesis, the interactions of MARCKSL1 and actin filaments were investigated in further details.

### 4.3. Stress and JNK signaling

JNK signaling has been implicated in various stress responses. Stress is the most potent inducer of JNK. Such forms of stress as heat shock, UV radiation, inflammatory cytokines, brain injury and ischemic stress activate JNK pathways. A large body of evidence has indicated that JNK signaling is required or involved in stress related responses (Devary *et al.* 1992; Derijard *et al.* 1994; Kyriakis *et al.* 1994; Ferrer *et al.* 1996). For instance, heat shock and anisomysin (a protein synthesis inhibitor) treatment of the cells leads to activation of JNK and phosphorylation of heat shock factor 1 (HSF1). In contrast, the inhibition of JNK reduced the phosphorylation of HSF1 after heat shock and anisomysin treatment (Park & Liu 2001). Similarly, UV induced apoptosis is also mediated by JNK activation. The mechanism underlying JNK activation has not yet been well elucidated, but overexpression of dominant negative JNK blocked the UV-induced apoptosis (Chen *et al.* 1996b). However, these studies did provide some insight by suggesting that the membrane related components and oxidative damage involved in UV-induced JNK activation. Also, some mitochondrial related cellular sensors might play a role in the heat shock induced JNK activation. In addition, the combination of environmental stress and inflammatory cytokines of the TNF (tumor necrosis factor) superfamily strongly activate the JNK pathway (Kyriakis & Avruch 2001). The JNK signaling pathway has therefore been considered as a potent target for novel anti-inflammatory drugs.

Brain injuries, such as stroke, traumatic brain injury, ischemic brain injury, that occur in living organisms and can be neurodegenerative, can

cause massive brain damage. Ischemic brain injury has been correlated with activation of JNK signaling. *In vivo* models of global ischemia have shown activation of JNK and other MAP kinases, including p38 and ERK within 5 min in CA1 and CA3 of the hippocampus. The activity of JNK and p38 was enhanced 72 hrs after reperfusion (Sugino *et al.* 2000), while the JNK inhibition in a transient model reduced apoptosis in the CA1 region of the hippocampus. Similar observations were made in another model of transient focal cerebral ischemia, where 60 min of cerebral occlusion of middle artery induced JNK activity while inhibition of JNK using SP600125 inhibitor reduced apoptotic death (Guan *et al.* 2005). JNK suppression has been considered as the prominent choice for treatment in stroke. The inhibition of JNK using a D-retroinverso peptide inhibitor (DJNKI-1) in an *in vivo* transient stroke model has led to significant reduction in the size of the lesion. Moreover, this reduction of lesion size also improved the animal behavior (Borsello *et al.* 2003). Additionally, Borsello *et al.*, showed that inhibition of JNK conferred protection against the excitotoxic stress induced by the NMDA administration in cortical cells. These results implicate the critical role of JNK signaling in excitotoxic and ischemic stress.

Recently, other forms of stress, such as psychological stress or physical stress, which are more relevant to psychological disorders, have been shown to induce JNK activation. Studies have shown that forced swim stress, which involved both physical as well as psychological distress, activates MKK4 and JNK. The activation of both kinases has been observed in different brain regions, including the hippocampus, hypothalamus, and amygdala. Another paradigm of restraint stress with less physical and more psychological stress components also induced activation of MKK4 and JNK in the mouse brain (Liu *et al.* 2004). More recently, Galeotti & Ghelardini demonstrated regionally differentiated activation of JNK along with other MAPK signaling in the hippocampus and the prefrontal cortex after forced swim stress and tail suspension stress paradigms (Galeotti & Ghelardini 2012). In addition, chronic isolation stress paradigm in rats compromised JNK signaling in the hippocampus and the prefrontal cortex (Filipovic *et al.* 2012). These studies clearly point out that anomalous activation of JNK signaling might be one of the underlying mechanisms in the pathogenesis of stress related disorders like anxiety and depression.

### 4.4. JNK in neuropsychiatric disorders

Neuropsychiatric disorders are a constant challenge for neurobiologists. With the advent of new technology, the psychiatric conditions have been associated with specific brain structures and some genetic abnormalities. Human genetic studies have revealed genes associated with JNK signaling that confer susceptibility to schizophrenia, intellectual disability and au-

tism spectrum disorders (Shoichet *et al.* 2006; Weiss *et al.* 2008; Kunde *et al.* 2013). A truncation in JNK3 has been found in patients with intellectual disability and seizures. It has been claimed that this truncation reduced the JNK signaling which results in deleterious effects on neuronal function (Kunde *et al.* 2013). A genetic association study identified two SNPs (single nucleotide polymorphisms) in the MKK7 (kinase directly upstream of JNK) that confer genetic susceptibility to schizophrenia. One of the polymorphisms was found in the 3´untranslated region of the MKK7 and is required for its targeting into the growth cones. Moreover, decreased expression of MKK7 was also observed in the prefrontal cortex of post-mortem brains of patients suffering from schizophrenia (Winchester *et al.* 2012). This suggests that reduced JNK signaling may be responsible for some of the neurochemical anomalies and core symptoms of the disorder.

Dendrite and spine structure anomalies are the hallmark of neuropsychiatric disorders. Recently, the microdeletion and microduplication of chromosomal region 16p11.2 have been associated with autism spectrum disorders (Weiss *et al.* 2008). The protein TAOK2 (thousand and one amino acid 2), is located on chromosome 16p11.2 and regulates basal dendrite formation in the mouse cortex. Downregulation of TAOK2 impairs the formation of basal dendrites (Weiss *et al.* 2008). Moreover, TAOK2 is reported to activate the JNK family kinases to modulate the basal dendrites in the cortex (de Anda *et al.* 2012). A study of the gene encoding IL1RAPL1 (interleukin-1 receptor accessory protein-like 1), provides an additional clue that JNK signaling may play a key role in the psychiatric disorders. A mutation in the IL1RAPL1 protein has been associated with intellectual disability. Recently, this mutation has been found to impair the synaptogenesis (Ramos-Brossier *et al.* 2015). In addition, the loss of this gene reduced the number of excitatory synapses as well as lead to defects in long term synaptic plasticity. Interestingly, the IL1RAPL1 mediates its action through JNK activation (Pavlowsky *et al.* 2010). Altogether, these findings highlight the relevance of JNK signaling in psychiatric disorders.

## 4.5. The JNK pathway as a therapeutic target

Due to the critical role of JNK in regulating events commonly associated with the pathogenesis of many human diseases, JNK inhibitors have gained considerable interest as therapeutics. In the last few decades, high throughput screening of chemical libraries for kinase activity and structure based drug design has revealed several ATP-competitive JNK inhibitors. The determination of X-ray structures of the MAPK family proteins also provided new approaches for designing potent and selective inhibitors of JNKs. The pyrazoloanthrone derivative SP600125 was the first

small molecule JNK inhibitor reported. SP600125 has been tested in animal models of stroke, where it showed neuroprotective effects, even when administered one hour after global transient ischemia (Guan *et al.* 2005). It also improved the functional recovery of mice subjected to spinal cord injury (Yoshimura *et al.* 2011). However, the specificity of SP600125 has been questioned. When SP600125 was tested under different conditions for a broad range of kinases, it was found to inhibit several other kinases with similar or greater potency (Bain *et al.* 2003).

Another chemical compound that has been reported is CEP-1347, which is a semi-synthetic MLK inhibitor. It is derived from the naturally occurring K52a and blocks the upstream components of the JNK pathway. CEP-1347 prevented neuronal death in *in vivo* models of Parkinsons and Alzheimer's disease (Wang *et al.* 2004; Silva *et al.* 2005). Recently, thiophene and aminopyridine based JNK inhibitors have been developed with enhanced specificity, selectivity and pharmacokinetics (Szczepankiewicz *et al.* 2006; Hom *et al.* 2010; Bowers *et al.* 2011). These chemical inhibitors showed increased potency in the nanomolar range and suitable pharmacokinetics for *in vivo* use. The pyridine based compounds showed a 1000 fold higher selectivity for the JNK1 and JNK2 isoforms than other MAP kinases *(*Szczepankiewicz *et al.* 2006*)*. The aminopyridine inhibitors are JNK3 specific and demonstrate good CNS permeability as well as bioavailability. However, the JNK inhibitors that are not isoform specific showed most neuroprotective effects *in vivo* (Graczyk 2013).

The ATP-competitive inhibitors have the potential disadvantage that their efficacy is reduced under conditions of high levels of endogenous ATP, as has been reported for SP600125 (Bennett *et al.* 2001). Therefore, alternative inhibitors have been developed to target the peptide substrate binding sites to inhibit the kinases. DJNKI-1 is one such peptide inhibitor (Bonny *et al.* 2001) that blocks the access of kinase to its targets, by causing an allosteric modulation in the JNK kinase. It prevents the protein-protein interaction between kinase and substrate without affecting the activity of kinase. It is made by linking 20 amino acid sequence from JIP1 (JNK inhibitory scaffold protein) to the 10 amino acid TAT peptide sequence (HIV-transport sequence). The JIP1 and JNK targets, including c-jun share the same binding motif, but JNK has a 100 fold higher binding affinity for JIP1 than for c-jun, which is the reason why this peptide blocks the access of the other substrates. The TAT delivery sequence helped in penetrating the membrane and D-amino acids confer stability to the peptide. DJNKI-1 confers strong neuroprotection in both *in vitro* and *in vivo* models. It completely inhibited neuronal death in cortical neurons, as well as in organotypic tissue cultures after excitotoxic stress with NMDA. In rodent models of ischemia, the peptide showed strong protection. In a transient ischemia model, the intracerebroventricular injection of DJNKI-1 after 6 hours of occlusion reduced the infarct volume significantly. Intraper-

itoneal and intravenous injection of the peptide after 6 hours in the permanent middle cerebral artery occlusion model (MCAO) also showed a significant reduction in infarct volume (Borsello *et al.* 2003; Wiegler *et al.* 2008). At the present, the only approved therapy for ischemic stroke is tissue plasminogen activator (tPA), which needs to be administered within 3 hours of onset of ischemia. Therefore, the protection observed after 6 hours following the onset of stroke makes DJNKI-1 a promising therapeutic option for treatment of stroke.

# Objectives

Architectural defects in the dendrites and altered distribution of spines have been reported in association with anxiety disorders. Recently, adult hippocampal neurogenesis has achieved considerable attention in the pathophysiology and treatment of anxiety disorders. Also, although the current parmacotherapies offer relief from the symptoms of these disorders, they do not reverse the underlying changes.

JNK signaling has been studied in both the physiological and pathological context, and JNKs have been shown to be involved in the maintenance of axodendritic architecture and have a role in neuronal death induced by cellular stress. Recently, human genetic data has shown that genes involved in the JNK pathway are deregulated in various neuropsychiatric disorders.

The role of JNK signaling has also been indicated, although not studied, in the context of mood disorders. Therefore, the main focus of the work presented in this thesis was to directly address whether JNK signaling regulates the structural and neurogenic changes associated with anxiety disorders.
This thesis focused on the following research questions:

- To validate that JNK signaling regulates anxiety behavior, (based on findings in a pilot study) by i) performing anxiety behavioral tests on *Jnk1-/-*mice and, ii) measuring anxiety in mice treated with a JNK inhibitor.
- To elucidate if JNK controls adult hippocampal neurogenesis, which is associated with anxiety behavior in mice.
- To investigate if JNK phosphorylation of actin bundling protein MARCKSL1 affects the migration of neurons
- To test whether the JNK1 alteration of dendrite architecture in the motor cortex correlates with motor behavior defects in *Jnk1-/-* mice.
- To investigate the role of JNK in regulating dendritic spines and to screen for potential downstream substrates.

In particular, the results from these experiments provide a deeper understanding of JNK signaling in anxiety-related behavior. These findings suggest that JNK is a major player in regulating the neurogenic changes associated with anxiety disorders. Moreover, the results also shed light on the role of JNK signaling in determining the dendrite and spine architecture, which are also impaired in anxiety related behavior.

# Experimental Procedures

The experimental material and methods are discussed briefly in this section. More details concerning the specific experiment are described in the publications and manuscripts (1-IV)

## 1. Materials

### 1.1. Plasmids

iMARCKSL1 wild type, iMARCKSL1$^{S120A,T148A,T183A}$, and iMARCKSL1 $^{S120D,T148D,T183D}$ insensitive mutants were prepared by insertional overlap into p1xCherry-N1(gift from C.Verhey, Department of Cell and Developmental Biology, University of Michigan, USA) and pEGFP-N1 (Clonetech) for Study I.

The wild type HMW-MAP2, GFP-HMW-MAP2-$^{T1619D,T1623D,T1625D}$, or GFP-HMW-MAP2-$^{T1619A,T1623A,T1625A}$ were prepared using the strategy described in more detail in Study II. For *in utero* electroporations, the CMV promoter was changed to a CAG promoter for better expression in the brain. pcDNA3-MKK7-JNK1, used in studies I and II, was a gift from Roger J Davis and pCMV from Harvey McMahon. The EGFP-DCX was bought from Clontech and EGFP-PSD95 was provided by Michael Courtney (A.I. Virtanen Institute, University of Eastern Finland, Kuopio) for Study IV. The EGFP-DCX-S334DT331D and PSD95-S295D mutants used were prepared in the lab using the mutagenesis protocol described in the in Study IV.

### 1.2. siRNAs and shRNAs

To silence MARCKSL1, two sequences (seq#5::-5´-GAACGGAACAGAUGA GGCA-3´ and seq#6::-5´GGCCUGUCCUUCAAGAGAA-3´) were used. One non-targeting (NT) shRNA was designed and cloned into the pGIPz lentivector (OpenBiosystems, Huntsville, AL-US) as described in Study I. The non-targeting shRNA and silencing shRNA for DCX used in Study IV were provided by Lo Turco, University of Connecticut, USA. The 5´-GCUAAUACCUAUCAAAU-3´non targeting and 5´UCUAGCUGCUAUCAU UGAA-3´siRNA sequences were used for SCG10 knockdown in neuronal cultures in Study IV.

### 1.3. Antibodies

List of antibodies used in the studies:

| Name | Source/Type | Supplier | Application |
|---|---|---|---|
| Phospho-JNK | Rabbit/polyclonal | Cell signaling | WB |
| pan-JNK | Rabbit/polyclonal | Upstate Biotechnology | WB |
| JNK1/2 | Mouse/monoclonal | Pharmingen | WB |
| Phospho PSD95 | Rabbit/polyclonal | Millipore | WB |
| PSD95 | Mouse/monoclonal | Millipore | WB |
| DCX | Rabbit/polyclonal | Cell Signaling | WB, IHC |
| BrdU | Mouse/monoclonal | Thermoscientific | IHC |
| Ki67 | Rabbit/polyclonal | Novacastra | IHC |
| $p27^{kip1}$ | Mouse/monoclonal | BD Biosciences | WB,IHC |
| SOX2 | Goat/polyclonal | R&D systems | IHC |
| GFAP | Rabbit/polyclonal | DAKO | IHC |
| MARCKSL1 | Rabbit/polyclonal | Coffey lab production | WB, IHC |
| MRPL11 | Rabbit/Polyclonal | Proteintech | WB,IHC |
| MAP2 | Mouse/monoclonal | Leinco Technologies | WB |
| SCG10 | Rabbit/polyclonal | Coffey lab production | IHC |
| β-Tubulin | Mouse/monoclonal | Millipore | WB |

WB: Western Blotting, IHC: Immunohistochemistry

### 1.4. Special reagents

Bromodeoxyurdine (BrdU) and Cytarabine (Ara-C) were purchased from Sigma. The peptide JNK inhibitor DJNKI-1 was ordered from GeneCust (Laboratoire de Biotechnologie du Luxembourg, SA). The JNK kinase inhibitor SP600125 was purchased from Calbiochem. Hoechst 33342 dye to stain nuclei and mounting medium Mowiol was ordered from Hoechst Marion Roussel (Frankfurt, Germany). Alexa 488 and 568 dyes were from Molecular probes. MARCKSL1 siRNA oligonucleotides were synthesized by MWG-Biotech AG.

## 2. Methods

**i) Cell cultures:** The human prostate cancer (PC-3) and COS-7 cell lines were used. The culturing and transfection methods are described in detail in the studies I and II.

The primary cultures of hippocampal and cortical cells were prepared from the newly born (P0) Sprague-Dawley pups for studies I and IV. The procedure for neuronal culture and transfection has been followed according to the previous studies (Tararuk *et al.* 2006), which involves dissection of hippocampus and cortices from P0 brains in the dissociation medium and maintenance in respective growth mediums.

**ii). Immunoblotting and quantification**: Study I, II and III.
**iii). Immunostaining and Immunohistochemistry**: Study I, II, III and IV.
**iv). Migration assays:** i) Transwell assay and ii) Wound healing assay are used in study I.

## 2.1. Animal experiments

The procedures for animal experiments were approved by the National Animal Experiment Board (ELLA) and the guidelines from Finnish (62/2006 Act and 36/2006) and European (86/609/EEC, 2010/63/EU, 1986/ETS 123) directives were strictly followed. C57BL/6J wild type and *Jnk1-/-* mice were used in these studies. The *Jnk1-/-* mice and C57BL/6J were a gift from Tuula Kallunki (Sabapathy *et al.* 2001) and were maintained as heterozygote colonies in the animal facility of the University of Turku. For stereotactic surgeries the wild type C57BL/6J were purchased from the commercial vendors, including Harlan Netherlands and Charles River Germany. All animals were group housed (except for the ones that had surgery), provided food and water *ad libitum*, and maintained on a 12hr light-dark cycle.

**i). Mouse anatomical measurements**: described in Study II
**ii). Mouse Perfusions**: described in Study II
**iii). Sholl analysis**: described in Study II
**iv). In vivo labeling of newborn cells with BrdU***:* described in Study III

### 2.1.1. Stereotactic surgery in mice

Stereotactic surgeries were performed for drug treatments in Study III. Animals were aneasthetized with isofluorane (4% for induction and 2% for surgery), and placed in a stereotactic device. A local anesthesia was given (0.1ml of 1 mg/1 µg lidocaine) subcutaneously around the incision point before initiating the surgery. The top of the head was shaved and an incision was made along the midline, the scalp was retracted and the area surrounding the bregma cleaned and dried. Thereafter, the cranium was drilled using a dentist's drill to generate holes in defined coordinates according to the mouse brain atlas (Paxinos G 2008).

### 2.1.2. Intracerebroventricular injections

For the short-term intracerebroventricular administration of compounds, a cannula (Agnthos) was implanted (anterior-posterior -0.6mm, medial lateral 1.5mm, dorsoventral -2.0mm to the lateral ventricle). The additional anchorage was provided via dental cement (Agnthos) and screws in the additional hole drilled closed to the cannulation site. After surgery the mice were single housed in the cages to avoid damage to the cannulas that might be caused by other inmates. After seven days of surgery, 2µl of JNK inhibitor or vehicle was injected at a rate of 0.25µl/min using CMA microdialysis for acute treatments.

### 2.1.3. Implantation of osmotic minipumps

For long term intracranial administration of the compounds (JNK inhibitor, Ara-C and vehicle) a small miniosmotic pump (Alzet model 2006, Cupertino, CA) was inserted subcutaneously at the back and attached to the brain infusion cannula implanted intra-cerebroventrically, according to the above coordinates. The screws were not used with the miniosmotic pumps. The additional support was provided by gluing the cannula to the skull using a small amount of dental cement. The incision point was sutured with absorbable silk. After surgery the mice were returned back to the cage and housed in isolation. Further care was taken by injecting saline and analgesics after 12 hrs of the surgery. The mini-pumps injected inhibitor at a rate of 0.15µl/hr for six weeks after which the mice were subjected to behavioral testing.

### 2.1.4. Animal Behavioral tests

In studies II and III, behavioral tests were performed to measure the motor coordination and anxiety, respectively. Here is a list of tests used in the thesis, more detail of the specific tests can be found in studies II and III. For all these procedures the observers were unaware of the treatments or genotypes.

***i) Suspended bar test:*** described in Study II
***ii) Beam test:*** described in Study II
***iii) Foot print analysis:*** described in Study II
***iv) Elevated plus maze:*** described in Study III
***v) Light-dark test:*** described in Study III
***vi) Open field test:*** described in Study III

## 2.2. Stereology

Stereology provides information about the 3-D structure by making measurements on the 2-D planar sections of tissues. It has been widely recognized as one of the best practices in biological research for quantifying different biological features. A stereological-based study design was followed in order to quantify the total number of adult born neurons and for measuring the volumetric changes in Study III.

### 2.2.1. Optical fractionator method for quantifying adult neurogenesis

The optical fractionator was used to quantify adult born neurons labeled with DCX, Ki67 and BrdU in Study III (West *et al.* 1991; Gould *et al.* 1999). The major considerations of the stereology method were to avoid counting labeled cells twice and to be consistent with the region in each section. Every fifth section throughout the rostral-caudal extent of the hippocampus (Bregma −1 to −4) (Paxinos G 2008) was processed for BrdU (Malberg *et al.* 2000) and Ki67 immunohistochemistry. Then the immunopositive cells were counted using the optical fractionator workflow of the Stereoinvestigator (MBF Biosciences). The tissue thickness was measured at five regions on a single slice in order to account for the shrinkage due to tissue processing. The final calculation was made according to the stereological equation.

$$(N=(1/tsf \times 1/ssf \times 1/asf) \times \Sigma Q)$$

where ΣQ is total number of cells counted using dissector in optical fractionator, tsf is the thickness sampling factor, ssf is the section sampling factor, and asf is the area sampling factor. The workflow of the optical fractionator takes into account these parameters and provides the final number N.

A modified unbiased stereology protocol was used to quantify the BrdU labeled cells in the dentate gyrus. To make BrdU a sufficient neural progenitor marker, the likelihood of over representation was reduced by limiting the quantification of +ve cells to the inner side of granular cell layer corresponding to SGZ of DG. On account of the small number of cells, exhaustive counting was performed for BrdU, which ruled out the area sampling factor from the equation.

For the phenotypic analysis of BrdU-labeled cells, every fifth section was selected from the dentate gyrus of animals and subjected to double immunolabeling for the BrdU and the neuronal phenotypic marker NeuN. The BrdU-positive cells were first identified and then checked by confocal microscopy for an overlapping fluorescent signal from NeuN (Zeiss LSM780). The images were sampled through a Z-series multitrack mode

with emission filters chosen to diminish the bleed-through from the fluorescent probes. This approach has been used in previous studies for the analysis of the phenotype of BrdU-labeled cells (Malberg *et al.* 2000; Madsen *et al.* 2005).

The maturity index of newborn neurons was also assessed using the optical fractionator. The DCX+ve cells were categorized into DCX+ve cells and DCX+cells with dendrites. During counting, the DCX+ve cells with dendritic branching were marked separately in the dissector to account for mature neurons. The branching was analyzed throughout the tissue thickness. The total numbers were quantified using the stereological parameters described above. The count of total DCX cells with dendrites provides an indication of the maturity of the neurons. A maturity index was calculated by dividing the former value by the total number of DCX+ve neurons in the dentate gyrus.

### 2.2.2. Volumetric measurements using Cavalieri method

The volumetric estimations of the dentate gyrus of wild type and *Jnk1-/-* mice were made using the Cavalieri method in Study III. The sections were stained with Cresyl violet in the same stereological manner with an interval of every fifth section. The dentate gyrus was traced in each section by placing contours with the Steroinvestigator software (MBF Bioscience). After tracing the dentate gyrus in all the slices, the set of slices of each animal was subjected to the Cavalieri workflow of volumetric analysis. The final numbers were averaged to estimate the total volumetric differences between the genotypes.

## 2.3. Statistical analysis

Analysis of variance (ANOVA) was used to analyze the dendritic architecture data. The Levene´s test has been performed to check the distribution of the data which was followed by one-way ANOVA or Student t-test to determine the significance levels in all the studies. In every study, the significance levels are indicated as follows: *$p<0.05$, **$p<0.01$, ***$p<0.001$.

# Results and Discussion

## 1. JNK phosphorylation of MARCKSL1 regulates actin stability and migration in neurons (Study I)

The role of JNK signaling in various physiological processes has been extensively reviewed in this thesis. There is convincing literature available which indicates that JNK activity mediates migration in many cell types including glioblastoma, epithelial and fibroblast cells (Johnson & Nakamura 2007). Moreover, JNK is also involved in regulation of neuronal migration during brain development (Westerlund *et al.* 2011). In the work presented in this thesis, MARCKSL1 was identified as a new JNK substrate affecting actin dynamics and migration.

### 1.1. MARCKSL1 identified as a novel substrate of JNK and its phosphorylation induces actin bundling and actin stabilization

A 40 kDa protein MARCKSL1 was identified as a novel physiological substrate of JNK by screening brain lysates using an *in vitro* phosphorylation assay. All the JNK isoforms phosphorylated MARCKSL1 *in vitro*, but phosphorylation was not detected with the other MAP kinases (Fig 1B, Study I). Three specific JNK phosphorylation sites (S120, T148, and T183) were found on the C-terminus by MS/MS sequencing of phospho-enriched MARCKSL1 (Fig.1D, Study I).

The MARCKS family proteins are known to bind actin filaments and induce actin bundling through their effector domain (Arbuzova *et al.* 2002). The isolated ED of MARCKSL1 also induces actin polymerization *in vitro*, but the full length intact MARCKSL1 does not (Wohnsland *et al.* 2000). It has been speculated that additional posttranslational modifications of MARCKSL1 may be required for actin binding. The functional consequences of JNK phosphorylation of MARCKSL1 on actin dynamics were tested. All the JNK sites on MARCKSL1 were mutated to aspartate to mimic phosphorylation. This phosphomimetic mutation of MARCKSL1 (MARCKSL1$^{S120D,T148D,T183D}$) resulted in the delayed initiation of the actin polymerization, as observed with the actin modifier cofilin (Fig.2A, Study I). On the contrary, the wild type MARCKSL1 and its alanine mutant (MARCKSL1$^{S120A,T148A,T183A}$) had no effect on actin polymerization. Further, by transmission electron microscopy, we discovered that the phosphomimetic mutant stimulated actin filament bundling. A dense and complex actin mesh-work with an increased thickness (up to 100nm in some areas) was observed in the presence of the MARCKSL1$^{S120D,T148D,T183D}$ mutant

(Fig.2B, Study I). These results indicated that JNK phosphorylation of MARCKSL1 induces actin bundling and delays growth of actin filament.

In addition, we also observed that wild type, alanine and aspartate mutants of MARCKSL1 bound to filamentous actin (F-actin) but not to monomeric actin (G-actin) (Fig.2C and 2D, Study I). Even though all the variants of MARCKSL1 bound to filamentous actin, only the phosphomimetic mutant of MARCKSL1 was capable of inducing actin polymerization.

Due to lack of crystallographic data, how MARCKSL1 facilitates actin bundling is not known, but our molecular modeling indicates that the JNK phosphorylation sites are surface accessible and surround the ED (Fig.S3 Supplemental material, Study I). Phosphorylation introduces a negative charge, which leads to a conformational change and exposes the ED to facilitate actin bundling via a mechanism similar to that explained by Tang & Janmey (Tang & Janmey 1998).

To evaluate if JNK influences the actin dynamics by phosphorylating MARCKSL1 in living cells, we expressed MARCKSL1 mutants along with the fluorescently labeled actin (venus-actin) in the MEF (mouse embryonic fibroblast) cells and measured the actin turnover using FRAP (Fluorescence recovery after photobleaching) . With this technique, the venus actin rich regions were photobleached and the recovery of fluorescence measured. We observed a faster actin turnover with the MARCKSL1$^{S120A,T148A,T183A}$ mutant, whereas the delayed actin turnover observed with the MARCKSL1$^{S120D,T148D,T183D}$ mutant (Fig.3, Study I) indicated decreased actin dynamics, which is most likely due to actin bundling caused by the phosphomimetic mutant.

To further examine this, we used digitonin permeabilization to compare the digitonin soluble and insoluble actin as an indicator for actin stabilization. The phosphomimetic mutant consistently resulted in a three-fold increase in the insoluble actin (Fig.4A and 4B, Study I). Furthermore, we addressed whether active JNK stabilizes actin by expressing the active JNK chimera (MKK7-JNK1/2) with wildtype MARCKSL1. The levels of stable actin were subsequently found to be increased in the cells expressing wildtype MARCKSL1. However, active JNK was unable to alter the actin turnover in the cells lacking MARCKSL1 (Fig 4C and 4D, Study I). These results firmly indicate that JNK is regulating actin dynamics by modulating MARCKSL1.

## 1.2. The JNK phosphorylation of MARCKSL1 inhibits migration in neurons

Cell migration is an event driven by active remodeling of the actin cytoskeleton at the leading edge of the cells (Horwitz & Webb 2003).

MARCKSL1 has been implicated in macrophage cell spreading and found to be colocalized with actin in the lamellipodia (Li *et al.* 1996).

Moreover, we found that MARCKSL1 is predominantly expressed in the brain and enriched in the neurons isolated from cortex and hippocampus (Fig.6A, Study I). To identify the role of JNK phosphorylation of MARCKSL1 in neuronal migration a transwell migration assay was used. The JNK phosphomimetic mutant of MARCKSL1 reduced the migration of neurons relative to the alanine mutant in fibroblast cells, but in contrast the wildtype MARCKSL1 mirrored the MARCKSL1$^{S120D,T148D,T183D}$ phenotype in neurons. This effect is most likely due to the high level of JNK activity in neurons (Coffey 2014), which phosphorylated the wild type MARCKSL1.

Consistent with this, the MARCKSL1$^{S120D,T148D,T183D}$ mutant was able to rescue the migration phenotype where JNK activity was suppressed using a JNK inhibitor, SP600125 and NES-JBD, (Fig.6C and 6D, Study I). In addition, on the basis of its selective knockdown in neuronal cells, MARCKSL1 was shown to be a critical regulator of neuronal migration. This resulted in increased migration of neurons in transwells, however, it was notable that iMARCKSL1 (a mutant of MARCKSL1 insensitive to the siRNA5 designed to knockdown MARCKSL1) rescued the normal migration phenotype. Together these results highlight the key role of MARCKSL1 in neuronal migration, which is further regulated by JNK phosphorylation.

## 2. JNK regulates dendritic field, cell soma and influences fine motor coordination (Study II)

The dendritic architectural abnormalities are a common hallmark of the neuropsychiatric disorders. Although the genetic basis of these disorders is largely unknown, there are, however, some indications that JNK pathway genes are deregulated (Weiss *et al.* 2008; Winchester *et al.* 2012; Kunde *et al.* 2013). Moreover, it has previously been demonstrated that JNK controls dendritic architecture in cerebellar (Bjorkblom *et al.* 2005) and hippocampal (Rosso *et al.* 2005) neurons. In Study 2, dendritic architecture was characterized in *Jnk1-/-* mice, and the critical role of JNK phosphorylation of MAP2 in mediating dendritic changes was established. In addition, the altered motor functions in mice devoid of *Jnk1* were observed.

### 2.1. JNK phosphorylates the proline rich domain (PRD) of MAP2 and influences the formation of protrusions and binding of MAP2 to microtubules

Both JNK1 and JNK3 were found to efficiently phosphorylate high molecular weight MAP2 (HMW-MAP2), although for JNK1 this was with slightly

higher efficacy (Fig.1A, Study II). Tandem mass spectrometry analysis of enriched phosphopeptides revealed three JNK phosphorylation sites (T1619,T1622 and T1625) on the proline rich C-terminal domain of rat GST-HMW-MAP2 (Fig.1B, Study II).

In addition *in vivo* phosphorylation was characterized using a commercial antibody that detects the phosphorylated PRD of HMW-MAP2. The antibody only recognized the GFP-HMW-MAP2 and did not recognize the GFP-HMW-MAP2-$^{T1619A,T1623A,T1625A}$ (Fig.1E, Study II), indicating its specificity for JNK phosphorylated MAP2. Using this antibody it was shown that the phosphorylation of these sites is decreased on HMW-MAP2 in the hippocampus and cortex of immature *Jnk1-/-*mice brains (Fig.1F and 1G, Study II).

It has been shown in previous work from our lab that exogenous expression of GFP-HMW-MAP2 in COS-7 cells (which lack MAP2) induces protrusions from the soma (Bjorkblom *et al.* 2005). In the light of these earlier observations the influence of JNK phosphorylation on the formation of these protrusions was investigated (Study II). The expression of the JNK phosphomimetic mutant GFP-HMW-MAP2-$^{T1619D,T1623D,T1625D}$ led to an increase in the number of COS-7 cells with processes. Moreover, the expression of active JNK along with the wild type HMW-MAP2 increased the number of protrusions in cells (Fig.2A and 2B, Study II). Whereas, the cells expressing GFP-HMW-MAP2-$^{T1619A,T1623A,T1625A}$ were not able to generate protrusions, even after JNK activation (Fig. 2A-C, Study II), indicating that JNK phosphorylation of HMW-MAP2 is required for the outgrowth of these protrusions. As an interpretation of this, it is speculated that this phosphorylation by JNK1 is somehow mediating the strong interaction of HMW-MAP2 with microtubules and supporting protrusion growth. Earlier it has been observed that a general increase in the HMW-MAP2 phosphorylation results in its increased interaction with microtubules (Sanchez *et al.* 2000). To confirm whether JNK phosphorylation of MAP2 alters the microtubule binding, wild type HMW-MAP2, GFP-HMW-MAP2-$^{T1619D,T1623D,T1625D}$, or GFP-HMW-MAP2-$^{T1619A,T1623A,T1625A}$ mutants were expressed along with venus tubulin in fibroblasts. Cells were lysed in a TX-100 containing medium, and the TX-100 soluble and insoluble fractions were analyzed. Microtubules are known to settle in the insoluble fraction, and wild type HMW-MAP2 and GFP-HMW-MAP2-$^{T1619D,T1623D,T1625D}$ were found together with the microtubules in the TX-100 insoluble pellet whereas GFP-HMW-MAP2-$^{T1619A,T1623A,T1625A}$ was found in the soluble fraction (Fig.2D and 2E, Study II). Moreover, the GFP-HMW-MAP2-$^{T1619D,T1623D,T1625D}$ also increased the proportion of stable microtubules (polymerized tubulin) in the TX-100 insoluble fraction, while the GFP-HMW-MAP2-$^{T1619A,T1623A,T1625A}$ reduced this significantly (Fig.2F, Study II). These results confirm that JNK phosphorylation of HMW-MAP2 increases its binding to the microtubules, thereby leading to it inducing protrusions.

## 2.2. *Jnk1-/-*mice show altered dendritic architecture and cell soma in the primary motor cortex

Previously, our lab has demonstrated that dendritic architecture is regulated by JNK in cerebellar granular neurons and in the cerebellum, and also indicated some alteration in the dendritic structure of the motor cortex by Golgi-Cox staining (Bjorkblom *et al.* 2005; Tararuk *et al.* 2006). In Study II, dendritic morphology was investigated in greater detail by labeling neurons using Lucifer yellow in the primary motor cortex of adult wild type and *Jnk1-/-*mice. Sholl analysis was performed on the 3-dimensional confocal images traced in Neurolucida software to analyze the dendritic architecture. This method places concentric circles around the cell soma and allows the measurement of intersections, nodes and the length of each dendritic arbor. Only basal dendrites of pyramidal neurons were considered for analysis due to incomplete labeling in the apical dendrites. In *Jnk1-/-* mice reduced dendritic complexity was found in the neurons of layer 2/3 of primary motor cortex (residing in the rostral and caudal forelimb areas, medial agranular cortex AGm, and lateral agranular cortex of the mouse primary motor cortex, M1) with fewer intersections, nodes and reduced dendritic length (Fig.3B-G, Study II). In striking contrast, the layer 5 (L5) neurons of *Jnk1-/-*mice showed a highly complex dendritic architecture with an increased number of intersections, nodes and overall dendritic length (Fig.3H-L and 3N, Study II). The increase in L5 dendritic complexity may be due to the reduced number of synaptic inputs from the L2/3 layer. The opposite effects may be partly due to different cellular contexts and most likely the effects depend on the substrate composition in the layer. Regardless, the JNK1 seems critical in shaping the M1 layer which is central in controlling motor functions.

In addition to the increased dendrite load in L5 M1 layer, we observed that the cell soma area in L5 M1 pyramidal neurons is 35% larger in *Jnk1-/-* mice. The cell perimeter and the cell soma were measured by stereological measurements of 3-dimensional confocal images of neurons labeled with Lucifer yellow. This increase is consistent with the previous studies which correlate the cell soma with dendrite load (van Pelt *et al.* 1996).

## 2.3. JNK phosphorylation of MAP2 *in vivo* increased the dendritic architecture of L2/3 pyramidal neurons

To study the effect of MAP2 phosphorylation on the dendritic structure during development, *in utero* electroporation of CAG-MAP2-WT and CAG-MAP2-$^{T1619D,T1623D,T1625D}$ was done for, targeting the developing cortex in the wild type embryos at E15. The L2/3 cells were efficiently labeled by the technique, but there was insufficient labeling of the deeper L5 layer.

The labeled neurons of L2/3 expressed GFP and were analyzed for dendritic architecture at postnatal day 21, when the basal dendrites have developed most of their adult features (Romand *et al.* 2011). The dendritic branching was noticeably increased, especially of 2nd and 3rd order in CAG-MAP2-$^{T1619D,T1623D,T1625D}$ (Fig.4, Study II), as compared to the CAG-MAP2-WT, which showed a pattern similar to the Lucifer yellow labeled L2/3 neurons. We also observed increased dendritic dendrite length of higher order dendrites (3rd and 4th order) in the L2/3 neurons expressing CAG-MAP2-$^{T1619D,T1623D,T1625D}$, although the length of the primary dendrites remained unaltered (Fig 4E-L, Study II). Overall, there was a 50% increase in the total dendrite length because of an extensive increase in branching. It may be worth noting that the CAG-MAP2-$^{T1619D,T1623D,T1625D}$ expressing neurons were located at a different distance from the pial surface as compared to CAG-MAP2-WT expressing neurons. It has been shown previously that JNK retards the radial migration (Westerlund *et al.* 2011), so JNK mediated HMW-MAP2 phosphorylations may halt migration prematurely.

## 2.4. Motor coordination skills are impaired in *Jnk1-/-* mice

The primary motor cortex is central in controlling the motor coordination functions. *Jnk1-/-*mice displaying alterations in the dendritic architecture in L2/3 and L5 of the motor cortex were subjected to a battery of behavioral tests to assess motor coordination and strength. The mice were first tested in the raised beam test, which evaluates motor activity and balance. The *Jnk1-/-* mice performed poorly and showed reduced latency to fall when compared to wild type mice (Fig.6A, Study II). Notably, while crossing the beam, *Jnk1-/-* mice had trouble in placing their hind paws and moving forward, which implies a defect in balance and motor coordination (Fig.6B, Study II). Further mice were subjected to the suspended bar test to analyze grip strength and fine motor skills. Mice were hung with their forelimbs on the coat hanger. The *Jnk1-/-*mice showed reduced latency to fall. Most of the *Jnk1-/-* mice did not complete the task, and those that completed took longer than wild type mice, which suggested weakened grip strength (Fig. 6C and 6D, Study II). The grip strength was also measured using the inverted grid test, where both forelimbs and hindlimbs are used to grip the inverted grip. The results were very similar, with reduced latency to fall in *Jnk1-/-* mice (Fig.6E, Study II). Taken together, the above tests indicated impaired motor coordination and grip strength in *Jnk1-/-* mice.

The motor coordination and synchrony of these mice was studied in more detail by measuring gait using footprint analysis (Brooks & Dunnett 2009). A reduction in stride width was observed in the 3-months old *Jnk1-/-*mice compared to wild type (Fig.6F, Study II). Interestingly, this defect was not apparent in the 7 month old *Jnk1-/-*mice where changes

were not significant. However, in 7 month old *Jnk1-/-*mice the stride length was significantly reduced. Our anatomical measurements of these mice identified a minor increase in the hindlimb length, which is unlikely to account for the reduction in stride length in old *Jnk1-/-*mice (Fig.6F and 6G, Study II). An osteoclast deficit may explain this difference, since JNK has been previously shown to regulate osteoclast differentiation (David *et al.* 2002), though no defects in bone structure has been reported in *Jnk1-/-* mice.

The mice were then subjected to a rotarod test to further look into their gross motor coordination and balance. The test revealed no phenotypic differences between knockouts and wild type on the accelerating rod (Fig.6I, Study II). The rotarod test is considered to be less sensitive to coordination because heavy mice perform poorly in this test (Brooks & Dunnett 2009). As the *Jnk1-/-*mice were observed to be lighter in weight than the wild type (Fig.6J, Study II), there is a possibility that the underlying defects might be masked due to this offset. Altogether, the battery of tests suggest defects in fine motor coordination in the *Jnk1-/-*mice, whereas in their home cages these mice showed no abnormalities, such as tremors, abnormal walking, or running behavior.

## 3. JNK regulates the adult hippocampal neurogenesis and structural changes underlying anxiety behavior in mice (Study III)

Numerous studies have reported structural abnormalities in the brain associated with anxiety-related behavior, such as altered dendrite architecture, spine distribution, and more recently adult hippocampal neurogenesis (Revest *et al.* 2009; Soetanto *et al.* 2010). MAP kinases have been implicated in stress related disorders, but the role of the JNK kinase subfamily has not been explored in context to anxiety behavior. In Study III, using *Jnk1-/-* mice and a pharmacological JNK inhibitor, a relationship was found between JNK and anxiety behavior, as well as its influence on the adult hippocampal neurogenesis.

### 3.1. *Jnk1-/-* mice display reduced anxiety-like behavior and increased adult hippocampal neurogenesis

The results from recent studies suggest the potential involvement of JNK subfamily in mood modulation (Galeotti & Ghelardini 2012). To address the contribution of the JNK pathway in anxiety-related behavior, *jnk1* deficient mice were subjected to three independent anxiety tests, i.e. the elevated plus maze, the light-dark test, and the open field test. A reduction in anxiety behavior was observed in *Jnk1-/-*mice compared to wild type

mice in all behavior paradigms. In the elevated plus maze, the *Jnk1-/-* mice spent more time in the open arms and also showed increased head dipping and rearing behavior when compared to wild type mice (Fig. 1a and 1b, Study III). These parameters suggest a high level of exploratory activity and reduced anxiety. Next we tested mice in the light-dark test, which explores the innate aversive behavior of rodents to illuminated areas and their competing exploratory instinct. The *Jnk1-/-* mice spent significantly more time and traveled longer distance in the light box as compared to the wild type mice (Fig. 1c, Study III) which further indicated reduced anxiety.

The Open field test, which measures normal activity and anxiety in mice, also showed similar results. *Jnk1-/-* mice entered more frequently in the center of the open field chamber (Fig.1d, Study III), and also traveled significantly longer distance in the centre. The total distance traveled in the box was also higher in *Jnk1-/-*mice in comparison to wild type mice (Fig.1d, Study III). Altogether, these results suggest that *Jnk1-/-* mice display reduced anxiety and hyperactivity in comparison to wild type mice.

Recent studies suggest that adult hippocampal neurogenesis is altered in anxiety behavior (Revest *et al.* 2009; Snyder *et al.* 2011). We therefore evaluated the basal adult hippocampal neurogenesis in *Jnk1-/-*mice. We used a gold standard method to measure neurogenesis where newborn cells are labeled with a thymidine analog BrdU (Bromodeoxyuridine), sacrificed after 28 days and immunostained with BrdU antibody to quantify neurogenesis in the dentate gyrus. The stereological estimation showed an increased number of BrdU positive cells in the dentate gyrus of *Jnk1-/-* mice compared to wild type mice (Fig.1e and 1f, Study III). The cells survived for 28 days and their neuronal identity was confirmed by co-immunostaining with the neuronal marker, NeuN and quantified by stereology (Fig.1i and 1k, Study III). Notably, there was no difference in the BrdU +ve cells after 24 hrs in wild type and *Jnk1-/-* dentate gyrus, which suggests that proliferation was not altered (data not shown). To further confirm our observations, we assessed young adult-born neurons in wild type and *Jnk1-/-*, which express doublecortin (DCX), a protein expressed for about one month after neuronal birth (Mendez-David *et al.* 2013). In line with the above results, we also found an increased number of DCX+ cells in the *Jnk1-/-*mice, compared to the wild type litter mates (Fig.1g and 1h, Study III). In conclusion, the results indicate that mice devoid of *Jnk1* have increased basal adult hippocampal neurogenesis, which might explain the reduced anxiety behavior as it is suggested elsewhere that adult hippocampal neurogenesis is reduced with the increase in anxiety (Revest *et al.* 2009).

In addition, the volumetric changes were also investigated in the dentate gyrus of *Jnk1-/-*mice, as volumetric alterations in the hippocampus have

been implicated in the anxiety disorders. Our stereological analysis revealed a minor increase in dentate gyrus volume in *Jnk1-/-*mice, although this was not significant (Fig.1j, Study III).

### 3.2. JNK inhibition shows the anxiolytic effect by increasing adult hippocampal neurogenesis in mice

Since mice devoid of JNK showed reduced anxiety and increased adult hippocampal neurogenesis, the therapeutic potential of the JNK inhibitor DJNKI-1 was tested (Fig.2a, Study III). Initially, acute treatment was made by infusing 100μM DJNKI-1 intracerebrally to the ventricles (Fig.2a, Study III). This was to avoid problems associated with blood brain barrier penetration. The behavioral tests were performed after 6 hours of infusion. This treatment did not alter the anxiety phenotype in mice when assessed in the elevated plus maze (EPM) and light-dark paradigms. In the EPM analysis, DJNKI-1 treated mice were not different from vehicle treated mice in any of the scored parameters (Fig.2d, Study III). Similarly, in the light-dark test the behavior of DJNKI-1-treated mice was almost similar to that of vehicle-treated mice (Fig 2e, Study III). However, the acute dose of 100μM DJNKI-1 efficiently reduced the phosphorylation of JNK substrate PSD95 (Fig.2b and 2c, Study III). In conclusion, the acute treatment of 100μM DJNKI-1 blocked the JNK activity but had no effect on anxiety behavior. On the basis of these observations, it was proposed that if adult hippocampal neurogenesis is an underlying mechanism the longer treatments are required.

To investigate this hypothesis, 6 week long treatments were performed using 100μM DJNKI-1 by infusing in ventricles *via* mini-osmotic pumps (Fig.3a, Study III). After 6 weeks of treatment mice were subjected to the elevated plus maze (EPM), which is a pharmacologically validated behavioral paradigm for testing anxiolytic drugs. Surprisingly, after 6 weeks of chronic DJNKI-1 treatment, significant changes were observed in almost all the parameters of the elevated plus maze test. DJNKI-1 treated mice spent more time in the open arms and showed increased frequency to enter into the open arms of the elevated plus maze (Fig. 3d-e and 3i Study III). Moreover, DJNKI-1 treated mice also showed increased head dipping and rearing (Fig. 3f, Study III) and in contrast, they showed reduced grooming and stretched attend postures (Fig. 3f, Study III), which overall suggests reduced anxiety in these mice. The treatment did not affect the body weight and mice showed no abnormal behavior such as increased running or tremors in their home cage.

Furthermore, the chronic DJNKI-1 treated mice were evaluated for adult hippocampal neurogenesis by quantifying BrdU labeled neuronal marker (NeuN) positive cells and the total number of young adult-born neurons

which express DCX. Interestingly, the DJNKI-1 treated mice showed an increased the number of BrdU-NeuN positive cells (Fig.3i-k, Study III), as well as DCX positive adult-born neurons (Fig.3g-h, Study III) in the dentate gyrus of the hippocampus. Finally, the efficacy of DJNKI-1 treatments was also tested in reducing JNK activity by quantifying the phosphorylation status of the JNK substrate PSD95. There was a significant reduction in phosphorylation levels in hippocampus and amygdala but, interestingly, the levels were unaffected in the prefrontal cortex (Fig.3b and 3c, Study III).

Altogether, these results indicate that chronic DJNKI-1 treatment facilitates adult hippocampal neurogenesis and shows an anxiolytic effect, suggesting adult hippocampal neurogenesis as an underlying mechanism. However, results can be confounded by hyperactivity which is limiting in above experiments even though there was no heightened activity in elevated plus maze and in their home cage.

### 3.3. Anxiolytic Effects of JNK inhibition are adult hippocampal neurogenesis dependent

Previous conclusions also raised the possibility that an increase in adult hippocampal neurogenesis might be a secondary effect of JNK inhibition. To investigate if the anxiolytic effects after genetic or pharmacological ablation of JNK are dependent on adult hippocampal neurogenesis, 2% cytarabine (Ara-C) was infused for 6 weeks to completely block neurogenesis in mice. After 6 weeks the mice were tested for the phenotype in the elevated plus maze and light-dark test (Fig.4a, Study III). The use of DJNKI-1 alone showed increased open arm entries, head dips and reduced stretched attend postures and grooming (Fig. 4b-e, Study III), suggesting reduced anxiety, as was observed in our previous experiments, whereas the addition of Ara-C diminished the above effects (Fig. 4b-e, Study III). Therefore, DJNKI-1 administration did not show any anxiolytic effect in the absence of adult hippocampal neurogenesis. Similarly, in the light-dark test there was a strong trend towards increased time spent in the light box and increased latency to enter in the dark box in mice injected with DJNKI-1 alone, in comparison to the vehicle, but no change in combination with Ara-C (Fig. 4f-g, Study III). Notably, the cohort of mice injected with Ara-C alone did not demonstrate any effect on anxiety parameters. This is consistent with other studies demonstrating that ablation of adult neurogenesis has no effect on basal anxiety (Santarelli *et al.* 2003; Saxe *et al.* 2006; Mendez-David *et al.* 2013). Also, these treatments did not alter the body weight, general activity and food intake in mice. Taken together, these results advocate that the effects of JNK inhibition are mediated by adult hippocampal neurogenesis.

### 3.4. JNK inhibition increased the adult neurogenesis in the ventral pole of the dentate gyrus

Recent studies show a functional dissociation of adult newborn neurons along the dorso-ventral axis of the dentate gyrus (Kheirbek *et al.* 2013; Wu & Hen 2014). This was also previously described by lesion studies showing that the dorsal pole of the DG regulates cognitive-related behavior and ventral pole modulates mood-related responses (Ferbinteanu & McDonald 2001; Bannerman *et al.* 2002; Kjelstrup *et al.* 2002). We analyzed dorsal and ventral sections separately to evaluate neurogenesis on both poles upon JNK inhibition. After 6 weeks of DJNKI-1 treatment, we observed a significant increase in BrdU labeled cells expressing NeuN in the ventral dentate gyrus, but no change in the dorsal pole (Fig.3j-k, Study III). Interestingly, we observed that JNK inhibition affects the process of adult neurogenesis at an early stage. The number of dividing Ki67 expressing precursor cells were increased in the dorsal as well as the ventral dentate gyrus of DJNKI-1 treated mice (Fig.4h-I, Study III). In addition, the total number of adult born neurons (DCX+ve cells) was increased in the ventral dentate gyrus upon DJNKI-1 treatment, whereas the dorsal pole remained unaffected (Fig.4j-k, Study III). The Ara-C treatment alone and in combination with DJNKI-1 resulted in almost complete ablation of Ki67 and DCX positive cells, which confirms the blockade of adult hippocampal neurogenesis (Fig.4h-l, Study III). These conclusions highlight that JNK regulates adult neurogenesis at the ventral pole and thus alters anxiety behavior.

We also subcategorized DCX +ve adult born neurons into DCX+ve cells and DCX+ve cells with dendrites, which are named tertiary DCX+ve cells as described by others to assess the maturity index (David *et al.* 2009; Mendez-David *et al.* 2014). DJNKI-1 treatment not only augmented the number of DCX+ve cells but also increased the proportion of DCX+ve cells displaying tertiary dendrites cells in the ventral dentate gyrus. However, there was no alteration in the tertiary DCX+ve cells in the dorsal dentate gyrus. The maturity of newborn neurons was assessed by dividing the number of tertiary DCX+ve cells by the total number of DCX+ve cells, as described elsewhere (David *et al.* 2009; Mendez-David *et al.* 2014). DJNKI-1 treated mice showed a higher maturation index in the ventral DG, which means more mature newborn neurons with tertiary dendrites in the ventral dentate gyrus than in vehicle treated mice (Fig.5a, Study III). These results indicate that DJNKI-1 induces maturity in adult born neurons by allowing dendritic growth in the ventral dentate gyrus, which may underlie the anxiolytic effects. However, these region-specific effects of DJNKI-1 in the ventral portion need to be further investigated. Our results lack the confirmation as to whether DJNKI-1 is inhibiting JNK activity equally on both poles of the dentate gyrus. Interestingly, it has been observed that pharmacological and non-pharmacological effectors have a

differential effect on neurogenesis in a region-specific manner. For example, fluoxetine preferentially enhances neurogenesis in the ventral pole, whereas environmental factors, such as exercise, augment the dorsal pole neurogenesis (Tanti *et al.* 2012). Our results are exciting and further experiments might indicate the underlying reasons and mechanism of these region-specific effects.

### 3.5. The genetic ablation of JNK1 increased the dendritic complexity in hippocampus of mice

JNK kinases have been implicated in regulating dendritic architecture (Coffey 2014), and we have shown previously that inhibition and knockout of JNK1 alter the dendritic architecture *in vitro* and *in vivo* (Bjorkblom *et al.* 2005; Komulainen *et al.* 2014). Moreover, the alterations in hippocampal CA3 region dendritic architecture have been associated with anxiety-depressive disorders (Soetanto *et al.* 2010). Stress being the major determinant also has been reported to induce a reduction in dendritic arborization in CA3 pyramidal neurons in the hippocampus (Vyas *et al.* 2002).

It is believed that mature and immature neurons of the dentate gyrus extend their projections towards the CA3 region in the hippocampal circuitry for information processing. To further explore the role of JNK1 on the integration of adult born neurons in this circuitry, CA3 pyramidal neurons of *Jnk1-/-*mice were labeled with lucifer yellow dye to trace dendritic projections and performed sholl analysis on 3D confocal sections. The intersections, nodes and length of dendritic arbors were mapped in both apical and basal dendrites. This analysis indicated increased complexity in apical and basal dendrites of the CA3 region in *Jnk1-/-*mice, with more intersections, a higher number of nodes and increased dendrite length (Fig. 5b-d, Study III). Interestingly, the differences were more prominent in the apical dendrites than in the basal dendrites of CA3 hippocampal neurons (Fig.5c-d). Increased neurogenesis may be an explanation for this increase in apical dendritic complexity due to more axonal input from newly born neurons in the dentate gyrus. It seems clear from these results that JNK1 regulates the innervations of newly born neurons in the hippocampal circuitry by maneuvering the dendritic skeleton of neurons.

## 4. Identification of JNK substrates regulating dendritic spine architecture (Study IV)

In this study, another structural change associated with the anxiety phenotype was explored. The small membranous protrusions on the dendrites are called dendritic spines and are critical for transmission of information through chemical and electrical synapses. The role of JNK in regulating the morphology and distribution of dendritic spines was investigated, in addition to screening for potential effectors.

### 4.1. *Jnk1-/-* mice display altered dendritic spine architecture in the hippocampal CA3 region

Dendritic spine morphological changes have been reported in various psychiatric disorders. The CA3 region of the hippocampus receives incoming projections from the dentate gyrus and is important in the context of mood-related behavior, as well as memory formation. The distribution and morphology of dendritic spines was evaluated with Lucifer yellow labeled CA3 pyramidal neurons. The different morphological forms of the spines were counted from the 3D confocal images using Neurolucida software. The density of mushroom spines was significantly reduced, whereas the density of immature, filamentous spines was increased in the CA3 neurons of *Jnk1-/-*mice as compared to wild type mice (Fig. 1a-c, Study IV). The increased dendritic architecture in the apical region of CA3 pyramidal neurons might be an explanation for the reduction of mushroom spines (Study III). As adult neurogenesis was observed to be increased in *Jnk1-/-* mice in Study III, more projections from the newborn neurons onto the CA3 might be a reason for increased immature spines. On the basis of these observations it is proposed that overall the hippocampal circuitry may be maintaining the balance of synaptic transmission by modulating the dendrite and spine architecture in *Jnk1-/-*mice. It seems that JNK1 is critical in maintaining the spine morphology and distribution.

### 4.2. Blocking JNK activity in neuronal cultures also disrupted the spine density

Neuronal cultures were used to further investigate the role of JNK in regulating spine density. The hippocampal neurons were transfected with a construct blocking JNK activity. The hippocampal cultures were transfected at DIV7 with control CAG-GFP and GFP-NES-JBD constructs targeting the cytosolic JNK activity and were fixed at DIV21. The GFP-NES-JBD allosterically binds to the substrate binding domain of JNK and interrupts the interaction with the downstream substrates. It was found that the density of total spines and mushroom spines has been decreased

drastically in the cultures. Similarly, there was a significant increase in the filamentous immature spines in the neurons transfected with NES-JBD (Fig. 2a-b, Study IV). The effects were more pronounced in cultures treated with the JBD inhibitor than in the CA3 neurons of *Jnk1-/-* mice *in vivo.* This is likely due to inhibition of other JNK isoforms, as the JBD inhibitor is not known to be JNK1-specific. Interestingly, this altered spine distribution after JNK inhibition also phenocopied the *in vivo* effects. We also tested the impact of nuclear JNK inhibition using NLS-JBD, although we did not observe any changes in spine density, which suggested that cytosolic JNK modulates spine changes.

### 4.3. Identification of potential JNK substrates regulating the dendritic spine architecture

On the basis of the previous observations, experiments were designed to study the events downstream of JNK, which might be responsible for this change in dendritic spines. Previously, it has been shown that JNK phosphorylates actin-associated and microtubule-associated proteins to regulate neuronal cytoskeleton (Chang *et al.* 2003; Bjorkblom *et al.* 2005; Tararuk *et al.* 2006; Coffey 2014). siRNA screening was performed to determine the potential downstream substrates that might affect dendritic spine distribution. siRNAs for DCX, MARCKSL1 and SCG10 have been designed and used in previous studies (Cohen *et al.* 2008; Björkblom *et al.* 2012), and with their transfection they efficiently reduced the expression of DCX, MARCKSL1 and SCG10 protein (Fig. 3a-c). It was found that DCX, MARCKSL1 and SCG10 were potential JNK substrates, and they resulted in a reduction in total and mushroom spine density when knocked down (Fig. 4a-b). The immature spines were also increased, particularly after knocking down DCX and MARCKSL1. This analysis suggests that JNK regulates dendritic spines via its substrates DCX, MARCKSL1 and SCG10.

## 5. Limitations and future directions

The results of this study highlight the novel underlying mechanism that might be involved in regulation of neurogenic changes associated with anxiety. There are promising anxiolytic effects in *Jnk1-/-* mice and upon JNK inhibition using DJNKI-1 inhibitor. In addition, the knockouts and inhibitor treatments both increased the adult hippocampal neurogenesis in mice. As recent studies have emphasized that adult neurogenesis is involved in anxiety (Revest *et al.* 2009) and also required for anxiolytic effect of antidepressants (Santarelli *et al.* 2003), the results presented in this thesis suggest that JNK signaling may be therapeutically relevant for anxiety disorders.

In keeping with recent behavioral studies, which have indicated increased exploratory behavior in *Jnk1-/-* mice (Reinecke *et al.* 2013), strong exploratory activity in *Jnk1-/-*mice was observed in the open field test. Hence, there is a possibility that DJNKI-1 inhibitor treatments lead to hyperactivity, which might induce neurogenesis and anxiolytic effects in mice. Due to experimental limitations of the presented studies, there is no data to support the exploratory behavior from open field test after JNK inhibition. However, it is noteworthy that there was no change in the normal activity and body weight of these inhibitor treated mice in home cage, whereas *Jnk1-/-* mice do differ in their body weight. Although no heightened activity was observed in the elevated plus maze and light dark tests, the results could be confounded by altered locomotor activity, which would need further investigation.

The *Jnk1-/-*mice show defects in fine motor coordination (Study II), and information about motor coordination as well as cognitive phenotypes in DJNKI-1 treated mice was also lacking. The altered motor functions and cognitive disability can influence the performance of mice in anxiety tests. Therefore, additional experiments are necessary to address the impact of JNK inhibition on motor functions and cognitive behavior to support the anxiolytic effect of DJNKI-1. Moreover, it is clear that JNK activity is basally high in brain and involved in key brain processes (Coffey 2014). It has been shown that JNK is regulating the neuronal cytoskeleton, which consequently affects the synaptic transmission and brain functioning (Bjorkblom *et al.* 2005; Westerlund *et al.* 2011; Coffey 2014; Komulainen *et al.* 2014). Thus, inhibiting JNK might have off-target effects that need to be elucidated in the future. For instance, the fear learning and anxiety circuits are overlapping, so fear conditioning tests after JNK inhibition are required to confirm these anxiolytic effects.

It seems clear that the location of adult neurogenesis in the hippocampus is vital, e.g., fluoxetine treatment enhances neurogenesis in ventral dentate gyrus, which induces emotional improvement but no effect on spatial learning (Satvat *et al.* 2012). The regional effects of DJNKI-1 increasing adult neurogenesis and maturation of new born neurons in the ventral pole are considerably intriguing. These results require additional region specific targeting of JNK in the future in order to confirm that they are not due to unequal JNK inhibition. New tools would be needed to efficiently dissect the dorsal and ventral pole of the dentate gyrus and measure the JNK inhibition on separate poles. Furthermore, studies are required to specifically inhibit JNK in the dorsal and ventral dentate gyrus to support its regional neurogenic and anxiolytic effects. The microenvironment of newborn neurons in the ventral and dorsal dentate gyrus might be one reason for such region-specific effects of JNK inhibition. The effect of DJNKI-1 on non-neuronal cells in both poles of the dentate gyrus should also be addressed.

Besides, the combinatorial treatments along with fluoxetine (neurogenesis -dependent) and rapidly acting drugs, such as benzodiazepines (neurogenesis-independent), will be valuable. It would be interesting to see if the anxiolytic effects are augmented or curtailed. Such experiments will also bring forth more knowledge on the downstream signaling of the antidepressants. In the future, more focused studies on JNK signaling in response to antidepressants like fluoxetine and stressors will be very useful.

In line with the other neuroplastic alterations reported in anxiety disorders dendrite and spine changes were observed in the hippocampus of *Jnk1-/-* mice and with JNK inhibiting constructs in *ex vivo* cultures. In *ex vivo* studies potential JNK substrates were screened, which might be regulating these structural changes. To follow up these *in vivo* studies it would be important to validate whether these JNK targets that have shown spine and dendrite changes are associated with anxiety disorders. In future studies, technological advancements, such as region specific fMRI (functional magnetic resonance imaging) and optogenetics in clinical and basic research, respectively, will be helpful in determining these neuroplastic changes in stress-related disorders.

On the perspective of using JNK inhibitors in therapeutics, significant progress has been made in the pharmaceutical industry and academia. As JNK pathways are induced by cellular stress and neuronal death, several pharmacological interventions have been tested to target such neurodegenerative disorders as stroke and Parkinson's disease (Kuan & Burke 2005; Chambers *et al.* 2011). Several JNK inhibitors have been designed and tested in preclinical studies, which have subsequently been tested in clinical trials (Koch *et al.* 2015). The peptide based inhibitors are the most potent that showed neuroprotective effects in stroke (Borsello *et al.* 2003; Repici *et al.* 2007). The future direction for targeting JNK pathways in neuropsychiatric disorders has gained attention and created excitement. The spotlights have also focused on developing JNK-isoform selective, non-ATP competitive inhibitors or to target interactions between JNKs and individual substrates (Koch *et al.* 2015). In addition to this, the compartment-specific JNK targeting is another step forward, since it is quite clear that the cellular localization of JNK drives its physiological function (Coffey 2014). In conclusion, novel and innovative strategies are required to develop and investigate these JNK inhibitors for the treatment of neurological disorders.

# Concluding Remarks

The work presented in this thesis was dedicated to understanding the molecular regulation of structural changes associated with anxiety behavior. The structural hallmarks of anxiety disorders are known, but the molecular events leading to such alterations in anxiety behavior are still unknown. The current anti-anxiety drugs are also either ineffective or lead to unnecessary side-effects. It is becoming important to understand the molecular signaling involved in anxiety behavior to develop better drugs. The research in the last decade has indicated that many anxiolytics show their anti-anxiety effects through adult hippocampal neurogenesis. There have been clear indications in various studies suggesting that neurogenesis is involved in anxiety disorders and anxiolytic effects are blocked in the absence of neurogenesis (Santarelli *et al.* 2003; Revest *et al.* 2009).

The MAP kinases are gaining attention with respect to their role in the molecular mechanism of anxiety-depressive behavior (Fumagalli *et al.* 2005; Engel *et al.* 2009; Todorovic *et al.* 2009; Wefers *et al.* 2012). The genetic and pharmacological inhibition of MAPKs, such as ERK and its upstream regulators, MEK (Mitogen activated and extracellular signal regulated kinase), PKA (protein kinase A) and PKC (protein kinase C), showed their potential involvement in the regulation of emotional behavior. The JNK family of MAP kinases has been previously discussed in this context (Galeotti & Ghelardini 2012) but has not been studied.

The results of this work gave novel insights into the role of JNK kinases in anxiety behavior. The mice lacking *Jnk1* displayed reduced anxiety in a battery of anxiety tests. Moreover, these *Jnk1-/-* mice also show increased adult hippocampal neurogenesis, which suggests that JNK1 regulates anxiety by controlling neurogenesis. Investigating further the link between JNK and anxiety, JNK was pharmacologically inhibited in adult mice and observed reduced anxiety behavior. After chronic inhibition, the adult hippocampal neurogenesis was also increased in the dentate gyrus. The anxiolytic effects of JNK inhibition were subsequently confirmed to be dependent on adult hippocampal neurogenesis. The blockade of neurogenesis completely abolished the anxiolytic effects of JNK inhibition, which is consistent with the prevailing hypothesis suggesting that antidepressants require adult hippocampal neurogenesis for their behavioral effects (Malberg *et al.* 2000; Santarelli *et al.* 2003).

The inhibition of JNK not only increased the number of adult born neurons but also facilitated maturation of newborn neurons. More mature newborn neurons with tertiary dendrites were observed in the ventral portion of dentate gyrus functionally connected with emotional behavior (Wu & Hen 2014). JNK may be controlling the neurogenesis as well as the in-

tegration of adult born neurons by manipulating dendritic growth in the hippocampus, which is critical for information processing. Previous data from the lab has shown that JNK inhibition enhances neurite outgrowth and branching (Bjorkblom *et al.* 2005). The role of JNK in dendritic innervations regulation is further supported by the data from CA3 hippocampal neurons *Jnk1-/-*mice. Increased dendritic branching was found in the CA3 region, which forms the trisynaptic connection and is vital for emotional information processing in hippocampus (Scharfman 2007). Stress, as one of the mediators of anxiety-depression disorders, has also reported to decrease the dendritic arborization in CA3 (Vyas *et al.* 2002), and JNK signaling is highly upregulated in stressed conditions. Taken together, these data indicate that JNK inhibition most likely reduces anxiety by enhancing neurogenesis as well as the integration of newborn neurons in the dentate gyrus.

While studying the dendritic architecture regulation by JNK, the dendrite architecture was found to be altered in the primary motor cortex of *Jnk1-/-*mice. The dendritic fields were strikingly deregulated in the cortical layers 2/3 and 5. These data indicate that JNK phosphorylation of microtubule stabilizing protein MAP2 *in vivo* might be underlying this alteration in the dendritic architecture. These layers constitute the major excitatory pathway in the motor cortex by receiving input from other cortical areas and sending the processed information to subcortical regions (Hooks *et al.* 2013; Kaneko 2013). The layer 2/3 and 5 are directly involved in affecting the voluntary movements and regulating motor functions in mice (Tennant *et al.* 2011; Levine *et al.* 2012). Therefore, when tested *Jnk1-/-* mice in commonly used behavioral tests to assess fine motor skills, these mice showed deficits in their fine motor coordination. These results further highlighted the importance of JNK1 in regulating the dendrite architecture and its influence on the behavior.

In addition to dendritic changes, the spine distribution and maturation has also been regarded as a hallmark in anxiety and other neuropsychiatric disorders. In keeping with this, altered spine distribution was found as well as maturation in the CA3 neurons of the hippocampus. Also, neuronal cultures transfected with plasmids inhibiting the JNK signaling showed altered spine distribution. The spines mainly consist of the actin cytoskeleton, which is regulated by actin associated proteins. These proteins regulate actin bundling and provide dynamicity and structural plasticity to dendritic spines. In one part of the work in this thesis, JNK1 phosphorylation of MARCKSL1 was found to induce actin bundling and regulate filopodial dynamics. This might be one of the downstream candidates by which JNK1 regulates spines. In addition, localization of postsynaptic protein PSD95 (Kim *et al.* 2007) and AMPA receptor subunits GluA2L and GluA4 (Thomas *et al.* 2008) has also been shown to be regulated by JNK mediated phosphorylation. We cannot rule out the microtu-

bule associated proteins which can also be responsible for entry of microtubules in developing spines. The dynamic microtubules and JNK are possibly involved in localization of PSD95 in spines (Jaworski *et al.* 2009; Hu *et al.* 2011). The altered density of spines similar to *Jnk1-/-* mice has also been observed in neurons lacking MAP1B (Tortosa *et al.* 2011).

To understand the role of JNK signaling in the regulation of dendritic spines, siRNA knockdown of potential downstream candidates was performed. Similar abnormalities were observed in spine distribution after knocking down JNK substrate microtubule associated protein SCG10 and DCX and actin bundling protein MARCKSL1 (Study IV). Further experiments targeting and rescuing the phosphorylation sites would be needed to confirm that the above JNK effectors are involved in the regulation of spine distribution and morphology.

To summarize, the results presented in this thesis suggest that JNK signaling is central in regulating the neurogenic changes associated with anxiety disorders. Additionally, it reinforces the fascinating hypothesis that adult hippocampal neurogenesis is pivotal for both the physiology and the pathophysiology of anxiety. Most importantly, this work highlights a new link between JNK and adult hippocampal neurogenesis in anxiety behavior. Additional studies on the molecular mechanism by which JNK regulates adult neurogenesis might provide new avenues of investigation for developing anxiety treatments.

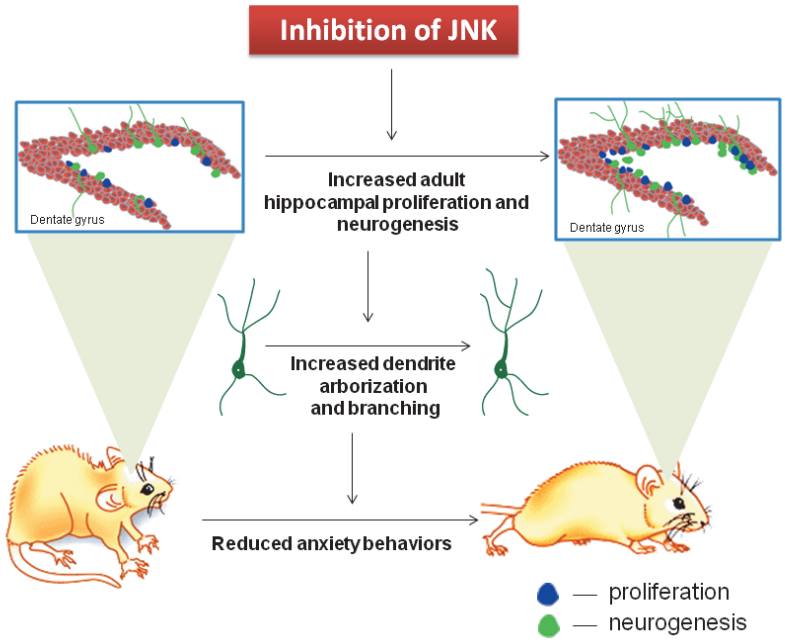



**Figure 5:** Schematic representation to summarize the results of this thesis. The genetic and pharmacological inhibition of JNK kinase reduced anxiety behavior in mice. This behavioral change is mediated by increased adult hippocampal neurogenesis and increased dendritic arborization (Mouse images are adapted from Nader K and Balleine B 2007 Nature Neuroscience 10:807-808).

**Table 1:** Summary of the results of this thesis.Major results of the thesis from *Jnk1-/-* mice and from DJNKI-1 and NES-JBD inhibitor treatments

| **Geno-type/Treatment** | **Results** | **Study** |
|---|---|---|
| *1) Jnk1-/-* | i) Reduced anxiety (EPM,LD,OF) | III |
| | ii) Increased adult hippocampal neuro-genesis | III |
| | iii) Altered dendritic complexity in primary motor cortex and CA3 pyramidal neurons | II, III |
| | iv) Altered fine motor coordination (SBT, BT, FPA) | II |
| | v) Altered spine architecture in CA3 neu-rons of hippocampus | IV |
| 2) DJNKI-1 | i) Reduced anxiety (EPM) | III |
| | ii) Increased adult neurogenesis in ventral DG | III |
| | iii) Increased proliferation in dorsal and ventral DG | III |
| | iv) Increased maturation of newborn neu-rons in ventral DG | III |
| 3)NES-JBD | i) Altered spine architecture in hippocam-pal neurons | IV |

EPM:elevated plus maze, LD:light dark, OF:open field, SBT:suspeded bar test, BT:beam test, FPA:footprint analysis, DG:dentate gyrus

# Acknowledgments

The work for this Ph.D. thesis would not have been possible without the outstanding research facilities at Turku Centre for Biotechnology, University of Turku and Åbo Akademi University. I am thankful to Professor Riitta Lahesmaa for providing state of the art instrumentation, skilled personnel, dedicated researchers and creative atmosphere. I deeply appreciate the invaluable support from Professor John Eriksson and Professor Kid Törnqvist in the last phase of my PhD.

I am very grateful to Professor Nuno Sousa for accepting the invitation to act as the opponent for my thesis. I am sincerely thankful to my reviewers Professor Esa R. Korpi and Docent Riikka Lund for their critical feedback, which has improved this thesis.

I wish to express gratitude to my supervisor Docent Eleanor Coffey for providing me an opportunity to be part of the lab. I am grateful for your intellectual as well as financial support during my PhD. I am thankful to my thesis advisors Professor Lea Sistonen and Dr. Eriika Savontaus for their helpful comments during annual project meetings and on my thesis draft. I am also thankful to Dr. Robert Moulder for reading my thesis and helping with the language.

I am extremely grateful to the collaborators who provided diverse expertise and ideas to shape up the projects. I would like to thank all the co-authors for their marvelous work. I believe this work would never have been completed without Susanna and Terhi. I would like to thank Susanna for making available every reagent required for experiments in time, and Terhi for being always ready to help during animal experiments. I am sincerely thankful to Jouko and Markku for sharing their expertise during imaging at CIC. Jouko, you are the coolest Bro in BTK. As a BTK MO Bro, I hope to see your moustache every Movember…)

The most significant people are lab members whom I worked with and spent a very important phase of my life. Thanks Artur Padzik, Justyna Zdrojewska, Emilia Komulainen, Hanna Heikelä, Raghavendra Mysore, Prasannakumar Deshpande, and Dani Flinkman. In these years, I have learnt a lot from each of you. Artur Padzik and Justyna Zdrojewska (hope I spelled correctly), both of you had been wonderful colleagues. I want to thank you for giving me strength in those difficult times. You guys were my rational and emotional gurus in those stressful phases of my PhD. Thanks Artur for board game sessions, although I lost most of the time..)) Justyna, I am never going to forget how to dissect brain for neuronal cultures and would be willing to learn *in utero* electroporation again…)). Prasanna, I am really going to miss your face on my right side. From the day one in the lab whenever I looked on my right side it was you. Thanks

for being my right hand during all these years..)). To the current lab members Patrik Hollos, Francesca Marchisella, Artemis Varidaki and Sylvia Ortega, I wish you luck for your future in the lab and in research. I wish to extend my gratitude to all my colleagues at BTK, MolBio, and BYoSF for scientific discussions and fun moments together.

Now, it is time to acknowledge my BACK-UP MAN, Dr. Kim Eerola. The best thing I learnt from you is to keep back-ups during experiments. It was great working with you as a collaborator which eventually led to our friendship. In coming years, we have a lot more to do as researchers as well as friends. Let's get to our common goals soon, because life is short and our dreams are big.

I was lucky to have some fantastic people around me whom I call my small 'Turku family'. Thanks to Raghu, Yashu, Subhash, Poonam, Prasanna, Amruta, Santosh, Preeti, for all those wonderful moments that we shared. And of course, now we have a new member in the family, little princess 'Lasya' to tell us that future is waiting…you know what I mean… hehe…) To Ms. Amanpreet Kaur, thanks my other half for supporting at every phase of this long journey. It would not have been possible for me to reach this day without you. The moments when it was unbearable for me to continue, your words kept me moving.

While working for PhD thesis, I also got some bad company in Turku. This includes my brown niggas Mohit, Vinay, Bhanu, and Shishir Ji and Arafat kaja Najamudin. Thanks buddies for splendid time, and the actual conclusion of my thesis is having you around keeps antidepressants away..)

I would like to express my gratitude and respect to my family. I know how many sacrifices they have made for me. Especially I am grateful to my father who always put education above everything and probably gave up some of his own desires for this day.

In the end, the financial support from Turku Doctoral Network of Molecular Biosciences, CIMO, Åbo Akademi University and finishing grant from the Rector of Åbo Akademi University, is gratefully acknowledged.

Hasan Mohammad

# References


Abraham I, Juhasz G, Kekesi KA, Kovacs KJ (1998). Corticosterone peak is responsible for stress-induced elevation of glutamate in the hippocampus. *Stress*. **2**, 171-181.

Abrous DN, Koehl M, Le Moal M (2005). Adult neurogenesis: from precursors to network and physiology. *Physiol Rev*. **85**, 523-569.

Aderem A (1992). The MARCKS brothers: a family of protein kinase C substrates. *Cell*. **71**, 713-716.

Adhikari A (2014). Distributed circuits underlying anxiety. *Front Behav Neurosci*. **8**.

Ahn SM, Choe ES (2010). Alterations in GluR2 AMPA receptor phosphorylation at serine 880 following group I metabotropic glutamate receptor stimulation in the rat dorsal striatum. *J Neurosci Res*. **88**, 992-999.

Airan RD, Meltzer LA, Roy M, Gong Y, Chen H, Deisseroth K (2007). High-speed imaging reveals neurophysiological links to behavior in an animal model of depression. *Science*. **317**, 819-823.

Akers KG, Martinez-Canabal A, Restivo L, Yiu AP, De Cristofaro A, Hsiang HL, Wheeler AL, Guskjolen A, Niibori Y, Shoji H, Ohira K, Richards BA, Miyakawa T, Josselyn SA, Frankland PW (2014). Hippocampal neurogenesis regulates forgetting during adulthood and infancy. *Science*. **344**, 598-602.

Alheid GF, Beltramino CA, De Olmos JS, Forbes MS, Swanson DJ, Heimer L (1998). The neuronal organization of the supracapsular part of the stria terminalis in the rat: the dorsal component of the extended amygdala. *Neuroscience*. **84**, 967-996.

Alonso J, Angermeyer MC, Bernert S, Bruffaerts R, Brugha TS, Bryson H, de Girolamo G, Graaf R, Demyttenaere K, Gasquet I, Haro JM, Katz SJ, Kessler RC, Kovess V, Lepine JP, Ormel J, Polidori G, Russo LJ, Vilagut G, Almansa J, Arbabzadeh-Bouchez S, Autonell J, Bernal M, Buist-Bouwman MA, Codony M, Domingo-Salvany A, Ferrer M, Joo SS, Martinez-Alonso M, Matschinger H, Mazzi F, Morgan Z, Morosini P, Palacin C, Romera B, Taub N, Vollebergh WA (2004). Prevalence of mental disorders in Europe: results from the European Study of the Epidemiology of Mental Disorders (ESEMeD) project. *Acta Psychiatr Scand Suppl*, 21-27.

Altman J, Das GD (1965). Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. *J Comp Neurol*. **124**, 319-335.

Alvarez-Buylla A, Garcia-Verdugo JM (2002). Neurogenesis in adult subventricular zone. *J Neurosci*. **22**, 629-634.

Anacker C, Zunszain PA, Carvalho LA, Pariante CM (2011). The glucocorticoid receptor: pivot of depression and of antidepressant treatment? *Psychoneuroendocrinology*. **36**, 415-425.

Andersch S, Rosenberg NK, Kullingsjo H, Ottosson JO, Bech P, Bruun-Hansen J, Hanson L, Lorentzen K, Mellergard M, Rasmussen S, et al. (1991). Efficacy and safety of alprazolam, imipramine and placebo in treating panic disorder. A Scandinavian multicenter study. *Acta Psychiatr Scand Suppl*. **365**, 18-27.

Arbuzova A, Schmitz AA, Vergeres G (2002). Cross-talk unfolded: MARCKS proteins. *Biochem J*. **362**, 1-12.

Arthur JS, Ley SC (2013). Mitogen-activated protein kinases in innate immunity. *Nat Rev Immunol*. **13**, 679-692.

Asaoka Y, Nishina H (2010). Diverse physiological functions of MKK4 and MKK7 during early embryogenesis. *J Biochem*. **148**, 393-401.

Bailey KR, Crawley JN (2009). Anxiety-Related Behavior in Mice.

Bain J, McLauchlan H, Elliott M, Cohen P (2003). The specificities of protein kinase inhibitors: an update. *Biochem J*. **371**, 199-204.

Baj G, Carlino D, Gardossi L, Tongiorgi E (2013). Toward a unified biological hypothesis for the BDNF Val66Met-associated memory deficits in humans: a model of impaired dendritic mRNA trafficking. *Front Neurosci*. **7**.

Baldwin DS, Ajel K, Masdrakis VG, Nowak M, Rafiq R (2013). Pregabalin for the treatment of generalized anxiety disorder: an update. In *Neuropsychiatr Dis Treat*), pp. 883-892.

Balu DT, Lucki I (2009). Adult Hippocampal Neurogenesis: Regulation, Functional Implications, And Contribution to Disease Pathology. *Neurosci Biobehav Rev*. **33**, 232-252.

Bannerman DM, Deacon RM, Offen S, Friswell J, Grubb M, Rawlins JN (2002). Double dissociation of function within the hippocampus: spatial memory and hyponeophagia. *Behav Neurosci*. **116**, 884-901.

Belzung C, Griebel G (2001). Measuring normal and pathological anxiety-like behaviour in mice: a review. *Behav Brain Res*. **125**, 141-149.

Bennett BL, Sasaki DT, Murray BW, O'Leary EC, Sakata ST, Xu W, Leisten JC, Motiwala A, Pierce S, Satoh Y, Bhagwat SS, Manning AM, Anderson DW (2001). SP600125, an anthrapyrazolone inhibitor of Jun N-terminal

kinase. *Proc Natl Acad Sci U S A*. **98**, 13681-13686.

Bergami M, Berninger B, Canossa M (2009). Conditional deletion of TrkB alters adult hippocampal neurogenesis and anxiety-related behavior. In *Commun Integr Biol*), pp. 14-16.

Bergink V, van Megen HJ, Westenberg HG (2004). Glutamate and anxiety. *Eur Neuropsychopharmacol*. **14**, 175-183.

Bessa JM, Ferreira D, Melo I, Marques F, Cerqueira JJ, Palha JA, Almeida OF, Sousa N (2009). The mood-improving actions of antidepressants do not depend on neurogenesis but are associated with neuronal remodeling. *Mol Psychiatry*. **14**, 764-773, 739.

Bjorkblom B, Ostman N, Hongisto V, Komarovski V, Filen JJ, Nyman TA, Kallunki T, Courtney MJ, Coffey ET (2005). Constitutively active cytoplasmic c-Jun N-terminal kinase 1 is a dominant regulator of dendritic architecture: role of microtubule-associated protein 2 as an effector. *J Neurosci*. **25**, 6350-6361.

Björkblom B, Padzik A, Mohammad H, Westerlund N, Komulainen E, Hollos P, Parviainen L, Papageorgiou AC, Iljin K, Kallioniemi O, Kallajoki M, Courtney MJ, Mågård M, James P, Coffey ET (2012). c-Jun N-Terminal Kinase Phosphorylation of MARCKSL1 Determines Actin Stability and Migration in Neurons and in Cancer Cells. In *Mol Cell Biol*), pp. 3513-3526.

Bodnoff SR, Suranyi-Cadotte B, Aitken DH, Quirion R, Meaney MJ (1988). The effects of chronic antidepressant treatment in an animal model of anxiety. *Psychopharmacology (Berl)*. **95**, 298-302.

Bogoyevitch MA, Kobe B (2006). Uses for JNK: the Many and Varied Substrates of the c-Jun N-Terminal Kinases. In *Microbiol Mol Biol Rev*), pp. 1061-1095.

Bonny C, Oberson A, Negri S, Sauser C, Schorderet DF (2001). Cell-permeable peptide inhibitors of JNK: novel blockers of beta-cell death. *Diabetes*. **50**, 77-82.

Borsello T, Clarke PG, Hirt L, Vercelli A, Repici M, Schorderet DF, Bogousslavsky J, Bonny C (2003). A peptide inhibitor of c-Jun N-terminal kinase protects against excitotoxicity and cerebral ischemia. *Nat Med*. **9**, 1180-1186.

Borsini A, Zunszain PA, Thuret S, Pariante CM (2015). The role of inflammatory cytokines as key modulators of neurogenesis. *Trends Neurosci*. **38**, 145-157.

Borsini F, Podhorna J, Marazziti D (2002). Do animal models of anxiety predict anxiolytic-like effects of antidepressants? *Psychopharmacology (Berl)*. **163**, 121-141.

Bowers S, Truong AP, Jeffrey Neitz R, Hom RK, Sealy JM, Probst GD, Quincy D, Peterson B, Chan W, Galemmo RA, Jr., Konradi AW, Sham HL, Toth G, Pan H, Lin M, Yao N, Artis DR, Zhang H, Chen L, Dryer M, Samant B, Zmolek W, Wong K, Lorentzen C, Goldbach E, Tonn G, Quinn KP, Sauer JM, Wright S, Powell K, Ruslim L, Ren Z, Bard F, Yednock TA, Griswold-Prenner I (2011). Design and synthesis of brain penetrant selective JNK inhibitors with improved pharmacokinetic properties for the prevention of neurodegeneration. *Bioorg Med Chem Lett*. **21**, 5521-5527.

Bremner JD (2006). Traumatic stress: effects on the brain. *Dialogues Clin Neurosci*. **8**, 445-461.

Bremner JD, Krystal JH, Southwick SM, Charney DS (1996). Noradrenergic mechanisms in stress and anxiety: I. Preclinical studies. *Synapse*. **23**, 28-38.

Broekkamp CL, Rijk HW, Joly-Gelouin D, Lloyd KL (1986). Major tranquillizers can be distinguished from minor tranquillizers on the basis of effects on marble burying and swim-induced grooming in mice. *Eur J Pharmacol*. **126**, 223-229.

Brooks SP, Dunnett SB (2009). Tests to assess motor phenotype in mice: a user's guide. *Nat Rev Neurosci*. **10**, 519-529.

Brown MD, Sacks DB (2009). Protein scaffolds in MAP kinase signalling. *Cell Signal*. **21**, 462-469.

Bruel-Jungerman E, Laroche S, Rampon C (2005). New neurons in the dentate gyrus are involved in the expression of enhanced long-term memory following environmental enrichment. *Eur J Neurosci*. **21**, 513-521.

Burghardt PR, Fulk LJ, Hand GA, Wilson MA (2004). The effects of chronic treadmill and wheel running on behavior in rats. *Brain Res*. **1019**, 84-96.

Calvo N, Volosin M (2001). Glucocorticoid and mineralocorticoid receptors are involved in the facilitation of anxiety-like response induced by restraint. *Neuroendocrinology*. **73**, 261-271.

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

Cameron HA, McEwen BS, Gould E (1995). Regulation of adult neurogenesis by excitatory input and NMDA receptor activation in the dentate gyrus. *J Neurosci*. **15**, 4687-4692.

Cameron HA, McKay RD (2001). Adult neurogenesis produces a large pool of new granule cells in the dentate gyrus. *J Comp Neurol*. **435**, 406-417.

Carrasco GA, Van de Kar LD (2003). Neuroendocrine pharmacology of stress. *Eur J Pharmacol.* **463**, 235-272.

Chambers JW, Pachori A, Howard S, Ganno M, Hansen D, Jr., Kamenecka T, Song X, Duckett D, Chen W, Ling YY, Cherry L, Cameron MD, Lin L, Ruiz CH, Lograsso P (2011). Small Molecule c-jun-N-terminal Kinase (JNK) Inhibitors Protect Dopaminergic Neurons in a Model of Parkinson's Disease. *ACS Chem Neurosci.* **2**, 198-206.

Chang L, Jones Y, Ellisman MH, Goldstein LS, Karin M (2003). JNK1 is required for maintenance of neuronal microtubules and controls phosphorylation of microtubule-associated proteins. *Dev Cell.* **4**, 521-533.

Charmandari E, Tsigos C, Chrousos G (2005). Endocrinology of the stress response. *Annu Rev Physiol.* **67**, 259-284.

Charney DS, Redmond DE, Jr. (1983). Neurobiological mechanisms in human anxiety. Evidence supporting central noradrenergic hyperactivity. *Neuropharmacology.* **22**, 1531-1536.

Chen AP, Ohno M, Giese KP, Kuhn R, Chen RL, Silva AJ (2006a). Forebrain-specific knockout of B-raf kinase leads to deficits in hippocampal long-term potentiation, learning, and memory. *J Neurosci Res.* **83**, 28-38.

Chen J, Chang S, Duncan SA, Okano HJ, Fishell G, Aderem A (1996a). Disruption of the MacMARCKS gene prevents cranial neural tube closure and results in anencephaly. *Proc Natl Acad Sci U S A.* **93**, 6275-6279.

Chen JT, Lu DH, Chia CP, Ruan DY, Sabapathy K, Xiao ZC (2005). Impaired long-term potentiation in c-Jun N-terminal kinase 2-deficient mice. *J Neurochem.* **93**, 463-473.

Chen YR, Wang X, Templeton D, Davis RJ, Tan TH (1996b). The role of c-Jun N-terminal kinase (JNK) in apoptosis induced by ultraviolet C and gamma radiation. Duration of JNK activation may determine cell death and proliferation. *J Biol Chem.* **271**, 31929-31936.

Chen ZY, Jing D, Bath KG, Ieraci A, Khan T, Siao CJ, Herrera DG, Toth M, Yang C, McEwen BS, Hempstead BL, Lee FS (2006b). Genetic variant BDNF (Val66Met) polymorphism alters anxiety-related behavior. *Science.* **314**, 140-143.

Christmas AJ, Maxwell DR (1970). A comparison of the effects of some benzodiazepines and other drugs on aggressive and exploratory behaviour in mice and rats. *Neuropharmacology.* **9**, 17-29.

Christoffel DJ, Golden SA, Russo SJ (2011). Structural and synaptic plasticity in stress-related disorders. *Rev Neurosci.* **22**, 535-549.

Clelland CD, Choi M, Romberg C, Clemenson GD, Jr., Fragniere A, Tyers P, Jessberger S, Saksida LM, Barker RA, Gage FH, Bussey TJ (2009). A functional role for adult hippocampal neurogenesis in spatial pattern separation. *Science.* **325**, 210-213.

Clemenson GD DW, Gage FH (2015). Environmental enrichment and neurogenesis: from mice to humans. **4**, 56–62.

Clynen E, Swijsen A, Raijmakers M, Hoogland G, Rigo JM (2014). Neuropeptides as Targets for the Development of Anticonvulsant Drugs. In *Mol Neurobiol*), pp. 626-646.

Coffey ET (2014). Nuclear and cytosolic JNK signalling in neurons. *Nat Rev Neurosci.* **15**, 285-299.

Coffey ET, Hongisto V, Dickens M, Davis RJ, Courtney MJ (2000). Dual roles for c-Jun N-terminal kinase in developmental and stress responses in cerebellar granule neurons. *J Neurosci.* **20**, 7602-7613.

Cohen D, Segal M, Reiner O (2008). Doublecortin supports the development of dendritic arbors in primary hippocampal neurons. *Dev Neurosci.* **30**, 187-199.

Conde C, Caceres A (2009). Microtubule assembly, organization and dynamics in axons and dendrites. *Nat Rev Neurosci.* **10**, 319-332.

Cook SC, Wellman CL (2004). Chronic stress alters dendritic morphology in rat medial prefrontal cortex. *J Neurobiol.* **60**, 236-248.

Correa SA, Eales KL (2012). The Role of p38 MAPK and Its Substrates in Neuronal Plasticity and Neurodegenerative Disease. *J Signal Transduct.* **2012**, 649079.

Courtney MJ, Coffey ET (1999). The mechanism of Ara-C-induced apoptosis of differentiating cerebellar granule neurons. *Eur J Neurosci.* **11**, 1073-1084.

Crawley J, Goodwin FK (1980). Preliminary report of a simple animal behavior model for the anxiolytic effects of benzodiazepines. *Pharmacol Biochem Behav.* **13**, 167-170.

Cryan JF, Holmes A (2005). The ascent of mouse: advances in modelling human depression and anxiety. *Nature Reviews Drug Discovery.* **4**, 775-790.

Cuevas BD, Abell AN, Johnson GL (2007). Role of mitogen-activated protein kinase kinase kinases in signal integration. *Oncogene.* **26**, 3159-3171.

Curlik DM, DiFeo G, Shors TJ (2014). Preparing for adulthood: thousands upon thousands of new cells are born in the hippocampus during puberty, and most survive with effortful learning. *Front Neurosci.* **8**.

David DJ, Klemenhagen KC, Holick KA, Saxe MD, Mendez I, Santarelli L, Craig DA, Zhong H, Swanson CJ, Hegde LG, Ping XI, Dong D, Marzabadi MR, Gerald CP, Hen R (2007). Efficacy of the MCHR1 antagonist N-[3-(1-{[4-

(3,4-difluorophenoxy)phenyl]methyl}(4-piperidyl))-4-methylphenyl]-2-m ethylpropanamide (SNAP 94847) in mouse models of anxiety and depression following acute and chronic administration is independent of hippocampal neurogenesis. *J Pharmacol Exp Ther*. **321**, 237-248.

David DJ, Samuels BA, Rainer Q, Wang JW, Marsteller D, Mendez I, Drew M, Craig DA, Guiard BP, Guilloux JP, Artymyshyn RP, Gardier AM, Gerald C, Antonijevic IA, Leonardo ED, Hen R (2009). Neurogenesis-dependent and -independent effects of fluoxetine in an animal model of anxiety/depression. *Neuron*. **62**, 479-493.

David JP, Sabapathy K, Hoffmann O, Idarraga MH, Wagner EF (2002). JNK1 modulates osteoclastogenesis through both c-Jun phosphorylation-dependent and -independent mechanisms. *J Cell Sci*. **115**, 4317-4325.

Davidson RJ (2002). Anxiety and affective style: role of prefrontal cortex and amygdala. *Biol Psychiatry*. **51**, 68-80.

Davis M (1997). Neurobiology of fear responses: the role of the amygdala. *J Neuropsychiatry Clin Neurosci*. **9**, 382-402.

Davis M, Whalen PJ (2001). The amygdala: vigilance and emotion. *Mol Psychiatry*. **6**, 13-34.

Davis RJ (2000). Signal transduction by the JNK group of MAP kinases. *Cell*. **103**, 239-252.

de Anda FC, Rosario AL, Durak O, Tran T, Graff J, Meletis K, Rei D, Soda T, Madabhushi R, Ginty DD, Kolodkin AL, Tsai LH (2012). Autism spectrum disorder susceptibility gene TAOK2 affects basal dendrite formation in the neocortex. *Nat Neurosci*. **15**, 1022-1031.

De Boer SF, Koolhaas JM (2003). Defensive burying in rodents: ethology, neurobiology and psychopharmacology. *Eur J Pharmacol*. **463**, 145-161.

Deckersbach T, Dougherty DD, Rauch SL (2006). Functional imaging of mood and anxiety disorders. *J Neuroimaging*. **16**, 1-10.

Deng W, Aimone JB, Gage FH (2010). New neurons and new memories: how does adult hippocampal neurogenesis affect learning and memory? *Nat Rev Neurosci*. **11**, 339-350.

Derijard B, Hibi M, Wu IH, Barrett T, Su B, Deng T, Karin M, Davis RJ (1994). JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell*. **76**, 1025-1037.

Devary Y, Gottlieb RA, Smeal T, Karin M (1992). The mammalian ultraviolet response is triggered by activation of Src tyrosine kinases. *Cell*. **71**, 1081-1091.

Dhaliwal J, Lagace DC (2011). Visualization and genetic manipulation of adult neurogenesis using transgenic mice. *Eur J Neurosci*. **33**, 1025-1036.

Dhillon AS, Hagan S, Rath O, Kolch W (2007). MAP kinase signalling pathways in cancer. *Oncogene*. **26**, 3279-3290.

Doetsch F, Caille I, Lim DA, Garcia-Verdugo JM, Alvarez-Buylla A (1999). Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell*. **97**, 703-716.

Dolcos F, LaBar KS, Cabeza R (2004). Interaction between the amygdala and the medial temporal lobe memory system predicts better memory for emotional events. *Neuron*. **42**, 855-863.

Dranovsky A, Hen R (2006). Hippocampal neurogenesis: regulation by stress and antidepressants. *Biol Psychiatry*. **59**, 1136-1143.

Dulawa SC, Hen R (2005). Recent advances in animal models of chronic antidepressant effects: the novelty-induced hypophagia test. *Neurosci Biobehav Rev*. **29**, 771-783.

Dulawa SC, Holick KA, Gundersen B, Hen R (2004). Effects of chronic fluoxetine in animal models of anxiety and depression. *Neuropsychopharmacology*. **29**, 1321-1330.

Duman CH, Schlesinger L, Kodama M, Russell DS, Duman RS (2007). A role for MAP kinase signaling in behavioral models of depression and antidepressant treatment. *Biol Psychiatry*. **61**, 661-670.

Duman RS, Monteggia LM (2006). A neurotrophic model for stress-related mood disorders. *Biol Psychiatry*. **59**, 1116-1127.

Dwivedi Y, Rizavi HS, Roberts RC, Conley RC, Tamminga CA, Pandey GN (2001). Reduced activation and expression of ERK1/2 MAP kinase in the post-mortem brain of depressed suicide subjects. *J Neurochem*. **77**, 916-928.

Egeland M, Zunszain PA, Pariante CM (2015). Molecular mechanisms in the regulation of adult neurogenesis during stress. *Nature Reviews Neuroscience*. **16**, 189-200.

Einat H, Yuan P, Gould TD, Li J, Du J, Zhang L, Manji HK, Chen G (2003). The role of the extracellular signal-regulated kinase signaling pathway in mood modulation. *J Neurosci*. **23**, 7311-7316.

Engel SR, Creson TK, Hao Y, Shen Y, Maeng S, Nekrasova T, Landreth GE, Manji HK, Chen G (2009). The extracellular signal-regulated kinase pathway contributes to the control of behavioral excitement. *Mol Psychiatry*. **14**, 448-461.

Eriksson PS, Perfilieva E, Bjork-Eriksson T, Alborn AM, Nordborg C, Peterson DA, Gage FH (1998). Neurogenesis in the adult human hippocampus. *Nat Med*. **4**, 1313-1317.

Ernst A, Alkass K, Bernard S, Salehpour M, Perl S, Tisdale J, Possnert G, Druid H, Frisen J

(2014). Neurogenesis in the striatum of the adult human brain. *Cell*. **156**, 1072-1083.

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

Farb DH, Ratner MH (2014). Targeting the modulation of neural circuitry for the treatment of anxiety disorders. *Pharmacol Rev*. **66**, 1002-1032.

Felmingham K, Williams LM, Whitford TJ, Falconer E, Kemp AH, Peduto A, Bryant RA (2009). Duration of posttraumatic stress disorder predicts hippocampal grey matter loss. *Neuroreport*. **20**, 1402-1406.

Ferbinteanu J, McDonald RJ (2001). Dorsal/ventral hippocampus, fornix, and conditioned place preference. *Hippocampus*. **11**, 187-200.

Ferrer I, Olive M, Ribera J, Planas AM (1996). Naturally occurring (programmed) and radiation-induced apoptosis are associated with selective c-Jun expression in the developing rat brain. *Eur J Neurosci*. **8**, 1286-1298.

Filipovic D, Zlatkovic J, Pavicevic I, Mandic L, Demajo M (2012). Chronic isolation stress compromises JNK/c-Jun signaling in rat brain. *J Neural Transm*. **119**, 1275-1284.

Finlay-Jones R, Brown GW (1981). types of stressful life event and the onset of anxiety and depressive disorders. *Psychol Med*. **11**, 803-815.

Fukushima N, Furuta D, Hidaka Y, Moriyama R, Tsujiuchi T (2009). Post-translational modifications of tubulin in the nervous system. *J Neurochem*. **109**, 683-693.

Fumagalli F, Molteni R, Calabrese F, Frasca A, Racagni G, Riva MA (2005). Chronic fluoxetine administration inhibits extracellular signal-regulated kinase 1/2 phosphorylation in rat brain. *J Neurochem*. **93**, 1551-1560.

Fuss J, Ben Abdallah NM, Vogt MA, Touma C, Pacifici PG, Palme R, Witzemann V, Hellweg R, Gass P (2010). Voluntary exercise induces anxiety-like behavior in adult C57BL/6J mice correlating with hippocampal neurogenesis. *Hippocampus*. **20**, 364-376.

Galeotti N, Ghelardini C (2012). Regionally selective activation and differential regulation of ERK, JNK and p38 MAP kinase signalling pathway by protein kinase C in mood modulation. *Int J Neuropsychopharmacol*. **15**, 781-793.

Gass P, Reichardt HM, Strekalova T, Henn F, Tronche F (2001). Mice with targeted mutations of glucocorticoid and mineralocorticoid receptors: models for depression and anxiety? *Physiol Behav*. **73**, 811-825.

Gilbertson MW, Shenton ME, Ciszewski A, Kasai K, Lasko NB, Orr SP, Pitman RK (2002). Smaller hippocampal volume predicts pathologic vulnerability to psychological trauma. *Nat Neurosci*. **5**, 1242-1247.

Goldwater DS, Pavlides C, Hunter RG, Bloss EB, Hof PR, McEwen BS, Morrison JH (2009). Structural and functional alterations to rat medial prefrontal cortex following chronic restraint stress and recovery. *Neuroscience*. **164**, 798-808.

Gould E, Beylin A, Tanapat P, Reeves A, Shors TJ (1999). Learning enhances adult neurogenesis in the hippocampal formation. *Nat Neurosci*. **2**, 260-265.

Gould E, McEwen BS, Tanapat P, Galea LA, Fuchs E (1997). Neurogenesis in the dentate gyrus of the adult tree shrew is regulated by psychosocial stress and NMDA receptor activation. *J Neurosci*. **17**, 2492-2498.

Gould E, Tanapat P, McEwen BS, Flugge G, Fuchs E (1998). Proliferation of granule cell precursors in the dentate gyrus of adult monkeys is diminished by stress. *Proc Natl Acad Sci U S A*. **95**, 3168-3171.

Graczyk PP (2013). JNK inhibitors as anti-inflammatory and neuroprotective agents. *Future Med Chem*. **5**, 539-551.

Gratzner HG (1982). Monoclonal antibody to 5-bromo- and 5-iododeoxyuridine: A new reagent for detection of DNA replication. *Science*. **218**, 474-475.

Griebel G, Holmes A (2013). 50 years of hurdles and hope in anxiolytic drug discovery. *Nat Rev Drug Discov*. **12**, 667-687.

Guan QH, Pei DS, Zhang QG, Hao ZB, Xu TL, Zhang GY (2005). The neuroprotective action of SP600125, a new inhibitor of JNK, on transient brain ischemia/reperfusion-induced neuronal death in rat hippocampal CA1 via nuclear and non-nuclear pathways. *Brain Res*. **1035**, 51-59.

Gupta S, Barrett T, Whitmarsh AJ, Cavanagh J, Sluss HK, Dérijard B, Davis RJ (1996). Selective interaction of JNK protein kinase isoforms with transcription factors. *EMBO J*. **15**, 2760-2770.

Ha HY, Cho IH, Lee KW, Song JY, Kim KS, Yu YM, Lee JK, Song JS, Yang SD, Shin HS, Han PL (2005). The axon guidance defect of the telencephalic commissures of the JSAP1-deficient brain was partially rescued by the transgenic expression of JIP1. *Dev Biol*. **277**, 184-199.

Haeusgen W, Herdegen T, Waetzig V (2011). The bottleneck of JNK signaling: molecular and functional characteristics of MKK4 and MKK7. *Eur J Cell Biol*. **90**, 536-544.

Hagg T (2009). From Neurotransmitters to Neurotrophic Factors to Neurogenesis. *Neuroscientist*. **15**, 20-27.

Hayes JP, LaBar KS, McCarthy G, Selgrade E, Nasser J, Dolcos F, Morey RA (2011). Reduced hippocampal and amygdala activity predicts memory distortions for trauma reminders in combat-related PTSD. *J Psychiatr Res.* **45**, 660-669.

Heine VM, Maslam S, Joels M, Lucassen PJ (2004a). Increased P27KIP1 protein expression in the dentate gyrus of chronically stressed rats indicates G1 arrest involvement. *Neuroscience.* **129**, 593-601.

Heine VM, Maslam S, Zareno J, Joels M, Lucassen PJ (2004b). Suppressed proliferation and apoptotic changes in the rat dentate gyrus after acute and chronic stress are reversible. *Eur J Neurosci.* **19**, 131-144.

Heisler LK, Chu HM, Brennan TJ, Danao JA, Bajwa P, Parsons LH, Tecott LH (1998). Elevated anxiety and antidepressant-like responses in serotonin 5-HT1A receptor mutant mice. *Proc Natl Acad Sci U S A.* **95**, 15049-15054.

Herman JP, Figueiredo H, Mueller NK, Ulrich-Lai Y, Ostrander MM, Choi DC, Cullinan WE (2003). Central mechanisms of stress integration: hierarchical circuitry controlling hypothalamo-pituitary-adrenocortical responsiveness. *Front Neuroendocrinol.* **24**, 151-180.

Hill AS, Sahay A, Hen R (2015). Increasing Adult Hippocampal Neurogenesis is Sufficient to Reduce Anxiety and Depression-Like Behavior. *Neuropsychopharmacology.*

Holmes A (2001). Targeted gene mutation approaches to the study of anxiety-like behavior in mice. *Neurosci Biobehav Rev.* **25**, 261-273.

Hom RK, Bowers S, Sealy JM, Truong AP, Probst GD, Neitzel ML, Neitz RJ, Fang L, Brogley L, Wu J, Konradi AW, Sham HL, Toth G, Pan H, Yao N, Artis DR, Quinn K, Sauer JM, Powell K, Ren Z, Bard F, Yednock TA, Griswold-Prenner I (2010). Design and synthesis of disubstituted thiophene and thiazole based inhibitors of JNK. *Bioorg Med Chem Lett.* **20**, 7303-7307.

Hooks BM, Mao T, Gutnisky DA, Yamawaki N, Svoboda K, Shepherd GM (2013). Organization of cortical and thalamic input to pyramidal neurons in mouse motor cortex. *J Neurosci.* **33**, 748-760.

Horwitz R, Webb D (2003). Cell migration. *Curr Biol.* **13**, R756-759.

Hu X, Ballo L, Pietila L, Viesselmann C, Ballweg J, Lumbard D, Stevenson M, Merriam E, Dent EW (2011). BDNF-induced increase of PSD-95 in dendritic spines requires dynamic microtubule invasions. *J Neurosci.* **31**, 15597-15603.

Jaworski J, Kapitein LC, Gouveia SM, Dortland BR, Wulf PS, Grigoriev I, Camera P, Spangler SA, Di Stefano P, Demmers J, Krugers H, Defilippi P, Akhmanova A, Hoogenraad CC (2009). Dynamic microtubules regulate dendritic spine morphology and synaptic plasticity. *Neuron.* **61**, 85-100.

Jeanneteau F, Garabedian MJ, Chao MV (2008). Activation of Trk neurotrophin receptors by glucocorticoids provides a neuroprotective effect. *Proc Natl Acad Sci U S A.* **105**, 4862-4867.

Johnson GL, Nakamura K (2007). The c-jun kinase/stress-activated pathway: regulation, function and role in human disease. *Biochim Biophys Acta.* **1773**, 1341-1348.

Johnson RS, van Lingen B, Papaioannou VE, Spiegelman BM (1993). A null mutation at the c-jun locus causes embryonic lethality and retarded cell growth in culture. *Genes Dev.* **7**, 1309-1317.

Jones BJ, Blackburn TP (2002). The medical benefit of 5-HT research. *Pharmacol Biochem Behav.* **71**, 555-568.

Kalisch R, Schubert M, Jacob W, Keßler MS, Hemauer R, Wigger A, Landgraf R, Auer DP (2005). Anxiety and Hippocampus Volume in the Rat. *Neuropsychopharmacology.* **31**, 925-932.

Kaneko T (2013). Local connections of excitatory neurons in motor-associated cortical areas of the rat. *Front Neural Circuits.* **7**, 75.

Kaplan MS, Hinds JW (1977). Neurogenesis in the adult rat: electron microscopic analysis of light radioautographs. *Science.* **197**, 1092-1094.

Katz RJ, Landau PS, Lott M, Bystritsky A, Diamond B, Hoehn-Saric R, Rosenthal M, Weise C (1993). Serotonergic (5-HT2) mediation of anxiety-therapeutic effects of serazepine in generalized anxiety disorder. *Biol Psychiatry.* **34**, 41-44.

Kempermann G, Jessberger S, Steiner B, Kronenberg G (2004). Milestones of neuronal development in the adult hippocampus. *Trends Neurosci.* **27**, 447-452.

Kempermann G, Kuhn HG, Gage FH (1997). More hippocampal neurons in adult mice living in an enriched environment. *Nature.* **386**, 493-495.

Kendler KS, Karkowski LM, Prescott CA (1999). Causal relationship between stressful life events and the onset of major depression. *Am J Psychiatry.* **156**, 837-841.

Kendler KS, Thornton LM, Gardner CO (2001). Genetic risk, number of previous depressive episodes, and stressful life events in predicting onset of major depression. *Am J Psychiatry.* **158**, 582-586.

Kennedy NJ, Martin G, Ehrhardt AG, Cavanagh-Kyros J, Kuan CY, Rakic P, Flavell RA, Treistman SN, Davis RJ (2007). Requirement of JIP scaffold proteins for NMDA-mediated

signal transduction. *Genes Dev.* **21**, 2336-2346.

Kessler RC, Aguilar-Gaxiola S, Alonso J, Chatterji S, Lee S, Ormel J, Üstün TB, Wang PS (2009). The global burden of mental disorders: An update from the WHO World Mental Health (WMH) Surveys. *Epidemiol Psichiatr Soc.* **18**, 23-33.

Kheirbek MA, Drew LJ, Burghardt NS, Costantini DO, Tannenholz L, Ahmari SE, Zeng H, Fenton AA, Hen R (2013). Differential control of learning and anxiety along the dorsoventral axis of the dentate gyrus. *Neuron.* **77**, 955-968.

Kim EK, Choi EJ (2010). Pathological roles of MAPK signaling pathways in human diseases. *Biochim Biophys Acta.* **1802**, 396-405.

Kim EK, Choi EJ (2015). Compromised MAPK signaling in human diseases: an update. *Arch Toxicol.* **89**, 867-882.

Kim M, McGaugh JL (1992). Effects of intra-amygdala injections of NMDA receptor antagonists on acquisition and retention of inhibitory avoidance. *Brain Res.* **585**, 35-48.

Kim MJ, Futai K, Jo J, Hayashi Y, Cho K, Sheng M (2007). Synaptic accumulation of PSD-95 and synaptic function regulated by phosphorylation of serine-295 of PSD-95. *Neuron.* **56**, 488-502.

Kirkpatrick LL, Brady ST (1999). Molecular Components of the Neuronal Cytoskeleton.

Kishimoto H, Nakagawa K, Watanabe T, Kitagawa D, Momose H, Seo J, Nishitai G, Shimizu N, Ohata S, Tanemura S, Asaka S, Goto T, Fukushi H, Yoshida H, Suzuki A, Sasaki T, Wada T, Penninger JM, Nishina H, Katada T (2003). Different properties of SEK1 and MKK7 in dual phosphorylation of stress-induced activated protein kinase SAPK/JNK in embryonic stem cells. *J Biol Chem.* **278**, 16595-16601.

Kjelstrup KG, Tuvnes FA, Steffenach HA, Murison R, Moser EI, Moser MB (2002). Reduced fear expression after lesions of the ventral hippocampus. *Proc Natl Acad Sci U S A.* **99**, 10825-10830.

Klempin F, Kempermann G (2007). Adult hippocampal neurogenesis and aging. *Eur Arch Psychiatry Clin Neurosci.* **257**, 271-280.

Koch P, Gehringer M, Laufer SA (2015). Inhibitors of c-Jun N-terminal kinases: an update. *J Med Chem.* **58**, 72-95.

Koen N, Stein DJ (2011). Pharmacotherapy of anxiety disorders: a critical review. *Dialogues Clin Neurosci.* **13**, 423-437.

Komulainen E, Zdrojewska J, Freemantle E, Mohammad H, Kulesskaya N, Deshpande P, Marchisella F, Mysore R, Hollos P, Michelsen KA, Magard M, Rauvala H, James P, Coffey ET (2014). JNK1 controls dendritic field size in L2/3 and L5 of the motor cortex, constrains soma size, and influences fine motor coordination. *Front Cell Neurosci.* **8**, 272.

Koo JW, Duman RS (2008). IL-1beta is an essential mediator of the antineurogenic and anhedonic effects of stress. *Proc Natl Acad Sci U S A.* **105**, 751-756.

Kowalczyk A, Filipkowski RK, Rylski M, Wilczynski GM, Konopacki FA, Jaworski J, Ciemerych MA, Sicinski P, Kaczmarek L (2004). The critical role of cyclin D2 in adult neurogenesis. *J Cell Biol.* **167**, 209-213.

Kuan CY, Burke RE (2005). Targeting the JNK signaling pathway for stroke and Parkinson's diseases therapy. *Curr Drug Targets CNS Neurol Disord.* **4**, 63-67.

Kuan CY, Yang DD, Samanta Roy DR, Davis RJ, Rakic P, Flavell RA (1999). The Jnk1 and Jnk2 protein kinases are required for regional specific apoptosis during early brain development. *Neuron.* **22**, 667-676.

Kuipers SD, Schroeder JE, Trentani A (2015). Changes in hippocampal neurogenesis throughout early development. *Neurobiol Aging.* **36**, 365-379.

Kunde SA, Rademacher N, Tzschach A, Wiedersberg E, Ullmann R, Kalscheuer VM, Shoichet SA (2013). Characterisation of de novo MAPK10/JNK3 truncation mutations associated with cognitive disorders in two unrelated patients. *Hum Genet.* **132**, 461-471.

Kyriakis JM, Avruch J (1990). pp54 microtubule-associated protein 2 kinase. A novel serine/threonine protein kinase regulated by phosphorylation and stimulated by poly-L-lysine. *J Biol Chem.* **265**, 17355-17363.

Kyriakis JM, Avruch J (2001). Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiol Rev.* **81**, 807-869.

Kyriakis JM, Avruch J (2012). Mammalian MAPK signal transduction pathways activated by stress and inflammation: a 10-year update. *Physiol Rev.* **92**, 689-737.

Kyriakis JM, Banerjee P, Nikolakaki E, Dai T, Rubie EA, Ahmad MF, Avruch J, Woodgett JR (1994). The stress-activated protein kinase subfamily of c-Jun kinases. *Nature.* **369**, 156-160.

Lagace DC, Benavides DR, Kansy JW, Mapelli M, Greengard P, Bibb JA, Eisch AJ (2008). Cdk5 is essential for adult hippocampal neurogenesis. *Proc Natl Acad Sci U S A.* **105**, 18567-18571.

Landgraf R, Wigger A (2002). High vs low anxiety-related behavior rats: an animal model of extremes in trait anxiety. *Behav Genet.* **32**, 301-314.

Lazarov O, Mattson MP, Peterson DA, Pimplikar SW, van Praag H (2010). When neurogenesis encounters aging and disease. *Trends Neurosci.* **33**, 569-579.

LeDoux JE (2000). Emotion circuits in the brain. *Annu Rev Neurosci.* **23**, 155-184.

Levine AJ, Lewallen KA, Pfaff SL (2012). Spatial organization of cortical and spinal neurons controlling motor behavior. *Curr Opin Neurobiol.* **22**, 812-821.

Levone BR JF, Olivia F O (2015). Role of adult hippocampal neurogenesis in stress resilience. **1**, 147–155.

Lewis PF, Emerman M (1994). Passage through mitosis is required for oncoretroviruses but not for the human immunodeficiency virus. *J Virol.* **68**, 510-516.

Li J, Zhu Z, Bao Z (1996). Role of MacMARCKS in integrin-dependent macrophage spreading and tyrosine phosphorylation of paxillin. *J Biol Chem.* **271**, 12985-12990.

Lian Y, Xiao J, Wang Q, Ning L, Guan S, Ge H, Li F, Liu J (2014). The relationship between glucocorticoid receptor polymorphisms, stressful life events, social support, and post-traumatic stress disorder. *BMC Psychiatry.* **14**, 232.

Liu YF, Bertram K, Perides G, McEwen BS, Wang D (2004). Stress induces activation of stress-activated kinases in the mouse brain. *J Neurochem.* **89**, 1034-1043.

Lucassen PJ, Meerlo P, Naylor AS, van Dam AM, Dayer AG, Fuchs E, Oomen CA, Czeh B (2010). Regulation of adult neurogenesis by stress, sleep disruption, exercise and inflammation: Implications for depression and antidepressant action. *Eur Neuropsychopharmacol.* **20**, 1-17.

Luddington NS, Mandadapu A, Husk M, El-Mallakh RS (2009). Clinical Implications of Genetic Variation in the Serotonin Transporter Promoter Region: A Review. In *Prim Care Companion J Clin Psychiatry*), pp. 93-102.

Luu P, Sill OC, Gao L, Becker S, Wojtowicz JM, Smith DM (2012). The role of adult hippocampal neurogenesis in reducing interference. *Behav Neurosci.* **126**, 381-391.

Lydiard RB (2003). The role of GABA in anxiety disorders. *J Clin Psychiatry.* **64 Suppl 3**, 21-27.

Madsen TM, Yeh DD, Valentine GW, Duman RS (2005). Electroconvulsive seizure treatment increases cell proliferation in rat frontal cortex. *Neuropsychopharmacology.* **30**, 27-34.

Maekawa T, Bernier F, Sato M, Nomura S, Singh M, Inoue Y, Tokunaga T, Imai H, Yokoyama M, Reimold A, Glimcher LH, Ishii S (1999). Mouse ATF-2 null mutants display features of a severe type of meconium aspiration syndrome. *J Biol Chem.* **274**, 17813-17819.

Magarinos AM, McEwen BS, Flugge G, Fuchs E (1996). Chronic psychosocial stress causes apical dendritic atrophy of hippocampal CA3 pyramidal neurons in subordinate tree shrews. *J Neurosci.* **16**, 3534-3540.

Malberg JE, Duman RS (2003). Cell proliferation in adult hippocampus is decreased by inescapable stress: reversal by fluoxetine treatment. *Neuropsychopharmacology.* **28**, 1562-1571.

Malberg JE, Eisch AJ, Nestler EJ, Duman RS (2000). Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. *J Neurosci.* **20**, 9104-9110.

Manev H, Uz T, Smalheiser NR, Manev R (2001). Antidepressants alter cell proliferation in the adult brain in vivo and in neural cultures in vitro. *Eur J Pharmacol.* **411**, 67-70.

Maren S (1996). Synaptic transmission and plasticity in the amygdala. An emerging physiology of fear conditioning circuits. *Mol Neurobiol.* **13**, 1-22.

Mathew SJ, Price RB, Charney DS (2008). Recent advances in the neurobiology of anxiety disorders: implications for novel therapeutics. *Am J Med Genet C Semin Med Genet.* **148c**, 89-98.

Mazzucchelli C, Vantaggiato C, Ciamei A, Fasano S, Pakhotin P, Krezel W, Welzl H, Wolfer DP, Pages G, Valverde O, Marowsky A, Porrazzo A, Orban PC, Maldonado R, Ehrengruber MU, Cestari V, Lipp HP, Chapman PF, Pouyssegur J, Brambilla R (2002). Knockout of ERK1 MAP kinase enhances synaptic plasticity in the striatum and facilitates striatal-mediated learning and memory. *Neuron.* **34**, 807-820.

McEwen BS (1999). Stress and hippocampal plasticity. *Annu Rev Neurosci.* **22**, 105-122.

McGaugh JL (2004). The amygdala modulates the consolidation of memories of emotionally arousing experiences. *Annu Rev Neurosci.* **27**, 1-28.

McGinn MJ, Sun D, Colello RJ (2008). Utilizing X-irradiation to selectively eliminate neural stem/progenitor cells from neurogenic regions of the mammalian brain. *J Neurosci Methods.* **170**, 9-15.

McNamara RK, Lenox RH (2000). Differential regulation of primary protein kinase C substrate (MARCKS, MLP, GAP-43, RC3) mRNAs in the hippocampus during kainic acid-induced seizures and synaptic reorganization. *J Neurosci Res.* **62**, 416-426.

Meltzer LA, Yabaluri R, Deisseroth K (2005). A role for circuit homeostasis in adult neurogenesis. *Trends Neurosci.* **28**, 653-660.

Mendez-David I, David DJ, Darcet F, Wu MV, Kerdine-Romer S, Gardier AM, Hen R (2014).

Rapid anxiolytic effects of a 5-HT(4) receptor agonist are mediated by a neurogenesis-independent mechanism. *Neuropsychopharmacology*. **39**, 1366-1378.

Mendez-David I, Hen R, Gardier AM, David DJ (2013). Adult hippocampal neurogenesis: an actor in the antidepressant-like action. *Ann Pharm Fr*. **71**, 143-149.

Merali Z, Levac C, Anisman H (2003). Validation of a simple, ethologically relevant paradigm for assessing anxiety in mice. *Biol Psychiatry*. **54**, 552-565.

Meshi D, Drew MR, Saxe M, Ansorge MS, David D, Santarelli L, Malapani C, Moore H, Hen R (2006). Hippocampal neurogenesis is not required for behavioral effects of environmental enrichment. *Nat Neurosci*. **9**, 729-731.

Milad MR, Quirk GJ (2002). Neurons in medial prefrontal cortex signal memory for fear extinction. *Nature*. **420**, 70-74.

Ming GL, Song H (2005). Adult neurogenesis in the mammalian central nervous system. *Annu Rev Neurosci*. **28**, 223-250.

Mirescu C, Gould E (2006). Stress and adult neurogenesis. *Hippocampus*. **16**, 233-238.

Mirescu C, Peters JD, Gould E (2004). Early life experience alters response of adult neurogenesis to stress. *Nat Neurosci*. **7**, 841-846.

Mitra R, Jadhav S, McEwen BS, Vyas A, Chattarji S (2005). Stress duration modulates the spatiotemporal patterns of spine formation in the basolateral amygdala. *Proc Natl Acad Sci U S A*. **102**, 9371-9376.

Mizumatsu S, Monje ML, Morhardt DR, Rola R, Palmer TD, Fike JR (2003). Extreme sensitivity of adult neurogenesis to low doses of X-irradiation. *Cancer Res*. **63**, 4021-4027.

Morgan MA, Romanski LM, LeDoux JE (1993). Extinction of emotional learning: contribution of medial prefrontal cortex. *Neurosci Lett*. **163**, 109-113.

Mukherjee PK, DeCoster MA, Campbell FZ, Davis RJ, Bazan NG (1999). Glutamate receptor signaling interplay modulates stress-sensitive mitogen-activated protein kinases and neuronal cell death. *J Biol Chem*. **274**, 6493-6498.

Myers KM, Davis M (2007). Mechanisms of fear extinction. *Mol Psychiatry*. **12**, 120-150.

Nacher J, Alonso-Llosa G, Rosell DR, McEwen BS (2003). NMDA receptor antagonist treatment increases the production of new neurons in the aged rat hippocampus. *Neurobiol Aging*. **24**, 273-284.

Nicolas LB, Kolb Y, Prinssen EP (2006). A combined marble burying-locomotor activity test in mice: a practical screening test with sensitivity to different classes of anxiolytics and antidepressants. *Eur J Pharmacol*. **547**, 106-115.

Olivier B, van Wijngaarden I, Soudijn W (2000). 5-HT(3) receptor antagonists and anxiety; a preclinical and clinical review. *Eur Neuropsychopharmacol*. **10**, 77-95.

Pan YW, Storm DR, Xia Z (2013). Role of adult neurogenesis in hippocampus-dependent memory, contextual fear extinction and remote contextual memory: new insights from ERK5 MAP kinase. *Neurobiol Learn Mem*. **105**, 81-92.

Park J, Liu AY (2001). JNK phosphorylates the HSF1 transcriptional activation domain: role of JNK in the regulation of the heat shock response. *J Cell Biochem*. **82**, 326-338.

Pavlowsky A, Zanchi A, Pallotto M, Giustetto M, Chelly J, Sala C, Billuart P (2010). Neuronal JNK pathway activation by IL-1 is mediated through IL1RAPL1, a protein required for development of cognitive functions. In *Commun Integr Biol*), pp. 245-247.

Paxinos G FK (2008). The Mouse Brain in Stereotaxic Coordinates, Compact | 978-0-12-374244-5 | Elsevier.

Pellow S, Chopin P, File SE, Briley M (1985). Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. *J Neurosci Methods*. **14**, 149-167.

Petrik D, Lagace DC, Eisch AJ (2012). The neurogenesis hypothesis of affective and anxiety disorders: are we mistaking the scaffolding for the building? *Neuropharmacology*. **62**, 21-34.

Pittenger C, Duman RS (2008). Stress, depression, and neuroplasticity: a convergence of mechanisms. *Neuropsychopharmacology*. **33**, 88-109.

Praag H, Shubert T, Zhao C, Gage FH (2005). Exercise Enhances Learning and Hippocampal Neurogenesis in Aged Mice. *J Neurosci*. **25**, 8680-8685.

Pulverer BJ, Kyriakis JM, Avruch J, Nikolakaki E, Woodgett JR (1991). Phosphorylation of c-jun mediated by MAP kinases. *Nature*. **353**, 670-674.

Quirk GJ, Russo GK, Barron JL, Lebron K (2000). The role of ventromedial prefrontal cortex in the recovery of extinguished fear. *J Neurosci*. **20**, 6225-6231.

Radley JJ, Rocher AB, Miller M, Janssen WG, Liston C, Hof PR, McEwen BS, Morrison JH (2006). Repeated stress induces dendritic spine loss in the rat medial prefrontal cortex. *Cereb Cortex*. **16**, 313-320.

Ramos-Brossier M, Montani C, Lebrun N, Gritti L, Martin C, Seminatore-Nole C, Toussaint A, Moreno S, Poirier K, Dorseuil O, Chelly J, Hackett A, Gecz J, Bieth E, Faudet A, Heron D, Frank Kooy R, Loeys B, Humeau Y, Sala C, Billuart P (2015). Novel IL1RAPL1

mutations associated with intellectual disability impair synaptogenesis. *Hum Mol Genet*. **24**, 1106-1118.

Ravindran LN, Stein MB (2010). The pharmacologic treatment of anxiety disorders: a review of progress. *J Clin Psychiatry*. **71**, 839-854.

Reinecke K, Herdegen T, Eminel S, Aldenhoff JB, Schiffelholz T (2013). Knockout of c-Jun N-terminal kinases 1, 2 or 3 isoforms induces behavioural changes. *Behav Brain Res*. **245**, 88-95.

Repici M, Centeno C, Tomasi S, Forloni G, Bonny C, Vercelli A, Borsello T (2007). Time-course of c-Jun N-terminal kinase activation after cerebral ischemia and effect of D-JNKI1 on c-Jun and caspase-3 activation. *Neuroscience*. **150**, 40-49.

Revest JM, Dupret D, Koehl M, Funk-Reiter C, Grosjean N, Piazza PV, Abrous DN (2009). Adult hippocampal neurogenesis is involved in anxiety-related behavior. *Mol Psychiatry*. **14**, 959-967.

Rojczyk-Gołębiewska E, Pałasz A, Wiaderkiewicz R (2014). Hypothalamic Subependymal Niche: A Novel Site of the Adult Neurogenesis. In *Cell Mol Neurobiol*), pp. 631-642.

Romand S, Wang Y, Toledo-Rodriguez M, Markram H (2011). Morphological Development of Thick-Tufted Layer V Pyramidal Cells in the Rat Somatosensory Cortex. *Front Neuroanat*. **5**.

Rossato JI, Bevilaqua LR, Lima RH, Medina JH, Izquierdo I, Cammarota M (2006). On the participation of hippocampal p38 mitogen-activated protein kinase in extinction and reacquisition of inhibitory avoidance memory. *Neuroscience*. **143**, 15-23.

Rosso SB, Sussman D, Wynshaw-Boris A, Salinas PC (2005). Wnt signaling through Dishevelled, Rac and JNK regulates dendritic development. *Nat Neurosci*. **8**, 34-42.

Rotzinger S, Lovejoy DA, Tan LA (2010). Behavioral effects of neuropeptides in rodent models of depression and anxiety. *Peptides*. **31**, 736-756.

Saari TI, Uusi-Oukari M, Ahonen J, Olkkola KT (2011). Enhancement of GABAergic activity: neuropharmacological effects of benzodiazepines and therapeutic use in anesthesiology. *Pharmacol Rev*. **63**, 243-267.

Sabapathy K, Jochum W, Hochedlinger K, Chang L, Karin M, Wagner EF (1999). Defective neural tube morphogenesis and altered apoptosis in the absence of both JNK1 and JNK2. *Mech Dev*. **89**, 115-124.

Sabapathy K, Kallunki T, David JP, Graef I, Karin M, Wagner EF (2001). C-Jun Nh2-Terminal Kinase (Jnk)1 and Jnk2 Have Similar and Stage-Dependent Roles in Regulating T Cell Apoptosis and Proliferation. In *J Exp Med*), pp. 317-328.

Sahay A, Scobie KN, Hill AS, O'Carroll CM, Kheirbek MA, Burghardt NS, Fenton AA, Dranovsky A, Hen R (2011). Increasing adult hippocampal neurogenesis is sufficient to improve pattern separation. *Nature*. **472**, 466-470.

Sajdyk TJ, Shekhar A (1997). Excitatory amino acid receptors in the basolateral amygdala regulate anxiety responses in the social interaction test. *Brain Res*. **764**, 262-264.

Salam JN, Fox JH, Detroy EM, Guignon MH, Wohl DF, Falls WA (2009). Voluntary exercise in C57 mice is anxiolytic across several measures of anxiety. *Behav Brain Res*. **197**, 31-40.

Samuels BA, Hen R (2011). Neurogenesis and affective disorders. *Eur J Neurosci*. **33**, 1152-1159.

Samuels IS, Karlo JC, Faruzzi AN, Pickering K, Herrup K, Sweatt JD, Saitta SC, Landreth GE (2008). Deletion of ERK2 Mitogen-Activated Protein Kinase Identifies Its Key Roles in Cortical Neurogenesis and Cognitive Function.

Sanchez C, Diaz-Nido J, Avila J (2000). Phosphorylation of microtubule-associated protein 2 (MAP2) and its relevance for the regulation of the neuronal cytoskeleton function. *Prog Neurobiol*. **61**, 133-168.

Santarelli L, Saxe M, Gross C, Surget A, Battaglia F, Dulawa S, Weisstaub N, Lee J, Duman R, Arancio O, Belzung C, Hen R (2003). Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. *Science*. **301**, 805-809.

Sapolsky RM (2000). Glucocorticoids and hippocampal atrophy in neuropsychiatric disorders. *Arch Gen Psychiatry*. **57**, 925-935.

Satvat E, Gheidi A, Voll S, Odintsova IV, Marrone DF (2012). Location is everything: neurons born during fluoxetine treatment accumulate in regions that do not support spatial learning. *Neuropharmacology*. **62**, 1627-1633.

Saxe MD, Battaglia F, Wang JW, Malleret G, David DJ, Monckton JE, Garcia AD, Sofroniew MV, Kandel ER, Santarelli L, Hen R, Drew MR (2006). Ablation of hippocampal neurogenesis impairs contextual fear conditioning and synaptic plasticity in the dentate gyrus. *Proc Natl Acad Sci U S A*. **103**, 17501-17506.

Scharfman HE (2007). The CA3 "backprojection" to the dentate gyrus. *Prog Brain Res*. **163**, 627-637.

Seger R, Krebs EG (1995). The MAPK signaling cascade. *Faseb j*. **9**, 726-735.

Sherrin T, Blank T, Hippel C, Rayner M, Davis RJ, Todorovic C (2010). Hippocampal c-Jun-

N-Terminal Kinases Serve as Negative Regulators of Associative Learning. In *J Neurosci*), pp. 13348-13361.

Shin LM, Rauch SL, Pitman RK (2006). Amygdala, medial prefrontal cortex, and hippocampal function in PTSD. *Ann N Y Acad Sci.* **1071**, 67-79.

Shoichet SA, Duprez L, Hagens O, Waetzig V, Menzel C, Herdegen T, Schweiger S, Dan B, Vamos E, Ropers HH, Kalscheuer VM (2006). Truncation of the CNS-expressed JNK3 in a patient with a severe developmental epileptic encephalopathy. *Hum Genet.* **118**, 559-567.

Shors TJ, Townsend DA, Zhao M, Kozorovitskiy Y, Gould E (2002). Neurogenesis may relate to some but not all types of hippocampal-dependent learning. *Hippocampus.* **12**, 578-584.

Sidman RL, Miale IL, Feder N (1959). Cell proliferation and migration in the primitive ependymal zone: an autoradiographic study of histogenesis in the nervous system. *Exp Neurol.* **1**, 322-333.

Silva RM, Kuan CY, Rakic P, Burke RE (2005). Mixed lineage kinase-c-jun N-terminal kinase signaling pathway: a new therapeutic target in Parkinson's disease. *Mov Disord.* **20**, 653-664.

Smith GW, Aubry JM, Dellu F, Contarino A, Bilezikjian LM, Gold LH, Chen R, Marchuk Y, Hauser C, Bentley CA, Sawchenko PE, Koob GF, Vale W, Lee KF (1998). Corticotropin releasing factor receptor 1-deficient mice display decreased anxiety, impaired stress response, and aberrant neuroendocrine development. *Neuron.* **20**, 1093-1102.

Smith SM, Vale WW (2006). The role of the hypothalamic-pituitary-adrenal axis in neuroendocrine responses to stress. *Dialogues Clin Neurosci.* **8**, 383-395.

Snyder JS, Hong NS, McDonald RJ, Wojtowicz JM (2005). A role for adult neurogenesis in spatial long-term memory. *Neuroscience.* **130**, 843-852.

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

Soetanto A, Wilson RS, Talbot K, Un A, Schneider JA, Sobiesk M, Kelly J, Leurgans S, Bennett DA, Arnold SE (2010). Association of anxiety and depression with microtubule-associated protein 2- and synaptopodin-immunolabeled dendrite and spine densities in hippocampal CA3 of older humans. *Arch Gen Psychiatry.* **67**, 448-457.

Song H, Kempermann G, Wadiche LO, Zhao C, Schinder AF, Bischofberger J (2005). New Neurons in the Adult Mammalian Brain: Synaptogenesis and Functional Integration.

Stanton RA, Gernert KM, Nettles JH, Aneja R (2011). Drugs That Target Dynamic Microtubules: A New Molecular Perspective. *Med Res Rev.* **31**, 443-481.

Stevens JC, Pollack MH (2005). Benzodiazepines in clinical practice: consideration of their long-term use and alternative agents. *J Clin Psychiatry.* **66 Suppl 2**, 21-27.

Sugino T, Nozaki K, Takagi Y, Hattori I, Hashimoto N, Moriguchi T, Nishida E (2000). Activation of mitogen-activated protein kinases after transient forebrain ischemia in gerbil hippocampus. *J Neurosci.* **20**, 4506-4514.

Surget A, Saxe M, Leman S, Ibarguen-Vargas Y, Chalon S, Griebel G, Hen R, Belzung C (2008). Drug-dependent requirement of hippocampal neurogenesis in a model of depression and of antidepressant reversal. *Biol Psychiatry.* **64**, 293-301.

Szczepankiewicz BG, Kosogof C, Nelson LT, Liu G, Liu B, Zhao H, Serby MD, Xin Z, Liu M, Gum RJ, Haasch DL, Wang S, Clampit JE, Johnson EF, Lubben TH, Stashko MA, Olejniczak ET, Sun C, Dorwin SA, Haskins K, Abad-Zapatero C, Fry EH, Hutchins CW, Sham HL, Rondinone CM, Trevillyan JM (2006). Aminopyridine-based c-Jun N-terminal kinase inhibitors with cellular activity and minimal cross-kinase activity. *J Med Chem.* **49**, 3563-3580.

Tada E, Parent JM, Lowenstein DH, Fike JR (2000). X-irradiation causes a prolonged reduction in cell proliferation in the dentate gyrus of adult rats. *Neuroscience.* **99**, 33-41.

Tanapat P, Hastings NB, Rydel TA, Galea LA, Gould E (2001). Exposure to fox odor inhibits cell proliferation in the hippocampus of adult rats via an adrenal hormone-dependent mechanism. *J Comp Neurol.* **437**, 496-504.

Tang JX, Janmey PA (1998). Two distinct mechanisms of actin bundle formation. *Biol Bull.* **194**, 406-408.

Tanti A, Rainer Q, Minier F, Surget A, Belzung C (2012). Differential environmental regulation of neurogenesis along the septo-temporal axis of the hippocampus. *Neuropharmacology.* **63**, 374-384.

Tararuk T, Ostman N, Li W, Bjorkblom B, Padzik A, Zdrojewska J, Hongisto V, Herdegen T, Konopka W, Courtney MJ, Coffey ET (2006). JNK1 phosphorylation of SCG10 determines microtubule dynamics and axodendritic length. *J Cell Biol.* **173**, 265-277.

Tennant KA, Adkins DL, Donlan NA, Asay AL, Thomas N, Kleim JA, Jones TA (2011). The organization of the forelimb representation of the C57BL/6 mouse motor cortex as defined by intracortical microstimulation and cytoarchitecture. *Cereb Cortex.* **21**, 865-876.

Thomas GM, Huganir RL (2004). MAPK cascade signalling and synaptic plasticity. *Nat Rev Neurosci.* **5**, 173-183.

Thomas GM, Lin DT, Nuriya M, Huganir RL (2008). Rapid and bi-directional regulation of AMPA receptor phosphorylation and trafficking by JNK. *Embo j.* **27**, 361-372.

Tiraboschi E, Tardito D, Kasahara J, Moraschi S, Pruneri P, Gennarelli M, Racagni G, Popoli M (2004). Selective phosphorylation of nuclear CREB by fluoxetine is linked to activation of CaM kinase IV and MAP kinase cascades. *Neuropsychopharmacology.* **29**, 1831-1840.

Todorovic C, Sherrin T, Pitts M, Hippel C, Rayner M, Spiess J (2009). Suppression of the MEK/ERK signaling pathway reverses depression-like behavior of CRF2-deficient mice. *Neuropsychopharmacology.* **34**, 1416-1426.

Tortosa E, Montenegro-Venegas C, Benoist M, Härtel S, González-Billault C, Esteban JA, Avila J (2011). Microtubule-associated Protein 1B (MAP1B) Is Required for Dendritic Spine Development and Synaptic Maturation. In *J Biol Chem*), pp. 40638-40648.

Tovote P, Fadok JP, Lüthi A (2015). Neuronal circuits for fear and anxiety. *Nature Reviews Neuroscience.* **16**, 317-331.

Tye KM, Prakash R, Kim SY, Fenno LE, Grosenick L, Zarabi H, Thompson KR, Gradinaru V, Ramakrishnan C, Deisseroth K (2011). Amygdala circuitry mediating reversible and bidirectional control of anxiety. *Nature.* **471**, 358-362.

Uchida S, Hara K, Kobayashi A, Fujimoto M, Otsuki K, Yamagata H, Hobara T, Abe N, Higuchi F, Shibata T, Hasegawa S, Kida S, Nakai A, Watanabe Y (2011). Impaired hippocampal spinogenesis and neurogenesis and altered affective behavior in mice lacking heat shock factor 1. *Proc Natl Acad Sci U S A.* **108**, 1681-1686.

van den Bout I, van Rheenen J, van Angelen AA, de Rooij J, Wilhelmsen K, Jalink K, Divecha N, Sonnenberg A (2008). Investigation into the mechanism regulating MRP localization. *Exp Cell Res.* **314**, 330-341.

van Pelt J, van Ooyen A, Corner MA (1996). Growth cone dynamics and activity-dependent processes in neuronal network development. *Prog Brain Res.* **108**, 333-346.

Verghese GM, Johnson JD, Vasulka C, Haupt DM, Stumpo DJ, Blackshear PJ (1994). Protein kinase C-mediated phosphorylation and calmodulin binding of recombinant myristoylated alanine-rich C kinase substrate (MARCKS) and MARCKS-related protein. *J Biol Chem.* **269**, 9361-9367.

Vivar C, van Praag H (2013). Functional circuits of new neurons in the dentate gyrus. *Front Neural Circuits.* **7**.

von Moltke LL, Greenblatt DJ (2003). Medication dependence and anxiety. *Dialogues Clin Neurosci.* **5**, 237-245.

Vyas A, Bernal S, Chattarji S (2003). Effects of chronic stress on dendritic arborization in the central and extended amygdala. *Brain Res.* **965**, 290-294.

Vyas A, Mitra R, Shankaranarayana Rao BS, Chattarji S (2002). Chronic stress induces contrasting patterns of dendritic remodeling in hippocampal and amygdaloid neurons. *J Neurosci.* **22**, 6810-6818.

Walf AA, Frye CA (2007). The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. *Nat Protoc.* **2**, 322-328.

Wang LH, Besirli CG, Johnson EM, Jr. (2004). Mixed-lineage kinases: a target for the prevention of neurodegeneration. *Annu Rev Pharmacol Toxicol.* **44**, 451-474.

Warner-Schmidt JL, Duman RS (2006). Hippocampal neurogenesis: opposing effects of stress and antidepressant treatment. *Hippocampus.* **16**, 239-249.

Watanabe Y, Gould E, McEwen BS (1992). Stress induces atrophy of apical dendrites of hippocampal CA3 pyramidal neurons. *Brain Res.* **588**, 341-345.

Wefers B, Hitz C, Holter SM, Trumbach D, Hansen J, Weber P, Putz B, Deussing JM, de Angelis MH, Roenneberg T, Zheng F, Alzheimer C, Silva A, Wurst W, Kuhn R (2012). MAPK signaling determines anxiety in the juvenile mouse brain but depression-like behavior in adults. *PLoS One.* **7**, e35035.

Weiss LA, Shen Y, Korn JM, Arking DE, Miller DT, Fossdal R, Saemundsen E, Stefansson H, Ferreira MA, Green T, Platt OS, Ruderfer DM, Walsh CA, Altshuler D, Chakravarti A, Tanzi RE, Stefansson K, Santangelo SL, Gusella JF, Sklar P, Wu BL, Daly MJ (2008). Association between microdeletion and microduplication at 16p11.2 and autism. *N Engl J Med.* **358**, 667-675.

Wellman CL (2001). Dendritic reorganization in pyramidal neurons in medial prefrontal cortex after chronic corticosterone administration. *J Neurobiol.* **49**, 245-253.

West MJ, Slomianka L, Gundersen HJ (1991). Unbiased stereological estimation of the total number of neurons in thesubdivisions of the rat hippocampus using the optical fractionator. *Anat Rec.* **231**, 482-497.

Westenbroek C, Den Boer JA, Veenhuis M, Ter Horst GJ (2004). Chronic stress and social housing differentially affect neurogenesis in male and female rats. *Brain Res Bull.* **64**, 303-308.

Westerlund N, Zdrojewska J, Padzik A, Komulainen E, Bjorkblom B, Rannikko E, Tararuk T, Garcia-Frigola C, Sandholm J, Nguyen L, Kallunki T, Courtney MJ, Coffey ET (2011). Phosphorylation of

SCG10/stathmin-2 determines multipolar stage exit and neuronal migration rate. *Nat Neurosci.* **14**, 305-313.

Wiegler K, Bonny C, Coquoz D, Hirt L (2008). The JNK inhibitor XG-102 protects from ischemic damage with delayed intravenous administration also in the presence of recombinant tissue plasminogen activator. *Cerebrovasc Dis.* **26**, 360-366.

Williamson DE, Forbes EE, Dahl RE, Ryan ND (2005). A genetic epidemiologic perspective on comorbidity of depression and anxiety. *Child Adolesc Psychiatr Clin N Am.* **14**, 707-726, viii.

Winchester CL, Ohzeki H, Vouyiouklis DA, Thompson R, Penninger JM, Yamagami K, Norrie JD, Hunter R, Pratt JA, Morris BJ (2012). Converging evidence that sequence variations in the novel candidate gene MAP2K7 (MKK7) are functionally associated with schizophrenia. *Hum Mol Genet.* **21**, 4910-4921.

Winner B, Kohl Z, Gage FH (2011). Neurodegenerative disease and adult neurogenesis. *Eur J Neurosci.* **33**, 1139-1151.

Wohnsland F, Steinmetz MO, Aebi U, Vergeres G (2000). MARCKS-related protein binds to actin without significantly affecting actin polymerization or network structure. Myristoylated alanine-rich C kinase substrate. *J Struct Biol.* **131**, 217-224.

Wong EY, Herbert J (2004). The corticoid environment: a determining factor for neural progenitors' survival in the adult hippocampus. *Eur J Neurosci.* **20**, 2491-2498.

Wong EY, Herbert J (2005). Roles of mineralocorticoid and glucocorticoid receptors in the regulation of progenitor proliferation in the adult hippocampus. *Eur J Neurosci.* **22**, 785-792.

Wong EY, Herbert J (2006). Raised circulating corticosterone inhibits neuronal differentiation of progenitor cells in the adult hippocampus. *Neuroscience.* **137**, 83-92.

Wu MV, Hen R (2014). Functional dissociation of adult-born neurons along the dorsoventral axis of the dentate gyrus. *Hippocampus.* **24**, 751-761.

Xu P, Das M, Reilly J, Davis RJ (2011). JNK regulates FoxO-dependent autophagy in neurons. *Genes Dev.* **25**, 310-322.

Yamasaki T, Kawasaki H, Nishina H (2012). Diverse Roles of JNK and MKK Pathways in the Brain. *J Signal Transduct.* **2012**.

Yang H, Courtney MJ, Martinsson P, Manahan-Vaughan D (2011). Hippocampal long-term depression is enhanced, depotentiation is inhibited and long-term potentiation is unaffected by the application of a selective c-Jun N-terminal kinase inhibitor to freely behaving rats. *Eur J Neurosci.* **33**, 1647-1655.

Yao B, Jin P (2014). Unlocking epigenetic codes in neurogenesis.

Yasuda J, Whitmarsh AJ, Cavanagh J, Sharma M, Davis RJ (1999). The JIP group of mitogen-activated protein kinase scaffold proteins. *Mol Cell Biol.* **19**, 7245-7254.

Yoshimura K, Ueno M, Lee S, Nakamura Y, Sato A, Kishima H, Yoshimine T, Yamashita T (2011). c-Jun N-terminal kinase induces axonal degeneration and limits motor recovery after spinal cord injury in mice. *Neurosci Res.* **71**, 266-277.

Yuan A, Rao MV, Veeranna, Nixon RA (2012). Neurofilaments at a glance. *J Cell Sci.* **125**, 3257-3263.

Yuan TF, Li J, Ding F, Arias-Carrion O (2014). Evidence of adult neurogenesis in non-human primates and human. *Cell Tissue Res.* **358**, 17-23.

Zencak D, Lingbeek M, Kostic C, Tekaya M, Tanger E, Hornfeld D, Jaquet M, Munier FL, Schorderet DF, van Lohuizen M, Arsenijevic Y (2005). Bmi1 loss produces an increase in astroglial cells and a decrease in neural stem cell population and proliferation. *J Neurosci.* **25**, 5774-5783.

Zhao C, Deng W, Gage FH (2008). Mechanisms and functional implications of adult neurogenesis. *Cell.* **132**, 645-660.

Zhen X, Du W, Romano AG, Friedman E, Harvey JA (2001). The p38 mitogen-activated protein kinase is involved in associative learning in rabbits. *J Neurosci.* **21**, 5513-5519.

## The Author

Hasan Mohammad was born on November 16, 1986 in Himachal Pradesh, India. He did his Bachelors in Microbiology from Panjab University, Chandigarh, India. He received Masters degree in Biotechnology from Jawaharlal Nehru University, New Delhi, India. Since 2010, he has been working for Ph.D. thesis in the laboratory of Dr Eleanor Coffey at Turku Centre for Biotechnology, University of Turku and Åbo Akademi University.

ISBN 978-952-12-3360-9