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221 00 Lund  
+46 46-222 00 00

# Lithium and Brain Plasticity

Studies on glial cell changes and electroconvulsive  
treatment-induced amnesia in rats

by

**Karin Orre**

## **Academic dissertation**

With the approval from the Faculty of Medicine at Lund University  
this thesis will be defended on January 26, 2013 at 9.00,  
in Segerfalksalen, Wallenberg Neuroscience Center, Lund, Sweden

## **Faculty opponent**

Professor Jerker Hetta

Department of Clinical Neuroscience, Karolinska Institute, Stockholm, Sweden



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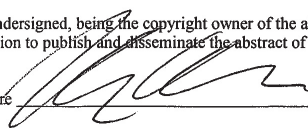
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Abstract <p>Depression and bipolar disorder, collectively known as mood disorders, are devastating, common and often chronic illnesses. Imaging studies of patients with mood disorders have demonstrated structural changes in several brain regions implicated in mood regulation. Furthermore, bipolar disorder is associated with white matter abnormalities and <i>post mortem</i> analysis of brain tissue from patients with mood disorders have shown glial cell pathology. Electroconvulsive therapy (ECT) and pharmacological treatment with lithium have been used in the treatment of mood disorders for over 70 respectively 60 years, but the mechanisms behind their therapeutic effects remain elusive.</p> <p>We have previously shown increased neurogenesis and NG2 cell proliferation in a rat model of ECT, electroconvulsive seizures (ECS). NG2 cells can differentiate into mature myelinating oligodendrocytes in the adult brain. Moreover, given the fact they are an abundant proliferative cell type in all areas implicated in mood disorders and with a unique capacity to respond directly to neuronal signalling changes through their specialized contacts with neurons, NG2 cells are highly interesting in the context of mood disorder-associated white and grey matter changes.</p> <p>In paper I we show that chronic lithium treatment unlike its stimulating effect on hippocampal neurogenesis, decreased NG2 cell proliferation in the rat dentate hilus of hippocampus, amygdala and corpus callosum. Decreased proliferation could reflect decreased oligodendrogenesis or possibly cell cycle arrest in favour of differentiation into oligodendrocytes. Thus, in paper II we investigated the effect of lithium on remyelination and oligodendrogenesis in corpus callosum after chemically induced demyelination. We found that lithium treatment during the recovery period after the demyelinating insult decreased remyelination and oligodendrogenesis. In addition, the demyelination-induced inflammation was decreased by lithium. Further studies are needed to investigate if those effects are specific for rats, the dose of lithium used and the brain region investigated.</p> <p>Studies from our laboratory have previously shown a low-grade glial cell activation following ECS. In paper III we show that blood-borne macrophages are recruited to the hippocampal vessel walls after ECS. It can represent the first step in an inflammatory process, but when no further signals are acquired further progression through the astrocytic end-feet layer into the brain parenchyma is halted.</p> <p>ECT's clinical practice and general acceptance has been limited by concerns about side effects, particularly regarding memory deficits. Certain pharmacological agents administered in association with ECT may protect against amnesia. During recent years, lithium has been shown to reduce memory deficits induced by stroke, stress, head trauma etc. in rodents. In paper IV, we investigated the effect of ECS and lithium treatment on spatial memory and demonstrated robust memory loss for a hippocampus-dependent navigational task learned during the week preceding ECS. This finding was consistent in four independent investigations. However the effect of lithium treatment on ECS-induced amnesia was not as conclusive. In two identically designed studies, lithium counteracted the ECS-induced amnesia, but was neither associated with reduced cell death nor reduced microglia activation. Importantly though, an anti-amnesic effect of lithium was not found in two following equally designed studies. Further investigations of ECS-related disturbances are currently ongoing in our research group.</p>	
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Date 2012-12-14

# Lithium and Brain Plasticity

Studies on glial cell changes and electroconvulsive  
treatment-induced amnesia in rats

**Karin Orre**

Psychiatric Neuromodulation Unit  
Department of Clinical Sciences  
Lund University  
Lund, Sweden



**LUND**  
UNIVERSITY

Academic Dissertation

Lund 2013

*Cover*

*An artistic illustration of two NG2 cells by Greta Mundt-Petersen*



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To my Family

*“The most beautiful thing we can experience is the mysterious.  
It is the source of all true art and science.”*

*Albert Einstein*



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# Original articles

This thesis is based on the following papers, which will be referred to by their roman numerals:

I

**Orre K.**, Wennström M., Tingström A.

Chronic lithium treatment decreases NG2 cell proliferation in rat dentate hilus, amygdala and corpus callosum.

*Prog Neuropsychopharmacol Biol Psychiatry*. 2009 Apr 30;33(3):503-10.

II

**Orre K.**, Tingström A.

Lithium decreases oligodendrogenesis and microglia activation after cuprizone-induced demyelination.

*Submitted*

III

Jansson L., **Orre K.**, Tingström A.

Repeated electroconvulsive seizures increase the number of vessel-associated macrophages in rat hippocampus.

*J ECT*. 2012 Sep;28(3):174-9.

IV

**Orre K.**\*, Jansson L.\*, Svensson M., Grahm M., Tingström A.

Effect of lithium treatment on electroconvulsive seizure-induced spatial memory loss.

*Manuscript*

\* These authors contributed equally to this work

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## Article not included in the thesis

Mohapel P, **Mundt-Petersen K**, Brundin P, Frielingsdorf H.

Working memory training decreases hippocampal neurogenesis.

*Neuroscience*. 2006 Oct 27;142(3):609-13

# Abbreviations

ACTH, adrenocorticotrophic hormone

ADC, apparent diffusion coefficient

AMPA, 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid

ANOVA, analysis of variance

BBB, blood-brain-barrier

BDNF, brain-derived neurotrophic factor

BrdU, 5-bromo-2'-deoxyuridine

CA, cornus ammonis

CNS, central nervous system

CRH, corticotropin-releasing hormone

DAB, 3,3'-diaminobenzidine

DSM-IV, the 4th edition of diagnostic and statistical manual of mental disorders

DTI, diffusion tensor imaging

DWI, diffusion weighted imaging

ECM, extra cellular matrix

EEG, electroencephalography

ECS, electroconvulsive seizures

ECT, electroconvulsive therapy

FA, fractional anisotropy

GABA,  $\gamma$ -aminobutyric acid

GCL, granule cell layer

GSK-3, glycogen synthase kinase-3

HCl, hydrochloric acid

HPA, hypothalamic-pituitary-adrenal  
ICD-10, international classification of diseases, 10th revision  
KPBS, potassium phosphate buffered saline  
LFB, luxol fast blue  
LPC, lysolecithin  
LTP, long-term potentiation  
MAOIs, monoamine oxidase inhibitors  
ML, molecular layer  
MRI, magnetic resonance imaging  
MS, multiple sclerosis  
MWM, Morris water maze  
NGF, nerve growth factor  
NMDA, N-methyl-D-aspartate  
OPCs, oligodendrocyte precursor cells  
PBS, phosphate buffered saline  
PFA, phosphate buffered paraformaldehyde  
PFC, prefrontal cortex  
SGZ, subgranular zone  
SNRIs, serotonin and noradrenaline reuptake inhibitors  
SSRIs, selective serotonin reuptake inhibitors  
STAT, signal transducer and activator of transcription  
TCAs, tricyclic antidepressants  
TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling  
VEGF, vascular endothelial growth factor  
WHO, World Health Organization  
WKY, Wistar-Kyoto



# Svensk populärvetenskaplig sammanfattning

Att ibland känna sig ledsen och nerstämd och vid andra tillfällen upprymd, kreativ och full av energi är en naturlig del av livet. Vid affektiva sjukdomar förändras emellertid humöret så mycket att yrkesliv, sociala aktiviteter och/eller nära relationer allvarligt drabbas. Oftast är då också vitala funktioner såsom aptit, sömn och sexuell lust rubbade. Affektiva sjukdomar delas grovt in i depression och bipolär sjukdom (tidigare kallad manodepressiv sjukdom). Bipolär sjukdom kännetecknas av omväxlande episoder av nedstämdhet (depression) respektive förhöjt eller irriterat stämningsläge med överdrivet självförtroende, rastlöshet och minskat sömnbehov (mani). Affektiva sjukdomar är allvarliga sjukdomar som i de svåraste fallen har dödlig utgång i form av självmord. Depression är en folksjukdom som drabbar ungefär var femte person någon gång under livet. Bipolär sjukdom drabbar ungefär 1 % av befolkningen och är oftare kronisk.

Orsaken till affektiva sjukdomar är inte känd, men de flesta är överens om att både arv och miljö är av betydelse. Med hjälp av bland annat magnetkameraundersökningar har man på senare år upptäckt strukturella förändringar i vissa av hjärnans så kallade limbiska områden, både hos patienter med bipolär sjukdom och depression. Limbiska hjärnområden, såsom hippokampus och amygdala är regioner med grå substans som är inblandade i regleringen av vårt känsloliv. Grå substans innehåller bland annat nervcellkroppar. Hos patienter med bipolär sjukdom har man sett att även den vita substansen är abnormal. I den vita substansen sker kommunikation mellan olika hjärnområden. Den består av nervcellstrådar som är isolerade med myelin, ett tunt fettinnehållande lager, som gör att signalerna genom nervcellstrådarna går fortare. Myelinet tillverkas inte av nervcellerna själva utan av speciella gliaceller, så kallade oligodendrocyter. När man har undersökt hjärnvävnaden hos avlidna personer med kända affektiva sjukdomar har man sett minskat antal av just gliaceller på vissa ställen.

Litium är det läkemedel man i första hand väljer för att behandla bipolär sjukdom. Det motverkar utvecklingen av både manier och depressioner. Detta upptäcktes av en slump i början på 1950-talet. *Hur* medicinen egentligen fungerar är ännu inte känt.



Nyligen upptäckte man att litium både skyddade mot nervcellsöd och ökade nybildningen av nervceller i hippocampus hos råttor. Man upptäckte också att volymsminskningarna man sett här med magnetkamera-tekniken hos patienter med bipolär sjukdom återställdes med litiumbehandling. Litiums effekt på den vita substansen är dock fortfarande ofullständigt utredd.

I den första uppsatsen i denna avhandling ville vi därför undersöka hur kontinuerlig litiumbehandling påverkade nybildningen och överlevnaden av så kallade NG2-positiva gliaceller. Dessa celler finns spridda i hela hjärnan, både i vit och grå substans och är den celltyp som nyproduceras mest i den vuxna hjärnan. NG2-celler kan utmognas till oligodendrocyter och därigenom alltså bidra till ökad hastighet i nervsignaleringen. Men NG2-celler verkar även kunna fungera som känsliga övervakare av nervcellskommunikationen. Dessa celler kan som enda gliacellstyp koppla sig direkt till nervceller och läsa av, påverkas av och möjligen påverka nervsignalleringen. I våra försök visade det sig att det nybildades ca 45 % färre NG2-celler i den vita substansen hos råttor som behandlats med litium. I de grå hjärnområdena amygdala och hippocampus minskade nybildningen med ca 20 respektive 40 %. Precis som andra tidigare visat i sina studier fann vi också att litium ökade nybildningen av nervceller i hippocampus.

Vi undrade om detta kunde betyda att NG2 cellerna slutade att dela sig och istället utvecklades till oligodendrocyter. Eller var det så att det istället blev färre oligodendrocyter i litiumbehandlade råttor? För att undersöka detta använde vi i den andra uppsatsen oss av råttor som först behandlats med en substans som bryter ner den vita substansen. Detta för att skapa ett behov av nya oligodendrocyter och därmed lättare kunna undersöka nybildningen av dem. När dessa råttor behandlades med litium visade det sig att den vita substansen inte återbildades lika snabbt. De nya mogna oligodendrocyter var färre i de litiumbehandlade råttorna. Likaså var intensiteten av det infärgade myelinet lägre. Däremot upptäckte vi att den inflammation som uppstått i den skadade vita substansen mildrades avsevärt med litium-behandling. Fler studier behövs för att utreda om våra resultat är specifika för råttor, det hjärnområden vi undersökt och den dos av litium vi använt. Med tanke på våra resultat är det dock viktigt att i framtida studier ta hänsyn till att litium-behandling har potential att påverka nybildningen av olika celltyper och att behandlingen inte har samma effekt på alla celltyper.

Vår forskargrupp har tidigare visat att elbehandling (ECT), som används vid behandling av allvarliga fall av affektiva sjukdomar, ökar nybildningen av NG2-celler i hippocampus och amygdala hos råttor. Samtidigt uppstår en låggradig inflammation där. NG2-cellynybildning skulle kunna vara en del av det inflammatoriska svaret. Vid allvarliga inflammationer i hjärnan kommer det via blodbanorna in immunförsvarsceller till hjärnan. I denna avhandlingens tredje uppsats, ville vi undersöka om detta skedde efter elbehandling. En speciell immunförsvarande celltyp,

makrofagen (storätarcellen) ansamlades i kärnväggen i hippokampus i elbehandlade djur, men vandrade inte vidare in i själva hjärnvävnaden.

Elbehandling är liksom andra medicinska behandlingar förknippad med vissa biverkningar. En del patienter får minnesstörningar. Speciellt påverkas då minnet för sådant som hände dagarna närmast elbehandlingen. I råttor har litiumbehandling visat sig kunna skydda mot minnesstörning som ses vid Alzheimers sjukdom, stress, stroke m.m. Detta har då varit kopplat till minskad nervcellsöd. I den fjärde uppsatsen undersökte vi om litiumbehandling kunde skydda mot elbehandlingsorsakade minnesstörningar och om detta var kopplat till celldöd och inflammation. Elbehandlade råttor hade en tydlig minnesstörning för en navigationsuppgift som de lärt sig veckan innan den första elbehandlingen, men de råttor som fått litiumbehandling tillsammans med elbehandling hade ingen sådan minnesstörning. Detta var dock inte kopplat till celldöd eller inflammation. Inom forskningen är det viktigt att kunna återupprepa resultat. Litiums skyddande effekt mot minnesstörningar efter elbehandling upprepades i en oberoende identisk studie. I två efterföljande likadana experiment såg vi också en tydlig minnesstörning hos de elbehandlade råttorna, men den påverkades inte av samtidig litiumbehandling. I kliniken har man tidigare undvikit att behandla patienter med litium och elbehandling samtidigt eftersom det finns rapporter om fall där patienter vid dubbelbehandling blivit mer förvirrade precis efter uppvaknandet efter elbehandling. Idag har flera studier visat att då litiumdosen hålls på den nivå som rekommenderas är samtidig behandling inget problem. Vi har i våra råttstudier visat att litiumbehandling inte verkar försämra minnesstörningarna efter elbehandling, utan möjligen under vissa omständigheter mildra dem.

Slutligen vill jag poängtera att affektiva sjukdomar skapar stort lidande hos människor i hela världen. De behandlingar som finns att erbjuda idag hjälper långt ifrån alla. För att utveckla nya mer effektiva behandlingar är det viktigt att utreda vad som orsakar sjukdomarna och hur befintliga behandlingar fungerar för att uppnå terapeutisk effekt. Studierna i den här avhandlingen är en liten del i det arbetet.

# Summary

Mood disorders are common, debilitating and often chronic disorders, which in severe cases can have a fatal outcome, suicide. Not only the state of mood is affected, but also vital functions such as appetite, sexuality, sleep and memory. Mood disorders are subdivided into depressive disorders and bipolar disorders (formerly known as manic-depressive illness). According to the WHO Global Burden of disease estimate, major depressive disorders will in year 2020 be the second leading cause of mortality and disability in the world. If considering disability alone, major depressive disorders is the leading cause and bipolar disorder is the sixth leading cause of disability in the world. The lifetime risk for major depression is around 15-20 % and women are more often affected than men. However, bipolar disorder affects men and women equally and the lifetime risk is circa 1 %.

Brain imaging and *post mortem* studies of patients with mood disorders provide strong evidence for morphological abnormalities in several brain regions including prefrontal cortex regions, amygdala and hippocampus. For bipolar disorder, white matter changes are also a consistent finding. Magnetic resonance imaging studies have repeatedly shown decreased size and abnormal shape and signal intensity of corpus callosum (the large white matter commissure connecting the cerebral hemispheres). In addition, diffusion tensor imaging studies show altered integrity in corpus callosum. A few post-mortem studies have reported reduced density of oligodendrocytes in amygdala, prefrontal cortex and adjacent white matter. Furthermore, decreased expression of oligodendrocyte-associated proteins has been shown in transcriptome studies.

Pharmacological treatment with lithium has for over 60 years been used in the treatment of bipolar disorder and is still the first-line choice. Recent magnetic resonance imaging meta-analyses conclude that lithium increase grey matter volume in limbic areas such as hippocampus and amygdala. This is in line with animal and *in vitro* studies showing strong neurotrophic and neuroprotective effects of lithium. Lithium is known to increase neurogenesis and decrease stress-induced dendritic atrophy in hippocampus. However, reports on the effect of lithium on white matter and glial cells are sparse.

Studies from our laboratory have focused on plastic changes following electroconvulsive seizures (ECS), an animal model of the psychiatric treatment electroconvulsive therapy (ECT). ECT is used to treat severe forms of depression, mania and catatonia. It is mainly considered when pharmacological treatments have failed or when there is an urgent need for a fast response. Our research group has previously shown increased hippocampal neurogenesis, gliogenesis and angiogenesis and in addition gliogenesis in amygdala following ECS. The glial cells proliferating in response to ECS are NG2 cells, a class of glial cells distinct from astrocytes, microglia and mature oligodendrocytes. They are widely spread throughout the grey and white matter and are the predominantly dividing cell type in adult rodent brain. It is well known that NG2 cells can differentiate into myelinating oligodendrocytes. However, the somewhat equal distribution of NG2 cells between white and grey matter, their complex morphology and the fact that they continue to proliferate after the peak of oligodendrogenesis, imply that they have other functions apart from being oligodendrocyte precursors. In recent years NG2 cells have been demonstrated to receive direct synaptic input from glutamatergic and GABAergic neurons. It has been proposed that neuron-NG2 cell synapses could regulate NG2 cell development in a neuronal activity-dependent manner. Finally these cells are also suggested to take part in axonal guidance, inflammatory response and synaptic function and structure.

Studies included in this thesis show that chronic lithium treatment in therapeutically relevant concentrations decrease NG2 cell proliferation in the rat dentate hilus, amygdala and corpus callosum. Interestingly, lithium had no effect on proliferation of NG2 cells in the molecular layer or CA-regions of hippocampus. A majority of the NG2 cells in corpus callosum differentiated into oligodendrocytes, whereas in the grey matter regions a majority remained in an NG2-positive state. Even though it is not possible to grasp the whole picture yet, direct or indirect effects on NG2 cell proliferation/fate/function have potential to modulate synaptogenesis, myelination, axonal outgrowth and other plastic changes important for changing the connectivity in the diseased human brain. Considering the increasing number of imaging studies showing white matter abnormalities in patients with bipolar disorder we found it interesting to investigate the effect of lithium on the oligodendrocyte fate of NG2 cells after a demyelinating insult. We show that lithium treatment decreased oligodendrogenesis in the rat corpus callosum after chemically induced demyelination. In association with the decreased oligodendrogenesis we also found decreased remyelination. This is opposed to other studies showing decreased demyelination in an animal model of an autoimmune demyelinating disease. However, in line with several other studies showing anti-inflammatory effects of lithium, we also show that lithium decreased the demyelination-induced microglia activation.

In addition to the low-grade glial cell activation previously shown in studies from our laboratory following ECS, we found that macrophages from the periphery were recruited to the blood vessel walls of hippocampus. However, we did not find any further progression of T-cells and macrophages into the brain parenchyma.

ECT's clinical practice and general acceptance has been limited by concerns about side effects, particularly regarding memory deficits. Certain pharmacological agents administered in association with ECT may protect against amnesia. During recent years, lithium has been shown to reduce memory deficits induced by stroke, stress, head trauma etc. In the last study in the thesis we investigated the effect of ECS and lithium treatment on spatial memory in rats and demonstrated robust memory loss for a hippocampus-dependent navigational task learned during the week preceding ECS. This finding was consistent in four independent studies and in line with previous studies by others. However the effect of lithium treatment on ECS-induced amnesia was not as conclusive. In two equally designed studies lithium counteracted the ECS-induced amnesia, but in two following studies no such effect was found. Further studies are needed to reveal the cause of the conflicting result.

Mood disorders are major contributors to human disability and mortality all over the world. The treatments available today fail to relieve the symptoms in many patients. To develop new more effective treatments, it is important to understand the mechanism behind the diseases and how the current treatments exert their effects. The studies included in this thesis are a small part of that work.

# Introduction

## Mood Disorders

### **What is a disorder of the mood?**

When can the state of mood be considered a disease? How can we distinguish abnormal mood from normal mood? Feelings of sadness, hopelessness and loss of energy are experienced by most people at some point. Equally we sometimes experience elevated mood with increased self-esteem and creativity. The precise boundaries between these normal reactions to life events and disease are hard to define, but it seems like people have noticed extreme variety of mood in all times and all cultures. At least, descriptions of mood disorders date back to the oldest writings of mankind (see Box 1).

Currently mood disorders are subdivided into depressive disorders and bipolar disorders (formerly known as manic-depressive illness). They are common, often chronic disorders, which in severe cases can have a fatal outcome, suicide. Not only the state of mood is affected but also vital functions such as appetite, sexuality, sleep and memory. The diagnoses are considered when the duration of elevated or depressed mood is longer than normal and the symptoms are sufficiently severe to cause marked impairment in working ability, in usual social activities or relationships with others (see Symptoms, diagnosis and classification below).

## BOX 1

Already the ancient Greeks...

The ancient Greeks and Romans described extreme manifestations of mood and coined the terms melancholia and mania. Hippocrates (460 to 357 BC), often called the father of medicine, described melancholia as a state of “aversion to food, despondency, sleeplessness, irritability and restlessness”; a description that very much resembles the criteria used to diagnose major depression today (see Box 2 below). Hippocrates also thought that mental disorders were disorders of the brain. However, this insight was lost under the influence of Galen’s humoral theory; according to which your temperament was set by the balance of four bodily fluids; blood, yellow bile, black bile and phlegm. Melancholia was thought to be due to an excess of black bile and mania either to an excess of blood or a mixture of black and yellow bile. Psychiatric disorders were not re-localised to the brain until the mid 17<sup>th</sup> century, when the English professor Thomas Willis stated that “melancholy is a complicated distemper of the brain and the heart” (Geddes et al., 2011).

### **Symptoms, diagnosis and classification of mood disorder**

Currently in Sweden, mood disorders are diagnosed based on the criteria stated in the tenth revision of the International Classification of Diseases (ICD-10) by the World Health Organization (WHO). However, a manual specific for psychiatric diseases, the fourth edition of Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) is also widely used along with the official diagnostic system ICD-10. The depressive disorders include major depressive disorder (also called unipolar disorder/depression) and dysthymia (chronic depression with less severe symptoms). Major depressive disorder can be further specified based on severity (mild/moderate/severe) and special features (psychotic features, catatonic depression, melancholic depression, atypical depression, postpartum depression and affective seasonal disorder). The DSM-IV criteria for a depressed episode are listed in Box 2. To summarise, a depressed episode is characterized by depressed mood, low energy, changes in appetite and sleep, feelings of guilt, loss of interest in things that were normally found pleasurable and sometimes thoughts about death and suicide (Geddes et al., 2011).

## BOX 2

Criteria for Major Depressive Episode (adopted from DSM-IV)

Five (or more) of the following symptoms have been present during the same 2-week period and represent a change from previous functioning; at least one of the symptoms is either 1) or 2).

1. Depressed mood
  2. Loss interest or pleasure
  3. Significant weight loss/weight gain or decrease/increase in appetite.
  4. Insomnia or hypersomnia
  5. Psychomotor agitation or retardation
  6. Fatigue or loss of energy
  7. Feelings of worthlessness or inappropriate guilt (which may be delusional)
  8. Diminished ability to think or concentrate, or indecisiveness
  9. Recurrent thoughts of death, suicidal plans or a suicide attempts
- The symptoms cause clinically significant distress or impairment in social, occupational, or other important areas of functioning.
  - The symptoms are not due to the direct physiological effects of a substance (e.g., a drug of abuse, a medication) or a general medical condition (e.g., hypothyroidism).
  - The symptoms are not better accounted for by bereavement, i.e., after the loss of a loved one, the symptoms persist for longer than 2 months or are characterized by marked functional impairment, morbid preoccupation with worthlessness, suicidal ideation, psychotic symptoms, or psychomotor retardation.

Even though the majority of patients with bipolar disorder experience both episodes of elevated and depressed mood, the strict criteria for bipolar disorder (I) is only the presence of a manic episode lasting for at least seven days. The DSM-IV criteria for a manic episode are listed in Box 3. Shortly, mania is characterized by elevated or irritable mood. Thoughts are racing and changing rapidly. Speech is forced and louder than normally. People in a manic episode are restless and have a decreased need for sleep, only a few hours per day or not at all for several days. Energy and self-



esteem is high. Increased sexual activities and many new social contacts are also common. Hypomania (literally, below mania) is a less severe form of mania.

DSM-IV lists three specific subtypes of bipolar disorder; 1) bipolar I (defined by one or more manic episodes) 2) bipolar II (defined by one or more hypomanic episodes and one or more major depressive episodes) and 3) cyclothymia (defined by hypomanic episodes with episodes of depression that do not meet criteria for major depressive episodes). However, several other subtypes of bipolar disorder have been proposed. Bipolar III is defined by hypomanic episodes induced by antidepressant treatment in depressed patients. Bipolar IV refers to depressed patients having a substance-induced hypomania. Just as in the case of depressive disorders, the diagnosis bipolar disorder can also be further specified based on severity (mild/moderate/severe) and on special characteristics (psychotic features, catatonic features, postpartum onset, rapid cycling). Rapid cycling is defined by at least four episodes during the last year. Otherwise, the mean number of episodes for patients with bipolar disorder is around 0,5 per year and the median length of an episode is three months. But it varies substantially between patients (Angst and Sellaro, 2000).

### BOX 3

Criteria for a Manic Episode (adopted from DSM-IV)

A distinct period of abnormally and persistently elevated, expansive or irritable mood, lasting at least 1 week (or any duration if hospitalization is necessary). During the period of mood disturbance, three (or more) of the following symptoms have persisted (four if the mood is only irritable) and have been present to a significant degree:

1. Inflated self-esteem or grandiosity
  2. Decreased need for sleep (e.g., feels rested after only 3 hours of sleep)
  3. More talkative than usual or pressure to keep talking
  4. Flight of ideas or subjective experience that thoughts are racing
  5. Distractibility (i.e., attention too easily drawn to irrelevant external stimuli)
  6. Increase in goal-directed activity (at work or sexually) or psychomotor agitation
  7. Increased risk taking behaviour (e.g., engaging in unrestrained buying sprees, sexual indiscretions, or foolish business investments)
- The mood disturbance is sufficiently severe to cause marked impairment in occupational functioning or in usual social activities or relationships with others, or to necessitate hospitalization to prevent harm to self or others, or there are psychotic features.
  - The symptoms are not due to the direct physiological effects of a substance (e.g., a drug of abuse, a medication or other treatment) or a general medical condition (e.g., hyperthyroidism)

### Epidemiology

Mood disorders are as said above common and debilitating, causing severe personal suffering and high costs for the society. WHO lists which diseases and injuries that cause the most mortality and disability all over the world in an analysis called Global Burden of Disease. According to the Global Burden of disease from 1990, projected to 2020, major depressive disorders will in year 2020 be the second leading cause of mortality and disability in the world. If considering disability alone, major depressive disorders is the leading cause and bipolar disorder is the sixth leading cause of disability in the world (Murray et al., 1996).

Reports about the lifetime risks for major depression varies from 4.5% up to 30%, but is most often reported to be around 17 % (Kessler et al., 2003). Women are affected twice as often as men and the mean age of onset is around the age of 25-35 (Weissman et al., 1996).

For bipolar I, the prevalence is mostly found around 1 %. The lifetime prevalence is also around 1%, reflecting the high degree of chronicity of the disease (Weissman et al., 1996). In contrast to depressive disorders, the prevalence in males and females is similar and the most common age of onset is the teenage years or the 20's (Weissman et al., 1996).

## Structural changes in brain regions implicated in mood disorders

Brain imaging and *post mortem* studies of patients with mood disorders provide strong evidence for morphological and morphometric abnormalities in several brain regions; prefrontal cortex (PFC) regions and in the classical limbic structures amygdala and hippocampus (Price and Drevets, 2012). For bipolar disorder, white matter changes is also a consistent finding (De Peri et al., 2012).

### **PFC**

As the name indicates, PFC is the most anterior part of the frontal lobes of the brain. PFC has a crucial role in executive function, planning complex cognitive behaviour, decision making, moderating social behaviour and personality expression (Kandel et al., 2000).

Abnormalities in the left anterior cingulate cortex of the PFC, ventral to the *genu* (knee) of corpus callosum have repeatedly been reported for patients with mood disorder. Magnetic resonance imaging (MRI) demonstrate over 40 % volume reductions in this specific area and positron emission tomographic images of cerebral blood flow and glucose metabolism show decreased activity in the depressive state of mood disorder (Drevets et al., 1997). These changes have been proposed to exist already at an early stage of the illness (Boes et al., 2008) but progression of the abnormalities have also been shown in patients with psychotic mood disorder (Koo et al., 2008). Chronic lithium treatment (Moore et al., 2009), but not antidepressant drugs (Drevets et al., 1997) has been shown to normalize PFC volumes. Volume

reductions in other PFC subregions, such as the orbitofrontal cortex have also been reported (for review see (Price and Drevets, 2012).

## **Amygdala**

Amygdala is an almond-shaped bilateral structure deep in the antero-medial part of the temporal lobes, rostral to hippocampus. It plays a key role in fear processing (Ohman, 2005), aggressive behaviour, emotional modulation of learning and memory (Phelps and LeDoux, 2005) and sexual behaviour (Hines, 2010). For instance, a very early experiment by Klüver and Bucy from 1939 demonstrated that the bilateral destruction of the anterior temporal lobe, including the amygdala, led to hypoemotionality, hypersexuality, hyperorality, and loss of anger or fear responses, together accordingly called the Klüver-Bucy syndrome (Klüver and Bucy, 1939). In humans, bilateral amygdala damage cause an impaired recognition of emotional facial expression (Adolphs et al., 1994).

Amygdala is composed of a number of sub-nuclei including the basal, lateral, central and the medial nucleus. The basal and lateral nuclei are often collectively referred to as the basolateral nucleus and is the main sensory input to the amygdala. It has major connections to higher-level sensory cortical regions, thalamus and hippocampus and is involved in adding emotional significance to complex stimuli. The central nucleus is the main output nucleus in amygdala. It is highly connected to hypothalamus and the brainstem, adding physiological expression to emotions (affecting cutaneous blood flow, heart rate, piloerection etc.) (Kandel et al., 2000).

Reports on volumetric changes in amygdala in patients with mood disorders have been inconsistent. Increased (Altshuler et al., 1998; Frodl et al., 2002), decreased (Sheline et al., 1998; Kalmar et al., 2009) and preserved volume (Kempton et al., 2009) have been reported. Differences in medication status of patients included in these studies may partly explain the conflicting results. A recent meta-analysis concludes that lithium treatment is associated with increased amygdala size (Hafeman et al., 2012). Functional imaging studies show increased activity in amygdala in mood disorders (Chen et al., 2011). Lithium treatment has been shown to prevent stress-induced dendritic hypertrophy in amygdala (Johnson et al., 2009).

## **Hippocampus**

The hippocampal formation, usually simply called hippocampus, is a bilateral structure located within the medial temporal lobe (in primates), underneath the cortical surface. It traditionally belongs to the limbic system and is a very well studied

structure of the brain. Hippocampus creates a spatial map of the environment (O'Keefe, 1990) and is also important for formation of declarative memories (Squire and Zola, 1996) and regulation of mood (Fanselow and Dong, 2010; Chen et al., 2011). London taxi drivers have larger posterior hippocampus, corresponding to dorsal hippocampus in rats (Maguire et al., 2000). Furthermore, the size correlated with the amount of time spent as a taxi driver. This is in line with the assumption that the posterior hippocampus stores a spatial representation of the environment. Lesions to the dorsal, but not ventral, hippocampus impair allocentric spatial memory in rats (Bannerman et al., 2002; Lee and Kesner, 2003). As opposed to egocentric spatial memory, allocentric spatial memory is independent of the position of the organism and rather depends on external cues.

The hippocampus comprises the hippocampus proper, the dentate gyrus and the subiculum. Sometimes the entorhinal cortex is also included. The hippocampus proper is also commonly known as Cornus Ammonis (CA) and is further divided into three main subareas: CA1, CA2, CA3. The dentate gyrus, consists of three layers – the granule cell layer (GCL), the polymorphic layer (or dentate hilus in which CA4 often is included), and the molecular layer (ML). The principal neurons in the CA regions are called pyramidal cells and in the dentate gyrus granule cells (Amaral and Witter, 1989).

The subgranular zone (SGZ) is the border between the dentate hilus and the GCL and is one of two areas in the adult mammalian brain where neurogenesis take place (Altman and Das, 1965; Eriksson et al., 1998; Gould et al., 1999); the other one being the subventricular zone lining the lateral ventricles. Hippocampal neurogenesis is influenced by learning, stress and antidepressant treatments (see the “The plasticity hypothesis of mood disorders” below).

Patients with mood disorders often have memory disturbances indicating the involvement of hippocampus (Frey et al., 2007). Most studies (Campbell et al., 2004; Kempton et al., 2011), but not all (Vakili et al., 2000), report reduced hippocampus volume in depressed patients. The inconsistent findings in bipolar disorder (Javadpour et al., 2010; Hartberg et al., 2011) have in a meta-study been shown to depend on lithium treatment, which increases/normalizes hippocampal volume (Hafeman et al., 2012). Hippocampal volume has been correlated with depression severity (Shah et al., 1998) and time spent with untreated disease (Sheline et al., 2003). However a recent meta-analysis report decreased hippocampal volume already in first episode of depression (Cole et al., 2011).

## **White matter**

Although historically grey matter changes have been the focus of neuropathological and imaging studies, white matter changes in psychiatric disorders have during the last decade gained interest (Brambilla et al., 2009; McIntosh et al., 2009). Firstly, structural MRI studies report decreased size of white matter tracts in patients with bipolar disorder (see the Corpus callosum section below and for review see De Peri et al., 2012). Further analysis of white matter integrity of the living brain was made possible 1985 through the development of MR diffusion weighted imaging (DWI). This technique is based on the fact that diffusion of water molecules in tissue is not free, but depends on the architecture of the tissue. The apparent diffusion coefficient (ADC) is a measure of water molecule diffusion. Myelinated axons are natural barriers to random water molecule diffusion and therefore give a low ADC. ADC is high in for instance the ventricles and demyelinated regions where water diffusion is unimpeded. Diffusion tensor imaging (DTI) is calculated from six or more different diffusion weighted acquisitions. In white matter, water can easily diffuse along the main axis of the myelinated fibres while the diffusion perpendicular to the fibres is restricted. Such preferentially oriented diffusion is called anisotropic diffusion. Fractional anisotropy (FA) is an index of white matter coherence. A high FA represent organized normally myelinated axons. Low FA (and high ADC measure) is a measure of axonal loss (and/or damaged axonal membrane or reduced intra-axonal microtubular integrity) and/or dys-, de-myelination. However, since white matter is highly myelinated, the density of myelin and axonal membranes contribute most to the FA and ADC values (Beaulieu, 2002).

DWI studies have revealed microstructural disruption in subcortical white matter area in bipolar disorder. Increased ADC values and decreased FA measures have been reported for several fronto-limbic tracts (for review see Brambilla 2009). However a few studies show increased white matter integrity in bipolar disorder for some white matter tracts (Yurgelun-Todd et al., 2007; Wessa et al., 2009) including fibre tracts between subgenual cingulate and the amygdalo-hippocampal complex (Houenou et al., 2007). More evidence on oligodendrocyte pathology in mood disorder comes from studies demonstrating decreased expression of oligodendrocyte-associated proteins in patients with bipolar disorder (Tkachev et al., 2003; Konradi et al., 2012).

## BOX 4

### White matter matters – white matter plasticity

Myelin, the multilamellar lipid-rich membrane made by oligodendrocytes in the central nervous system (CNS) to insulate axons, is evolutionary a late development appearing in vertebrates (Zalc et al., 2008). The proportion of myelin is higher in primates than in other mammals and the human brain in turn has approximately 20% more white matter in PFC compared to chimpanzees (Schoenemann et al., 2005). Myelin enables so called saltatory neurotransmission and increases signalling speed more than 10-fold (Hartline and Colman, 2007). By decreasing refractory time to about 1/30 it also increases number of action potentials that can be transmitted per time unit (Quandt and Davis, 1992; Felts et al., 1997; Bartzokis, 2011). Myelination has been proposed to continue in man till the age of 50 (Bartzokis et al., 2001). A maintained capacity for myelination allows neuronal networks to remain plastic. Thus, specifically localised increases in FA has been found following juggling training (Scholz et al., 2009). White matter plasticity is crucial to continually optimize the timing of action potentials and network oscillations on which learning, cognition and behaviour depend (Salami et al., 2003; Bartzokis, 2011). Asynchronous arrival of action potentials can contribute to synaptic loss/pruning (Purves and Lichtman, 1980). Myelin thickness has been proposed to change in response to axonal firing rate (Fields, 2008). A very recent study showed that social isolation of adult mice impaired adult prefrontal cortex myelination and caused behavioural changes. The changes were reversed by social reintegration (Liu et al., 2012). Hence, myelination increase/decrease in response experience. Dysregulation of the increasingly complex stages of myelination that continue during the first half century of life in humans may contribute to the onset of psychiatric diseases during those years.

### *Corpus callosum*

Corpus callosum is the thick mass of white matter that mediates communication between the right and left brain hemispheres. It is the largest white matter tract of the brain and is found in all placental mammals. Corpus callosum can be subdivided into at least five regions along its rostro-caudal extension. The middle part is called the body of corpus callosum. The most posterior part is called the splenium and the anterior-most, slightly bent, region is the genu (“knee”). A thinner part of corpus callosum between the body and splenium is called isthmus. The topology of the corpus callosum matches brain regions, i.e. the anterior part of corpus callosum connects the right and left frontal lobes, the median section part connects the temporo-parietal structures and so on (Kandel et al., 2000).

Decreased size of corpus callosum in adults with bipolar disorder has repeatedly been shown in original MRI studies (Hauser et al., 1989; Coffman et al., 1990; Brambilla et al., 2003) and in meta-analyses (Arnone et al., 2008). Also youths with early-onset bipolar disorder have reduced callosal volume indicating white matter abnormalities early in disease (Lopez-Larson et al., 2010). Moreover, abnormal MRI signalling intensity from corpus callosum has been reported for both adult (Brambilla et al., 2004) and paediatric patients (Caetano et al., 2008). In addition, DTI studies show altered integrity in corpus callosum in both adults (Yurgelun-Todd et al., 2007; Wang et al., 2008; Benedetti et al., 2011; Emsell et al., 2012) and paediatric patients (Frazier et al., 2007). A failure to integrate information across the hemispheres has been suggested to contribute to the pathophysiology of bipolar disorder (Arnone et al., 2008).

## Neurobiological etiology/pathology of mood disorders

Mood disorders are a heterogeneous group of complex illnesses that make studies on the underlying mechanisms troublesome. Though the pathophysiology is not clear, most agree on mood disorders to be multifactorial diseases depending on an interaction between genetic and environmental factors. A commonly used strategy to validate the contribution of genetic and environmental factors is comparing disease concordance among twins. A higher concordance in monozygotic twins (35-75%) compared to dizygotic twins (0-35%) indicates for example a high heritability for bipolar disorder (Kiesepfä et al., 2004).

### **The monoamine hypothesis of depression**

The monoamine hypothesis of depression has been dominant for the last 50 years. It postulates a deficiency or imbalance in the monoaminergic neurotransmitters serotonin, noradrenaline and possibly dopamine. The hypothesis was initiated by two pharmacological observations. 1) It was revealed that Reserpine (used to treat hypertension and psychosis), known to give rise to depression-like symptoms, depleted monoamines in synapses in the brain. 2) The first two groups of antidepressant drugs – monoamine oxidase inhibitors (MAOIs) and tricyclic antidepressants (TCAs) – both turned out to increase the amount of monoamine neurotransmitters available in the synaptic cleft (Schildkraut, 1965). Consistent with the monoamine hypothesis, the newer and most prescribed antidepressants today; the selective serotonin reuptake inhibitors (SSRIs), also increase the levels of serotonin at the nerve terminal (Geddes et al., 2011). Furthermore, a longitudinal study from



2003 showed that the risk of getting depressed after stressful life events depended on differences in the promoter region of the serotonin transporter gene (Caspi et al., 2003).

However, despite intensive research, no convincing evidence of a primary deficiency of monoamines in specific brain structures has been shown to be necessary or sufficient for the occurrence of mood disorders. The main problem with the monoamine hypothesis in its original form is the fact that even though the pharmacological effects of antidepressant drugs are immediate (minutes, hours), several weeks of chronic treatment is required for the therapeutic effect (Racagni and Popoli, 2008).

## BOX 5

### The HPA axis and mood disorders

The hypothalamic-pituitary-adrenal (HPA) axis is the interaction between hypothalamus, the anterior pituitary gland and the adrenal gland. Cells in the hypothalamus release corticotropin-releasing hormone (CRH), which stimulates cells in the anterior pituitary gland to secrete adrenocorticotrophic hormone (ACTH), which in turn stimulates the adrenal glands to secrete the stress hormones – glucocorticoids (cortisol, corticosterone and cortisone). Secretion is regulated via negative feedback, the sleep/wake cycle (circadian rhythm), physical activity, illness and stress. Dysregulation of the HPA-axis, as revealed by non-suppression of the serum cortisol following dexamethasone suppression test, is found in about 50 % of patients with mood disorders (Nemeroff, 1998; Daban et al., 2005; Rybakowski and Twardowska, 1999). Abnormalities have been observed at several levels along the axis in patients with depression: increased numbers of CRH-producing neurons (Raadsheer et al., 1994), elevated levels of CRH (Nemeroff et al., 1984) that is reduced upon treatment (Brady et al., 1991), raised plasma levels of ACTH (Pfohl et al., 1985), pituitary (Krishnan et al., 1991) and adrenal gland enlargements (Rubin et al., 1995).

## The plasticity hypothesis of mood disorders

The lag of several weeks for antidepressant drugs to exert their effect suggests indirect and adaptive changes for the therapeutic effect. The plasticity hypothesis proposes that impairments in brain plasticity are key factors in the pathophysiology of mood disorders. Brain plasticity – basically the brain's ability to adapt – includes diverse processes such as alteration of dendritic function, synaptic remodelling, long-term

potentiation (LTP), neurite extension, synaptogenesis and neurogenesis (Racagni and Popoli, 2008). Myelination, not included in the classical neuroplasticity concept, has recently been shown to be an important part of adult brain plasticity (Liu et al., 2012). Diverse observations during the last two decades underpin the plasticity hypothesis.

Firstly, as described above, volume reductions have repeatedly been found in limbic structures and white matter in patients with mood disorders. *Post-mortem* studies support the idea of cell loss and/or atrophy (see above and below).

Secondly, the HPA axis is often dysregulated in both depression and bipolar disorder (See Box 5) and stressful life events can trigger mood disorders (Geddes et al., 2011). Moreover, in a condition with elevated levels of the stress hormone cortisol – Cushing’s syndrome – mood disorders are common (Sonino and Fava, 2001) and associated with hippocampal volume loss (Starkman et al., 1992). Most studies of atrophy in response to stress have focused on the hippocampus, however it is plausible that stress hormones also influence atrophy and survival of cells in other brain regions. In experimental animal models, stress (Willner, 2005) and exogenous administration of glucocorticoids (Kalynchuk et al., 2004) induces depressive-like behaviour. Stress and glucocorticoids administration also cause atrophy in several brain regions, reduces hippocampal neurogenesis (Cameron and Gould, 1994; Czéh et al., 2001), lead to glial cell pathology (Banasr et al., 2010) and dendritic atrophy (McEwen, 1999; Holmes and Wellman, 2009). Antidepressant treatments can counteract those effects (Czéh et al., 2001; Hellsten et al., 2002). Glucocorticoids also attenuate myelination (Chari et al., 2006).

Finally, all the commonly used treatments for mood disorders: TCAs (Santarelli et al., 2003), SSRIs (Malberg et al., 2000), serotonin and noradrenaline reuptake inhibitors (SNRIs) (Xu et al., 2006), MAOIs (Malberg et al., 2000), lithium (Chen et al., 2000) and ECT (Madsen et al., 2000) increase neurogenesis in hippocampus. An often cited article by Santarelli et al. (2003) reinforced the hypothesis when showing that there was no antidepressant effect of fluoxetine in mice if the hippocampal neurogenesis was blocked by local irradiation of hippocampus. However, other cell types including glial cells must naturally also have been damaged by the irradiation.

A growing body of data suggests that increased glutamate neurotransmission together with excessive glucocorticoids may explain, at least in part, the observed atrophic changes (Popoli et al., 2012). Microdialysis studies have shown increased levels of extracellular glutamate during stress in several brain regions (Moghaddam, 1993; Reznikov et al., 2007). Overactivation of the glutamate ionotropic receptors give rise to high levels of intracellular calcium, which is neurotoxic and glucocorticoids

potentiate the excitotoxicity and increase glutamatergic signalling. Moreover, glial regulation of glutamate transmission in a rat model of depression has been shown to be dysfunctional (Gómez-Galán et al., 2012). The particular sensitivity of hippocampal neurons to glucocorticoids and excitotoxicity may contribute to hippocampal dysfunction in mood disorders (Lee et al., 2002; Popoli et al., 2012). Interestingly, as opposed to the slow-acting monoamine targeting antidepressant drugs, clinical trials with ketamine, an N-methyl-D-aspartate (NMDA) receptor antagonist, shows antidepressant effect within hours (Berman et al., 2000; Zarate et al., 2006b; Diazgranados et al., 2010). Importantly, this is associated with induction of synaptogenesis and reversal of chronic stress induced atrophy (Li et al., 2010; 2011b).

### **Glial cell pathology in mood disorder**

Neurons account for only circa 10 % of the cells in the human brain, while glia cells, present in both grey and white matter, account for the remaining 90 %; astrocytes (45 %), oligodendrocytes (35 %), microglia (5 %) and NG2 cells (5 %) (Bartzokis, 2011). There is a continuous cross talk between neurons and glial cells, which is thought to be crucial for the function of the neuronal network. Humans have dramatically enlarged neocortex compared to other primates. Due to the high metabolic demands related to the support of the expansive dendritic arbors and long projecting axons in the enlarged brain we also have a higher neuron-glia ratio (Sherwood et al., 2006). It is possible that the enhanced capacity of the human brain makes it more sensitive to glial abnormalities and oxidative stress. Moreover, rats raised in an enriched environment had higher glia-neuron ratio compared to those living in a deprived environment (Diamond et al., 1964) and in a study from 2008, Banasr et al. showed that glial loss in the prefrontal cortex of rats is sufficient to induce depressive-like behaviour (Banasr and Duman, 2008).

*Post mortem* studies of patients with mood disorder demonstrate reductions in number and/or density of glial cells in several brain regions. Reduced glial density and neuronal size in PFC has been reported (Ongür et al., 1998; Rajkowska et al., 1999; Cotter et al., 2001; Rajkowska et al., 2001). In a study by Bowley et al., reduced glial cell density was reported in amygdala and entorhinal cortex in depressed patients and non-treated bipolar patients. However there was no change in the number of neurons. Lithium and valproate seemed to reverse glial pathology (Bowley et al., 2002). Moreover, some studies have failed to show reduced number of neurons in hippocampus of depressed patients (Muller et al., 2001), but Stockmeier et al. propose in study from 2004 reduced neuropil in hippocampus of depressed patients (Stockmeier et al., 2004). Many of these studies are based on stereological analysis of

Nissl-stained sections in which it is difficult to distinguish glial subtypes. Nevertheless, some investigators have found oligodendrocytes to be responsible for the observed glial cell loss in certain areas (Hamidi et al., 2004; Uranova et al., 2004; Vostrikov et al., 2007).

### *NG2 cells*

NG2 cells are glial cells distinct from astrocytes, microglia and mature oligodendrocytes. They are widely spread throughout the brain (Butt et al., 2005) and make up approximately 5 % of all cells in the adult brain. Furthermore, they are the predominantly dividing celltype in adult rodent brain (Dawson et al., 2003). NG2 cells are characterised by their expression of the chondroitin sulphate proteoglycan Neuron-Glia 2 (hence, the name NG2) and their typical stellate morphology. The only other cells known to express NG2-proteoglycan in the adult brain are pericytes lining blood vessels (Ozerdem et al., 2001) but those cells have both a different morphology and a distinct location making them easy to tell apart from NG2 cells. In this thesis, NG2 cells will not refer to the pericytes. In grey matter, NG2 cells have a centrally placed cell body surrounded by numerous symmetrically distributed processes that bifurcate at least three times. However, in white matter, NG2 cells are often polarized, due to preferential extension of processes along the axonal axis (Butt et al., 2005). In this thesis these glial cells will be referred to as NG2 cells, but others have used different names: O-2A progenitor cells, beta astrocytes, synantocytes, polydendrocytes, and adult oligodendrocyte precursor cells (OPCs) (for review see Nishiyama 2007). NG2 cells are capable of differentiate into myelinating oligodendrocytes, in both grey and white matter of the adult brain (Polito and Reynolds, 2005). However, the somewhat equal distribution of NG2 cells between white and grey matter, their complex morphology and the fact that they continue to proliferate after the peak of oligodendrogenesis, imply that they have other functions apart from being OPCs (Butt et al., 2005; Nishiyama, 2007).

Interestingly, NG2 cells express  $\gamma$ -aminobutyric acid (GABA)-A receptors and the glutamatergic ionotropic 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid (AMPA) receptors. In recent years NG2 cells have been demonstrated to receive direct synaptic input from glutamatergic neurons in all areas investigated: hippocampus (Bergles et al., 2000; Mangin et al., 2008), cortex (Kukley et al., 2008), brainstem (Müller et al., 2009), cerebellar cortex (Lin et al., 2005), cerebellar white matter (Káradóttir et al., 2008) and corpus callosum (Kukley et al., 2007; Ziskin et al., 2007). In addition, they have been shown to receive GABAergic synaptic input from neurons in hippocampus (Lin and Bergles, 2004a; Mangin et al., 2008) and cortex (Tanaka et al., 2009). Since NG2 cells keep their synaptic connections throughout cell division (kukley 2008), it has been proposed that neuron-NG2 cell

synapses could regulate NG2 cell development in a neuronal activity-dependent manner (Paukert and Bergles, 2006; Mangin and Gallo, 2011).

Furthermore, some, but not all (Zhu et al., 2008), argue that NG2 cells have the capacity to differentiate into neurons (Belachew et al., 2003; Aguirre et al., 2004) and a subtype of grey matter astrocytes (Alonso, 2005; Zhu et al., 2008). Finally, NG2 cells can also regulate axonal outgrowth (Ughrin et al., 2003; Huang et al., 2005; Tan et al., 2006; Yang et al., 2006) and by their close contacts with neurons, astrocytes, oligodendrocytes and myelin they may contribute to an integrative neuron-glia communication pathway (Butt et al., 2005).

NG2 cell proliferation decreases after prolonged treatment with glucocorticoids (Alonso, 2000; Wennström et al., 2006) and increases in response to electroconvulsive therapy (Wennström et al., 2006). Given the fact that NG2 cells are an abundant proliferative cell type in all areas implicated in mood disorders and with a unique capacity to respond directly to neuronal signalling changes through their specialized contacts with neurons, NG2 cells are highly interesting in the context of mood disorder-associated white and grey matter changes.

## Treatment of mood disorders

There are different treatment strategies for management of mood disorders: psychotherapy, pharmacological treatment and diverse physical treatments (electroconvulsive therapy (ECT), bright light, transcranial magnetic stimulation and deep brain stimulation). Which treatment or combination of treatments to choose depends mostly on the type of mood disorder and the severity of the disease. Psychotherapy is primarily used in milder cases of mood disorders or in combination with other treatments. ECT on the other hand is often first considered when pharmacotherapy and psychotherapeutic interventions have failed and the depressive or manic episode is severe. Bright light is mainly used for seasonal depression and deep brain stimulation is a new treatment so far is only used in small clinical trials (Geddes et al., 2011).

### **Pharmacological treatment of unipolar depression**

For unipolar depression the pharmacological treatments include reuptake inhibitors (TCAs, SNRIs and SSRIs) and MAOIs. All these pharmacological treatments have the common effect of acutely enhancing monoamine function (see “The monoamine hypothesis of depression” above).

## Pharmacological treatment of bipolar disorder

The risk of treating bipolar depression with antidepressants is precipitation of mania and rapid cycling. Bipolar disorder is instead treated with mood stabilising drugs. Lithium was the first mood stabilising drug introduced and is still the first line of choice (see under the heading Lithium below). Other mood stabilisers are the anticonvulsive drugs sodium valproate, lamotrigine and carbamazepine. The term mood stabilizing drugs can be somewhat misleading since sodium valproate and carbamazepine are predominantly antimanic and lamotrigine is most effective in preventing depressive episodes. Lithium, however has effect on both manic and depressive episodes (Goodwin and Malhi, 2007). Atypical antipsychotics, for example olanzapine, can also be effective during manic episodes. If antidepressant drugs are needed they should be given in combination with a mood-stabilizing drug (Geddes et al., 2011).

## Lithium

Lithium, a monovalent cation, has long been used as a first-line treatment for bipolar disorder. It is effective in treating acute mania and preventing recurrent manic and depressive episodes (Goodwin and Malhi, 2007). Long-term treatment reduces the risk of suicide by 80% in patients with bipolar disorder (Baldessarini et al., 2006). Lithium has also been shown to potentiate the effect of other mood stabilizers and antidepressant drugs (Geddes et al., 2011).

### *History*

Lithium was first used in the 19<sup>th</sup> century to treat gout and later to treat mania and depression. During this time it was used in mineral spring water and believed to cure many disorders. Interestingly, lithium citrate was added in the soft drink 7-up till 1948! The use of lithium to treat mania was rediscovered by the Australian psychiatrist John Cade in 1949, but the breakthrough for lithium treatment came when the Danish psychiatrist Mogens Schou from Aarhus presented his results from a randomly controlled trial in 1954. The United States Food and Drug Administration approved lithium treatment against mania in 1970 and in 1974 it was also approved for bipolar disorder (Shorter, 2009).

### *Pharmacokinetics*

Lithium is given orally as lithium salts (lithium-carbonate/-citrate/-chloride/-sulphate). It is almost completely absorbed in the upper gastrointestinal (GI) tract and

distributed in total body water without being bound to proteins or metabolised. According to a microdialysis study in rat, it passes the blood-brain-barrier (BBB) fast, followed by a slower entry into brain cells where it concentrates (Hillert et al., 2012). It has been suggested that lithium competes with sodium for transport across plasma membranes (Hillert et al., 2012; Malhi et al., 2012). Steady-state levels of lithium in humans are reached after approximately one week. Lithium is freely filtered by the glomerulus of the kidneys and mainly reabsorbed in the proximal convoluted tubule. During sodium-restricted conditions, more lithium is reabsorbed in the distal nephron. Thus, adequate sodium consumption is important for lithium clearance (Malhi et al., 2012).

### *Dose and side effects*

Lithium has a narrow therapeutic window. Initial studies indicated the need for serum levels between 0.8-1.2 mM, but today, especially in Europe, lower levels in the range of 0.5-0.8 mM are more commonly used for maintenance treatment of bipolar disorder. One study indicated that the higher doses were approximately 3-times as effective as doses achieving blood levels in the lower range; < 0.6 mM (Gelenberg et al., 1989), but higher lithium doses are associated with more side effects and lower patient compliance with the treatment. Common side effects include fine hand tremor, nausea, diarrhea, weight-gain and hypothyroidism. Reversible diabetes insipidus, characterized by polyuria, polydipsia and reduced ability to concentrate urine, is also commonly associated with lithium treatment. This is mainly due to counteracting effect of lithium on the intracellular vasopressin signalling via adenylate cyclase (Trepiccione and Christensen, 2010). Serum levels above 1.5 mM are toxic and prolonged exposures to toxic levels of lithium affects multiple organs including the brain, kidneys, liver, GI tract, thyroid gland etc. Serum levels above 2.5 mM can cause seizures, coma, cardiac dysrhythmia and permanent neurological sequelae (Geddes et al., 2011).

### **Lithium and plasticity**

Neurotrophic and neuroprotective properties of lithium have been proposed to underlie the therapeutic effect in treatment of bipolar disorder (Machado-Vieira et al., 2009). Firstly, a recent meta-study conclude that lithium treatment normalise or increase grey matter volume, particularly in the hippocampus, amygdala, anterior and subgenual cingulate cortex (Hafeman et al., 2012). Lithium's positive effect on grey matter volume correlated with treatment response (Moore et al., 2009; Lyoo et al., 2010). A study by Moore et al. showed that four weeks of lithium treatment was enough to induce increased grey matter volume (Moore et al., 2000) and recent studies have shown increased serum levels of brain-derived neurotrophic factor

(BDNF) in lithium-treated patients (de Sousa et al., 2011). As the name indicates, BDNF is a secreted neurotrophic protein that supports survival of neurons and encourage the growth and differentiation of new neurons and synapses.

Secondly, in animal studies lithium has been shown to increase hippocampal neurogenesis (Chen et al., 2000), increase expression of the neurotrophic factors including BDNF (Fukumoto et al., 2001; Hashimoto et al., 2002), nerve growth factor (NGF) (Hellweg et al., 2002) and vascular endothelial growth factor (VEGF) (Guo et al., 2009). Furthermore, lithium increases expression of for example the cytoprotective protein Bcl-2 whereas reducing levels of the pro-apoptotic proteins such as p53 and Bax (Chen and Chuang, 1999) and protects against excitotoxicity by inhibiting NMDA receptor-mediated  $Ca^{2+}$  influx (Nonaka et al., 1998). Additionally, chronic lithium treatment prevents stress-induced decreased dendritic length in CA3 pyramidal neurons in hippocampus (Wood et al., 2004), but interestingly reduces stress-induced dendritic branching of amygdalar pyramidal neurons (Johnson et al., 2009). Pretreatment with lithium have been demonstrated to reduce infarct size after an experimental model of stroke by 56 % (Nonaka and Chuang, 1998). Finally, lithium treatment reduces memory deficits in animal models of Alzheimer's disease (De Ferrari et al., 2003) and memory deficits induced by chronic stress (Vasconcellos et al., 2003), stroke (Yan et al., 2007), ethanol (Sadrian et al., 2012) and traumatic brain injury (Zhu et al., 2010). Regarding white matter, lithium has been shown to reduce demyelination, microglia activation and leukocyte infiltration in an animal model of multiple sclerosis (MS) (De Sarno et al., 2008).

Due to its neuroprotective effects, lithium has been proposed for use in treatment of several neurodegenerative disorders and positive results have been reported from clinical trials on patients with Alzheimer's disease (Forlenza et al., 2012) and amyotrophic lateral sclerosis (Fornai et al., 2008).

### **Molecular pathways underlying the effects of lithium**

A series of mechanisms for lithium's therapeutic effects have been proposed during the last 50 years. These include: 1) effects on adrenergic release and reuptake (1960s), effects on postsynaptic receptor modulation (1970s), effects on second messenger system; adenylate cyclase and G-proteins (1980), effects on phosphoinositol turnover (the inositol depletion model), protein kinas C and glycogen synthase kinase-3 (GSK-3) (1990s). During the last years the neurotrophic and neuroprotective effects of lithium have gained much interest and multiple signalling pathways, sometimes overlapping, are suggested to account for this effect. For a review see (Chiu and Chuang, 2010). One of the most acknowledged pathways to date will be further discussed below.



### *Inhibition of GSK-3*

GSK-3, is a protein kinase that acts as an intermediary in several intracellular signalling pathways. In general, GSK-3 is considered to be constitutively active under non-stimulated conditions. It has a number of different effects and is considered a key regulator of apoptosis, cellular plasticity (Zarate et al., 2006a) and inflammation (Beurel et al., 2010). GSK-3 activity is regulated by a variety of kinases and systems including Akt, protein kinase A/C, MAP kinases, and the Wnt pathway. Lithium inhibits GSK-3 in several ways: 1) directly via competition with magnesium for a binding site (monovalent lithium and divalent magnesium has interestingly very similar ionic radius), 2) indirectly by affecting upstream targets (Jope, 2003) and 3) by decreasing its transcription (Mendes et al., 2009). Inhibition of GSK-3 leads to activation of cell-survival transcription factors (such as CREB,  $\beta$ -catenin, heat-shock factor-1) and inhibition of pro-apoptotic factors such as p53 (Grimes and Jope, 2001). GSK-3 is also inhibited by valproate (Chen et al., 1999) and ECT (Roh et al., 2003). Moreover, direct pharmacologic and genetic inhibition of GSK-3 produces mood stabilizer-like behaviour in rodents (Gould and Manji, 2005). Interestingly, GSK-3 has also been suggested to play a critical role in regulating circadian rhythm (Kaladchibachi et al., 2007).

## ECT

As mentioned above, ECT is used to treat severe forms of depression, mania and catatonia. It is mainly considered when pharmacological treatments have failed. However, it is also used as first-line treatment when there is an urgent need for fast response; for instance when the suicide risk is a high, the patient doesn't eat and drink etc. (Mankad et al., 2010). ECT has been shown to be especially effective in treatment of depression with psychotic features. In a multi-site collaboration study the remission rate for those patients was 95% after just two treatments (Petrides et al., 2001).

### *History*

In the 1930s it was noticed that patients with psychosis improved after epileptic seizures. The Hungarian psychiatrist von Meduna therefor chemically induced seizures (by injecting Camphor) in a man with a catatonic form of schizophrenia in 1934. After five treatments the man started to talk and eat for the first time in years and after eight treatments he went home and back to work. Chemically induced seizures were painful and in 1938 the Italian psychiatrists Cerletti and Bini demonstrated the efficacy of electrically induced seizures – ECT (Mankad et al., 2010).

### *The treatment procedure*

Since its introduction in 1938, the ECT procedure has been refined considerably. Nowadays patients are anaesthetised, hyperoxygenated and given muscle relaxants before the treatment. In Sweden today, right unilateral treatment is most commonly used. This means that one electrode is placed on the crown of the head and the other one on the right temple. After a brief electrical stimulation, a symmetric epileptic seizure (Grand Mal seizure) is initiated. The seizure is monitored with electroencephalography (EEG). Occasionally a single or a few treatments is sufficient to induce remission, but in depression, typically 6-12 treatments (sometimes more) – given two to three times a week – are necessary to relieve the condition (Mankad et al., 2010). It has been estimated that 1-2 million people worldwide receive ECT in one year (Abrams, 2002). In Sweden approximately 45 000 ECTs are given per year.

### *Mechanism of action*

The mechanism through which ECT exerts its therapeutic effect is elusive but there are many theories. A few of these will be mentioned here. Firstly, the neuroendocrine hypothesis states that normalization of endocrine dysfunction might explain the therapeutic effect of ECT. It has in several studies been shown that hypothalamic peptides and hormones are released in response to ECT. Repeated treatments have for instance been shown to restore the HPA-axis, which also correlated to clinical improvements (Kunugi et al., 2006).

Secondly, the “anticonvulsant hypothesis” postulates that increased inhibitory GABA:ergic neurotransmission is crucial for the therapeutic effect of ECT. Occipital cortex GABA concentrations increase two-fold following ECT (Sanacora et al., 2003). As a consequence, ECT may be a useful adjunctive treatment in intractable epilepsy (Sackeim et al., 1983). Further support for this hypothesis comes from EEG recordings of patients receiving ECT. Sustained postictal suppression (suppression of brain activity directly following the seizure) has repeatedly been shown to positively correlate with good therapeutic outcome (Azuma et al., 2007).

Thirdly, the monoamine explanation is based on theories about sensitisation of the serotonergic and dopaminergic receptors and normalisation of noradrenalinergic receptor sensitivity (Ottosson, 1983).

Finally, the plasticity hypothesis of ECT is based on several observations from clinical and preclinical studies. First of all, as described above mood disorders are associated with structural changes in several brain regions and ECT has been shown to increase hippocampal volume (Nordanskog et al., 2010). However, another study found no effect of ECT on the hippocampal-amygdala complex (Coffey et al., 1991). Using an animal model of ECT, electroconvulsive seizures (ECS), neurogenesis has been shown

to increase two to three times after both acute and repeated ECS (Madsen et al., 2000; Malberg et al., 2000). This is a more robust increase compared to what is seen after treatment with pharmacological antidepressants which typically increase neurogenesis by 20-30% (Malberg et al., 2000). However, not only neurogenesis is affected by ECS. Gliogenesis is increased in hippocampus (Wennström et al., 2003), amygdala (Wennström et al., 2004) and prefrontal cortex (Madsen et al., 2005) following ECS. In addition, decreased gliogenesis in a depression model was counteracted in response to ECS (Wennström et al., 2006). Moreover ECS induces hippocampal angiogenesis (Hellsten et al., 2005), synaptogenesis (Chen et al., 2009) and axonal sprouting (Vaidya et al., 1999). In line with the structural changes, expression of growth factors including BDNF (Balu et al., 2008), VEGF (Newton et al., 2003) and NGF (Conti et al., 2009) have been shown to be up-regulated following ECS. Increased levels of BDNF have also been demonstrated in patients following ECT (Taylor, 2008).

### **Side effects**

Minor and short-lived side effects of ECT such as headaches nausea and muscle soreness are common. Confusion directly after awakening is also commonly occurring and linked to bilateral electrode placement, higher dosages, older age, and the presence of neurological disease. The time to recovery of full orientation has been associated with other more long-lasting cognitive deficits (Sobin et al., 1995). Long-lasting confusion after ECT, post-ECT deliriums, is an uncommon side effect. Nevertheless, after regaining orientation, anterograde amnesia, i.e. deficits in the ability to learn and remember new things after the course of ECT, have repeatedly been reported (Sackeim et al., 1986; Steif et al., 1986). Substantial recovery of anterograde amnesia takes place over the first few days and weeks and by one-month anterograde memory has for the most part recovered to baseline (Calev et al., 1991). Some studies actually show improved anterograde memory two months after ECT compared to baseline (Sackeim et al., 1993; Semkowska and McLoughlin, 2010). Non-declarative forms of memory such as procedural memory and priming have not been studied much after ECT, but according to the few studies performed, they seem to be unaffected (Vakil et al., 2000). However, ECT can also result in transient retrograde amnesia, i.e. inability to recall information acquired before the ECT. There are also indications of some degree of persistent retrograde amnesia, mainly for information acquired temporally close to ECT (Squire et al., 1981; Calev et al., 1991; Lisanby et al., 2000). Concerns about memory deficits have limited clinical application and general acceptance and several refinements of electrode placements and stimulus parameters have been made in an attempt to reduce side effects. Unfortunately, procedures accompanied with reduced risk of side effects are sometimes at the cost of efficacy (McCall et al., 2000).

## **Potential mechanisms behind the cognitive side effects of ECT**

As with the mechanism for the therapeutic effect of ECT, the molecular process behind the memory deficits are poorly understood. However, several mechanisms – involving cell death, structural changes, inflammation, the glutamatergic, cholinergic and glucocorticoid systems – have been proposed (for review see Pigot et al., 2008 and further comments in the discussion section).

### *Does ECT cause structural brain damage?*

Whether ECT induces structural brain damage has been a controversy since ECT was introduced. However, scientific studies have failed to find any ECT-induced abnormalities in patients (Coffey et al., 1991; Devanand et al., 1994; Ende et al., 2000). A few animal studies have shown discrete neuronal loss within specific cell populations in hippocampus (Lukoyanov et al., 2004; Zarubenko et al., 2005; Cardoso et al., 2008) after ECS. However, most preclinical reports do not find cell death after ECS in rodents (Dalby et al., 1996; Vaidya et al., 1999; Chen et al., 2009) and primates (Dwork et al., 2004; 2009). Our research group has previously shown a mild microglia cell activation following ECS (Jansson et al., 2009). Microglia are key regulators of neuroinflammation and have traditionally been associated with brain pathology. However recent studies suggest a dual role of microglia; apart from being a cleaning squad, these cells – together with other cells from the immune system – also orchestrate neuronal differentiation and have a role in neurogenesis and other plastic changes in the brain (Ziv et al., 2006; Ekdahl et al., 2009; Graeber and Streit, 2010).

### *The effect of ECT on LTP*

LTP is a widely accepted mechanism for learning and memory. It means that a fixed amount of presynaptic stimulation gives long-lasting enhanced postsynaptic response after high-frequency stimulation of an afferent fibre (Shors and Matzel, 1997). One of the most well studied types of LTP is NMDA receptor-dependent LTP. During simultaneous depolarization of a neuron and glutamate binding to its NMDA receptors, influx of  $\text{Ca}^{2+}$  into the post-synaptic neuron activates signalling pathways that eventually lead to synaptic changes (Woodside et al., 2004). Repeated ECS treatments have been shown to disrupt hippocampal LTP (Anwyl et al., 1987; Stewart and Reid, 1993; Stewart et al., 1994) and induce memory deficits. During seizures there is a massive release of glutamate and it has been hypothesized that ECS induce  $\text{Ca}^{2+}$  influx and LTP in an indiscriminative manner and thereby disrupts the fine-tuning pattern of strengthened synapses (Reid and Stewart, 1997; Brun et al., 2001). Support for this theory comes from studies showing anti-amnesic effects of NMDA receptor antagonists (Stewart and Reid, 1994; McDaniel et al., 2006) and calcium channel blockers (Zupan et al., 1996; Sushma et al., 2004).



# Aims of the thesis

The main objective of this thesis was to study glial changes and effects on spatial memory in response to two of the most powerful treatments for mood disorders – lithium treatment and electroconvulsive treatment. The specific aims were:

- To investigate the effect of lithium treatment on NG2 cell proliferation, survival and differentiation (Paper I)
- To investigate the effect of lithium treatment on oligodendrogenesis after a demyelinating insult (Paper II)
- To investigate the effect of lithium treatment on remyelination and inflammation after a demyelinating insult (Paper II)
- To investigate whether blood-borne immune cells are recruited to the hippocampus in response to ECS (Paper III)
- To investigate the effect of lithium treatment and ECS treatment on spatial memory (Paper IV)

# Materials and Methods and comments

## Animals

Adult male Wistar (paper I and II), Sprague-Dawley (paper III and IV) or Lister Hooded (paper IV) rats were used. All experiments were carried out according to guidelines set by the Malmö-Lund Ethical Committee for the use and care of laboratory animals. The rats were kept on 12 hour light-dark cycle and experiments were carried out during the light period. Rats were housed three per cage with *ad libitum* access to food and water. Sprague-Dawley rats were chosen in paper IV due to good performance in the Morris water maze (MWM) task (D'Hooze and De Deyn, 2001). Pigmented rat strains, like the Lister Hooded rat, have superior visual acuity compared to albino rats, like Sprague-Dawley rats (Prusky et al., 2002). Thus, in study four in paper IV we turned to use the pigmented Lister Hooded rats, hypothesising that their better visual ability would result in better performance in the MWM task and thereby lead to more pronounced groups differences. A second reason for using Lister Hooded rats was further future investigations of ECS-induced amnesia using other memory tests with high demands on visual acuity.

## Treatments

### **Chronic lithium treatment**

Lithium was given in the diet. Lithium-chloride (Sigma Aldrich, St Louis, MO, USA) was homogenously mixed into standard rat chow (produced by Lantmännen, Kimstad, Sweden). During lithium treatment all rats (including control rats) had free access to sodium chloride (NaCl) – either as saline solution, blocks of NaCl hanging from the roof of the cage or both. As mentioned in the introduction satisfactory lithium clearance in the kidneys requires adequate sodium intake. In paper I, rats were treated with lithium for four weeks, sufficient of time to study the differentiation and survival of newborn cells. In patients, lithium treatment is often initiated with a low dose, which is then raised to the desired level. When treating rats

with lithium for more than three weeks it is recommended to start the treatment at a lower dose and then increase the dose after one week (personal communication with Arne Mörk; Lundbeck A/S, Denmark). This was done in paper I. In paper II and IV however, the period of lithium treatment was shorter and the targeted dose was therefore used from start. The full-dose of lithium was 60 mmol/kg food (equivalent to 2.5 g lithium chloride/kg) in the proliferation study in paper I. Serum levels of lithium, 1.0 mM, was within the recommended therapeutic range (0.6-1.2 mM) but since the rats ate little and gained very little weight, lower doses (40 mmol/kg and 50 mmol/kg) were used in a few following studies (data not published). The dose 40 mmol/kg gave serum levels at the lower range of the therapeutic range. Thus, the dose 50 mmol/kg (equivalent to 2 g lithium chloride /kg) was used for all following lithium studies.

### **Cuprizone treatment**

To induce demyelination in rats the cuprizone model was used. It is a well-established demyelination model in mice. Although some studies indicate that that demyelination is restricted to mice, it has during the last years also successfully been used in young rats (Adamo et al., 2006). When given in the diet, demyelination and microglia activation occurs in corpus callosum in a time frame of weeks. After cuprizone withdrawal remyelination occurs within weeks. The toxin specifically induces apoptosis of mature oligodendrocytes, without damage to other cell types. The mechanism of action is not known, but it's been hypothesised that the binding of cuprizone to copper leads to copper deficiency, which in turn negatively affects copper requiring enzymes in the mitochondria. Since mature oligodendrocytes maintain vast expanse of myelin, they have extraordinary high metabolic demands making them preferentially susceptible (Matsushima and Morell, 2001). In paper II, 0.6 % (w/w) cuprizone (Bis(cyclohexanone)oxaldihydrazone; Sigma Aldrich) was mixed into standard rat chow (produced by Lantmännen, Kimstad, Sweden) and fed to rats for four weeks.

### **Bromodeoxyuridine administration**

The thymidine-analogue 5-bromo-2'-deoxyuridine (BrdU) (B5002; Sigma Aldrich) is incorporated into dividing cells during the S-phase and thus serves as a marker for proliferating cells (Cooper-Kuhn and Kuhn, 2002). BrdU (20 mg/mL), dissolved in phosphate buffered saline (PBS), was administered intraperitoneally (100 mg/kg), twice daily, at 12-hour intervals. In paper II and in the survival study in paper I, BrdU was injected during five days. However, in the proliferation study in paper I,



BrdU was only given during three days since it was important to minimize the days during which BrdU was administered to avoid survival effects on the newborn cells.

## **ECS treatment**

Rats were given bilateral ECS once daily for five (paper IV) or ten (paper III) consecutive days (24 h intervals). Electrical current was delivered via ear clips with a pulse generator applying unidirectional square wave pulses (current 50 mA, frequency 50 Hz, pulse width 10 ms and pulse train duration 0.5 s). In study four in paper IV a different set of parameters with shorter pulse width was used; current 99 mA, frequency 100 Hz, pulse width 0.5 ms and pulse train duration 0.5 s. The length of the tonic seizure was defined as the time from the start of the motor seizure to the point where the forelimbs of the rat reached a position perpendicular to the body (Jansson et al., 2009). All rats given ECS were monitored during wake-up phase to ensure their well-being. Control rats were sham-treated, i.e. handled identically to the ECS rats except that no current was applied.

## **Behavioural tests**

### **MWM navigation task**

In paper IV, spatial memory and learning abilities were evaluated using the MWM task, a hippocampus-dependent task (Morris, 1984). The maze consisted of a 45 cm deep, circular tank (180 cm in diameter) filled with water ( $20 \pm 1^\circ\text{C}$ ) to a depth of 30 cm. Imaginary lines divided the water-filled maze into four equal-sized quadrants. A platform was submerged by 1.5 cm in the centre of one of the quadrants. The water was made opaque by addition of white paint. Visual extramaze cues were kept strictly constant during the whole experiments. The swim path was recorded by a computerized video-tracking system (Ethovision 3.1, Noldus, The Netherlands). In study one and two (from paper IV), a pre-trial was performed the day before the training period, to familiarize the rats to the swimming task. During the pre-trial, each rat was given one trial (60 s) during which no extramaze cues were present.

Hidden platform training was performed for eight consecutive days with four trials per day and 20 s between trials. On each trial, the rat was placed in the water at one of four randomly varied starting points. Rats finding and climbing onto the platform had to stay on it for 20 s. If rats failed to find the escape platform within 90 s, the experimenter guided them to it. Escape latency – i.e. the time required to reach the platform – was recorded. Data from the four daily trials were pooled to provide

averaged data per rat per day. A retention test was performed 24 h after the last ECS (Fig 1). For the retention test (90 s), the platform was removed and the behaviour of the rats in the maze examined. Time spent in the quadrant where the platform used to be (platform quadrant), mean distance to the former position of the platform (distance to platform), latency to first crossing over the former position of the platform (first platform crossing) and number of crossings over the former platform area (platform crossings) were evaluated.

Apart from true spatial learning about the location of the platform, the MWM task also includes learning about the task; learning to swim away from the sidewall, find a hidden platform and stay on it (Morris, 1984). When first introduced to the water maze, most rats swim around the pool close to the sidewall (thigmotaxis). Under normal conditions they soon learn to swim to the inner part of the pool. Thigmotaxis, i.e. time spent in the area 0–15 cm from the sidewall was analysed for the ECS group during the retention test and compared with the first learning trial.

## Tissue preparation

### **Myelin isolation and determination of myelin yield**

To determine if cuprizone treatment had induced demyelination, myelin was isolated from whole brain homogenate and subsequently quantified (paper II). The rats were anesthetized with sodium pentobarbital and then killed by decapitation. The entire brain (except the olfactory bulbs) was carefully excised and weighed. The tissue was homogenized at 10% (w/v) in ice-cold 0.32 M sucrose using a 40 ml Dounce homogenizer. Pure myelin from the total homogenate was isolated by ultracentrifugation (Beckman ultracentrifuge, SW 32 Ti) according to the method of Norton and Poduslo (1973) with slight modifications. To remove sucrose and concentrate the sample after discontinuous sucrose gradient centrifugation, three final washes in water at 75,000 g were performed. All steps were carried out in 0–4 °C. Samples were then dried until a constant weight was obtained. Myelin yield was calculated as milligram dry weight myelin per gram of fresh tissue.

### **Histological procedures**

For histological analysis of brain tissue the rats were anesthetized with sodium pentobarbital and in the absence of nociceptive reflexes transcardially perfused with 0.9% saline (100 ml) followed by ice-cold 4% phosphate buffered paraformaldehyde (PFA; 200 ml). The brains were then post-fixed in 4% PFA at 4°C overnight (three

hours in paper III) and subsequently cryoprotected in sucrose in PBS. Coronal serial 40  $\mu\text{m}$  (paper I and IV) or 30  $\mu\text{m}$  (paper II and III) thick sections were cut and stored in antifreeze cryoprotectant solution at  $-20\text{ }^{\circ}\text{C}$ .

#### *Luxol fast blue staining*

Luxol fast blue stain (LFB) is a conventional method to visualise myelin under light microscopy. The stain reacts with the base of lipoprotein in myelin making myelin fibres blue. In paper II, sections were mounted on glass slides and incubated in 0.1% solvent blue solution 38 (Sigma Aldrich) over night at  $58^{\circ}\text{C}$ , rinsed in 95% ethanol, differentiated with 0.05% lithium carbonate and then 70% ethanol, rinsed with dd- $\text{H}_2\text{O}$ , dehydrated and coverslipped.

#### *Immunofluorescence stainings*

Indirect immunofluorescence staining, used in paper I, II, III and IV, allows for visualization of a specific antigen in the tissue by binding a labelled secondary to a primary antibody specific for the targeted antigen. Specific antibody data are presented in Table 1. Detailed protocol for the immunohistochemical procedures can be found in papers I-III. Basic protocol for all immunofluorescence stainings follows.

Free-floating brain sections were rinsed in potassium phosphate buffered saline (KPBS) and then preincubated (one hour in room temperature) in blocking solution containing normal sera from the animal specie in which the secondary antibodies were raised. Subsequently, sections were incubated with primary antibodies in blocking solution 24-40 h at  $4^{\circ}\text{C}$  according to Table 1. After rinsing in KPBS, sections were incubated with the secondary antibodies (in KPBS containing 0.25% Triton X-100; KPBS<sup>+</sup>) at  $4^{\circ}\text{C}$  for 24 h according to Table 1. When biotinylated secondary antibodies were used, sections were again rinsed and then incubated with streptavidin Alexa 488 (1:200, Molecular Probes, Eugene, Oregon) in KPBS<sup>+</sup> (over night, at  $4^{\circ}\text{C}$ ) to fluoro label the secondary antibody. Finally, sections were rinsed and mounted on Super Frost slides, air-dried, and coverslipped with glycerolbased mounting medium.

Double stainings with BrdU and the cell-specific markers were performed sequentially. After labelling with the cell-specific markers (NG2/Rip/APC), sections were fixed in 4 % PFA for 15 min in room temperature and then, after rinsing, incubated in 1 M hydrochloric acid (HCl) for 30 min at  $65^{\circ}\text{C}$  in order to denature DNA and expose the antigen.

#### *Immunoperoxidase staining*

Immunoperoxidase staining, conducted for Ki67 staining in paper I, is an immunostaining in which the antibodies are visualized via a peroxidase-catalysed reaction. Sections were rinsed in KPBS and then pretreated with 3%  $\text{H}_2\text{O}_2$  in 10%

methanol for 20 min to quenched endogenous peroxidase activity. After additional rinsing, the sections were preincubated in blocking solution, then with the Ki67 antibody and finally with the biotinylated secondary antibody according to Table 1 and the procedure described above. This was followed by incubation with avidin-biotinylated-horseradish-peroxidase complex (Vectastain Elite ABC kit; Vector Laboratories Inc.) for one hour in room temperature. Nickel-enhanced peroxidase detection was then performed by incubation for 2 min in PBS with 0.5 mg/mL 3,3'-diaminobenzidine (DAB), 0.03% NiCl<sub>2</sub> and 0.01% H<sub>2</sub>O<sub>2</sub>. The peroxidase activity thereby converts the chromogenic substrate DAB into a coloured precipitate at the sites of antigen localization. Sections were then mounted, dehydrated and coverslipped with Pertex (Histolab Products AB, Göteborg, Sweden).

The terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) method was used to detect DNA fragmentation in paper IV (unpublished data). DNA fragmentation reflects apoptotic cell death (Gavrieli et al., 1992). The staining was performed on brain sections mounted on glass slides. After the quenching procedure as above, sections were incubated with proteinase K (Roche Diagnostics, Mannheim, Schweiz; 10µg/ml in 10 mM Tris-HCl) for 12 min, fixed for 4 min in PFA and pretreated with ice-cold 0.1% Triton X-100 in 0.1% sodium citrate for 2 min; with KPBS rinses between each step. Subsequently, sections were incubated at 37° C for 60 min in terminal deoxynucleotidyl transferase reaction solution containing fluorescein-conjugated nucleotides (Roche Diagnostics). After rinsing in KPBS, sections were incubated in blocking solution for 30 min at 37° C and then horseradish peroxidase-conjugated anti-fluorescein antibody (Roche Diagnostics) for 30 min at 37° C. This was followed by rinsing in KPBS and peroxidase detection as above.

**Table I. Antibodies**

Primary antibodies Working dilution; incubation time; manufacturer	Detected structures Targeted antigen Reference	Secondary antibodies Working dilution; manufacturer
<b>Immunofluorescence stainings</b>		
Rabbit anti-Ng2 1:250; 40 h; Chemicon	NG2 cells The chondroitin sulphate proteoglycan NG2 Nishiyama et al. (1999)	Biotinylated goat anti-rabbit 1:200; Vector Laboratories
Mouse anti-Ng2 1:400; 40 h; Chemicon	NG2 cells The chondroitin sulphate proteoglycan NG2 Nishiyama et al. (1999)	Cy-3-conjugated goat anti-mouse 1:400; Jackson ImmunoResearch
Mouse anti-Rip 1:1000; 40 h; Hybridoma Bank	Oligodendrocytes and myelin sheaths Cyclic nucleotide phosphodiesterase (CNP) Friedman et al. (1989)	Biotinylated horse anti-mouse 1:200; Vector Laboratories
Mouse anti-APC 1:200; 40 h; Chabiochem	Oligodendrocytes Adenomatous polyposis coli protein Bhat et al. (1996)	Cy-3-conjugated goat anti-mouse 1:400; Jackson ImmunoResearch
Rat anti-BrdU 1:100; 40 h; Oxford Biotechnology	Proliferating cells BrdU Cooper-Kuhn and Kuhn et al. (2002)	Cy-3-conjugated donkey-anti-rat 1:400; Jackson ImmunoResearch / Alexa Fluor 488-conjugated goat anti-rat 1:400; Invitrogen, Molecular Probes
Rabbit anti-Iba-1 1:1000; 40 h; Wako Chemicals	Microglia (macrophages) Ionized calcium binding adaptor protein; an actin-linking molecule Sasaki et al. (2001)	Alexa Fluor 488-conjugated goat anti-rabbit 1:400; Invitrogen, Molecular Probes
Mouse anti-ED1 1:200; 40 h; AbD Serotec,	Reactive phagocytic microglia (macrophages) A glycoprotein in the lysosomal membrane Damoiseaux et al. (1994)	Cy-3-conjugated goat anti-mouse 1:400; Jackson ImmunoResearch
Mouse-anti MHC II 1:500; 40 h; AbD Serotec	Reactive microglia (macrophages) Major histocompatibility complex Class II (MHC II) Collawn and Benveniste (1999)	Cy-3-conjugated goat anti-mouse 1:400; Jackson ImmunoResearch
Mouse anti-CD4 1:500; 24 h; AbD Serotec	T <sub>H</sub> /T <sub>reg</sub> cells/subpopulations of activated microglia/macrophages/monocytes The cell surface glycoprotein CD4 Perry and Gordon (1987)	Cy3-conjugated goat anti-mouse 1:800; Jackson Immunoresearch
Mouse anti-CD163 1:500; 24 h; AbD Serotec	Mature blood-borne macrophages CD163, a scavenger receptor Polfliet et al. (2006)	Cy3-conjugated goat anti-mouse 1:800; Jackson Immunoresearch
Rabbit anti-laminin 1:1000; 24 h; Sigma-Aldrich	Capillary walls Laminin; a matrix protein in the basal lamina of blood vessels Colognato and Yurchenco (2000)	Alexa Fluor 488-conjugated goat anti-rabbit 1:1000; Invitrogen; Molecular Probes
<b>Immunoperoxidase staining</b>		
Mouse anti-Ki67 1:50; 40 h; Novocastra	Proliferating cells Ki67, a nuclear protein expressed during late G1, S-phase, M-phase and G2 Scholzen and Gerdes (2000)	Biotinylated donkey anti-mouse 1:200; Vector Laboratories

# Microscopical analysis

## **Epifluorescence microscopy**

For characterisation and quantification of immunofluorescence-labelled cells an Olympus AX70 epifluorescence microscope (Olympus Optical Co., Ltd, Tokyo, Japan) was used. Stained tissue sections were randomly mixed and coded; thus, the person analysing the sections was blinded to the treatment conditions. Number of cells per section and region was determined using “modified unbiased stereology” (Malberg et al., 2000), i.e., counting anatomically defined regions between rostrocaudal landmarks (according to Paxinos and Watson, 1986) and with the same sampling frequency (in this case, every tenth section) in all animals.

In paper I, number of newborn cells was analysed in three main regions; i) middorsal hippocampus, ii) caudal part of the body of corpus callosum (2.8 - 4.52 mm posterior to bregma), iii) and the mid basolateral nuclei of amygdala (2.56 - 3.5 mm posterior to bregma). Cells in five different subregions of the middorsal hippocampus were counted separately: the GCL, dentate hilus, ML, CA1, CA2 and CA3. The SGZ was included in the GCL and BrdU positive cells extending into the dentate hilus, but being part of clusters of BrdU positive cells in the GCL were included. Total number of BrdU positive cells as well as cells double positive for BrdU together with NG2, APC or Rip was counted. The number of BrdU+/Rip cells in corpus callosum could not be determined due to intense Rip staining of myelin in the fibre tracts, concealing the stained cell bodies. Instead APC, an antigen expressed in the cell bodies of oligodendrocytes, was used.

In paper II, glial cells positive for BrdU, MHC II or double positive for BrdU/APC and ED1/Iba-1 respectively, were quantified in the frontal parts of corpus callosum (between bregma and 1.6 mm anterior to bregma). In paper III, blood vessel-associated cells positive for CD163, CD4 and MHC II respectively were quantified in the five subregions of middorsal hippocampus mentioned above. In paper IV, number of TUNEL-positive and MHC II-positive cells in hippocampus was analysed (unpublished data).

## **Brightfield microscopy**

An Olympus AX70 light microscope with a 40x objective was used to quantify number of cells positive for Ki67 in middorsal dentate hilus and GCL of hippocampus, caudal corpus callosum and basolateral nuclei of amygdala according to above (paper I).

To evaluate the intensity of the LFB staining in paper II, microphotographs were taken on every 10<sup>th</sup> section through out the targeted part of corpus callosum using the same light conditions for all sections (on average six sections per animal). The micrographs were converted to grey-scale and the corpus callosum outlined on the micrographs using Adobe Photoshop (11.0). The intensity of LFB staining was expressed as mean grey-scale level of the selected area (0=black, 255=white), using Adobe Photoshop (11.0). The staining intensity was averaged within each animal.

### **Confocal microscopy**

With regular fluorescence microscopy the tissue section is completely illuminated by the excitation light, meaning that the whole depth of the section is fluorescing at the same time. The highest intensity of the excitation light is at the focal point, but other parts of the sample also fluoresce. Accordingly it can be hard to separate a truly double positive cell from two cells that are positioned on top of each other on different depths of the section. Using confocal microscopy, out of focus fluorescent light is evaded. By scanning many thin sections through the tissue, it is possible to reconstruct a three-dimensional image of cells.

Before starting to count double-labelled cells in the epifluorescence microscope in paper I and II, we confirmed that our judgment of a double-labelled cell was correct using a confocal laser-scanning microscope (Leica TCS SL), with a 100x oil immersion lens objective and Leica Confocal Software (version 2.61; Leica Microsystems, Mannheim, Germany). We concluded, that for these stainings, it was possible to assess double labelling in the epifluorescence microscope. Though, in paper one, the proportion of BrdU+ cells expressing APC was evaluated with confocal microscopy. Three hundred BrdU-positive cells in each treatment group (about 30 cells per animal) were analysed for double labelling with APC.

### **Statistical analysis**

Statistical significance was set at  $p < 0.05$  and data are presented as mean  $\pm$  standard error of the mean (SEM). Analysis of differences between two groups was carried out using student's t-test (paper I, II and IV). For comparisons of three or more groups, one-way analysis of variance (ANOVA) followed by Fisher's PLSD post hoc test (paper II, III and IV) was used. For time-course experiments repeated measures ANOVA was performed (paper IV). All statistical analyses were performed with Statview software, version 5.0 (Abacus Concepts, Berkeley, CA, USA).

# Results and comments

## Lithium treatment decreases formation of new NG2 cells in both grey and white matter areas implicated in bipolar disorder (Paper I and unpublished data)

Imaging and *post mortem* studies have shown structural brain abnormalities and glial pathology in white and grey matter regions of patients with bipolar disorder (Vostrikov et al., 2007; Hafeman et al., 2012). Lithium, the recommended first-line treatment of bipolar disorder has been shown to increase grey matter volume (Moore et al., 2000), be neuroprotective (Chen and Chuang, 1999) and increase neurogenesis in hippocampus (Chen et al., 2000). However, the effect of lithium on the most proliferating cell type in the adult brain, the NG2 cell had not yet been investigated. NG2 cells proliferate in both white and grey matter, are known to differentiate into oligodendrocytes (Polito and Reynolds, 2005), but can also stay in an NG2-positive state receiving synaptic input from glutamatergic neurons (Paukert and Bergles, 2006) and affect axonal outgrowth (Ughrin et al., 2003; Yang et al., 2006). ECT, a highly effective treatment mainly used for otherwise treatment resistant severe depression and mania has been shown to increase NG2 cell proliferation in hippocampus (Wennström et al., 2003) and amygdala (Wennström et al., 2004).

In paper I, the proliferation, survival and differentiation of NG2 cells was investigated in regions that have been implicated in the pathophysiology of bipolar disorder. Two grey matter limbic regions (hippocampus and basolateral amygdala) and the main white matter tract in the brain, the corpus callosum, were analysed using BrdU to label newborn cells. During two hours after injection, BrdU labels cells in S-phase of the cell cycle. After cell division, BrdU will be present in the daughter cells of the initially labelled cell (Cameron and McKay, 2001). In paper I we injected BrdU just before or in the end of a four-week long lithium treatment period to assess proliferation and survival respectively. Brain sections were stained for BrdU, Ki67 (a nuclear protein expressed in proliferating cells) and markers specific for NG2 cells and oligodendrocytes.

Most of the proliferating cells were NG2 cells (80-90%) in all areas investigated except for the GCL where hardly none of the proliferating cells were NG2-positive.



In the GCL, clusters of BrdU-positive cells, were typically found, whereas in all other areas BrdU-positive cells were distributed in pairs. The morphology of the NG2-positive cells agreed with that described for NG2 cells (Butt et al., 2005). For micrographs see paper I. In therapeutically relevant levels (here 1.0 mM), lithium treatment significantly decreased the proliferation of NG2 cells in corpus callosum and dentate hilus of hippocampus with 45 and 40 % respectively and in amygdala by 20%. However in the ML and CA-regions of hippocampus lithium had no effect on proliferation. In the GCL on the other hand, lithium treatment significantly increased proliferation by 45%. Most cells in GCL are from other studies known to be neuronal precursors both during normal conditions and at lithium treatment. Furthermore, lithium has in previous studies been shown to increase neurogenesis (Chen et al., 2000). Thus, in accordance with previous studies we also found increased GCL- cell proliferation in response to chronic lithium treatment. The decreased/increased proliferation rates assessed by BrdU labelling, was confirmed using another proliferation marker, Ki67. The magnitude of proliferation decrease in dentate hilus, amygdala and corpus callosum was the same for both proliferation markers.

### **Confirming results in additional studies**

Decreased proliferation of NG2 cells in dentate hilus of hippocampus was repeatedly found in two additional independent studies (unpublished data). Proliferation in corpus callosum and amygdala was not analysed in those two studies. Both studies were designed in accordance with the proliferation study in paper I with a few exceptions. In the first study, a somewhat lower dose of lithium was used (40 mmol/kg instead of 50mmol/kg; giving serum levels of 0.7 mM instead of 1mM). The decreased proliferation rate in dentate hilus was significantly decreased, but only by 20% (on average 24 vs 19 cells per section;  $p=0.005$ ; unpublished data). In the second study, the Wistar-Kyoto (WKY) rat strain was used. WKY rats were initially developed as the normotensive control strain for the spontaneously hypertensive rat, but has been proposed as an animal model of depression. They demonstrate hormonal, behavioural, and physiological measures that correspond to those found in depressive patients. WKY rats are hyper-reactive to stress and show dysregulation of the HPA-axis (Solberg et al., 2001; Rittenhouse et al., 2002). After four weeks of lithium treatment, serum levels of lithium were 0.9 mM and proliferation of NG2 cells in dentate hilus was significantly decreased by 40 % compared to control (on average 12 vs 7 cells per section;  $p=0.015$ ; unpublished results). We can not exclude that reduced food intake in rats treated with lithium could influence the proliferation. In an attempt to create an appropriate control group NaCl (instead of LiCl) was added in the diet to one group of rats. However, sodium chloride supplemented diet did not influence the food intake (unpublished data). A reduced amount of rat chow

could have been given to the control group to equalise the groups, but there are a few obstacles with doing so. When housed together, there is a risk that the dominant rat in the cage eat more the other rats and that all food is consumed at once and not spread out over the day as it naturally should (Kasanen et al., 2009). Single housing bypasses those problems but is stressful for the animals.

### **Lithium treatment does not effect the survival or oligodendrocyte differentiation of cells born before the treatment start (Paper I)**

Cells labelled with BrdU just before lithium treatment was initiated survived for a month to the same extent as in the control group in GCL, dentate hilus, amygdala and corpus callosum. In grey matter, (dentate hilus and amygdala) around 75- 80 % of the BrdU-positive cells were still positive for NG2 after four weeks and a few percent were oligodendrocytes. However in white matter (corpus callosum) only around 10 % of the BrdU cells were still NG2-positive. On the other hand, 70 % of the BrdU cells were now oligodendrocytes. Lithium treatment did not significantly affect the proportion of BrdU cells double positive for NG2 or oligodendrocytes although there was a trend towards decreased proportion of BrdU-positive oligodendrocytes in corpus callosum after lithium treatment.

To summarise, chronic lithium treatment increases hippocampal neurogenesis but robustly decreases proliferation of NG2 cells in corpus callosum, dentate hilus of hippocampus and amygdala. However, lithium treatment had no effect on NG2-cell proliferation in ML and CA-regions. The effect was most pronounced in corpus callosum and dente hilus. Futhermore, lithium treatment had no effect on the survival or oligodendrocyte differentiation of cells born before treatment start.

### **Lithium treatment decreases oligodendrogenesis and microglia activation in white matter in a demyelination model (Paper II)**

Some argue, that the function of NG2 cells in the adult CNS is to replace oligodendrocytes upon demand. In paper I we found that lithium induced decreased NG2 cell proliferation but had no effect on survival or oligodendrocyte differentiation for cells born before treatment. We hypothesised that in intact brain, there is already an optimal turnover rate of oligodendrocytes and that a demyelinating insult would be needed in order to study lithium's effect on oligodendrogenesis and remyelination.

## **The cuprizone model was chosen to induce demyelination (paper II)**

The cuprizone model was used to induce demyelination in adult rats. For information about the model see the material and method section. After four weeks of cuprizone treatment, significantly less myelin was isolated from whole brain homogenate compared to control. Two weeks of cuprizone treatment was enough to induce demyelination in three-week old rats, but not in adult rats (unpublished data).

Lithium treatment was given for three weeks beginning directly after the cuprizone withdrawal. Giving lithium at the same time as cuprizone would have been convenient since successful spontaneous remyelination occurs during the weeks after cuprizone withdrawal and thus aggravates the evaluation of additional effect of lithium on physiological remyelination. It is also possible that the neurotrophic and anti-apoptotic effects of lithium could have decreased demyelination if administered at the same time as the toxin. However, simultaneous treatment was not possible as rats treated with lithium eat less. Accordingly it would be hard to evaluate the results from such a study since less intake of cuprizone chow naturally would give rise to decreased demyelination.

By choosing a demyelination model that doesn't depend on diet administration, lithium treatment during the demyelinating insult would be possible. A separate study, using lyssolecithin (LPC) -induced demyelination was therefore performed. Stereotaxic injections of LPC in white matter tracts induce focal demyelination. LPC is a membrane solubilising agent with a particular toxicity to myelinating cells (Woodruff and Franklin, 1999). Rats were treated with control or lithium diet for ten days before LPC-injections into the anterior part of the genu of the corpus callosum. Lithium treatment was continued till the end of the study eight days after the LPC-injections. However, due to vast interindividual variance within the groups in terms of lesion size (and to some degree localisation of the expansion), the analysis of the effect of lithium was not possible (unpublished data). Accordingly, the cuprizone model, giving more reproducible demyelination, was used for further studies. Recently, the cuprizone model has been used for study of psychiatric disorders (Herring and Konradi, 2011; Chandran et al., 2012; Zhang et al., 2012).

## **Lithium treatment decreases oligodendrogenesis in a demyelination model (Paper II)**

Labelling of proliferating cell with BrdU was performed during the first five days of lithium treatment (i.e. directly after cuprizone withdrawal; see paper II for experimental timeline).

Demyelination (i.e. cuprizone treatment) gave rise to a significantly increased number of BrdU-positive cells. Thus, cells proliferating and then surviving for three weeks were increased after cuprizone treatment alone compared to all other groups. Oligodendrogenesis during the same period; i.e. number of BrdU-positive cells also positive for APC was likewise significantly increased in the cuprizone group compared to all other groups; notice, also compared to the cuprizone group treated with lithium during the recovery period. Increased oligodendrogenesis after demyelinating insults is a well-known fact (Matsushima and Morell, 2001). Thus, after cuprizone-induced demyelination, spontaneous oligodendrogenesis was increased whereas lithium treatment during the recovery period attenuated the cuprizone-induced oligodendrogenesis at the time point studied. Though, both cuprizone and lithium significantly increased the proportion of BrdU-positive cells also being APC-positive. The combination of lithium and cuprizone treatment did not have an additive effect on the proportion of newborn cells differentiating to oligodendrocytes. The proportion of BrdU-positive cells in the control group was lower in paper II than in paper I. The most likely explanation for this discrepancy is that the rats in Paper II were somewhat older than in Paper I at the time for BrdU-labelling and oligodendrogenesis decreases with age. Moreover, in paper II the most frontal part of corpus callosum was studied whereas in paper I a more caudal part was investigated. In line with the decreased oligodendrogenesis after lithium treatment, the intensity of myelin staining (LFB staining) was significantly weaker in the two lithium treated groups (lithium and cuprizone + lithium) compared to the control group. For details and graphs see paper II.

### **Lithium treatment decreases demyelination-induced microglia activation (Paper II)**

Accumulation of microglia/macrophages in the lesion sites after a few weeks of demyelinating cuprizone treatment is well characterised in the mouse cuprizone model. In agreement with those studies, we also found substantially increased numbers of phagocytic microglia identified using Iba-1/ED1-labeling, in the cuprizone treated rats. Likewise, the number of activated microglia, recognised using MHC II-labelling, was also increased by cuprizone treatment. Interestingly and in accordance with other studies stating anti-inflammatory effects of lithium (De Sarno et al., 2008), we also detected decreased microglia activation after lithium treatment in the cuprizone treated rats. Hence, lithium decreased cuprizone-induced microglia activation in corpus callosum. For details see paper II.

## Repeated electroconvulsive seizures recruit macrophages to the hippocampal blood vessel walls (Paper III)

Studies from our group have previously shown a number of cellular changes in hippocampus after ECS treatment including angiogenesis (Hellsten et al., 2005) and low-grade glial cell activation (Jansson et al., 2009). The blood-brain-barrier (BBB) strictly controls entrance of immune cells into the CNS. During angiogenesis and blood vessel growth, BBB permeability transiently increases (Rigau et al., 2007). Others have shown a transient increase in blood barrier permeability after ECT (Mander et al., 1987). Furthermore, in many pathological conditions microglia activation is accompanied by recruitment of immune cells from the blood (Rezai-Zadeh et al., 2009). Under normal conditions only a small number of T-cells and macrophages enter the brain (Hickey, 1991). After damage to the brain parenchyma, leukocytes are recruited to the brain in a highly graded manner (Raivich et al., 1999). In severe brain pathology such as infections and ischemia there is an influx of large numbers of T-cells, monocytes and granulocytes. Infiltration of leucocytes into the brain parenchyma has traditionally been regarded as harmful, however also positive protective effects of infiltrating T-cells has been shown (Ziv et al., 2006). Jansson et al. (2009) have shown that the microglia activation in response to repeated ECS treatments was most pronounced in hippocampus. In paper III, we therefore wanted to investigate if ECS treatments also led to increased recruitment and infiltration of blood born macrophages and T-cells into the hippocampal parenchyma.

Rats were given one daily ECS treatment for ten consecutive days. After the last ECS, rats were allowed to survive for either two hours or two, four or eight days. Analyses of brain sections stained for laminin (stains blood vessel walls) and CD163 (stains macrophages) demonstrated several CD163-positive cells within the vasculature of all animals. However, in animals treated with ECS, the number was increased by approximately 30% at all survival time points. Nevertheless, no CD163-positive cells were found in the brain parenchyma in any of the treatment groups. Most of the cells were found in vessels within the ML of hippocampus. CD-163 has convincingly been shown to be expressed by perivascular macrophages or meningeal macrophages under both normal and pathological conditions in the brain (Graeber et al., 1989; Polfliet et al., 2006). The meningeal macrophages are largely localized on the surface of the pia mater (Hickey et al., 1992) and the perivascular macrophages are recognized by their location in the perivascular space of arteries, medium sized vessels, and capillaries (Graeber et al., 1992). The perivascular space is a tightly regulated microenvironment between the endothelial basement membrane and the foot processes of astrocytes.

CD4 is a cell surface glycoprotein expressed on T helper cells, T regulatory cells, monocytes and macrophages (Stewart et al., 1986) but also on a subset of activated

microglial cells (Perry and Gordon, 1987). Hardly any CD4-positive cells with morphology typical for T lymphocytes (small round cells) were found in any of the rats. Instead, the morphology of the CD4-positive cells was very similar to that found for CD163-positive cells; i.e. large, irregular elongated cells often with protruding processes or with a crescent-shaped appearance (as often seen in perpendicularly intersected blood vessels). Those cells were also seen in association with the blood vessels and their number was also increased by 30 % following ECS. It is highly likely the increased number of CD163-positive and CD4-positive cells within the blood vessel walls both represent increased number of perivascular macrophages, most likely recruited from the blood.

CD4-positive cells with another type of morphology were found outside the vessel walls in the ECT treated animals, preferentially in the dentate hilus. Those cells had morphology very similar to that of microglia. Furthermore, ECS treatment gave rise to a transient increase in the number of MHC II-positive cells within blood vessel wall. Eight days after the last ECS, the number of these cells had returned to baseline levels.

## Possible effect of lithium treatment on ECS-induced spatial memory loss (Paper IV and unpublished data)

ECT is one of the most effective treatments for severe mood disorders (Pagnin et al., 2004). However its clinical use has been limited by concerns about side-effects, particularly amnesia. The mechanism behind ECT-induced amnesia is not understood. Drugs targeting diverse molecular pathways have been administered in association with ECT in the clinic as well as in animal models in an attempt to reduce amnesia and clarify the mechanisms. Clinical data are still sparse and inconsistent and have not led to any change of practice. During recent years, lithium treatment has been shown to attenuate amnesia in several different animal models of memory deficits. In paper IV we investigated the effect of chronic lithium treatment on spatial retrograde memory after ECS.

A hippocampus-dependent spatial navigation test, the MWM test, was used to evaluate learning and memory abilities (Morris, 1984). Rats were trained to navigate and find an escape platform in the maze for eight consecutive days. Typically, performance was improved during the first four days and was then stable for the rest of the days. Lithium treatment did not affect learning. One daily ECS was then given for five days starting on the last day of MWM training. Lithium had been given for ten days before the first ECS and the treatment was continued throughout the whole

study. The day after the last ECS, a retention test was performed to test if the rats remembered the position of the platform learnt during the week preceding ECS.

### **Lithium treatment attenuates ECS-induced spatial retrograde amnesia**

The rats in the control group had a significant preference for the platform quadrant. The group treated with ECS alone on the other hand, spent only around 20% in the platform quadrant. That is significantly less than the control group (40%) and moreover less than they would spend there by chance (25%). Instead they spent more time in the area where they were put down in the pool. Furthermore, in all studies, the group treated with lithium solely performed in level with the control group. The group treated with lithium before and during the ECS treatment performed significantly better than the non-lithium treated ECS group and additionally in level with the control group and lithium group. For details and graphs see study one and two in paper IV.

### **Procedural memory**

During the first trial of the learning period the rats mostly swim around the pool along the sidewalls, thigmotaxis. After a few trials they start to spend more time exploring the inner parts of the pool. Since the rats in the ECS group did not have any spatial bias for the platform quadrant during the retention test, we investigated whether they behaved as naive rats by comparing thigmotaxis in the first learning trial with the same behaviour during the retention test. Interestingly, even though the rats in the ECS group showed no signs of remembering the position of the escape platform in the maze they did not behave as they did in the beginning of the training period. During the first learning trail they spent around 80 % of their time along the side walls, whereas during the retention test they spent only around 30 % there.

### **No correlation between spatial memory and cell death or microglia activation in hippocampus**

An equal anti-amnesic effect of lithium was found in a second, independent, identical study. We next sought to find a mechanism underlying the protective effect of lithium. Lithium has neuroprotective properties and since anti-amnesic effects of lithium after stroke had been explained by decreased cell death in the lithium treated group (Yan et al., 2007) we analysed the number of apoptotic cells (TUNEL-positive cells) in hippocampus. Very few TUNEL-positive cells were found in all treatment groups and no group differences were found when the whole hippocampus region was

analysed. No analysis of the hippocampal subregions separately could explain the differences detected in the memory task either (unpublished data).

Furthermore, our group has previously found a low-grade inflammation following ECS, efficiently detected by immuno-labelling a marker for activated microglia, MHC II (Jansson et al., 2009). Lithium has in several studies, including paper II in this thesis, been shown to reduce inflammation response in the CNS (De Sarno et al., 2008). In accordance with our previous studies, ECS increased the number of activated microglia in hippocampus. However, this activation was equally large in the group treated with lithium and ECS simultaneously. Thus, as opposed to demyelination-induced microglia activation (paper II), lithium did not significantly reduce the ECS-induced microglia activation and could accordingly not explain the memory differences seen between the two ECS-groups (unpublished data).

### **The anti-amnestic effect of lithium on ECS-induced amnesia was not reproducible in two additional studies.**

When further investigating the anti-amnestic effect of lithium treatment, new MWM studies were conducted. During those studies, investigating for example the contribution of postictal confusion, our results were inconsistent. Consequently, an additional study, again identical with the first study was conducted. In this study (called study three in paper IV) the ECS-group, just as in the earlier studies had robust spatial reference memory deficits. However, in this study, lithium treatment did not attenuate the amnesia.

We next turned to use the pigmented Lister Hooded rat (study four in paper IV). They have superior visual acuity compared to albino rats, like Sprague-Dawley rats (Prusky et al., 2002), and we hypothesised that their better visual ability would give better performance in the MWM task and thereby give more pronounced groups differences. A second reason for using Lister Hooded rats was further future investigations of ECS-induced amnesia using other memory tests with high demands on visual acuity. However, the learning and performance during the retention test did not differ significantly for the Lister Hooded rats compared to the performance of Sprague-Dawley rats used in the other studies. In this study the stimulus parameters to induce seizures was slightly changed (see material and method section). Most notably a shorter pulse width was used. Shorter pulse widths are associated with less cognitive side effects in the clinic, but clinically even shorter pulse widths are used (Sackeim et al., 2008). Here it should be mentioned that pulse frequency, pulse width, stimulation current, factors that all affect length and characteristics of the epileptic seizures are dependent on several factors such as cranial geometry, size etc. It



is therefore not possible to directly translate stimulation parameters used for clinical ECS to those used in experimental rat studies. Nevertheless, performance during the retention test was the same for the ECS group in this study as in previous studies using longer pulse widths; i.e. clear amnesic effect was seen in the ECS group. However, as in study three and opposed to study one and two (paper IV), there was no effect of lithium treatment on ECS-induced amnesia. For details and graphs see paper IV. When pooling data from all four studies, there was a significant difference between the ECS group and the lithium + ECS group using student t-test ( $p=0.01$ ). However, the mean value for the lithium + ECS group is when pooling data only 25%.

To summarise, in four independent but equally designed studies, we repeatedly found robust spatial reference memory deficits following five ECS treatments for a navigational task learnt the week preceding the first ECS. However procedural memory was not affected. Furthermore, in two of the four studies lithium treatment attenuated the ECS-induced amnesia but the anti-amnesic effect was not associated with either reduced cell death or reduced microglia activation. In the additional two studies lithium had no effect on the ECS-induced amnesia.

# General discussion

## Why does lithium treatment decrease NG2 cell proliferation and what does it mean?

Our research group have previously shown that the proliferation of NG2 cells in hippocampus and amygdala is substantially increased by ECS. It is now well established that 1) NG2 cells represent the most proliferating cell type in the adult grey and white matter (Dawson et al., 2003) and 2) these cells are indeed the main and probably sole source of oligodendrocytes in the adult brain (Zhu et al., 2008; Kang et al., 2010) and 3) they receive synaptic input from GABAergic and glutamatergic neurons even while undergoing mitosis (Kukley et al., 2008; Tanaka et al., 2009). Other features of NG2 cells are still poorly understood: 1) Can they differentiate into neurons (Belachew et al., 2003; Zhu et al., 2008), 2) what is the function of NG2 cells that remain in their NG2-positive state (sometimes called synantocytes) and 3) what is the effect of synaptic input to NG2 cells?

With this background, we found it highly interesting to study NG2 cell proliferation and differentiation in response to lithium treatment. We found that lithium treatment decreased the proliferation of NG2 cells in dentate hilus of hippocampus, amygdala and even more so in corpus callosum, but not in ML and the CA-regions of hippocampus. In grey matter regions most NG2 cells stayed in an NG2-positive state whereas in white matter most of them differentiated to oligodendrocytes.

### **Direct effects of lithium on NG2 cell proliferation**

Lithium has been demonstrated to stimulate neurogenesis by inhibiting GSK-3 $\beta$  and increasing Wnt/ $\beta$ -catenin (Wexler et al., 2008; Boku et al., 2009). In contrast, inhibition of proliferation (in astrocytes) (Pardo et al., 2003; Kim et al., 2004; Zhu et al., 2011) has in a recent study been proposed to be GSK-3 $\beta$  independent and instead be due to inhibition of the STAT-3 pathway (Zhu et al., 2011). Whether that holds true also for other glial cell types (such as NG2 cells) remains to be investigated.

Lithium can freely pass through voltage-gated sodium channels in excitable cells. However, it is not pumped out by the Na<sup>+</sup>/K<sup>+</sup>-ATPase and thus tends to accumulate

inside cells (Rang et al., 2003). It has been demonstrated that at therapeutically relevant concentrations lithium entry and accumulation reduces outward current (most importantly outward potassium currents) and depolarizes neurons (Butler-Munro et al., 2010). Whether lithium can induce NG2 cell depolarisation is not known, but since these cells express voltage gated-sodium channels this is a possibility (Lin and Bergles, 2004b). Depolarization of NG2 cells is known to decrease their proliferation rate (Knutson et al., 1997; Butt et al., 2005) and taken together these findings may suggest a possible mechanistic explanation for our results.

## **NG2 cells respond to inflammation and brain damage**

Following stroke (Ohta et al., 2003), stab wounds (Hampton et al., 2004) and excitotoxic damage (Ong and Levine, 1999), NG2 cell proliferation is enhanced and they migrate towards the injured site and up-regulate expression of the NG2 proteoglycan (Ong and Levine, 1999; Tanaka et al., 2001; Jones et al., 2002). Thus, in this context, the increased proliferation of NG2 cells seen after ECS could be part of the mild inflammatory response seen after ECS whereas the decreased proliferation rate of NG2 cells after lithium treatment is in line with anti-inflammatory and neuroprotective effects associated with lithium treatment (Nonaka et al., 1998; Rapaport and Manji, 2001; De Sarno et al., 2008; Rao et al., 2008).

## **Indirect effects of NG2 cell proliferation via changes in neuronal signalling**

As mentioned above, several independent research groups have shown that NG2 cells receive synaptic glutamatergic and GABAergic input from neurons; a feature that has been shown in all grey and white matter regions investigated (for review see Mangin et al. 2011). The function of this fast and direct signalling is not yet clear, but is proposed to accommodate NG2 cell function to neuronal activity. For instance, glutamatergic activation of NG2 cell AMPA receptors has been shown to inhibit their proliferation (Yuan et al., 1998) and differentiation (Gallo et al., 1996) and to stimulate their migration (Gudz et al., 2006). Interestingly, some clinical studies indicate a dysfunction of the glutamatergic system in patients with bipolar disorder (Friedman et al., 2004; Hashimoto et al., 2007; Ongür et al., 2008; Fountoulakis, 2012) and a modulating role of lithium (Friedman et al., 2004; Shibuya-Tayoshi et al., 2008). Different techniques were used to estimate glutamate levels in patients. For instance, in the study by Hashimoto et al., levels of amino acids in *post mortem* brains from patients with bipolar disorders was analysed while Ongür et al. used proton magnetic resonance spectroscopy to measure glutamine/glutamate ratio. There are also animal studies reporting increased levels of glutamate after lithium treatment (Gottesfeld, 1976; Marcus et al., 1986) and inhibiting effects on NMDA receptors

(Nonaka et al., 1998; Basselin et al., 2006). In addition, a lithium-induced change in AMPAR trafficking has been reported (Du et al., 2008). The effects of lithium on the glutamatergic system have been suggested to vary in a region, dose, and time dependent manner (Gottesfeld, 1976; Dixon and Hokin, 1998; Antonelli et al., 2000). A change in neuronal signalling pattern during lithium treatment seems therefore compatible with different levels of proliferation and differentiation of NG2 cells in various brain regions.

### **Effects of decreased NG2 cell proliferation**

#### *Effect of NG2 cells on axonal guidance and synaptogenesis – NG2 as a synantocyte*

The NG2 molecule is a chondroitin sulphate proteoglycan, a class of molecules that are known to inhibit axonal outgrowth. The NG2 proteoglycan has also been demonstrated to cause growth cone collapse and inhibit neurite outgrowth (Dou and Levine, 1994; Fidler et al., 1999; Berry et al., 2003; Ughrin et al., 2003; Huang et al., 2005). In addition, blocking the NG2 molecule promotes regeneration in the spinal cord (Tan et al., 2006). Thus, a reduction in NG2 cell numbers may for example facilitate axonal outgrowth from GCL neurons through hilus (where decreased NG2 cell proliferation was seen after lithium treatment) to CA3 targets. However, a study by Yang et al. (2006) showed that NG2 cells provided an adhesive substrate for axonal growth cones and promoted their growth *in vitro*. Either way, it seems like NG2 cell might guide neurite outgrowth and depending on the expression of different surface and extra cellular matrix (ECM) molecules the effect might either be inhibiting or permissive. Indeed, it has been shown that the relative levels of repellent versus adhesive interactions is important for a precise regulation of axonal outgrowth (Grumet et al., 1996). Furthermore, NG2 cells form close contacts with astrocytes, neurons, oligodendrocytes and myelin. The term “tripartite synapse” was coined when astrocytes turned out to be an important component at synapses. Butt et al. (2005) propose the term “tetrapartite synapse” to illustrate that also NG2 cells play an important roll in synaptic function. It is known that NG2 cells produce ECM molecules, which stabilizes the synapses. Thus, apart from taking part in axonal guidance, NG2 cells may regulate both synaptic function and structure.

#### *Decreased NG2 cell proliferation reduced oligodendrogenesis and remyelination – NG2 cells as oligodendrocyte precursor cells*

It is well established that NG2 cells can differentiate into myelinating mature oligodendrocytes in the adult brain (Levison et al., 1999; Dawson et al., 2003; Polito and Reynolds, 2005). It is not far fetched to hypothesize that lithium induced decrease in NG2 cell proliferation, as described in paper I, could be followed by a decreased formation of mature oligodendrocytes. An opposite scenario in which NG2

cell proliferation is blocked in favour of differentiation, leading to more mature oligodendrocytes, is however also possible (Jablonska et al., 2007). In paper I there was no effect on the survival or differentiation of NG2 cells born before lithium treatment started. Considering the increasing number of imaging, *post mortem* and transcriptome studies indicating white matter abnormalities associated with bipolar disorder, we wanted to investigate if the decreased proliferation of NG2 cell in paper I actually reflected a change in oligodendrogenesis. We hypothesized that lithium inhibited NG2 cell proliferation in favour of oligodendrocyte differentiation and that effects on oligodendrogenesis and myelination would be more pronounced when investigated after a demyelinating insult where there is a need for new oligodendrocytes (Bu et al., 2004). We turned to the cuprizone model to induce demyelination (see the material and method section and result section for further information about the model). Recently, the cuprizone model has been used as an animal model for schizophrenia (Xu et al., 2009; Herring and Konradi, 2011) – a mental disorder which shares certain characteristics with bipolar disorder, for example in terms of white matter changes (Bartzokis, 2012; Konradi et al., 2012).

Unexpectedly, we found that chronic lithium treatment during the recovery period after a demyelinating insult decreased oligodendrogenesis and remyelination measured by 1) the number of newborn mature oligodendrocytes and 2) intensity of a myelin staining. Decreased number of newborn mature oligodendrocytes could either reflect decreased differentiation or decreased proliferation. Considering our results from paper I showing decreased proliferation, we find that explanation most likely. Although it is possible that differentiation and proliferation at other time-points than studied here could be differently affected. Nevertheless, the decreased intensity of the myelin staining indicates that the inhibitory effect of lithium actually decreases oligodendrogenesis and myelination. In rats not treated with cuprizone, lithium *per se* increased the proportion of newborn cells also being oligodendrocytes (note that in paper II, rats were injected with BrdU during lithium treatment and not before as in the survival study in paper I) but again, the total number of cells born was lower to begin with and thus, the number of newborn oligodendrocytes ends up to be lower also in this group. Analysis of total number of oligodendrocytes and electron microscopy measurements of myelin thickness would be required for further analysis.

### **Our results compared to other preclinical studies on lithium and white matter**

Our results are in contrast to a study by De Sarno et al. (2008) showing reduced demyelination in an animal model of MS after lithium treatment. Lithium may act differently in two separate demyelination models and the authors in De Sarno et al. (2008) speculate that lithium in their model may act by inhibiting the immune

response. Also opposed to our results is a recent study where lithium injected directly into the ventricles during three days promoted normal myelination process (Azim and Butt, 2011). However the short-term treatment they used differs substantially from our chronic systemic treatment. Furthermore, lithium has been shown to enhance remyelination of peripheral nerves (Makoukji et al., 2012). Nevertheless, in line with our results is a study by McQuillin et al. (2007) showing decreased expression of many oligodendrocyte-associated genes in mice after lithium treatment. In that study however, the lithium dose given and the serum levels of lithium obtained were very high. After the publication of our study, Zhu et al. (2011) showed that lithium decreased the number of A2B5 cells (a name used in cell culture for a cell type similar to NG2 cells) *in vitro*.

### **Imaging studies on the effect of lithium**

A recent meta-analysis conclude that there is indeed a positive effect of lithium on grey matter volume and that the effect on white matter abnormalities is more uncertain due to non-conclusive results (Hafeman et al., 2012). To summarise, most studies show no effect of lithium treatment on white matter (McIntosh et al., 2008; Wang et al., 2008; Zanetti et al., 2009), but some demonstrate normalizing (Macrithie et al., 2010) or negative effects (Benedetti et al., 2011). Interestingly, the same review point out that it is especially during depression episodes that bipolar patients have decreased coherence and directionality of white matter tracts (corpus callosum as well as tracts connecting the frontal lobe to other parts of the brain; uncinate fasciculus, superior longitudinal fasciculus, anterior thalamic radiation, and anterior cingulum).

### **Can the adverse effects of lithium affect myelination?**

Thyroid hormone administration has been shown to increase remyelination after cuprizone-induced demyelination in rats (Franco et al., 2008). Thus, the decreased remyelination in response to lithium treatment could possibly be due to hypothyreosis associated with lithium treatment of rats (Allagui et al., 2005).

We can not rule out, but find it unlikely that the effects we have seen on proliferation and myelination should be due to toxic effects of lithium. In paper I, the serum level of lithium was within the targeted range and in paper II the serum levels were slightly higher, but still far below levels associated with acute toxic effects (Timmer and Sands, 1999). Besides, the dose given is not considered high for oral administration to rats (O'Donnell and Gould, 2007). Furthermore, high toxic levels of lithium have

been shown to increase proliferation of oligodendrocyte progenitor cells *in vitro*, not decrease as we have shown (Uluitu et al., 1999).

Can the attenuated weight gain in the lithium groups affect the remyelination? Oligodendrocytes have high metabolic demands and starvation during development is associated with decreased myelination (Wiggins and Fuller, 1979; Royland et al., 1992) but this can hardly be equated with the decreased food intake seen in our rats. Moreover, studies on calorie restriction have actually been shown to repress demyelination (Piccio et al., 2008) indicating that the decreased oligodendrogenesis we demonstrate are more likely due to the lithium treatment *per se*, than the flattened weight curve.

Taken together, lithium could either affect NG2 cell proliferation directly or indirectly via effects on neurons or due to side effects of lithium such as weight loss, toxicity or hypothyroidism. Effects of lithium on neuronal plasticity/neurotransmission/connectivity can influence NG2 cell proliferation indirectly via neuron-NG2 synapses. Even though it is not possible to grasp the whole picture yet, direct or indirect modulation of NG2 cell proliferation/fate/function have potential to modulate synaptogenesis, myelination, axonal outgrowth and other plastic changes important for changing the connectivity in the diseased human brain.

## Lithium treatment modulates the inflammatory response in the brain

In paper II we show that chronic lithium treatment reduces microglia activation after cuprizone-induced inflammation measured as number of MHC II-expressing microglia. Microglia MHC II expression is up-regulated in response to inflammatory stimuli in the brain enhancing antigen presentation. An explanation to why lithium treatment reduced cuprizone-induced inflammation in paper II but not ECS-induced microglia activation in paper IV can not be found in the literature but the effect of lithium on the immune system is complex. Lithium has for long been known to cause granulocytosis (Tisman et al., 1973; Watanabe et al., 1974) and inhibit T-cell production (Fernandez and MacSween, 1980) but enhance T-cell activity (Ridgway et al., 1986). Lithium has also been shown to modulate the cytokine profile. Rapaport and Manji 2001 demonstrate that lithium treatment shifted the cytokine production in human whole blood culture from cytokines traditionally regarded as T-helper lymphocyte type-1 ( $T_H1$ ) immune response (IL-2 and IFN- $\gamma$ ) to a  $T_H2$  response (IL-4, IL-10). The same held true for the brain in a recent study on mouse (Sudduth et

al., 2012). Beurel et al., 2010 emphasize that the anti-inflammatory effects of lithium are due to inhibition of GSK3.

In line with our results are numerous studies during the last decade showing anti-inflammatory effects of lithium in diverse animal models of brain disease. Decreased microglia activation have been demonstrated after transplantation (Su et al., 2007), stroke (Kang et al., 2012), hypoxic (Li et al., 2011a) and traumatic (Yu et al., 2012) brain injury, neuroinflammation (Basselin et al., 2007) and MS (De Sarno et al., 2008).

## What do the perivascular macrophages do in the vessel after ECS?

We find it likely that the increased number of CD163- and CD4-positive cells we saw, two hours after repeated ECS treatments and then for at least eight days, are perivascular macrophages (see result section) coming from the blood circulation. Perivascular macrophages are located between the endothelial basement membrane and the foot processes of astrocytes; i.e. in the perivascular space and express CD163 (Polfliet et al., 2006) and CD4 (Jefferies et al., 1985). Furthermore, using green-fluorescent protein-transfected bone marrow cells to study the turnover rate and origin of perivascular macrophages it had been demonstrated that new perivascular macrophages are due to infiltration of blood-borne monocytes and not to mitotic activity (Bechmann et al., 2001). Under pathological conditions, the renewal rate increases in a graded manner depending on the severity of the insult (Thomas, 1999). Immune cells are suggested to enter the brain parenchyma by two differently regulated steps: transmigration through the vascular wall into the perivascular space followed by migration across the glia limitans membrane into the brain parenchyma (Owens et al., 2008). We did not detect increased numbers of CD4+ cells with T-cell morphology or cells phenotypically known as blood-born macrophages, within the brain parenchyma following ECS. It is still possible that macrophages invade the brain parenchyma but down-regulate the expression of CD163 upon entry, but if there were to be a substantial infiltration of macrophages we believe that we would have seen CD163-positive cells outside the vessel wall at some time point. Taken together, ECS recruit perivascular macrophages to the vessel walls of hippocampus, but we detected no further progression through the astrocytic end-feet layer. What does it mean?

The brain was for long considered an “immune-privileged” organ, but it is now known that there is a constant communication between the CNS and the immune system (Quan and Banks, 2007). The exact function of perivascular macrophages is



not fully clear, but their strategic position between blood and brain suggest key function in communication between the CNS and the immune system. They produce immune regulatory mediators (Schiltz and Sawchenko, 2002), recognize, take up and present antigens from the blood and brain (Kida et al., 1993; Polfliet et al., 2002; Fabriek et al., 2005). Thus, they play a pivotal role as scavengers of the CNS as well as in the regulation of both innate and adaptive immune responses in the brain.

Accumulation of perivascular macrophages seen during the week following ECS might be part of the weak ECS-induced inflammation previously reported from our group. It can represent the first step in an inflammatory process, but when no further signals are acquired, full neuroinflammation is evaded. It is tempting to speculate that a transient low-grade inflammation might instead be involved in regulating neuronal plasticity. As discussed in the introduction, growth factors are up-regulated and neurogenesis and total number of synapses are increased after ECS. Indeed, activated microglia and recruited macrophages can release neurotrophic factors and have neuroprotective effects (Correale and Villa, 2004; Ekdahl et al., 2009) and mild acute inflammation has been shown to stimulate neurogenesis (Whitney et al., 2009). Further studies are needed to sort out the role of the immune response after ECT and its connection to antidepressant and adverse effects. The fact that we did not find decreased microglia activation in the ECS treated rats with sustained spatial memory function (due to lithium treatment) indicates that hippocampal microglia activation might not be the key factor behind ECS-induced amnesia.

## Spatial reference memory loss in well-trained rats following ECS

Memory deficits are one of the greatest concerns among patients receiving ECT. The animal model of ECT, ECS has been widely used to study cognitive disturbances. Even though amnesia is a generally accepted phenomena after ECS in several species and memory tasks (Kopp et al., 1966; Beatty et al., 1985; McClintock et al., 2012), there is still no consensus regarding which kind of memory that are disrupted and the time frames for the memory deficits.

A single ECS delivered directly (seconds to minutes) after a single training trial in a passive (inhibitory) avoidance task induces amnesia in rodents (Chorover and Schiller, 1965; Quartermain et al., 1965). When several ECS are given, memories acquired up to two weeks before ECS treatment have been shown to be affected in the same kind of task. As in humans, the amnesia is temporally graded, with memories acquired the days prior to ECS being most affected (Spanis and Squire, 1987). Amnestic effect of ECS on spatial memory using appetitively (radial maze) and aversively motivated

tasks (MWM) remain argumentative. Bohbot et al. (1996) conclude that spatial working memory can only be disrupted by ECS within 30 seconds after learning, whereas others show amnesic effect when ECS is delivered two to four hours after learning, but not when it is given directly after a learning trial (15 min) (Beatty et al., 1985; Maki, 1985). Effect on well established spatial reference memory has been shown in some studies (Yao et al., 2010), but not all (Holzhäuer and Bures, 1986).

We show powerful retrograde amnesic effect on spatial reference memory in well-trained rats in a MWM task. This is a consistent finding in four independent studies and in line with earlier reports about ECS-induced amnesia (Kopp et al., 1966; Beatty et al., 1985; McClintock et al., 2012). It is also in accordance with a recent study showing amnesia in the same kind of task as used in our study, the MWM (Yao et al., 2010). A shorter pulse width used in one of the studies gave equal memory loss. However, as opposed to our results, Beatty et al., 1985, conclude that only spatial working memory is affected and that reference memory remains intact in the radial maze. Furthermore, Holzhäuer and Bures, (1986) report only short-term amnesia (lasting for only two hours) in well-trained rats using reference MWM task. However, in contrast to our study where repeated ECS are given, the effect of a single ECS is evaluated in the study by Holzhäuser et al (1986) and Beatty et al. (1985). In the passive avoidance task repeated ECS have been shown to give more retrograde amnesia than a single ECS (Chorover and Schiller, 1965; Spanis and Squire, 1987).

### **Is only hippocampal dependent memory disrupted?**

In accordance with Beatty et al. (1985), we do find that procedural memory (in this case searching for the platform in the inner part of the pool instead of swimming along the side-walls) is intact in ECS treated rats. This behaviour could reflect memory of the task (Devan et al., 1996; 1999) or possibly be due to other factors such as less anxiety (Simon et al., 1994). Searching for the platform in the correct quadrant is hippocampal dependent, whereas ability to change search strategy from the unfavourable thigmotaxis is dependent on dorsomedial striatum (Devan et al., 1996). The behaviour of the ECS treated rats could thus reflect hippocampal, but not striatal, ECS-induced deficits.

## **Can lithium treatment attenuate ECS-induced spatial memory deficits?**

Since the introduction of ECT, modulation of electrode placement and stimulation parameters have been made in an attempt to reduce amnesia with sustained

therapeutic effect. Pharmacological treatment in association with ECT is another strategy aiming to evade side effects. Diverse substances including ketamine (McDaniel et al., 2006), cholinesterase inhibitors (Levin et al., 1987) and thyroid hormone (Stern et al., 1991) have shown promising results but the studies are few and small and have not yet led to any change of clinical practice.

Lately lithium treatment has been shown to attenuate spatial memory deficits in animal models of Alzheimer's disease (De Ferrari et al., 2003; Toledo and Inestrosa, 2010) chronic stress (Vasconcellos et al., 2003), stroke (Yan et al., 2007) ethanol intoxication (Sadrian et al., 2012) and traumatic brain injury (Zhu et al., 2010).

In two out of four independent and equally designed studies we found that lithium significantly reduced ECS-induced retrograde spatial amnesia. However in the other two studies no such effect of lithium was found. In small studies with low power there is a risk of type I and type II errors. In other word there is a risk that the positive effects of lithium treatment in the first two studies was found by chance without any true effect. Equally, there is a risk that a true effect of lithium treatment in the last two studies was by chance not found. A third explanation for the discrepancy is of course differences between studies. One way to increase the power of the analysis is to pool the data from all four studies. When doing that the group treated with lithium and ECS simultaneously performed significantly better than the group treated with ECS only (t-test). Statistical significance is not reached when comparing the pooled data for all four groups in an ANOVA. Furthermore, when analysing all four studies together, the group treated with ECS + lithium only spent 25% of their time in the target area. The maze is divided into four equally sized areas. A common way to analyse spatial memory is to measure the time spent in the quadrant where an escape platform used to be during the training sessions. If the quadrants are equally preferred, rats will by chance spend 25% of their time in each quadrant. Thus, even though lithium increased the time spent in the platform quadrant, it could be argued that it does not represent memory. However, since the rats in the ECS-group only spent 20% of their time in the platform quadrant, it is possible that the quadrants are not equally preferred when there is no memory of the location of the platform, but rather that the platform quadrant is less attractive than it would be by chance. This could possible be due to a slightly brighter light in the platform quadrant and the fact that it is further away from the starting position during the retention test. Thus, the differences between the ECS-group and the ECS + lithium could still represent a slightly better memory in the ECS + lithium group.

### **Are there any differences between the studies that can explain the results?**

Even though our studies were equally designed, they differ somewhat. In study four, using another rat strain, the serum lithium concentration was considerably lower and

polyuria was not as pronounced as in the rest of the studies. It is possible that this level of lithium was too low to exert any effect on ECS-induced amnesia. However, in study three (where lithium had no anti-amnestic effect), the serum lithium levels were within the targeted range and in level with study one and two. Though, in study three the seizure length in the lithium + ECS group was longer than in all other studies and also significantly longer than the seizures in the ECS group. Longer seizures are known to produce more amnesia (Mankad et al., 2010). It is possible that the amnestic effect of longer seizures in study three override the possible protective effects of lithium. Nevertheless, in study one, where lithium had an anti-amnestic effect, the seizures in the lithium + ECS group was also significantly longer than in the ECS group, even though the difference was not as great. Lithium in combination with pilocarpine and other cholinergic drugs exerts proconvulsant properties in rats (Morrisett et al., 1987). It has also been shown to reduce hippocampal seizure threshold in kindling studies (Minabe et al., 1988).

In study one and two a pre-trial was performed the day before the training period, to familiarize the rats to the swimming task. During the pre-trial, each rat was given one trial (60 s) during which no extra-maze cues were present. Even though a pre-trial definitely can improve performance during the first day of “real” maze training, it is hard to see how one extra trial out of additionally 32 trials during the training sessions can alter the specific effect of lithium on ECS-induced amnesia two weeks later. The room where the MWM training/testing was performed was rebuilt between study two and three. Animal behavioural testing is sensitive and a rebuilt room could very well affect the learning procedure, but again, it is hard to see why only the effect of lithium would be affected. One could speculate that in a room in which it is harder to navigate, a weak anti-amnestic effect would be more difficult to find. However, we do not find this explanation very likely. We can not rule out that the effects on memory can be due to side effects of lithium, such as decreased weight gain/slight weight loss (Bruce-Keller et al., 1999). But, side effects would be similar in all studies. However, it is still possible that lithium only under certain circumstances, that we have not been able to apprehend, exert anti-amnestic effects.

Even though lithium treatment is nowadays maintained during ECT under certain circumstances and there are several new reports of safe concurrent treatment (Dolenc and Rasmussen, 2005; Thirthalli et al., 2010), the combination of lithium treatment and ECT has for long been avoided in the clinic due to early reports on excessive postictal confusion (Hoenig and Chaulk, 1977; Small et al., 1980). Duration of postictal confusion is highly correlated with the extent of retrograde amnesia (Sobin et al., 1995). However, many of the reports are case reports in which it is hard to distinguish between adverse effects related to the disorder, one agent alone or the combination of them. Furthermore, in some of the earlier reports, lithium levels have been above today’s recommended doses. We have shown that in an animal model of

ECT at least spatial memory is not negatively affected by simultaneous lithium treatment. Instead, lithium treatment might have an attenuating effect on amnesia. We are now turning to other memory tests to further investigate the mechanisms behind ECT-induced amnesia and strategies to avoid it.

### **Possible mechanisms behind ECS-induced amnesia and an effect of lithium**

ECT affects many physiological systems in the brain and several different pathways have been hypothesised to contribute to the cognitive impairments. Chronic lithium treatment is known to influence many of them.

#### *Cell death*

In a study by Yan et al. (2007) the anti-amnesic effect of lithium treatment in an animal model of stroke was proposed to be due to reduced cell death in the lithium treated group. More specifically, cell death of the sensitive CA1 pyramidal cells in hippocampus. We did not find any apoptotic cells within the pyramidal cell layer in CA1 (assessed by TUNEL-method) following ECS or lithium treatment. Overall, TUNEL-positive cells were very few in all sub-regions of hippocampus in all treatment groups. Furthermore, no differences between the groups could explain the changed performance in the MWM task (unpublished data). However, apoptosis is a fast process (Gavrieli et al., 1992) and we can not rule out that cell death takes place at other time-points after ECS.

#### *Inflammation*

Our group had previously found a low-grade inflammation following ECS, efficiently detected with immunolabelling of a marker for activated microglia, MHC II (Jansson et al. 2009 and paper III in this thesis). Lithium has in several studies, including paper II in this thesis, been shown to reduce inflammation response in the CNS (Yuskaitis and Jope, 2009). In accordance with our previous studies, ECS increased the number of activated microglia in hippocampus. However, this activation was equally large in the group treated with lithium and ECS simultaneously. Thus, as opposed to demyelination-induced microglia activation (paper II), lithium did not significantly reduce the ECS-induced microglia activation and could accordingly not explain the memory differences seen between the two ECS-groups (unpublished data). Microglia are proposed to be involved in the process of neurogenesis (Ekdahl et al., 2009) and synapse remodelling (Gehrmann et al., 1995; Trapp et al., 2007). The different results may reflect that lithium affects a subset of microglial activities.

## *LTP*

Induction of hippocampal LTP is a widely accepted cellular mechanism for learning and memory (Bliss and Collingridge, 1993). One common hypothesis is that ECT release glutamate and induce LTP in an indiscriminative manner, which disrupts the precisely controlled synaptic remodelling required for learning (Reid and Stewart, 1997; Brun et al., 2001). LTP in the Schaffer collateral pathway of the hippocampus is NMDA receptor-dependent. During simultaneous depolarization of a neuron and glutamate binding to the NMDA receptor influx of  $\text{Ca}^{2+}$  into the post-synaptic neuron activates signalling pathways directing synaptic changes (Woodside et al., 2004). Seven days of lithium treatment, in therapeutically relevant concentrations, has been shown to attenuate  $\text{Ca}^{2+}$  influx in hippocampal neurons after NMDA receptor activation and decrease intracellular calcium concentration (Nonaka et al., 1998; Sourial-Bassillious et al., 2009). Support for disrupted LTP-induction as a mechanism behind ECT-induced amnesia comes from studies showing protective roles of NMDA receptor antagonists (Stewart and Reid, 1994; McDaniel et al., 2006) and calcium channel blockers (Zupan et al., 1996; Kamath et al., 1997; Sushma et al., 2004).

## *The cholinergic system*

The brain cholinergic systems play an important role in memory function. ECT-induced amnesia is associated with decreased activity of the cholinergic system (Lerer et al., 1984; Lerer, 1985; Levin et al., 1987) and chronic lithium enhances the cholinergic function on several levels (Lerer, 1985; Morrisett et al., 1987; Liles and Nathanson, 1988). Furthermore, in clinical trials, a cholinesterase inhibitor in combination with ECT has been shown to attenuate memory disturbances

## *Other possible pathways*

Lithium inhibits GSK-3 $\beta$ . Inhibition of GSK-3 $\beta$  was shown to enhance long-term memory (Dewachter et al., 2009). The effect has been suggested to be mediated through GSK-3 $\beta$ 's regulation of the transcription factor  $\beta$ -catenin (Maguschak and Ressler, 2008). In a recently published study, the protective role of lithium on anesthesia-induced amnesia was explained by inhibition of GSK-3 $\beta$  (Liu et al., 2010).

Glucocorticoids have a dual effect on learning and memory (McEwen and Sapolsky, 1995). Increased glucocorticoid levels after ECT correlates with cognitive deficits (Neylan et al., 2001). Moreover, glucocorticoid receptor antagonist prevents ECS-induced retrograde amnesia (Nagaraja et al., 2007). Some studies show that lithium can normalize dysregulation of glucocorticoid levels (Peiffer et al., 1991; Semba et al., 2000).



# Concluding remarks

Studies included in this thesis show that chronic lithium treatment in therapeutically relevant concentrations decrease NG2 cell proliferation in the rat dentate hilus, amygdala and corpus callosum. A majority of the NG2 cells in corpus callosum differentiated into oligodendrocytes, whereas in the grey matter regions a majority stayed in an NG2-positive state. Even though it is not possible to grasp the whole picture yet, direct modulation of NG2 cell proliferation/fate/function have a large potential to modulate synaptogenesis, myelination, axonal outgrowth and other plastic changes important for changing the connectivity in the diseased human brain. On the other hand, effects of lithium on neuronal signalling can also influence NG2 cell proliferation indirectly via neuron-NG2 synapses. Considering the increasing number of imaging studies showing white matter abnormalities in patients with bipolar disorder we found it interesting to investigate the effect of lithium on the oligodendrocyte fate of NG2 cells after a demyelinating insult. We show that lithium treatment decreased oligodendrogenesis in the rat corpus callosum in a demyelination model and additionally decreased the intensity of the myelin staining. In line with several other studies showing anti-inflammatory effects of lithium, we also found that lithium decreased the demyelination-induced microglia activation.

Furthermore, in addition to the low-grade glial cell activation previously shown in our laboratory following ECS, we found that macrophages from the periphery were recruited to the blood vessel walls of hippocampus. However, we did not find any further progression of these cells into the brain parenchyma. Finally, we investigated the effect of ECS and lithium treatment on spatial memory and demonstrated robust memory loss for a hippocampus-dependent navigational task learned during the week preceding ECS. This finding was consistent in four independent studies. However the effect of lithium treatment on ECS-induced amnesia was not as conclusive. In two equally designed studies lithium counteracted the ECS-induced amnesia, but in two following studies no such effect was found. Further studies are needed to reveal the cause of the conflicting result.

Mood disorders are major contributors to human disability and mortality all over the world. The treatments available today fail to relieve the symptoms in many patients. To develop new more effective treatments, it is important to understand the mechanism behind the diseases and how the current treatments exert their effects. The studies included in this thesis are a small part of that work.



# Acknowledgements

Ever since the first thesis from our research group (Hellsten, 2005) it has been a tradition to write the acknowledgement section in Swedish. The good reasons for doing so are stated in Hellsten 2005. So for you non-swedish-speaking-guys, find a Swedish fellow and make him/her translate!

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