Studies on cellular changes and amnesia in a rat model of electroconvulsive therapy

Jansson, Linda

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Linda Jansson

Molecular Psychiatry Unit
Department of Clinical Sciences
Lund University
Lund, Sweden

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Original articles

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

I: Jansson L, Hellsten J, Tingström A. Region specific hypothalamic neuronal activation and endothelial cell proliferation in response to electroconvulsive seizures. *Biol Psychiatry*. 2006 Oct 15;60(8):874-81.

II: Jansson L, Wennström M, Johanson A, Tingström A. Glial cell activation in response to electroconvulsive seizures. *Prog Neuropsychopharmacol Biol Psychiatry*. 2009 Oct 1;33(7):1119-28.

III: Jansson L, Tingström A. Repeated electroconvulsive seizures increase the number of vessel-associated macrophages within rat hippocampus. *Manuscript in preparation.*

IV: Orre K, Jansson L, Elfving B, Wegener G, Tingström A. Chronic lithium treatment prevents electroconvulsive seizure-induced spatial memory loss in rats. *Submitted to European Neuropsychopharmacology 2010.*

Abbreviations

ACTH	adrenocorticotropin hormone
ANOVA	analysis of variance
ArcN	arcuate nucleus
AVP	arginine vasopressin
BDNF	brain-derived neurotrophic factor
BrdU	bromodeoxy uridine
CA1-3	cornu ammonis 1-3
COX	cyclooxygenase
CRH	corticotropin-releasing hormone
DAB	diaminobenzidine
DMH	dorsomedial nucleus
ECS	electroconvulsive seizures
ECT	electroconvulsive therapy
FGF-2	fibroblast growth factor
GABA	y-aminobutyric-acid
GCL	granular cell layer
GFAP	glial fibrillary acidic protein
HPA	hypothalamic pituitary-adrenal
Iba-1	ionized calcium binding adaptor molecule 1
IFN-γ	interferon γ
IGF-1	insulin-like growth factor 1
IL	interleukin
KPBS	potassium phosphate-buffered saline
LPS	lipopolysaccharide
LTP	long term potentiation
MHC	major histocompatibility complex
ML	molecular layer
MWM	morris water maze
NGS	normal goat serum
NG2	neuron-glia 2
NMDA	N-metyl-D-aspartat
NPY	neuropeptide Y

PBS	phosphate-buffered saline
PVN	paraventricular nucleus
RECA-1	rat endothelial cell antigen 1
RUL	right unilateral
SGZ	subgranular zone
SON	supraoptic nucleus
SVZ	subventricular zone
TNF-α	tumor necrosis factor α
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling
VEGF	vascular endothelial growth factor
VMH	ventromedial hypothalamic nucleus

Summary

Electroconvulsive therapy (ECT) is one of the most efficient treatments for severe depressive disorders. However, its clinical practice has been limited by concerns about cognitive side effects, most notably anterograde and retrograde amnesia. The principle behind ECT is the induction of a short generalized grand mal seizure. This results in an intense neuronal activation and subsequently a myriad of alterations at biochemical, cellular and network levels. However, the mechanisms that underlie the therapeutic and adverse effects of ECT are not fully understood. In addition to regulation of monoamines, neuropeptides and endocrine hormones, enhanced brain plasticity (for example neurogenesis, angiogenesis, and synaptic reorganization), has been suggested to contribute to the therapeutic effects of ECT.

In the first part of my thesis work, we investigated whether electroconvulsive seizures (ECS), an animal model of ECT, induced cellular plasticity in the hypothalamus, a brain region involved in the regulation of basal functions (sleep, appetite, sex drive, stress response etc) that are often disturbed in depression. We found that ECS treatment leads to an induction of neuronal activation and endothelial cell proliferation in specific hypothalamic nuclei. The endothelial cell proliferation correlated with neuronal activity, both spatially and in magnitude, and was found to increase in nuclei that have been implicated in the neuroendocrine pathophysiology of depression. The increased endothelial cell proliferation might reflect an expansion of the vascular tree in order to meet the raised metabolic demand secondary to ECS-induced neuronal activation. The newly formed hypothalamic endothelial cells should however not just be seen as simple building blocks of blood vessels. Most likely they also play an important role in a multicellular cross talk, exchanging paracrine factors with neurons and glial cells. However, angiogenesis also occurs following insults, and it is possible that the increased endothelial cell proliferation seen in the hypothalamus is part of an inflammatory response to ECS.

A large number of investigations have failed to detect cellular damage in response to ECT, but under certain circumstances limited cell loss might occur. Cell damage is often accompanied by a glial cell response, where the degree of glial cell activation depends on the severity of insult. To elucidate this issue further we have, in the second paper of my thesis work, investigated the glial cell response to ECS within different limbic regions, and further compared two different stimulus parameter protocols. We found that ECS elicited a low-grade glial cell activation, irrespective of stimulus protocol. This was seen as morphological changes of microglia, astrocytes and NG2(+) cells, and increased expression of certain glial cell activation markers. The antigen-presenting molecule major histocompatibility complex II (MHC II) and the lysosomal protein ED1 were upregulated in microglia, preferentially in the hippocampus, while expression of nestin was induced in astrocytes, being most pronounced in the piriform and entorhinal cortex. No increased expression of MHC II or ED1 could be detected in the hypothalamus, the region where ECS induced a marked endothelial cell proliferation. We further (in paper III) demonstrated that ECS stimulates recruitment of peripheral CD163(+) macrophages to the hippocampus. The observed low-grade glial cell activation is compatibe with local clearance of dead cells or debris, but it is also possible that the activated glial cells exert other functions, such as facilitating ECS-induced neuronal plasticity.

In the final paper we tried to block the glial cell activation in order to investigate whether this affected memory function. We also measured the level of apoptotic cell death after ECS. It was clear that animals treated with ECS showed significant retrograde amnesia in Morris water maze (a test for spatial, hippocampus-dependent, memory), and that ECS induced a slightly increased apoptotic cell death in the hippocampus. Treatment with the neuroprotective and anti-inflammatory agent lithium abolished ECS-induced retrograde amnesia. We hypothesized that this might be associated with an attenuation of the ECS-induced glial cell activation and cell death in hippocampus, but found, on the contrary, that lithium treatment in combination with ECS slightly enhanced apoptotic cell death and activation of astrocytes, while the degree of microglia activation was unchanged.

All together, the results of my thesis suggest that ECS induces endothelial cell proliferation in the hypothalamus, apparently without concurrent glial cell activation in the same region. The low-grade glial cell activation in other limbic regions, most notably in the hippocampus, might reflect the minor increase of apoptotic cell death following ECS. However, that lithium could abolish retrograde amnesia concomitant to slightly increased cell death and astrocyte activation indicates that the amnestic effects of ECS are independent of these processes. The functional significance of endothelial cell

proliferation and glial cell activation in relation to the effects of ECS remains to be shown. Further studies of lithium's effect on ECS-induced memory disturbances could be of major interest as part of ongoing attempts of refining and optimizing ECT.

Svensk populärvetenskaplig sammanfattning

Depression är en av de stora folksjukdomarna och drabbar varannan kvinna och var fjärde man någon gång under deras livstid. Vid djupa depressioner, då självmordsrisken är hög eller då patienten hamnat i ett livshotande tillstånd på grund av oförmåga att äta och dricka används elektrokonvulsiv terapi (ECT), eller som det ofta kallas, ”elbehandling”. Ungefär 45 000 patienter behandlas med ECT varje år i Sverige, och i de flesta fall ger behandlingen god effekt ganska omgående. Nackdelen är att ECT kan ge biverkningar i form av minnesstörningar. Detta har, tillsammans med den negativa bild som media ofta ger av ECT, lett till att många betraktar ECT som en kontroversiell behandling.

Trots att ECT använts i över 70 år, vet man ännu inte exakt vilka mekanismer som ligger bakom den antidepressiva effekten respektive minnesstörningarna. Enligt nyare hypoteser kan den antidepressiva effekten bero på att ECT ökar nybildningen av nervceller, samtidigt som kopplingarna mellan nervcellerna blir fler. Man menar att denna tillväxt skulle kunna bidra till att motverka den volymminskning av vissa hjärnområden (bl.a. hippokampus) som ofta ses hos deprimerade patienter. Vår forskargrupp har tidigare visat att ECT-behandling, som i djurförsök benämns elektrokonvulsiv stimulering (ECS), ger upphov till blodkärlsnybildning i hippokampus. Detta skulle kunna vara direkt avgörande för tillväxten genom att ge en ökad blodförsörjning.

I min avhandling har jag visat att ECS dessutom kan ge upphov till nybildning av blodkärlsceller i hypotalamus, ett hjärnområde som styr basala funktioner, t ex sömnreglering, aptit och det hormonella svaret på stress. Dessa är funktioner som ofta är störda vid depression. Den ökade kärlnybildningen i hypotalamus kan vara ett svar på den samtidiga nervcellaktivering som sker där efter ECS. Det är också möjligt att kärlnybildningen utgör ett delfenomen i en inflammatorisk process kopplad till cellskada.

Det är helt klarlagt att ECT inte ger hjärnskador på samma vis som långvariga epileptiska anfall – men under vissa omständigheter skulle begränsad celldöd kunna förekomma. Vid cellskada aktiveras gliaceller som en del i en neuroinflammatorisk process. Aktiverade gliaceller kan även ha betydelse för reglering av nybildning och utmognad av nervceller.

I arbete II och III har jag undersökt gliacellsaktivering i hypotalamus och andra delar av det limbiska systemet (strukturer som är kopplade till känsloreglering). Vi fann aktiverade gliaceller i alla regioner förutom just hypotalamus, vilket skulle kunna tolkas som att nybildningen av blodkärlscellerna i hypotalamus inte är ett led i en neuroinflammatorisk process. I övriga limbiska områden var gliacellsaktiveringen så pass diskret att denna inte heller nödvändigtvis tyder på cellskada, utan istället kan vara ett svar på de tillväxtprocesser man ser efter ECS.

I det avslutande arbetet ville jag undersöka kopplingen mellan den observerade gliacellsaktiveringen och ECS-inducerade minnesstörningar. Vi behandlade djuren med litium, som har nervcellsskyddande och antiinflammatoriska egenskaper. Litiumbehandling motverkade helt de minnesstörningar som uppkommer efter ECS. Gliacellsaktiveringen och nivån av celldöd var däremot oförändrad, eller till och med något förhöjd, efter litiumbehandling. Detta tyder på att den minnesskyddande effekten av litium inte beror på minskning av celldöd eller inflammation utan måste förklaras av andra mekanismer. Fortsatta studier av litiums hämmande effekt på de ECS-inducerade minnesrubbningarna kan på sikt få stor betydelse för utvecklingen av en ECT-behandling med färre biverkningar.

INTRODUCTION

The pathophysiology of depression

Major depression is a mental disorder characterized by an all-encompassing low mood, accompanied by low self-esteem and loss of interest or pleasure in normally enjoyable activities. Additional symptoms include disturbed sleep and appetite, weight loss, feelings of guilt and worthlessness, lack of energy, decreased ability to think or concentrate and suicidal thoughts. According to WHO (2010) depression is among the leading causes of disability worldwide, affecting 121 million people per year.

The monoamine hypothesis of depression

One of the most well known biological hypotheses regarding the pathophysiology of depression is "the monoamine hypothesis", which states that disturbances of the monoaminergic neurotransmission cause the disease. Support for this theory stems from the observation that all major antidepressant medications share the common effect of modulating the levels of monoamines (serotonin, noradrenalin, and dopamine) in the brain. However, the monoamine hypothesis has its limitations and did for example in its original form not take into account the fact that antidepressant drugs typically need to be taken severeal weeks before clinical effect is observed. It has been proposed that the monoamine systems influence other neurobiological systems with a more primary role in depression (Heninger et al., 1996).

Neuroendocrine disturbances in depression

Disturbed appetite, sleep, and sexual interest are frequent findings in depression. These symptoms emanate from dysregulation of hypothalamic neuroendocrine systems (Holsboer, 2001). In particular, a dysregulated stress response is a well-acknowledged condition implicated in depressive disorders, first described in the 1950s (Board et al., 1956). The stress response is mediated by the hypothalamic pituitary-adrenal (HPA) system.

Neurons in the hypothalamic paraventricular nucleus (PVN) produce the corticotropin-releasing hormone (CRH), which is released into the portal blood system of the anterior pituitary gland. CRH induce release of adrenocorticotropic hormone (ACTH) from the pituitary gland, which subsequently stimulate release of the glucocorticoid stress hormone cortisol from the adrenal cortex. Arginine vasopressin (AVP) potentiates the effects of CRH and is coexpressed by the CRH-producing neurons (for review see Bao et al., 2008). Elevated levels of CRH in depressed patients tend to normalize after antidepressant treatment (Nemeroff et al., 1984; Nemeroff et al., 1991). Various other indications point to a causal and primary role for a hyperactive HPA axis in depression (Pariante, 2003; Bao et al., 2008). For example, increased numbers of CRH-producing neurons (Raadsheer et al., 1994), changes in CRH binding sites (Nemeroff et al., 1988), and pituitary and adrenal enlargements (Krishnan et al., 1991; Axelson et al., 1992; Nemeroff et al., 1992; Rubin et al., 1995) have been reported in depressed subjects. Furthermore, pharmacological treatment with glucocorticoids often induces depression. Some studies show that abnormalities in HPA-axis function already exist prior to the onset of the clinical symptoms, suggesting that such abnormalities not only correlate, but also precipitate depressive episodes (Holsboer, 2000).

The role of brain plasticity in depression

A more recent hypothesis suggests that depression partly could be explained by a failure of adult hippocampal neurogenesis (Duman et al., 2000; Jacobs et al., 2000; D'Sa and Duman, 2002; Jacobs, 2002; Kempermann, 2002). Neurogenesis is the generation and integration of new neurons, postnatally confined to two neurogenic zones in the brain – the subventricular zone (SVZ) lining the lateral ventricles and the subgranular zone (SGZ), part of the dentate gyrus of the hippocampus. There are basically two lines of evidence that give support to the neurogenesis hypothesis of depression. Firstly, all known antidepressant treatments stimulate the proliferation of hippocampal neurons in animal models (Madsen et al., 2000; Malberg et al., 2000; Scott et al., 2000; Chen et al., 2000; Chen et al., 2009). It has been proposed that the latency to the onset of an antidepressive effect could reflect the maturation period of a new neuron. The neurogenesis hypothesis gained ground when Santarelli et al., (2003) in a mouse model demonstrated that local irradiation of the hippocampus blocked both neurogenesis and the antidepressant effect induced by fluoxetine, an antidepressant medication. However, since irradiation affects several other cellular processes in addition to neurogenesis, the validity of this study has subsequently been questioned

(Henn and Vollmayer, 2004). Further support for the neurogenesis hypothesis of depression comes from morphometric and morphologic analyses of the hippocampus, which have revealed volume loss and gray matter alterations in depressed patients (Sheline, 2000). Such volume loss is thought to reflect a decrease in neurogenesis, and tends to be reversible upon remission of the disease (Frodl et al., 2002). It has further been suggested that volume loss and changes of the hippocampus are related to HPA dysfunction. The neurons of the hippocampus express high levels of glucocorticoid receptors (GR), which are activated during stress. Prolonged exposure of animals to a high level of corticosterone suppresses neurogenesis and presumably causes neuronal degeneration (Gold et al., 1988; Sapolsky 2000).

Although depressive psychopathology involves some hippocampal symptoms, major depression has not generally been considered a hippocampal disorder. It has lately been stated that decreased neurogenesis might predispose to depression without being causative (Perera et al., 2008). Kempermann and Kronenberg, (2003) propose that adult hippocampal neurogenesis is merely one aspect of a more general cellular plasticity, involving also glial cell proliferation and cellular plasticity in areas outside the hippocampus.

Angiogensis and depression

Angiogenesis – the growth of new blood vessels from preexisting vessels – has recently gained interest as being relevant for the pathophysiology of depression. It is well known that there is comorbidity between vascular disease and depression (Halaris, 2009), and it has been shown that angiogenesis is a crucial part of neuronal plasticity (Black et al., 1989). Generation and maturation of new neurons, expansion of dendritic trees and remodeling of synapses, are all energy demanding processes that require supplies of nutrients and oxygen from the blood. When the metabolic demand exceeds the availability, angiogenesis is triggered in order to meet raised metabolic demands. Furthermore, adult neurogenesis has been described as occurring within an “angiogenic niche”, where neuronal and glial precursors divide in close proximity to proliferating endothelial cells (Palmer et al., 2000). Growth factors such as BDNF, fibroblast growth factor-2 (FGF-2), neuropeptide Y (NPY) and vascular endothelial growth factor (VEGF) are known to stimulate neurogenesis as well as angiogenesis (Ghosh and Greenberg 1995; Slavin, 1995; Zigova et al., 1998; Zukowska-Grojec et al., 1998; Pencea et al., 2001; Jin et al., 2002; Kim et al., 2004; Howell et al., 2005), indicating a possible coregulation. Moreover, antidepressant treatments have been shown to induce angiogenic factors (Newton et al., 2003; Greene et al., 2009) and proliferation of endothelial cells in the hippocampus (Hellsten et

al., 2004; Perera et al., 2007) and prefrontal cortex (Kodama et al., 2004; Madsen et al., 2005; Czéh et al., 2007).

Brain regions implicated in depression

Several limbic regions, important for emotional regulation, executive functioning, and memory, are implicated in the pathophysiology of depression and targets for antidepressant treatment (Berton and Nestler, 2006; Krishnan and Nestler, 2008). Postmortem and neuroimaging studies of depressed patients have revealed reductions in volume and glial cell density in the prefrontal cortex and the hippocampus (Drevets, 2001; Sheline, 2003). The prefrontal cortex is important for executive functions, while the hippocampus, together with the entorhinal cortex, is involved in the processes of learning and memory storage. Neuronal activity within the amygdala and subgenual cingulate cortex (a subregion within the prefrontal cortex) is increased in healthy subjects experiencing transient sadness and chronically increased in depressed individuals, but is normalized after successful treatment (Drevets, 2001; Ressler and Mayberg, 2007). Basal functions such as sleep, appetite, sexual drive, and stress response are regulated from the hypothalamus and amygdala, and are often disturbed in depression. However, it should be noted that the limbic regions are highly interconnected and although evidence points to involvement of certain regions in the etiology of depression, it is likely to be a simplistic view to localize a function to one anatomical area.

Current treatments of depressive disorders

Three major types of antidepressant treatment are used in psychiatry: psychotherapy, treatment with antidepressant drugs, and electroconvulsive therapy (ECT). Psychotherapy is primarily used in milder cases of depression. The first choice of treatment for moderate and severe types of depression is usually antidepressant drugs. There are several different classes of antidepressant drugs, but the majority of these have in common an effect on serotonergic or noradrenergic neurotransmission, or both (Kalat, 2001). Many of the antidepressant drugs are efficacious in only 60%-70% of patients (APA 2000). The main indications for ECT are severe major depression with psychotic features, if the person is not eating and drinking or if there is a high risk of suicide, but ECT is also effective in manic and catatonic states. With correct indication, ECT is effective in 80%-90% of all cases (APA, 2000). A major advantage of ECT is that the clinical response is faster compared with other currently available antidepressant treatments. Antidepressant drugs

normally alleviate the symptoms after approximately three to five weeks, while patients treated with ECT can experience symptom relief after one to two weeks, and sometimes already after a single ECT session.

ECT

The principle behind ECT is the induction of a short generalized epileptic seizure via electrical stimulation. ECT has been used clinically since the 1930s, but the technique of administering ECT has been refined considerably over the years. In the mid-1950s, muscle relaxation and anesthesia along with hyperoxygenation were introduced as routine measures for ECT. Further modifications in order to minimize the side effects of ECT were unilateral electrode placement and brief-pulse stimulation (for review see Fink, 1999). Despite these improvements, the use of ECT began to decline in the mid-1950s and continued to do so for many years (Babigian and Guttmacher, 1984; Shorter and Healey, 2007), mainly because of the steady growth of antidepressant drugs along with the negative descriptions of ECT in the mass media. When the screen version of Ken Kesey's novel "One Flew Over the Cuckoo's Nest" was released 1975 and depicted ECT as a tool of terror, used by the staff at a psychiatric ward to control insubordinate patients, it undoubtedly left the public with an unfavorable view of ECT. Along with many other presentations of ECT in the media as a cruel and inhumane treatment, ECT soon fell into disrepute. The use of ECT further declined until the 1980s, when there was a growing awareness of its benefits for treating severe depression. The interest in ECT was revived, and the use of the treatment has continued to grow since then. Richard Abrams, author of "Electroconvulsive Therapy" – considered to be the standard textbook of the field - estimates that 1-2 million people receive ECT annually worldwide (Abrams, 2002). In Sweden, around 45 000 patients are treated with ECT each year, which is twice the number 10 years ago.

The mortality rate is low, 2-10 per 100 000 treatments, comparable to that of anesthesia for minor surgery (Shiwach et al., 2001). However, cognitive side effects, most notably retrograde and anterograde amnesia, are sometimes seen in patients treated with ECT. The exact mechanisms behind ECT's therapeutic and adverse effects are only partially understood.

Theories of ECT's therapeutic mechanisms

ECT has a wide range of effects, many involving cellular and molecular alterations that antagonize or normalize the neurobiological disturbances seen in depression. The antidepressant actions of ECT have been suggested to include regulation of the monoamine neurotransmitter systems and the neuroendocrine systems. The anticonvulsant response to ECT and the enhanced cellular plasticity have also been proposed to underlie the therapeutic effect of ECT (reviewed in Mann, 1998 and Merkl et al., 2009).

Actions on monoamine transmitter systems, neuroendocrine effects and the anticonvulsant hypothesis

Plausible therapeutic effects of ECT via the monoamine neurotransmitter systems include sensitization of serotonergic and dopaminergic receptors, as well as a decreased release of noradrenalin and a normalization of the noradrenergic receptor sensitivity (Ottosson, 1983). A number of neuropeptides and hormones that are regulated from the hypothalamus, such as prolactin, NPY, AVP, oxytocin, and CRH, have also been implicated in the antidepressant actions of ECT (Whalley et al., 1987; Herman et al., 1989; Stenfors et al., 1989; Smith et al., 1994; Scott and Dinan, 1998), although their relation to clinical improvements is inconsistent (Smith et al., 1994; Devanand et al., 1998). Most notably is perhaps the ECT-induced restoration of HPA-axis abnormalities, which actually have been correlated to clinical improvements (Kunugi et al., 2006).

The "anticonvulsant hypothesis" suggests that ECT exerts its therapeutic effects through an increase in inhibitory gamma-aminobutyric-acid (GABA) neurotransmission (Sackeim et al., 1983; Sanacora et al., 2003). Relationships between clinical improvements and sustained postictal suppression – a suppression of electrical brain activity immediately following ECT – further reinforce this hypothesis (Nobler et al., 1993; Krystal et al., 1995; Suppes et al., 1996; Azuma et al., 2007).

The neuroplasticity hypothesis

ECT stands out among antidepressant treatments by having a particularly strong effect on neuroplasticity and neuroprotection (Kondratyev et al., 2001; Taylor, 2008). Both acute and chronic electroconvulsive seizures (ECS), an animal model of ECT, enhance hippocampal neurogenesis in the range of two- to threefold (Madsen et al., 2000; Malberg et al., 2000; Scott et al., 2000). Chronic, but not acute, treatment with antidepressive drugs increases neurogenesis by approximately 20%-25% (Chen et al., 2000; Malberg et al., 2000). There is also enhanced gliogenesis in the hippocampus (Wennström et

al., 2003), amygdala (Wennström et al., 2004), and prefrontal cortex (Madsen et al., 2005) as well as increased hippocampal endothelial cell proliferation (Hellsten et al., 2004) leading to an increased vascular density (Hellsten et al., 2005) in response to ECS. Furthermore, ECS has been shown to increase the total number of synapses (Chen et al., 2009) and to stimulate axonal sprouting (Vaidya et al., 1999; Lamont et al., 2001) in the hippocampus. All these actions may derive from changes in the availability of neurotrophic factors, including upregulation of growth factors such as BDNF, VEGF, NGF, and FGF-2 (Nibuya et al., 1995; Gwinn et al., 2002; Newton et al., 2003; Balu et al., 2008; Conti et al., 2009) and downregulation of proapoptotic agents (Kondratyev et al., 2001; Zarubenko et al., 2005). It is intriguing to speculate that these ECS-induced neuroplastic processes might alter the neural microenvironment in such ways that cellular atrophy and dysfunctional neuronal circuits in depressed patients are revoked. In line with the view of ECT as a growth-promoting treatment, a recent clinical study showed increased hippocampal volumes in patients treated with ECT (Nordanskog et al., 2010). Neuroprotective effects of ECS has further been demonstrated, as pretreatment with ECS protects against subsequent neurodegeneration induced in rodent models of adrenalectomy (Masco et al., 1999), epilepsy (Kondratyev et al., 2001), forebrain ischemia (Mishima et al., 2005) and Parkinson's disease (Anastasia et al., 2007).

Adverse effects of ECT

The clinical use of ECT has been limited by concerns about side effects, such as retrograde and anterograde amnesia (recall of memories and learning, respectively). Immediately after ECT, most patients experience retrograde amnesia involving events shortly before the treatment, but improvements are usually seen within a few months. However, significant loss of recall, i.e. retrograde amnesia that lasts for more than 4 weeks or is even persistent, has been reported (Lisanby et al., 2000; Sackeim et al., 2000; Sackeim et al., 2007). Anterograde amnesia, on the other hand, has not been reported to persist for more than 4 weeks after the ECT treatment according to a review by Nobler and Sackeim, (2008). Memory disturbances following ECT are usually more likely to affect the elderly and patients already experiencing cognitive deficits (Mulsant et al., 1991; Donahue, 2000).

There have been many attempts to minimize adverse effects without losing the efficacy of ECT. Manipulations of electrode placements, seizure threshold, and electrical pulse width during ECT have been tried in order to reduce the cognitive side effects (Loo et al., 2006). Originally, ECT was administered bilaterally (with electrodes placed on each side of the skull), but a right

unilateral (RUL) electrode placement (stimulation of the right hemisphere of the brain) was later introduced. RUL ECT at high, but not low dosage has been shown to be as effective as bilateral ECT at a standard dosage, while causing less cognitive side effects (Sackeim et al., 2000; Stoppe et al., 2006). Furthermore, RUL ECT with an ultra brief pulse width (0.3 ms) compared with standard pulse width (1 ms) further diminished memory impairments, particularly regarding the retention of verbal and visual information as well as retrograde autobiographical memory (Loo et al., 2008). Although RUL ECT with an ultra brief pulse width seems to be associated with less side effects, studies reporting reduced efficacy and slower treatment response compared with bilateral ECT suggest a need for further investigation (McCormick et al., 2009; Ottosson and Odeberg, 2011).

Potential mechanisms underlying the cognitive side effects of ECT

The molecular mechanisms behind ECT-induced memory disturbances are poorly understood. Persuasive evidence for the involvement of glutamatergic, cholinergic, glucocorticoid, and cyclooxygenase (COX) related mechanisms in ECT-induced amnesia have been presented (for review see Pigot et al., 2008). The hippocampus plays a major role in memory consolidation and spatial navigation, and the ability to form and retain memories is dependent on the integrity of the hippocampus (Squire et al., 2004).

Long-term potentiation (LTP) is a glutamate/Ca^{2+} dependent mechanism, widely accepted as the primary process underlying learning and memory (Bliss and Collingridge, 1993). During seizures, there is a substantial increase in intracellular Ca^{2+} through the glutamate-induced activation of NMDA receptors (Dubovsky et al., 2001), likely to cause an indiscriminate LTP induction resulting in saturation of the system. Thus, the capacity of further induction of LTP is exhausted and the process of memory recall and learning is thus impaired (Stewart and Reid, 1994). In support of this theory, the NMDA receptor antagonist ketamine has been shown to reduce ECT-induced amnesia (McDaniel et al., 2006), and the majority of calcium channel blockers preserve memory retention in rat models of ECS (Zupan et al., 1996; Kamath et al., 1997; Sushma et al., 2004). However, clinical trials of calcium channel blockers have not been able to show reduced memory-related side effcts of ECT (Cohen and Swartz, 1990; Dubovsky et al., 2001).

Acetylcholine, cortisol, and COX-2 are all involved in the processes of memory and learning, and the increased levels following ECT have been suggested to impair memory mechanisms (Lerer et al., 1984; Florkowski et al., 1996;

Lipsky, 1999; Newton et al., 2003; for review see Pigot et al., 2008). In support, coadministration of inhibitors of these factors was shown to prevent or attenuate amnesia following ECS (Levin et al., 1987; Rao et al., 2002; Prakash et al., 2006; Nagaraja et al., 2007; Andrade et al., 2008a; Matthews et al., 2008). Further research to improve our understanding of the mechanisms involved in the cognitive adverse effects of ECT, or finding new treatments with the potential to attenuate these, could be of great significance for future clinical use of ECT.

Cellular damage following ECS?

The cognitive deficits have raised concerns to whether ECT induces neuronal damage in the hippocampus. Hippocampal neurons are particularly vulnerable to seizures, i.e. more readily excited and thus more sensitive to excitotoxicity. A few studies have shown that ECS leads to loss of neurons in the hippocampus as well as the entorhinal cortex (Lukoyanov et al., 2004; Zarubenko et al., 2005; Cardoso et al., 2008) but in contradiction, it has repeatedly been stated that ECT/ECS does not cause any harm to neurons (Coffey et al., 1991; Devanand et al., 1994; Dalby et al., 1996; Ende et al., 2000; Dwork et al., 2004; Dwork et al., 2009). The opinion of most experts is that animal studies, at worst, show minimal damage, and that under conditions approximating modern clinical ECT, brain damage is unlikely.

Enhanced plasticity and glial cell activation might indicate cellular damage

Although many studies speaks against cellular damage following ECT/ECS, the response to ECS seems to have some similarities to a reaction to brain insult. Firstly, increased cellular plasticity such as neurogenesis and angiogenesis – observed after ECS – is often seen in association with regrowth and repair following brain injury. Furthermore, cellular damage is accompanied by glial cell activation. Previous studies have reported glial cell activativation following ECS, seen as increased glial cell proliferation (Wennström et al., 2003; Wennström et al., 2004; Madsen et al., 2005), upregulation of GFAP in astrocytes (Dwork et al., 2004) as well as morphological alterations of microglia in the hippocampus (Jinno and Kosaka, 2008).

Glial cell activation is also referred to as neuroinflammation, a term often associated with the more chronic, detrimental process of glial cell activation. However, neuroinflammation in its correct meaning refers to a restorative

process unless it becomes chronic (Graeber and Streit, 2010). Neuroinflammation is driven by the activation of resident microglia, astrocytes and infiltrating peripheral macrophages, which release a plethora of anti- and proinflammatory cytokines, chemokines, neurotransmitters, neurotrophic factors and reactive oxygen species. It seems to be commonly acknowledged that the profile of the inflammatory reaction is context-dependent and that microglia, as the key regulators of the neuroinflammatory response, adapt specifically to the given microenvironmental changes (Hanisch and Kettenmann, 2007; Ekdahl et al., 2009; Streit and Xue, 2009). Microglia are very responsive and react to virtually any change in the microenvironment, and depending on the severity of the insult they aquire various intermediate states of activation. There is also growing evidence for the involvement of microglia and the immune system in the processes of neurogenesis and synaptic plasticity (Schwartz, 2001; Ziv and Schwartz, 2008; Ekdahl et al., 2009; Whitney et al., 2009; Ben Achour and Pascual 2010; Graeber and Streit 2010).

AIMS OF THE THESIS

To investigate whether ECS induces endothelial cell proliferation in the hypothalamus, and its relation to neuronal activation.

To determine if ECS causes glial cell activation and whether this is dependent on different ECS stimulus parameters.

To investigate whether blood-borne immune cells are recruited to the hippocampus in response to ECS.

To investigate whether celecoxib and lithium have the potential to attenuate the memory disturbances seen after ECS and investigate whether this is correlated to hippocampal cell death and glial cell activation.

MATERIALS AND METHODS

Animals

Adult Wistar (papers I and II) and Sprague–Dawley (papers III and IV) male rats were used in the studies. The rats were housed three per cage, had free access to food and water and were kept on a 12 h light–dark cycle during all experiments. Experimental procedures were conducted according to the guidelines set by the Malmö/Lund ethical committee for the use and care of laboratory animals.

Treatments

Induction of ECS

Rats were given ECS once daily for one (paper I), five (papers I and IV) or ten (papers I, II and III) consecutive days. Electrical current was delivered through silver electrode 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 paper III, we also used a different set of stimulus parameters (current 100 mA, frequency 70 Hz, pulse width 0.5 ms, and pulse train duration 1 s). In paper I, the rats were monitored after the ECS treatment to ensure that clonic movements of the face and forelimbs, which are indicative of limbic seizures, occurred for a minimum of 20 s. In paper II, we introduced a standardized way of estimating the duration of tonic seizures. Tonic seizure length was then 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. We found this method straightforward and reproducible, and continued to use it throughout the studies. All rats given ECS were monitored during their wake-up phase and continuously, to ensure their well-being after the seizure. Control rats were sham treated, i.e. handled identically to the ECS rats, but without delivery of electrical current.

Bromodeoxyuridine (BrdU) administration

BrdU (B5002; Sigma Aldrich) is a thymidine analogue that is incorporated into dividing cells during the S-phase and thus serves as a marker for proliferating cells. BrdU was dissolved in phosphate buffered saline (PBS) (20 mg/mL) and administered by intraperitoneal injections (50 mg/kg) at 12 h intervals for 5 days (paper I).

Celecoxib administration (unpublished result)

Celecoxib, a selective COX-2 inhibitor, was administered through chow containing 187.5 mg or 625 mg celecoxib/kg (produced by Lantmännen). This dose has been calculated to correspond to a daily dose of about 15 mg or 50 mg/kg rat. The lower dose has been used by others to attenuate ECS-induced retrograde amnesia (Andrade et al., 2008a). Administration of celecoxib was started 10 days prior to the ECS course and was continued throughout the study.

Chronic lithium treatment

Lithium (paper IV) was administered through chow containing 2 g lithium chloride/kg (produced by Lantmännen). This lithium dose has previously been used in our lab and shown to give lithium serum concentrations within the recommended therapeutic range for humans (Orre et al., 2009). Rats had free access to chow, water, and saline throughout the experiments. To reach a "steady state" of lithium levels, the lithium administration started 10 days prior to the ECS treatment.

Behavioral tests

Morris water maze (MWM) navigation task for study of retrograde and anterograde memory

In study IV, we used the hippocampus-dependent MWM test to assess spatial retrograde and anterograde memory (the recall of acquired memories and ability to learn, respectively) (Morris 1984). The maze consisted of a 45 cm deep, circular tank (180 cm in diameter) filled with water (20 ± 1°C) to a depth of 30 cm and was divided into four equal-sized quadrants with imaginary lines. The water was made opaque by addition of nontoxic white paint, and a platform (15 cm in diameter) was submerged 1.5 cm at a fixed position in the center of one of the quadrants. Visual extramaze cues,

consisting of large black-and-white abstract figures on the walls, were kept constant during the experiment. To familiarize the animals with the maze, we conducted a pretrial 1 day before the first day of training during which each rat swam for 60 s while no extramaze cues were present.

During the learning trials (four trials per day for eight consecutive days), the rats were trained to find the hidden platform by navigation using the extramaze cues. On each trial, the rat was placed in the water facing the pool wall at one of the four fixed starting points. The order of starting points was randomly varied. Rats finding and climbing onto the platform had to remain on it for 20 s. If rats failed to find the escape platform within 90 s, the experimenter guided them. The rats were allowed to rest for 20 s between the four daily trials. The swim path was recorded using a computerized video-tracking system (Ethovision 3.1, Noldus, The Netherlands). Escape latency (i.e. time required to reach the platform) and distance swum were evaluated. Data from the four daily trials were pooled to provide averaged data per rat and day.

To test whether ECS influences retrograde memory (recollection of acquired memories), the learning trials were conducted before ECS-treatment. At 24 h after the last ECS treatment, a probe trial (90 s) was performed. To test anterograde memory (learning ability), the learning trials were conducted after the course of ECS, and analysed for differences between groups. As an additional test of anterograde memory, we conducted a probe trial 24 h after the last anterograde learning trial. Before the probe trials, the platform was removed and time spent in the quadrant of the former platform position (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.

Memory of the task

In addition to spatial navigation, the MWM test also included learning about the task, i.e. 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 – this natural proclivity to stay near the perimeters of a novel environment is known as thigmotaxis. However, they soon learn to swim to the inner part of the pool. In the retrograde memory study, thigmotaxis – i.e. time spent in the area 0–15 cm from the sidewall – was analyzed for the ECS group during the first learning trial and compared with the probe trial (paper IV).

Histological procedures

Tissue preparation

Animals were anaesthetized with sodium pentobarbital and ensured to be without any nociceptive responses before they were transcardially perfused with 0.9% saline (100 mL), followed by cold 4% paraformaldehyde (200 mL). Brains were postfixed in 4% paraformaldehyde overnight (papers I and IV) and for 3 or 4 hours (papers II and III, respectively) at 4°C and then cryoprotected in sucrose in PBS. Coronal sections of the brains (40 μm in papers I and IV or 30 μm in papers II and III) were cut on a freezing microtome and stored in an antifreeze cryoprotectant solution at –20°C until the immunohistochemical procedure.

Immunofluorescence techniques

Immunofluorescence labeling of brain sections was performed in papers I–III. Specific antibody data is presented in Table 1, but the basic protocol of all immunofluorescence staining procedures is as follows. Brain sections were rinsed (3 x 10 min) in 0.02 mol/L potassium phosphate buffered saline (KPBS) and then incubated in blocking solution (KPBS + 5% normal donkey serum (Harlan Sera-Lab) or 5% normal goat serum (NGS; Harlan Sera-Lab) + 0.25% Triton X-100 (Sigma Aldrich)) for 30–60 min at room temperature. Sections were then incubated with primary antibodies for 24–72 h at 4°C. Sections were rinsed with KPBS and subsequently incubated with secondary antibodies for 24–48 h at 4°C. Sections were rinsed in KPBS and when tertiary antibodies were applied, incubated with Alexa 488 (1:250, Molecular Probes) for 24 h at 4°C. After washing with KPBS, sections were mounted on Super Frost slides, air dried, and coverslipped with glycerol-based mounting medium.

The staining of RECA-1/BrdU was performed sequentially, with labeling of RECA-1 first. Before incubation with anti-BrdU, the sections were fixed in 4% paraformaldehyde for 15 min at room temperature, rinsed in KPBS, incubated in 1 mol/L hydrochloric acid at 65°C for 30 min, rinsed in KPBS, and exposed to blocking solution for 1 h at room temperature.

Table 1. Double immunofluorescense staining protocol	
Primary antibodies (concentration; incubation time; manufacturer)	**Secondary antibodies** (concentration; incubation time; manufacturer)
Immunofluorescence labeling	
Mouse anti-Rat Endothelial Cell Antigen 1 (RECA-1) (1:100; 48-72 h; Serotec)	**biotinylated donkey anti-mouse** (1:200; 2-24 h; Jackson Immunoresearch)
Rat anti-Bromodeoxy Uridine (BrdU) (1:100; 40 h; Oxford Biotechnology)	**Cy3-conjugated donkey anti-rat** (1:200; 24 h; Jackson Immunoresearch)
Rabbit anti-c-Fos (1:2000; 72 h; Sigma Aldrich)	**Cy3-conjugated goat anti-rabbit** (1:200; 2 h; Jackson Immunoresearch)
Mouse anti-NeuN (1:100; 72 h; Chemicon)	**biotinylated donkey anti-mouse** (1:200; 2 h; Jackson Immunoresearch)
Rabbit anti-Iba-1 (1:1000; 24 h; Wako)	**Alexa Fluor 488-conjugated goat anti-rabbit** (1:1000; 24 h; Invitrogen, Molecular Probes)
Mouse anti-Major Histocompatibility Complex II (MHC II) (1:500; 24 h; AbD Serotec)	**Cy3-conjugated goat anti-mouse** (1:800; 24 h; Jackson Immunoresearch)
Mouse anti-ED1 (1:200; 24 h; Serotec)	**Cy3-conjugated goat anti-mouse** (1:800; 24 h; Jackson Immunoresearch)
Rabbit anti-Glial Fibrillary Acidic Protein (GFAP) (1:2000; 24 h; Dakocytomation)	**Alexa Fluor 488-conjugated goat anti-rabbit** (1:1000; 24 h; Invitrogen, Molecular Probes)
Mouse anti-nestin (1:1000; 24 h; BD Bioscience Pharmingen)	**Cy3-conjugated goat anti-mouse** (1:800; 24 h; Jackson Immunoresearch)
Rabbit anti-Laminin (1:1000; 24 h; Sigma-Aldrich)	**Alexa Fluor 488-conjugated goat anti-rabbit** (1:1000; 24 h; Invitrogen, Molecular Probes)
Mouse anti-CD163 (1:500; 24 h; AbD Serotec)	**Cy3-conjugated goat anti-mouse** (1:800; 24 h; Jackson Immunoresearch)
Mouse anti-CD4 (1:500; 24 h; AbD Serotec)	**Cy3-conjugated goat anti-mouse** (1:800; 24 h; Jackson Immunoresearch)
Immunoperoxidase labeling	
Rabbit anti-c-Fos (1:2000; 40 h; Sigma Aldrich)	**biotinylated goat anti-rabbit** (1:500; 2 h; Vector Laboratories)
Rabbit anti-Iba-1 (1:1000; 24h; Wako)	**biotinylated goat anti-rabbit** (1:1000; 24 h; Vector Laboratories)
Rabbit anti-Glial Fibrillary Acidic Protein (GFAP) (1:2000; 24 h; Dakocytomation)	**biotinylated goat anti-rabbit** (1:1000; 24 h; Vector Laboratories)
Rabbit anti-NG2 (1:1000; 24 h; Chemicon)	**biotinylated goat anti-rabbit** (1:1000; 24 h; Vector Laboratories)

Immunoperoxidase staining

Immunoperoxidase antibody labeling was conducted for c-Fos in paper I and for Iba-1, GFAP, and NG2 in paper II. Specific antibody data is presented in Table 1, but the basic protocol of the immunoperoxidase staining is described below.

Sections were rinsed (3 x 10 min) in KPBS followed by quenching in 3% H_2O_2 and 10% methanol. After rinsing, the sections were incubated in blocking solution (KPBS, 0.25% Triton X-100, and 5% NGS (Chemicon)) for 1 h at room temperature, followed by incubation with primary antibodies at 4°C. Sections were rinsed with KPBS and incubated with biotinylated secondary antibodies at 4°C. Sections were then rinsed in KPBS before incubation with an avidin-biotin-peroxidase complex (Vectastain Elite ABC Kit, Vector Laboratories Inc.) in KPBS for 1 h at room temperature. After rinsing, nickel-enhanced diaminobenzidine (DAB-Ni) was applied for antibody-complex detection, followed by rinsing in KPBS. Sections were mounted on Super Frost slides, dehydrated, and coverslipped with xylene-based mounting medium.

In paper IV, apoptotic cells were visualized through terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL). A TUNEL-POD kit (Roche Diagnostics) was used and the staining was performed according to the manufacturer's manual for fixed tissue. Sections were pretreated with proteinase K (Roche Diagnostics) for 12 min and DAB was applied for peroxidase detection.

Microscopical analysis

Characterization and quantification of cells using epifluorescence microscopy

An Olympus AX70 fluorescence microscope (Olympus Optical) with a 40x objective was used for all analyses of immunofluorescence staining. Stained tissue sections from different treatment groups were randomly mixed and coded; thus, the person analyzing the sections was blinded to the treatment group.

To assess hypothalamic cell proliferation in response to ECS (paper I), BrdU-labeled cells in the mid-hypothalamus were quantified in five major nuclei; PVN, SON, ventromedial hypothalamic nucleus (VMH), arcuate nucleus (ArcN), and dorsomedial nucleus (DMH). The PVN was subdivided into the parvocellular and magnocellular areas, identified by their characteristic

neuronal cell size. The total number of BrdU-labeled cells as well as the number of proliferating endothelial cells (BrdU/RECA-1-labeled cells) were determined in each area.

In paper II, glial cells double-positive for MHC II/Iba-1, ED1/Iba-1 and nestin/GFAP, respectively, were quantified in the prefrontal cortex, hippocampus, amygdala, hypothalamus, piriform, and entorhinal cortex. In paper III, the number of cells double-positive for laminin and CD163, CD4, or MHC II, respectively, were determined in five different subregions of the mid-dorsal hippocampus; granular cell layer (GCL), hilus, molecular layer (ML), CA1, and CA2/CA3. For all cell quantifications, we used "modified unbiased stereology" (Malberg et al., 2000), thus, we identified all regions by anatomically landmarks (according to Paxinos and Watson 1986) and analyzed sections with the same sampling frequency. All cell numbers were averaged and expressed as cell numbers per section.

Characterization and quantification of cells using brightfield microscopy

The number of c-Fos(+) cells in paper I was determined through computer-assisted image analysis (ImageJ version 1.34s, NIH Image, NIH, Bethesda, MD, USA). Three sections per animal and hypothalamic nuclei were photographed at 20x with an Olympus AX70 brightfield microscope (Olympus Optical). The sections were carefully selected to represent the same position relative to bregma for each animal and nuclei. The image analysis program was set to count the number of c-Fos(+) cells in each mid-hypothalamic nucleus.

In paper II, the morphology of cells labeled with Iba-1, GFAP and NG2 respectively, was analyzed in different limbic regions (prefrontal cortex, hippocampus, amygdala, hypothalamus, piriform, and entorhinal cortex) with an Olympus AX70 brightfield microscope. The sections were coded; thus, the person evaluating cell morphology was blinded to the treatment group.

TUNEL-stained apoptotic cells (paper IV) were counted in the GCL, hilus, ML, CA1, and CA2-3 regions of the hippocampus using an Olympus AX70 light microscope with a 40x objective. The same "modified unbiased stereology" method as described above was used. All counts were pooled together for each rat and region and reported as the mean cell number per section.

Statistical analysis

Data are presented as means ± standard error of the mean for papers I, III, and IV. Differences in endothelial cell proliferation and c-Fos expression were

analyzed using Student's t–test (paper I), while one-way analysis of variance (one-way ANOVA) followed by Fisher's PLSD post hoc test was used in paper III. Two-way ANOVA was used to test the effect of lithium- and ECS-treatments on the number of TUNEL-positive cells and performance in the MWM probe trial in paper IV. Repeated-measures ANOVA was used to assess differences in seizure length (papers II and IV) as well as escape latency during MWM learning trials (paper IV). Because of the unequal variances between groups, differences in cell numbers in paper II were analyzed using the nonparametric Kruskal-Wallis test followed by Dunn's post hoc test for multiple pairwise comparisons. These values are presented as boxplots revealing the sample minimum, the 25th percentile, the median, the 75th percentile, and the sample maximum of all groups.

Statistical significance was set at $p \leq 0.05$ for all studies. Kruskal-Wallis and Dunn's tests (paper II) were performed using Matlab software. All other statistical analyses were performed with Statview software, version 5.0 (Abacus Concepts, Berkeley, CA, USA).

RESULTS AND COMMENTS

ECS-induced endothelial cell proliferation is region-specific and correlates with neuronal activation in the hypothalamus (paper I)

Our group had previously shown that ECS enhances endothelial cell proliferation in the hippocampus concomitant with an increase in neurogenesis (Hellsten et al., 2004). Angiogenesis have also been demonstrated in other brain areas following an intense neuronal activity (Black et al., 1987; Black et al., 1990) and ECS has been shown to induce neuronal activation in several specific limbic nuclei (Samoriski et al., 1997). This has raised the question as to whether ECS–induced endothelial cell proliferation occurs in brain regions outside the hippocampus, and if such an angiogenic response could be correlated to neuronal activation. The important role of the hypothalamus in the pathophysiology of depression led us to investigate endothelial cell proliferation and its possible relationship to neuronal activation following ECS in the major mid-hypothalamic subnuclei (PVN, SON, VMH, ArcN, DMH).

To analyze the pattern of neuronal activation after ECS, rats were given one ECS treatment daily for 1, 5, or 10 days and were killed 2 h after the last ECS treatment. Neuronal activation was analyzed by measuring the expression of the immediate early gene c-Fos. To assess cell proliferation, rats were given BrdU (which labels dividing cells) during a course of five ECS treatments. We detected a basal proliferation of endothelial cells in control animals in the PVN, SON and to a lower extent in the VMH. In rats treated with ECS, the proliferation was increased two-to threefold in the PVN and SON, and more than 20-fold in the VMH, but no significant increase was seen in the ArcN or DMH. The fraction of dividing endothelial cells constituted approximately 75% of all proliferating cells in the PVN and SON in both control- and ECS-treated animals. In the VMH of control rats, only 10% of the dividing cells were endothelial cells, but this fraction increased to nearly 60% after ECS treatment. ECS treatment gave rise to increased numbers of activated neurons in all hypothalamic nuclei investigated, but the strongest increase was seen in the VMH (20-fold), followed by the PVN and SON (sevenfold) and

then the ArcN and DMH (two-to threefold). We also confirmed that the c-Fos expressing cells were indeed neurons and not endothelial or glial cells.

To summarize, there seems to be a convincing correlation between the hypothalamic endothelial cell proliferation and neuronal activation in response to ECS, both spatially and in regard to the magnitude of proliferation and activation.

ECS gave rise to a low-grade glial cell activation in several limbic regions of the brain, but this was not dependent on the stimulus parameters (paper II)

The cognitive side effects of ECT have raised concern as to whether ECT can cause cellular damage in vulnerable brain regions. Cellular damage is followed by an inflammatory reaction, seen as an activation of glial cells that proliferate, go through morphological alterations, and aquire novel skills in response to insult (Raivich et al., 1999; Butt et al., 2002; Hampton et al., 2004). The angiogenic response is another important aspect of the inflammatory process, and inflammatory mediators have been shown to directly or indirectly promote angiogenesis (Jackson et al., 1997). Previous work by our group, which had shown that ECS increases endothelial cell proliferation in the hippocampus (Hellsten et al., 2004) and hypothalamus (paper I), as well as gliogenesis in the hippocampus (Wennström et al., 2003) and amygdala (Wennström et al., 2004), prompted us to further characterize glial cell activation in response to ECS in the limbic system (prefrontal cortex, hippocampus, amygdala, hypothalamus, piriform cortex, and entorhinal cortex). Could the gliogenesis and angiogenesis seen following ECS be part of an inflammatory reaction? Studies have lately shown that ECT with shorter electrical pulse width gives rise to less cognitive side effects (Sackeim et al., 2008). We hypothesized that less adverse effects might be reflected in the intensity of glial cell activation. Thus, in addition we wanted to investigate whether a shorter pulse width could attenuate any glial cell activation.

Rats were given a course of 10 ECS-treatments, with two different sets of stimulus parameters, and were killed either 2 h or 4 weeks after the last ECS treatment. Glial cell activation was evaluated by morphological alterations of microglia, astrocytes, and NG2(+) cells, and immunoreactivity for the widely used inflammatory markers MHC II and ED1 in microglial cells and for nestin in astrocytes.

We observed morphological changes of microglia, astrocytes and NG2(+) cells at 2 h following ECS, with the most pronounced alterations seen in the hippocampus and piriform cortex. Microglia underwent the most marked morphological changes, while the alterations of the NG2(+) cells were very subtle. Four weeks later, glial cell morphology in ECS-treated rats could not be distinguished from that of control animals.

No or single scattered MHC II(+) microglia were found in the control animals, but 2 h following ECS, there were elevated numbers of MHC(+) cells in all investigated regions except the hypothalamus. The highest density of MHC(+) microglia was found in the SGZ, hilus, and CA1 and CA3 regions of the hippocampus. After 4 weeks, the levels of MHC(+) microglia almost had returned to control levels, but a slight elevation of MHC(+) cells in the hippocampus of ECS-treated rats remained.

ED1 is expressed by phagocytic microglia, and was found sparsely in sham-treated animals. Two hours after ECS-treatment, there was a slight increase in the expression of ED1 in hippocampal microglia, preferentially seen along the SGZ and in the CA1 and CA3 regions. The ED1 expression was seen as small granules in the microglial cell bodies, and most ED1(+) microglia had the morphology typical of intermediate activated microglia (Lehrmann et al., 1997). No differences in ED1 expression was found between control animals and animals killed 4 weeks after ECS treatment.

Nestin is an intermediate filament type IV protein expressed by activated astrocytes. In sham-treated animals, no or very few activated astrocytes were found. Two hours after ECS treatment, there was a marked increase of nestin(+) astrocytes in the prefrontal cortex, piriform cortex, entorhinal cortex, and the hippocampus. The activated cells were most abundant in the piriform and entorhinal cortex, where a large fraction of the astrocytes expressed nestin. However, 4 weeks after the ECS treatment, no significant differences in the number of nestin(+) astrocytes were seen compared with control animals.

The two sets of ECS stimulus parameters used in the study gave rise to similar patterns of glial cell activation. In general, stimulation using a short pulse width (ECS_S), induced slightly higher numbers of activated glial cells, but there were no statistically significant differences between the ECS groups, except for in hypothalamus. Here, the expression of nestin was significantly higher for the ECS group with long pulse width (ECS_L), compared to ECS_S. However, since the numbers of nestin(+) astrocytes in the hypothalamus were very low (in avarage 0.4 cells per section), no clear conclusions can be effectively drawn from this analysis.

In conclusion, in paper II we show that a course of 10 ECS treatments gives rise to moderate glial cell activation, seen as morphological alterations in the glial cells and an upregulation of certain inflammatory markers. The activation of microglia was most pronounced in the hippocampus, while most activated astrocytes were found in the piriform and entorhinal cortex. The intensity of glial cell activation was not dependent on the different sets of stimulus parameters.

The number of peripheral macrophages adhering to vessels in the hippocampus is increased following ECS (paper III)

The results from paper II clearly demonstrate that ECS-treatment leads to a low-grade inflammatory response in most limbic regions. Many glial cell reactions include recruitment and infiltration of blood-derived immune cells, such as monocytes/macrophages and lymphocytes. We now wanted to determine whether such leukocyte recruitment and infiltration also occurred in response to ECS. Because we had shown that the microglial response following ECS was most intense in the hippocampus, we chose to investigate this region.

Rats were given one ECS treatment daily for 10 consecutive days, and were then allowed to survive for 2 h, 2 days, 4 days, or 8 days after the last ECS treatment. Recruitment and infiltration of macrophages were estimated by analyzing the location and numbers of cells expressing CD163 – a widely used macrophage marker. In control animals, we found several CD163(+) macrophages within the vasculature, but no CD163(+) cells within the brain parenchyma. At all the investigated time points following ECS, there was a significant increase, by approximately 30%, of the vessel-associated macrophages, but there were no signs of further migration into the brain parenchyma of these CD163(+) cells. In addition, we analyzed the number of CD4(+) and MHC II(+) cells within the hippocampal vasculature. CD4 is expressed by T helper cells and T regulatory cells, but can also be expressed by monocytes, macrophages, and activated microglia. We saw extremely few CD4(+) cells with the small round morphology that is typical of T lymphocytes. Instead, the CD4(+) cells had a morphology similar to the CD163(+) cells, suggesting that the CD4(+) cells in our study might be macrophages and not T lymphocytes. ECS increased the number of CD4(+) cells by around 30% compared with baseline levels in control animals, just as for the number of CD163(+) cells. In ECS-treated animals, we also found CD4(+) cells within the brain parenchyma, preferentially in the hilus of the

hippocampus. However, these cells had the morphology of microglia, and could be activated microglia and not infiltrating macrophages. ECS further gave rise to a marked but transient increase in the number of MHC II(+) cells within the blood vessel wall. The number of these cells had returned to baseline levels 8 days after the course of ECS.

To summarize, we show in study III that the inflammatory response to ECS includes recruitment of macrophages to the hippocampus, but that no convincing signs of CD4(+) T lymphocytes or tissue infiltration were detected.

No effect of COX-2 inhibitors on memory performance after ECS (unpublished data)

In the previous papers (II and III), we have shown that ECS induces a mild inflammatory reaction, seen as modest glial cell activation in limbic areas and increased macrophage recruitment in the hippocampus. We next wanted to find out whether this low-grade inflammatory response could be associated with the adverse effects of ECS.

Inflammation has been associated with memory deficits in several other conditions, such as Alzheimer's disease (Agostinho et al., 2010) and multiple sclerosis (Mandolesi et al., 2010). Moreover, others have shown that ECS-induced retrograde amnesia is attenuated in rats treated with anti-inflammatory COX inhibitors such as indomethacin and celecoxib (Rao et al., 2002; Andrade et al., 2008a). ECS upregulates COX-2 activity, and has been suggested to impair learning and memory (Andrade et al., 2008b). We reasoned that the COX-inhibitors might prevent the ECS-induced memory deficits through a concomitant attenuation of the low-grade inflammatory response. Thus, we performed repeated experiments where rats were treated with ECS and celecoxib simultaneously. We analyzed their memory performance in the MWM and the glial cell activation in the hippocampus, but our results were inconclusive. ECS clearly impaired retrograde memory, but there was no significant attenuation of the retrograde amnesia when celecoxib was administered. Neither could we see any clear effect on the ECS-induced glial cell activation in the hippocampus of celecoxib-treated rats. We came to the conclusion that we could not, in our model of ECT, repeat the results by Andrade et al., (2008a), regarding attenuation of memory disturbances through administration of celecoxib.

Lithium prevents the memory deficits induced by ECS, but there is no clear correlation to glial cell activation or cell death (paper IV and unpublished data)

After the negative results with celecoxib, we searched for another agent that has the potential to reduce the glial cell activation and attenuate the ECS-induced retrograde amnesia. Lithium is the classic therapeutic treatment for bipolar disorder and has been proven to exert neuroprotective and neurotrophic actions and a broad range of anti-inflammatory effects (Beurel et al., 2010; Quiroz et al., 2010). It has been shown that lithium abolishes memory disturbances in an animal model of stroke (Yan et al., 2007), in chronic stress (Vasconcellos et al., 2003), and after brain injury (Zhu et al., 2010). This memory protective effect has been suggested to be linked to attenuated cell death in hippocampus (Yan et al., 2007). We hypothesized that lithium might attenuate ECS-induced amnesia, possibly through decreasing the inflammatory response and/or hippocampal cell death.

We conducted two studies–a "retrograde memory study" and an "anterograde memory study"–where lithium administration was begun 10 days prior to a course of five ECS treatments. To assess retrograde amnesia, the rats were trained to localize a hidden platform in a water maze with the aid of extramaze visual cues before the ECS treatments started. One day after the last ECS treatment, their ability to remember the platform location was tested. The results clearly showed that rats given ECS did not remember the platform location, but that cotreatment with lithium abolished the ECS-induced retrograde memory disturbances. That the ECS-treated rats did not behave as naïve animals – they swam more in the center of the pool than along the pool wall – suggests that ECS impairs spatial memory, but not necessarily the memory of the task *per se*. To assess anterograde memory, the water maze training started 3 days after the ECS course. The course of progress made in learning for the ECS or lithium groups did not differ from the control group in escape latency or distance swum. Neither did we see any differences between the groups in the test probe following 8 days of training.

To investigate whether the memory-sparing effect of lithium is associated with attenuated cell death and glial cell activation, we performed immunohistochemical stainings on the brain tissue from the retrograde study. Two-way ANOVA analyses on TUNEL staining data revealed a slight increase of apoptotic cell death in the CA regions following ECS, and in the ML in lithium-treated animals. Group comparisons using one-way ANOVA showed no differences in apoptosis when the whole hippocampal region was

analysed. In one subregion, the ML, rats simultaneously treated with ECS and lithium had increased apoptotic cell death compared with the nonlithium treated groups. However, it should be noted that there were very few apoptotic cells regardless of treatment (2–3 per section in the entire hippocampus).

There was a general and significant increase in nestin(+) astrocytes in the hippocampus following ECS, in accordance with the results from paper II. However, lithium treatment in combination with ECS resulted in an increased nestin expression in hippocampal astrocytes (ECS + litium 58 ± 7; ECS 29 ± 9; $p < 0.0001$) compared with ECS treatment alone. Analysis of subregions revealed increases in hilus (ECS + litium 19 ± 2; ECS 8 ± 2; $p < 0.0001$) and in ML (ECS + litium 23 ± 3; ECS 9 ± 6; $p < 0.001$) (unpublished data).

MHC II was increased after ECS in all hippocampal subregions, also in line with paper II, but no differences between the ECS group and the ECS + lithium group were found (unpublished data).

In summary, we have shown that lithium treatment in combination with ECS clearly prevents spatial retrograde amnesia, but that ECS and lithium – alone or in combination – have no significant effect on anterograde memory. In addition, we have shown that microglial MHC II expression is unchanged, while apoptotic cell death and astrocytic nestin expression are slightly increased in hippocampal subregions, when lithium is given in combination with ECS.

GENERAL DISCUSSION

In the current thesis, we have investigated previously unknown cellular changes following ECS, and further found a novel approach to attenuate ECS-induced memory deficits. First, we show that ECS stimulated angiogenesis in certain hypothalamic subnuclei, and that this correlated with an increased neuronal activity. Possible implications of an increased endothelial cell proliferation were considered. The enhanced endothelial cell proliferation might lead to an expansion of the vascular tree to adapt to an increased metabolic need. The newly formed endothelial cells might also palay an important role in a local multicellular cross talk. The question of whether the angiogenic response to ECS is part of an inflammatory reaction was also raised. The following studies in this thesis examined this last question in somewhat more detail, and glial cell activation and recruitment of blood borne macrophages were analysed. In addition, we investigated whether two sets of ECS stimulus parameters affected the glial cell activation differentially, but no such difference was found. Next, we aimed to block the glial cell reaction to ECS and test whether the ECS-induced memory deficits concomitantly could be attenuated. We failed to see any clear effects on glial cell activation or spatial memory of the anti-inflammatory substance celecoxib, but found that treatment with the neuroprotective and anti-inflammatory agent lithium abolished ECS-induced amnesia. To our surprise, the glial cell activation and the minimal increase in apoptotic cell death following ECS were unchanged or even elevated when cotreatment with lithium was given. The following discussion will first assess the plausible implications of an increased endothelial cell proliferation and then turn to possible functions of the ECS-induced glial cell activation and macrophage recruitment. Finally, the actions of lithium on the amnestic effects of ECS/ECT will be addressed.

Possible functions of the ECS-induced increase in hypothalamic endothelial cell proliferation

We have shown that endothelial cell proliferation and neuronal activity are increased by ECS in specific hypothalamic nuclei in a correlated way: the

more neuronal activity, as measured by c-Fos expression, the more endothelial cell proliferation. Endothelial cell proliferation, leading to the formation of new blood vessels, is known to occur during development, in physiological situations when the metabolic demand exceeds the availability, and during repair and regrowth in pathological conditions.

Vascular expansion to meet an increased metabolic demand

We have previously reported that ECS-induced endothelial cell proliferation in the hippocampus leads to a subsequent increase in vascular density (Hellsten et al., 2005). Thus, it is plausible that the increased hypothalamic endothelial cell proliferation following ECS also contributes to vessel growth. The basic function of blood vessels is to supply oxygen and nutrients to the tissue. The observed enhancement of endothelial cell proliferation may reflect an adaptation to an increased metabolic demand, following the neuronal activation induced by ECS. Similar angiogenic processes have been demonstrated in other brain regions, following increased neuronal activation through physical exercise, increased sensory input and environmental enrichment - a widely used animal model of experience-induced plasticity (Black et al., 1987; Black et al., 1990; Swain et al., 2003; Ding et al., 2004; Ekstrand et al., 2008).

Endothelial cells as part of a multicellular cross talk

Apart from serving as building blocks of blood vessels, endothelial cells have been shown to communicate with other cell types, as part of a "neurovascular unit" (Lok et al., 2007). This functional unit includes neurons, glial cells, and endothelial cells, that all interact with each other through positive and negative feedback mechanisms (Lo et al., 2004; Hawkins and Davis, 2005; Allan, 2006). It is possible that the hypothalamic neuronal activation and endothelial cell proliferation we observed following ECS are two independently triggered events, but there may also be a reciprocal cross talk between endothelial cells and neurons that synchronize their activity. ECS induces the expression of many growth factors and neuropeptides such as VEGF, BDNF, NPY, and FGF-2; all with both neurogenic and angiogenic properties (Nibuya et al., 1995; Zachrisson et al., 1995; Kondratyev et al., 2001; Newton et al., 2003; Altar et al., 2004). Indeed, the processes of neurogenesis and angiogenesis have many commonalities in addition to shared growth factors and receptors, including cell migration, and cell differentiation (Carmeliet, 2008). Neurogenesis has further been shown to occur in a vascular niche, where neuronal precursors divide in close

proximity to proliferating endothelial cells (Palmer et al., 2000). The hypothalamus is generally not considered to be a neurogenic zone, but postnatal neurogenesis has been demonstrated in the adult hypothalamus of several species (Pencea et al., 2001; Fowler et al., 2002; Kokoeva et al., 2005; Xu et al., 2005; Kokoeva et al., 2007; Migaud et al., 2009; Pérez-Martín et al., 2010), although not yet described in nonhuman primates or humans. Similarly to the neurogenesis in the SVZ and SGZ, the adult hypothalamic neurogenesis process can be modulated by various physiological and pharmacological stimuli (Pencea et al., 2001; Kokoeva et al., 2005; Xu et al., 2005; Matsuzaki et al., 2009; Pérez-Martín et al., 2010). However, whether ECS induces neurogenesis in the hypothalamus is yet to be investigated. Endothelial cells have also been shown to be important regulators of neuronal and glial function in other ways. De Seranno et al., (2004) has reported that endothelial cells directly influence hormone release from neurosecretory axons in the hypothalamic median eminence by regulating to which extent specialized glial cells wrap around the nerve endings, releasing hormone into the blood stream. Another study showed that endothelial cells are able to depolarize neuronal axons through production of nitric oxide *in vitro* (Garthwaite et al., 2006). It is tempting to speculate that the increase in endothelial cell numbers could play a role in the cellular interplay that may assist in mediating the therapeutic effects of ECT.

Increased endothelial cell proliferation in brain regions implicated in depression

The ECS-induced increase in endothelial cell proliferation was region-specific and only seen in the PVN, the SON and the VMH of the mid-hypothalamus. As described in the introduction, the hypothalamus is thought to play a key role in the pathophysiology of depression, and hyperactivity of the HPA axis is perhaps the most acknowledged neuroendocrine dysfunction in depression. The HPA axis is controlled from the parvocellular neurons of the PVN, which release CRH and AVP into the hypothalamo-pituitary portal system. ACTH is subsequently released from the pituitary gland into the blood stream, stimulating cortisol release from the adrenal cortex. The PVN also consists of magnocellular neurons, which together with the magnocellular neurons of the SON project to the posterior pituitary where they release oxytocin and AVP. ECS increased the endothelial cell proliferation two- to threefold within both the magnocellular and parvocellular areas. In the VMH, the endothelial cell proliferation following ECS was increased twenty-fold, and the fraction of dividing endothelial cells increased from 10% to approximately 60% of the total number of proliferating cells. The VMH is involved in the regulation of

appetite, and neurons in this region secrete NPY (Dube et al., 1995). Plasma levels of NPY are reduced in patients with major depression (Hashimoto et al., 1996) and ECT is known to increase the production of NPY (Stenfors et al., 1989). Interestingly, NPY also has angiogenic properties, suggesting that the strong increase in endothelial cell proliferation following ECS in the VMH may be caused by the release of NPY.

Angiogenesis as part of an inflammatory reaction

Enhanced cerebral angiogenesis is also seen in pathological conditions such as brain injury (Frontczak-Baniewicz and Walski, 2002), stroke (Greenberg, 1998) and epilepsy (Rigau et al., 2007). These are all conditions where normal oxygen and glucose homeostasis is compromised, and angiogenesis is triggered in an attempt to alleviate the detrimental effects of the trauma. Such insults often lead to massive neuronal death and an accompanying inflammatory reaction (Fujikawa, 2005; Vezzani and Granata, 2005; Doyle et al., 2008; Kriz and Lalancette-Hébert, 2009; Wang and Qin, 2010) There are several factors that coregulate angiogenesis and inflammation. For example, VEGF and COX-2, both markedly upregulated following ECS (Newton et al., 2003), are known to be angiogenic as well as inflammatory mediators (Croll et al., 2004; Patrignani et al., 2005). We considered that the angiogenic response to ECS might be part of an inflammatory reaction and thus investigated the expression of a few but well established markers for glial cell activation (MHC II, ED1, and nestin) in several limbic regions including the hypothalamus. However, we were not able to detect an increased expression of MHC II or ED1 in the hypothalamus. There was a statistically significant increased expression of nestin following one of the ECS protocols (ECS_L), but because the number of the nestin(+) astrocytes was extremely low, no clear conclusions could be correctly drawn from this analysis. Although we cannot account for the possible presence of other markers of inflammation, our findings speaks in favor of an endothelial cell proliferation uncoupled from an inflammatory response.

The glial cell response to ECS - associated with cell damage and/or brain plasticity?

Is the glial cell reaction reflecting cell death?

In study II, we show that ECS – regardless of which of the two different stimulus parameters used – induces a glial cell response in all investigated limbic regions, except the hypothalamus. This glial cell response was

characterized by morphological alterations and an increased expression of ED1, MHC II, and nestin, widely used as markers of glial cell activation (Pekny and Nilsson, 2005; Graeber and Streit, 2010). In study III, we further show that ECS enhances macrophage recruitment to hippocampal blood vessels. The response of glial cells, and microglia in particular, depends on the severity of insult and results in numerous intermediate states of activation. The glial cell activation following ECS appears to be very subtle. For instance, compared with a rat model of epilepsy, where a prolonged (> 30 minutes) generalized grand mal seizure (status epilepticus) was induced and hippocampal neuronal damage was widespread, the profile of glial cell activation in response to ECS was very different. Several weeks after the induction of status epilepticus, the majority of microglia in the hippocampus were phagocytic, i.e. expressing ED1, and had an amoeboid morphology (Bonde et al., 2006). Two hours after a course of ten ECS, we observed around ten ED1/Iba-1(+) cells with intermediate activated morphology per total hippocampus.

Nevertheless, the slight increase in ED1-expressing microglia in the hippocampus of ECS-treated animals is consistent with local phagocytosis of cellular debris. We found a small, but significant increase in apoptotic cell death following a course of five ECS treatments in the CA regions of the hippocampus (study IV). Although the number of apoptotic cells was very low for all groups, it should be remembered that apoptosis is a rapid process (1–3 h from initiation to cell elimination) and thus difficult to survey (Gavrieli et al., 1992). The TUNEL method only detects cells that are apoptotic at the moment of perfusion, and we cannot exclude higher levels of apoptosis at other time points after a course of ECS.

A generalized grand mal seizure is associated with massive release of glutamate. Excessive stimulation of glutamate receptors can induce neuronal cell death or damage through excitotoxicity (Olney, 1969), and the neurons of the hippocampus, piriform cortex and entorhinal cortex are particular vulnerable to seizures (Loscher and Ebert, 1996; Scharfman, 2000). As previously mentioned, most studies show that there is no cell loss or damage after ECS (Coffey et al., 1991; Devanand et al., 1994; Dalby et al., 1996; Ende et al., 2000; Zachrisson et al., 2000; Dwork et al., 2004; Busnello et al., 2006; Dwork et al., 2009; Palmio et al., 2010). However, there are a few reports showing that ECS can induce neuronal loss in the entorhinal cortex and the hippocampus (Lukoyanov et al., 2004; Zarubenko et al., 2005; Cardodso et al., 2008). It should be noted however, that the studies by Lukoyanov et al., (2004) and Cardoso et al., (2008) used an ECS paradigm with only 2 h spacing between the two last ECS treatments in order to maximize the extracellular glutamate levels and thus increase the vulnerability of the neurons to

excitotoxic damage (Rowley et al., 1997). However, in the study by Zarubenko et al., (2005), a seemingly standard ECS protocol was used, with 8 ECS treatments given 48 h apart. Thus, it seems that under certain circumstances, ECS can cause neuronal loss.

Could the ECS-induced neuroimmunological mechanisms be involved in brain plasticity?

Enhanced brain plasticity, including stimulation of neurogenesis, gliogenesis, angiogenesis, synaptic reorganization and axonal sprouting, has been reported in response to ECS. It has become increasingly evident that the immune system plays a central role in neural plasticity (for review see Yirmiya and Goshen, 2011). It is tempting to speculate that the ECS-induced activation of microglia – the intrinsic immune cells of the brain – might be associated with the processes of neural plasticity.

Microglia have been referred to as "the electricians of the brain" (Graeber and Streit, 2010), and proposed to have an instructive and supportive role in neurogenesis as well as a role in synaptic plasticity (Walton et al., 2006; Cullheim and Thams, 2007; Thored et al., 2009). A role of microglia in synapse elimination was first suggested by Blinzinger and Kreutzberg, (1968) in their report on microglia-mediated synaptic stripping of axotomized facial motoneurons. More recent studies corroborate these early findings by showing that the highly branched processes of microglia in the healthy brain make transient connections with neuronal synapses, and that this microglia–synapse contact is prolonged following ischemic insult, resulting in synaptic elimination (Wake et al., 2009). Activated microglia have also been shown to contribute to synaptic stripping of cortical neurons after an inflammatory lesion (Trapp et al., 2007). Synaptic stripping leads to a reduction of synaptic activity (Yamada et al., 2008) and a functional impairment of neuronal circuits (Graeber et al., 1993), but helps promote regrowth and remapping of damaged neural circuitry (Gehrmann et al., 1995). ECS has been shown to influence the expression of synaptic vesicle protein and increase the total number of hippocampal synapses (Elfving et al., 2008; Chen et al., 2009). On the other hand, Cardoso et al., (2008) showed that ECS, when given as previously described with 2 hours between the last two ECS-treatments, led to a reduced number of synapses in the entorhinal cortex. It is plausible that activated microglia may be involved in ECS-induced synaptic reorganization (Trapp et al., 2007).

Astrocytes have also been shown to play an important role in neural and synaptic functioning (Volterra and Meldolesi, 2005; Halassa and Haydon,

2010; Henneberger and Rusakov, 2010). Astrocytic processes ensheath most synapses in the brain, express receptors for several neurotransmitters and secrete various gliotransmitters that modulate neuronal excitability and synaptic strength (Perea and Araque, 2007; Halassa and Haydon, 2010). There seems to be a close correlation between changes in astrocyte morphology and synape formation (Jones and Greenough, 1996; Ullian et al., 2001). To protect neurons from excessive levels of glutamate, surrounding astrocytes rapidly remove glutamate at the synaptic cleft via excitatory amino acid transporters (Bernardinelli et al., 2004; Benarroch, 2005).

Besides taking part in synapse remodeling, microglia in the adult brain have been proposed to be involved in the process of neurogenesis (Ekdahl et al., 2009). *In vivo* evidences suggests that activated microglia regulate neurogenesis in the hippocampus (Battista et al., 2006; Ziv et al., 2006), and in an *in vitro* model of neural stem cell coculture with microglial cells or microglia-conditioned medium, microglia provided secreted factors essential for neurogenesis (Walton et al., 2006). On the other hand, blockade of microglia activation has been shown to restore neurogenesis after cranial irradiation (Monje et al., 2003). The unique profile of inflammatory factors, depending on the severity of inflammation, can have varying consequences on neurogenesis (Whitney et al., 2009). The discrepancies between the pro- and antineurogenic properties of inflammation may depend on the means by which microglia, macrophages, and astrocytes are activated, as well as the duration of the inflammation. For instance, microglia activated by systemic LPS injection have been reported to reduce neurogenesis (Ekdahl et al., 2003) while IL-4 activated microglia promote neurogenesis (Butovsky et al., 2006). Ziv et al., (2006) demonstrated that environmental enrichment stimulated microglia to express MHC II, and proposed that this specific microglia phenotype was required for the concomitant increase in neurogenesis, through the activation of T lymphocytes. The impact of CD4(+) T lymphocytes on neurogenesis have been supported by others (Wolf et al., 2009; Huang et al., 2010), but contradictive results have shown that mice subjected to running had a dramatic increase in neurogenesis, although no signs of MHC II(+) microglia were found in the region (Olah et al., 2009). The exact microglial phenotype induced by ECS, and whether the expression of MHC II may be a requisite for the ECS-induced increase in neuronal proliferation, differentiation, integration and/or survival, remains to be determined however.

In addition to assisting microglia in the clearance of dead cells and debris, blood-recruited macrophages have been suggested to mediate neuroprotection and support neurogenesis (Borders et al., 2007; London et al., 2011). The recruited macrophages following ECS in our study were

defined by their expression of CD163. Interestingly, the anti-inflammatory cytokine IL-10 has been reported to strongly upregulate CD163 mRNA in monocytes and macrophages, while pro-inflammatory mediators, such as IFN-γ, LPS and TNF-α, have been reported to suppress CD163 expression (Buechler et al., 2000). Consequently, CD163 has been indicated as a possible marker for so-called alternatively activated macrophages (M2), which have been suggested to possess immunosuppressive activity (Gordon, 2003; Mosser, 2003; Verreck et al., 2006).

So far, we have discussed whether the glial cell response to ECS might reflect cell death and/or assist in the process of neural plasticity. It should also be considered, however, that a small amount of cell death might be a natural part of the neural plasticity process. It is possible that increased apoptosis in response to ECS might reflect the enhanced cell proliferation, as in an increased cell turnover (Gould et al., 2001). This means that despite an enhanced cell death, the total number of cells might still be increased if the level of newly born cells exceeds the level of apoptosis.

Protection against ECS-induced retrograde amnesia

In study IV, we show that a course of 5 ECS impairs retrograde spatial memory, evaluated by the Morris water maze test and in line with previous studies (Andrade et al., 2008a; Yao et al., 2010). However we found no significant anterograde amnesia following ECS, although others show learning deficits after ECS (Khan et al., 1994; Lukoyanov et al., 2004). Andrade et al., (2008a) have reported that anti-inflammatory COX-2 blockade with celecoxib abolishes ECS-induced memory deficits. The authors suggested that celecoxib protect against ECS-induced retrograde amnesia by attenuating ECS-induced COX-2-mediated glutamatergic excitotoxicity. In their study, a passive avoidance test was used to assess retrograde amnesia. We hypothesized that COX-2 inhibition in addition to protecting against retrograde amnesia might reduce the ECS-induced glial cell activation through downregulation of inflammatory mediators. However, we could see neither any reduced glial cell activation nor any protective effect of celecoxib on retrograde memory when testing the animals in Morris water maze. The discrepancies in results may depend on the different memory tests or other variations in the experimental design.

Lithium-induced attenuation of ECS-induced retrograde amnesia

Treatment with lithium in rodent models has been shown to protect against memory deficits induced by stroke (Yan et al., 2007), traumatic brain injury (Zhu et al., 2010) and chronic stress (Vasconcellos et al., 2003). Furthermore, lithium alone has been demonstrated to enhance spatial working memory and promote long-term retention, and was consequently suggested as a potential cognitive enhancer (Tsaltas et al., 2007). It has been proposed that lithium exerts its protective effects on cognition through reducing neurodegeneration by upregulating neurotrophic factors and antiapoptotic factors and downregulating proapoptotic agents and proinflammatory cytokines such as IL-1β (Yan et a 2007; Zhu et al., 2010). Lithium has further been shown to attenuate proinflammatory and enhance antiinflammatory properties of microglia (Yuskaitis and Jope, 2009). We show in paper IV, that lithium treatment abolishes the ECS-induced retrograde memory disturbances. We investigated whether this was associated with changes in hippocampal apoptotic cell death or glial cell activation. ECS and lithium individually increased apoptotic cell death in separate subregions of the hippocampus (the CA regions and ML, respectively), and group comparisons revealed that lithium in combination with ECS slightly enhance the apoptotic cell death in the ML of hippocampus. A significant increase of nestin-expressing astrocytes was also found in the hilus and ML in animals cotreated with lithium and ECS, but the number of MHC II(+) microglia was unchanged. Thus, lithium appears to exert protective effects against ECS-induced amnesia without reducing apoptosis or attenuating the glial cell response, which also indicates that the amnestic effects of ECS may be independent of these mechanisms.

The memory impairments following ECS has been proposed to depend on glutamate release and induction of long term potentiation (LTP) in an indiscriminative manner, which disrupts the delicately controlled synaptic remodeling required for learning (Reid and Stewart, 1997; Brun et al., 2001). This theory is supported by the findings that NMDA receptor antagonists and calcium channel blockers reduce ECS-induced amnesia (Stewart and Reid, 1994; Zupan et al., 1996; Krystal et al., 2003). Lithium treatment has also been shown to reduce glutamate-mediated Ca2+ response in hippocampal neurons (Nonaka et al., 1998; Sourial-Bassillious et al., 2009), and it is plausible that inhibition of ECS-induced excessive Ca2+ influx may attenuate the saturation of the LTP system, thus leaving the system intact. However, lithium modulates a variety of different biochemical pathways, and further investigation of lithium's actions on the glutamatergic system is needed for a better understanding of its protective effect.

Excessive postictal confusion and prolonged awakening time when combining ECT with lithium treatment have raised some concerns (Hoenig and Chaulk, 1977; Small et al., 1980), but there are several new reports of safe concurrent treatment (Dolenc and Rasmussen, 2005; Thirthalli et al., 2010). Our findings suggest that lithium has potential as an agent for attenuation of ECT-induced amnesia.

CONCLUDING REMARKS

The results of this thesis show that ECS, an animal model of the antidepressant treatment ECT, induces endothelial cell proliferation in correlation with neuronal activation in specific nuclei of the hypothalamus. This angiogenic process might be a response to an increased metabolic demand following intense neuronal activation, or a factor contributing to the hypothalamic cellular cross talk, but could also be a part of an inflammatory reaction to ECS. An inflammatory response is partly seen as a glial cell reaction, and it is further demonstrated in this thesis that there is low-grade glial cell activation in several limbic regions and a concomitant recruitment of macrophages to the hippocampus. Such a low-grade glial cell response might reflect the slight increase in apoptosis we found following ECS, but we also propose that the activated glial cells may assist in the process of the ECS-induced brain plasticity, including, for example, neurogenesis, angiogenesis, and synaptic sprouting. Further, we demonstrate in this thesis that lithium treatment attenuates ECS-induced retrograde amnesia, without an accompanying reduction of hippocampal glial cell activation or cell death. Further studies on lithium's effect on ECS-induced amnesia could be of great value for the development of an ECT protocol with less cognitive side effects.

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sion, ECT is known to regulate neurotransmitters (for review, see Mann 1998), growth factors expression (Altar et al 2004), axonal sprouting (Lamont et al 2001; Vaidya et al 2000), and

From the Molecular Psychiatry Unit (LJ, JH, AT), Wallenberg Neuroscience Center, Lund, Sweden.
Address reprint requests to Anders Tottenborg, Molecular Psychiatry Unit, Wallenberg Neuroscience Center, BMC A13, S-22184, Lund, Sweden. E-mail: anders.tottenborg@mpsy.lu.se.
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start of the studies. A first study, "the proliferation study," was carried out to assess endothelial cell proliferation in response to ECS treatment (Figure 1), and rats were assigned to the following groups: Control (n = 7) and 5 ECS (n = 7). A second study, "the neuronal activation study," was performed on another brood of rats, assigned to the following groups: Control (n = 6), 1 ECS (n = 6), 5 ECS (n = 6), and 10 ECS (n = 6) (Figure 1).

A third study [illegible] treatment on drinking and feeding behavior and on serum [illegible] (n = 6) and 5 ECS treatment (n = 6) for 5 days, and their food and water consumption was monitored during these 5 days. Serum osmolality was

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