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*DOI (link to publication from Publisher):*
10.5278/vbn.phd.med.00024

*Publication date:*
2015

*Document Version*
Publisher's PDF, also known as Version of record

# THE INFLUENCE OF DNA METHYLATION ON GENE EXPRESSION INVOLVED IN THE ETIOLOGY AND TREATMENT OF PSYCHIATRIC DISORDERS

**BY**
**MADS DYRVIG**

DISSERTATION SUBMITTED 2015

AALBORG UNIVERSITY
DENMARK

# THE INFLUENCE OF DNA METHYLATION ON GENE EXPRESSION INVOLVED IN THE ETIOLOGY AND TREATMENT OF PSYCHIATRIC DISORDERS

PhD dissertation

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Dissertation submitted January 2015

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| PhD Series: | Faculty of Medicine, Aalborg University |

ISSN (online): 2246-1302
ISBN (online): 978-87-7112-214-5

Published by:
Aalborg University Press
Skjernvej 4A, 2nd floor
DK – 9220 Aalborg Ø
Phone: +45 99407140
aauf@forlag.aau.dk
forlag.aau.dk



Printed in Denmark by Rosendahls, 2015

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**Publications (before PhD)**

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| 2012 | Henningsen, K.*, **Dyrvig, M.***, Bouzinova, E. V., Christiansen, S., Christensen, T., Andreasen, J. T., Palme, R., Lichota, J. and Wiborg, O. (2012). Low maternal care exacerbates adult stress susceptibility in the chronic mild stress rat model of depression. Behavioural pharmacology, 23(8), pp. 735–743. |
| 2011 | Rasmussen, C., **Johannesen, M.D.**, Peters, N.D (2011). Biologisk behandling af reumatologiske sygdomme - status over anvendelsen af biologiske lægemidler. BestPractice Reumatologi, 13, s. 24-30. |

# PREFACE

Increasing evidence proves that epigenetic mechanisms are importantly involved in the pathophysiology of psychiatric disorders. This is primarily based on findings of epigenetic regulation in post mortem brains and the working mechanisms of currently used pharmaceuticals. Epigenetic dysregulation caused by genetic variants and environmental exposures are potentially reversible. However, our knowledge on epigenetic regulation in the brain is still limited.

This dissertation: ”The influence of DNA methylation on gene expression involved in the etiology and treatment of psychiatric disorders” has been submitted to the Faculty of Medicine, Aalborg University, Denmark. The dissertation presents experiments that investigate the influence of genetic variants on DNA methylation, correlations between DNA methylation and gene transcription in brain biopsies, activity-induced methylation changes in neurons, and pharmacological interventions targeting epigenetic mechanisms.

The majority of experiments presented in this dissertation were conducted at the Laboratory of Neurobiology, Aalborg University, under the supervision of Associate Professor Jacek Lichota. The experiments involving *BRD1* were conducted at Professor Anders Børglums laboratory, Department of Human Genetics, Aarhus University, under supervision of Associate Professor Jane H. Christensen.

During my PhD study I have supervised students at the educations MedIS and Medicine, and participated in PhD courses corresponding to 30.65 ECTS points. Together these activities correspond to nearly a full year of my PhD study.

The dissertation is based on five manuscripts of which two have been published and the remaining three are in preparation. The thesis is composed of a general introduction encompassing the primary topics being explored in the manuscripts, objectives, manuscripts, and a general discussion.

# ENGLISH SUMMARY

Psychiatric disorders such as schizophrenia and depression have high life time prevalences and large costs for individuals as well as society. Common for both diseases is that the current medications target the same basic mechanisms as those discovered by serendipity several decades ago. The primary reason is that our knowledge on pathophysiology of psychiatric disorders is still sparse.

Family studies have revealed high heritability for schizophrenia and moderate heritability for depression. These observations have been succeeded by genetic studies that have revealed a high number of genetic variants associated with both diseases. In addition, a number of environmental risk factors have been identified. Both genetic variations and environmental exposures are known to influence epigenetic modifications. Adverse epigenetic modifications can potentially cause long-term dysregulation of genes and be implicated in pathophysiology. Importantly, these mechanisms can be modulated by pharmaceuticals and hence offer an alternative strategy for treating and preventing psychiatric disorders.

The first part of this PhD dissertation focus on a single nucleotide polymorphism, rs138880, which is located in the *BRD1* promoter region and has repeatedly been linked with schizophrenia. BRD1 is a transcription factor essential during embryogenesis and CNS development and is widely expressed in the adult brain. It is first established that DNA methylation plays a central role in regulating *BRD1* transcription. We then determine that the schizophrenia-risk allele of rs138880 is associated with increased DNA methylation. Importantly, we demonstrate that the affected regions undergo dynamic changes in DNA methylation levels during fetal brain development. This suggests that *BRD1* may be dysregulated in carriers of the rs138880 risk allele in both the developing and mature brain.

In the second part, we focus on the *CHRNA7* gene, encoding the α7 nicotinic acetylcholine receptor, which is considered a promising target for treatment of

cognitive dysfunction in schizophrenia. DNA methylation of the *CHRNA7* core promoter has previously been implicated in transcriptional regulation and we identify two other important regions. Clinical trials with the α7 nicotinic acetylcholine receptor have been challenged by relatively modest effect sizes and large inter-individual response variations. These variations have been suggested to result from genetic variations and we use human brain biopsies to investigate if DNA methylation could be involved. Finally, we use cell lines to demonstrate the potential of pharmacologically modulating receptor expression.

In the final part, we concentrate on electroconvulsive therapy (ECT), one of the most effective treatments of major depression. Electroconvulsive stimulation (ECS), an animal model of ECT, has been extensively studied in attempts to identify novel treatment mechanisms. Unfortunately, ECT is also characterised by side effects including memory deficits, which are reported by some patients. In three consecutive studies we study both positive and adverse expression changes caused by ECS. Recent studies have identified neuronal activity-dependent methylation changes and in this relation we focus specifically on the gene *Arc*, which plays an important role in long-term synapse-specific modifications important for memory processes.

Findings of this research emphasise the importance and potential of studying epigenetic mechanisms in psychiatric disorders. They indicate how a genetically associated risk variant can lead to dysregulation, how DNA methylation is involved in transcriptional regulation in the brain, and how neuronal activity leads to active methylation and demethylation that can be involved in both beneficial and adverse processes.

# DANSK RESUME

Psykiske sygdomme såsom skizofreni og depression har høje livstidsprævalenser og store omkostninger for både individet og samfundet. Fælles for begge sygdomme er, at de nuværende behandlinger er rettet mod de samme mekanismer, som tilfældigt blev opdaget for adskillige årtier siden. Den primære årsag, til at behandlingen ikke har udviklet sig, er, at vores viden om patofysiologien af psykiatriske lidelser til stadighed er begrænset.

Familiestudier har for årtier siden afsløret, at arveligheden for skizofreni er høj og moderat for depression. Efterfølgende har genetiske studier afsløret en lang række genetiske risikovarianter for begge sygdomme. Derudover er en række miljømæssige risikofaktorer blevet identificeret. Både genetiske variationer og miljøet kan påvirke epigenetiske modifikationer. Skadelige epigenetiske modifikationer kan potentielt forårsage langvarig dysregulering af genekspression og være involveret i sygdommenes patofysiologi. Disse epigenetiske modifikationer kan dog moduleres med lægemidler og kan derfor potentielt bruges som en alternativ strategi til at behandle og forhindre psykiatriske lidelser.

Den første del af denne afhandling fokuserer på en enkeltnukleotidpolymorfi, rs138880, der er lokaliseret i promoterregionen af *BRD1* genet, og som gentagne gange er blevet associeret med skizofreni. BRD1 er en transskriptionsfaktor, der er essentiel for embryogenesen samt centralnervesystemets udvikling og den er højt udtrykt i den voksne hjerne. Først påvises det, at DNA metylering spiller en central rolle i reguleringen af *BRD1* transskriptionen. Dernæst afslører vi, at skizofreni-risiko-allelen af rs138880 er associeret med øget DNA metylering i bestemte regioner. Endvidere påviser vi, at de ramte regioner normalt gennemgår dynamiske ændringer i metyleringsgrad i løbet af fosterets hjerneudvikling. Dette indikerer, at *BRD1* kan være dysreguleret i både fosterstadiet og den voksne hjerne hos bærere af rs138880 risiko-allelen.

I den anden del fokuserer vi på α7 nikotinreceptoren, der betragtes som en lovende kandidat for behandling af kognitiv dysfunktion hos skizofrene. Det er tidligere blevet vist, at DNA metylering omkring transkriptionsstartstedet for α7 nikotinreceptor-genet er vigtig for transkriptionel regulering, og vi identificerer yderligere to vigtige områder. Klinisk afprøvning af α7 nikotinreceptor-agonister er blevet vanskeliggjort af begrænsede effektstørrelser og store inter-individuelle responsvariationer. Det er tidligere blevet foreslået, at disse variationer skyldes genetiske varianter, og vi bruger humane hjernebiopsier til at undersøge, om DNA metylering også kan spille en rolle. Endvidere bruger vi cellelinjer til at demonstrere, at α7 nikotinreceptor-ekspressionen potentielt kan moduleres ved farmakologisk intervention.

I den sidste del fokuserer vi på elektrokonvulsiv terapi (ECT), som er en af de mest effektive behandlinger af depression. Elektrokonvulsiv stimulation (ECS), en dyremodel for ECT, er hyppigt blevet brugt i forsøg på at identificere nye potentielle behandlingsmekanismer. ECT er imidlertid også karakteriseret af bivirkninger, herunder hukommelsesproblemer, som rapporteres af nogle patienter. I tre fortløbende studier undersøger vi både virknings- og bivirkningsrelaterede ekspressionsændringer forårsaget af ECS. Nyere studier har afsløret, at neuronal aktivitet resulterer i metyleringsændringer, og i den sammenhæng fokuserer vi på *Arc* genet, som spiller en vigtig rolle i langvarige synapse-specifikke modifikationer, der er vigtige for hukommelsesprocesser.

Resultaterne af denne forskning understreger vigtigheden og potentialet af at studere epigenetiske modifikationer i relation til psykiatriske sygdomme. Ydermere indikerer de, hvordan en genetisk risikovariant kan føre til dysregulering af genekspressionen, hvilken rolle DNA metylering spiller i regulering af genekspression i hjernen, og hvordan neuronal aktivitet kan føre til aktiv metylering og demetylering, som både kan være gavnlig og uønsket.

# SUPERVISOR AND ASSESSMENT COMMITTEE

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# ACKNOWLEDGEMENTS

First of all I would like to sincerely thank my supervisor Associate Professor Jacek Lichota. Not only for guiding me throughout my PhD studies but also for supervision on the projects that preceded and were done during my M.Sc.. You have been a great inspiration and I highly appreciate your advice and comments. The first project I did under your supervision was immediately after you had been employed at Biomedicine and started your own laboratory. You gradually introduced me to all the basic laboratory skills and through the years involved me in setting up most new methodologies. There is no doubt that this has immensely impacted my creativity, way of thinking, and problem solving. It has been a true pleasure to feel how our relationship has gradually developed from a supervisor-student relationship to something much closer to a partnership.

Secondly, I express my gratitude to Associate Professor Jane H. Christensen and Professor Anders Børglum. Professor Anders Børglum for welcoming me to your laboratory and for providing the financial basis for extending my stay at your laboratory by redeeming me from remaining teaching obligations at Aalborg University. Associate Professor Jane H. Christensen for teaching me new techniques involving cell cultures and for scientific guidance.

I would like to extend my gratitude to my group members both at Aalborg University and Aarhus University. Both places provided an inspiring working environment and great atmosphere. I owe a special thanks to Merete Fredsgaard and Anne Hedemand for managing the laboratories at Aalborg and Aarhus University, respectively.

In terms of collaboration I would like to thank Professor Jens Damsgaard Mikkelsen at Neurobiology Research Unit, Rigshospitalet, for involving me in the Cognito project. I thank Associate Professor David Woldbye at Laboratory of Neural Plasticity, Department of Neuroscience and Pharmacology, University of

Copenhagen, and members of your group for setting up the animal experiments involving electroconvulsive stimulation and for discussions regarding these studies.

Finally and most importantly I highly value the help and support from my family and friends. There is no doubt that the love and upbringing from my parents taught me to be stubborn and diligent, which have helped me greatly to cope with challenges and the numerous failures experienced in the laboratory. I cannot express enough my love and gratitude to Mette and our son Christian. You are always in my mind and a constant reminder of what is most important in life. You have always helped and supported me, particularly during the last and tough period of the PhD. First of all by taking care of all the practical tasks but more importantly you both distracted me from thinking about work all the time and cheered me up with relaxation and play.

# LIST OF STUDIES

1. **DNA methylation regulates *BRD1* and is increased by the schizophrenia associated SNP rs138880**

Mads Dyrvig, Per Qvist, Jacek Lichota, Knud Erik Larsen, Mette Nyegaard, Anders D. Børglum, and Jane H. Christensen

We intend to send this manuscript to Neuropsychopharmacology

2. **DNA methylation regulates *CHRNA7* transcription in human cortical tissue and can be modulated by HDAC inhibitor valproate in human cell lines**

Mads Dyrvig, Jens D. Mikkelsen, and Jacek Lichota

We intend to send this manuscript to Clinical Epigenetics

3. **Epigenetic regulation of *Arc* and *c-Fos* in the hippocampus after acute electroconvulsive stimulation in the rat**

Mads Dyrvig, Henrik H. Hansen, Søren H. Christiansen, David P.D. Woldbye, Jens D. Mikkelsen, Jacek Lichota

Published in: Brain Research Bulletin 88 (2012) 507–513

4. **Temporal gene expression profile after acute electroconvulsive stimulation in the rat**

Mads Dyrvig, Søren H. Christiansen, David P.D. Woldbye, Jacek Lichota

Published in: Gene 539(1) (2014) 8–14

5. **Decitabine attenuates *Dnmt3a* upregulation after electroconvulsive stimulation but does not prevent expression and epigenetic changes for the *Arc* gene**

Mads Dyrvig, Casper René Gøtzsche, David P.D. Woldbye, Jacek Lichota

This manuscript has been submitted to Neuropharmacology

# LIST OF ABBREVATIONS

| | |
|---|---|
| *ARC* | activity-regulated cytoskeleton-associated protein gene |
| *BDNF* | brain-derived neurotrophic factor |
| *BRD1* | bromodomain containing 1 gene |
| *CHRNA7* | cholinergic receptor, nicotinic, alpha 7 |
| CGI | CpG island |
| CNS | central nervous system |
| DNMT | DNA (cytosine-5-)-methyltransferase |
| ECS | electroconvulsive stimulation |
| ECT | electroconvulsive therapy |
| GWAS | genome wide association study |
| HAT | histone acetyltransferase |
| HDAC | histone deacetylase |
| HDACi | histone deacetylase inhibitor |
| HMT | histone methyltransferase |
| HDM | histone demethylase |
| HPA | hypothalamic-pituitary-adrenal |
| MBD | methyl binding domain |
| NMDA | N-methyl-D-aspartate |
| PK | protein kinase |
| PP | protein phosphatase |
| *PP1* | protein phosphatase 1 gene |
| SNP | single nucleotide polymorphism |
| TET | ten-eleven translocation |
| TSS | transcription start site |
| 5-azaC | 5-azacytidine |
| 5-azaCdR | 5-aza-deoxycytidine (decitabine) |
| 5mc | 5-methylcytosine |
| 5hmc | 5-hydroxymethylcytosine |

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# 1. INTRODUCTION

This PhD dissertation is centered around five manuscripts that collectively focus on DNA methylation alterations involved in the etiology and treatment of psychiatric disorders. While the studied mechanisms are conjoint, the diseases are distinctive although having common features. The primary focus of the five included papers are: 1) a single nucleotide polymorphism (SNP) that has been linked to schizophrenia 2) regulation of the α7 nicotinic acetylcholine receptor (nAChR), which is a promising target for treating cognitive dysfunction in schizophrenic patients 3) effects and side-effects resulting from electroconvulsive therapy (ECT), a treatment commonly used for major depression. The purpose of this chapter is to introduce the topics most relevant for this dissertation. Epigenetic mechanisms and schizophrenia will be thoroughly introduced whereas the presentation of depression is more restricted and focus primarily on molecular mechanisms relevant for ECT.

## 1.1. EPIGENETIC MECHANISMS

Gene function may be altered by either a change in DNA sequence or changes in epigenetic programming of the gene. The following sections will start by introducing DNA methylation and histone modification and although described in separate sections these mechanisms are highly interconnected. The traditional definition of epigenetic mechanisms requires that the mechanisms must be heritable either across the germ line or cell divisions (Bird 2007). As neurons cannot divide and are not germ cells, the mechanisms occurring in the adult central nervous system (CNS) do not qualify as being epigenetic by this definition (Day & Sweatt 2010). However, several studies have demonstrated that modification of chromatin structure and DNA methylation are critical for functions in the CNS. These processes can be described as neuroepigenetic to distinguish them from traditional heritable epigenetic marks involved in development and cell differentiation (Day & Sweatt 2010). A separate section will describe neuroepigenetics. Finally, the basic principles of drugs targeting epigenetic mechanisms relevant for psychiatric

disorders will be described as these have the potential to alter gene expression profiles.

### 1.1.1. DNA METHYLATION

In 1975 it was suggested for the first time that methylation of cytosine residues in a CpG dinucleotide context could serve as an epigenetic mechanism in vertebrates (Holliday & Pugh 1975; Riggs 1975). It was proposed that CpG sites could be methylated *de novo*, that enzymes recognising hemimethylated DNA can allow inheritance through somatic cell division, that methyl groups can be interpreted by DNA-binding proteins, and that DNA methylation silences genes. Although these assumptions have turned out correct, the relationship between DNA methylation, gene silencing, and responsible mechanisms has been difficult to unravel (Jones 2012). The highly stable covalent modification of DNA produces potentially life long changes in gene expression making it essential for maintaining stable cellular identities (Gavin et al. 2013). So far most studies have been focused on 5-methylcytosine in a CpG context and have particularly focused on CpG islands located in promoter regions of approximately 60% of genes in the vertebrate genome (Wang & Leung 2004). More recently, the focus has expanded to include low density CpG promoters and methylation at gene bodies, enhancers, and insulators (Jones 2012).

#### 1.1.1.1. Enzymes catalyse methylation and demethylation

The function of DNA methylation is inevitably linked to the mechanisms responsible for establishing, maintaining, and removing the methyl group. The functions of DNA methyltransferases: DNMT1, DNMT3a, and DNMT3b have been extensively studied and it has long been known that *de novo* methyltransferases DNMT3a and DNMT3b are essential for setting up DNA methylation patterns in early development (Jones & Liang 2009). It was originally thought that DNMT1 could maintain an established methylation pattern, but later it was discovered that DNMT3a and DNMT3b are also actively involved (Jones & Liang 2009). However, the mechanisms that guide a DNMT to a specific CpG have not been fully

elucidated, but most likely it occurs through interactions with transcription factors and chromatin proteins (Gavin et al. 2013).

The existence of active DNA demethylation mechanisms was controversial for many years (Ooi & Bestor 2008). However, now both direct and indirect evidence points for their existence. The abundant expression of DNMTs in neurons implies a DNA demethylation pathway (Sharma et al. 2008), because otherwise aging should gradually result in accumulating DNA methylation and this is not the case (Numata et al. 2012). It has also been demonstrated that activity dependent demethylation occurs in neurons (Miller & Sweatt 2007; Levenson et al. 2006; Feng et al. 2010a). Further, many DNMT inhibitors are cytosine analogs and functions by incorporating into DNA where they trap DNMTs and this mechanism also occurs in non-dividing cells (Yamagata et al. 2012). The most consistent observation is that DNA demethylation may occur by a base excision repair process. This process initially involves oxidisation of 5-methylcytosine (5mc) to form 5-hydroxymethylcytosine (5hmc) in a process catalysed by ten-eleven translocation (TET) enzymes (Guo et al. 2011a; Ito et al. 2010; Koh et al. 2011). 5hmc may further be oxidised by TET enzymes to form 5-formylcytosine (5fc) and subsequently 5-carboxylcytosine (5cac) (Ito et al. 2011). Alternatively 5mc or 5hmc can be deaminated by activation-induced cytidine deaminase (AICDA) or apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC) forming either thymidine or 5-hydroxymethyluracil (5hmu). Overall this may result in mismatches 5cac:G, T:G, or 5hmu:G that are removed through a process involving thymidine or uracil glycosylases (Guo et al. 2011a). In this demethylation process it is suspected that Gadd45 proteins bind to and direct the enzymatic activities to specific gene promoters (Cortellino et al. 2011; Rai et al. 2008).

#### 1.1.1.2. Characteristics of CGI and non-CGI promoters

The CpG site density varies considerably in mammalian promoters and although the density follows a bimodal distribution, some promoters have intermediate CpG density (Takai & Jones 2002). In somatic cells most CGI-promoters are unmethylated. The active CGI-promoters are characterised by nucleosome-depleted

regions that are flanked by the histone variant H2A.Z and marked with the histone modification H3K4me3 (Kelly et al. 2010) (Figure 1). H2A.Z and H3K4me3 are anti-correlated with DNA methylation (Zilberman et al. 2008; Conerly et al. 2010). The activating histone marks H3K4me2 and H3K4me3 that are usually found near TSSs of active genes have also been found to block *de novo* methylation (Ooi et al. 2007). In addition, it has been found that DNMT binding requires nucleosomes as a substrate (Ooi et al. 2007).

For the subset of promoters that do become highly methylated at a CGI, this usually occurs when there is long-term stabilisation of the repressed state such as during imprinting. The mechanism by which DNA methylation represses transcription of a CGI-promoter has been well described. The promoter has nucleosomes at the TSS that are marked with repressive H3K9me3 (Lin et al. 2007) and bound by methylated DNA binding proteins that recruit histone deacetylases (HDACs) (Wade & Wolffe 2001). Methylated DNA is recognised by methyl binding domain proteins (MBDs), such as MeCP2 that are part of large protein complexes containing HDACs and histone methyltransferases (HMTs) resulting in further transcriptional repression (Tsankova et al. 2007). Consequently the DNA is assembled to nucleosomes and this prevents initiation of transcription at the TSS (Venolia & Gartler 1983; Hashimshony et al. 2003; Kass et al. 1997).

In contrast to the stability of methylation at CGI-promoters of silent genes, substantial fluctuations occur at non-CGI promoters. While the consequences of methylation of non-CGI promoters have been less well-studied evidence suggests that it is also important for repressing transcription. Indeed genome-wide analysis has revealed that there is an inverse relationship between methylation of non-CGI promoters and expression (Gal-Yam et al. 2008). However, because research up to this point has been highly focused on CGIs, little is known about the specific functions. However, it seems most likely that DNA methylation and repressive histone modifications work in concert to cause chromatin condensation and transcriptional repression.

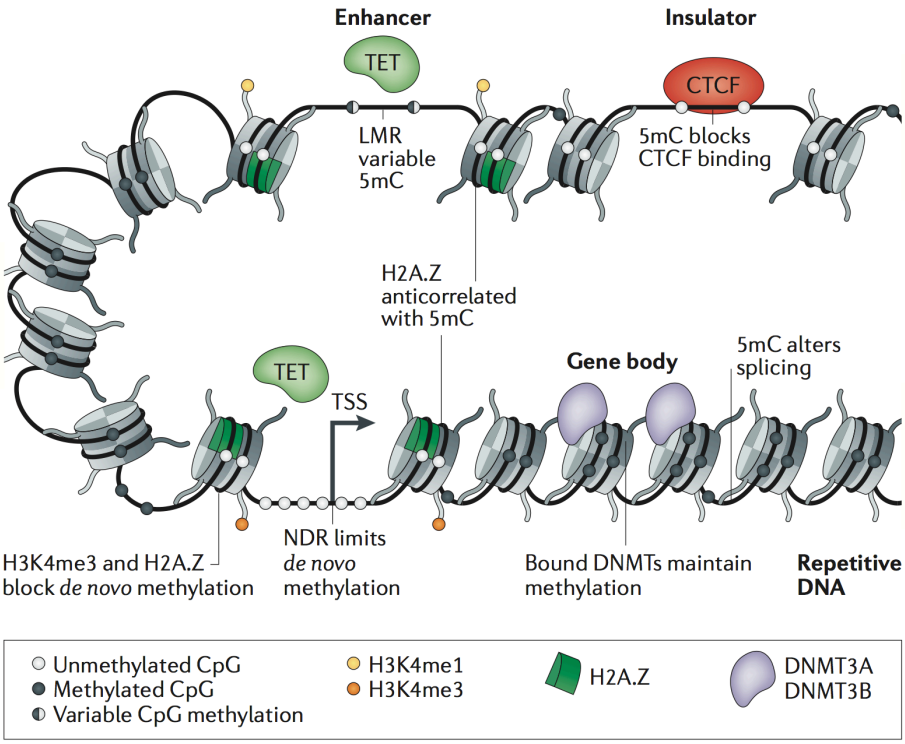


**Figure 1. Molecular structure of chromatin illustrating the role of CpG methylation in regulating gene expression.** More than half of human genes have CpG islands (CGIs) at their promoters. Active genes are characterised by nucleosome-depleted regions (NDR) at the transcriptional start site (TSS). DNMTs need nucleosomes to bind and the nucleosomes flanking the TSS are marked with H3K4me3 and the histone variant H2A.Z which prevents DNMT binding. The gene body is mostly CpG depleted and the CpG sites are mostly methylated. Gene body methylation patterns have revealed that exons are more highly methylated than introns and that this shift occurs at the exon-intron boundary, suggesting a possible role in splicing. DNA methylation is maintained by DNMT1 and DNM3A and/or DNMT3B. Enhancers are usually CpG poor and show variable methylation, which suggests that levels may be dynamically regulated. When active, these regions are also nucleosome-depleted and flanking nucleosomes are marked with H3K4me1 and H2A.Z. Proteins such as CTCF are known to bind to insulators. When these are unmethylated the region is nucleosome-depleted, whereas increased methylation changes this and is known (in some cases) to block binding of CTCF. CpG-poor promoters and silenced CGIs are not illustrated but are both in the silent state associated with nucleosomes at the TSS. LMR: Low-methylated region (LMR). Modified from (Jones 2012).

#### 1.1.1.3. Changes in transcriptional activity lead to methylation or demethylation

As described above, methylation of a CGI causes the region to assemble to nucleosomes and this prevents initiation of transcription at the TSS (Venolia & Gartler 1983; Hashimshony et al. 2003; Kass et al. 1997). However, it still remains controversial whether silencing or methylation comes first.

Experiments have shown that methylation of *HPRT* occurred as a secondary event after inactivation of one X chromosome (Lock et al. 1987) and in cancer cells there is strong evidence that CGI promoters that are silenced by Polycomb complexes are much more likely to become methylated (Gal-Yam et al. 2008; Ohm et al. 2007; Schlesinger et al. 2007; Widschwendter et al. 2007). In addition, DNA mutations that affect promoter activity have shown to affect DNA methylation. By studying promoter SNPs that alter transcriptional drive, it has been found that a CpG island in a less active allele was more likely to become *de novo* methylated (Hitchins et al. 2011) whereas the opposite was observed for an allele that had an additional binding site for an activating transcription factor (Boumber et al. 2008).

While not all promoters are *de novo* methylated, the observation above predicts that a promoter is more likely to become methylated when gene transcription decreases. However, these observations suggest that DNA methylation is probably not an initial silencing method and this notion is supported by studies on *de novo* methyltransferases. By doing experiments with cells expressing DNMT3L (a catalytically inactive homologue of DNMT3a and DNMT3b) it was found that *de novo* methylation was achieved by a tetrameric complex of two molecules each of DNMT3A2 and DNMT3L and that a nucleosome was required for binding of the complex (Ooi et al. 2007). This sequence of events has also been observed for specific genes during cellular differentiation. Here transcription factor binding is lost, which results in occurrence of a nucleosome, followed by DNMT3A binding, and subsequently *de novo* methylation (You et al. 2011) (Figure 2).

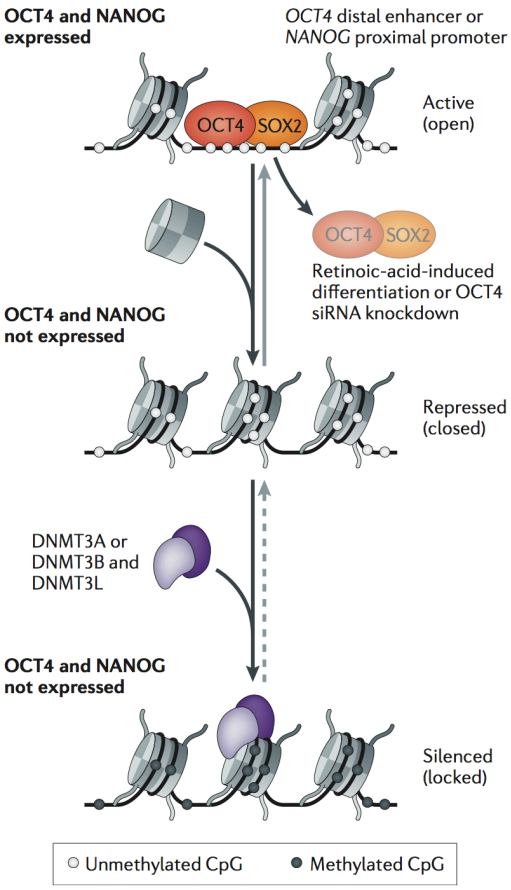


**Figure 2. A decrease in promoter activity occurs before DNA methylation.** Active promoters and enhancers are characterised by nucleosome-depleted regions where transcription factors (in this case OCT4 and SOX2) and chromatin remodellers bind. NANOG may bind in the proximal promoter (not illustrated). Loss of transcription factor binding as occurs during embryonic carcinoma cell differentiation where OCT4 and NANOG are downregulated, or in situations where SNPs disrupt a transcription factor binding site, nucleosome occupancy at the region increases. This provides a substrate for *de novo* methyltransferases and silencing of the gene. Modified from (Jones 2012).

For non-CGI promoters the causality of methylation changes has still not been fully resolved. It is known that some transcription factors can bind strongly to methylated DNA which leads to passive regional demethylation (Hsieh 2000) and hence it is not known if methylation is merely present to stabilise transcriptionally incompetent states. However, there are large variations in how methylation affects transcription factor binding. For example it is known that SP1 is unaffected by methylation (Harrington et al. 1988) whereas binding of MYC is directly inhibited (Prendergast & Ziff 1991). Epigenome-wide scans show that transcription factor binding is strongly influenced by methylation within their recognition sites (Chen et al. 2011). In some cases methylation within 100 bp in each side of the target sequence is sufficient for hindering binding (You et al. 2011). In general the strength of

repression correlates with the extent of DNA methylation and while heavily methylated genes are irreversibly silenced, strong activators may overcome lower degrees of methylation (Williams et al. 2011; Bell & Felsenfeld 2000).

#### 1.1.1.4. Gene body methylation correlates with transcriptional activation

Gene bodies are most frequently CpG poor with occasional CGIs and are extensively methylated (Jones 1999). It has long been known that active gene transcription is associated with gene body methylation (Wolf et al. 1984) and these observations have more recently been confirmed (Hellman & Chess 2007; Feng et al. 2010b). When CGIs are located in gene bodies they mostly remain unmethylated, however this is different in the human brain where 34% intragenic CGIs are methylated (Jones 2012). In cases with high intragenic CGI methylation this does not block transcription elongation, despite that these regions are marked with H3K9me3 and bound by MECP2 that represses transcription when present at the TSS (Nguyen et al. 2001). Therefore, only initiation of transcription is sensitive to methylation whereas elongation is not. Gene body methylation outside CGIs may be important for silencing repetitive DNA elements including retroviruses (Yoder et al. 1997). Studies on gene body methylation patterns have revealed that exons are more highly methylated than introns and that this shift occurs at the exon-intron boundary, suggesting a possible role in splicing (Laurent et al. 2010). Indeed, methylation is known to prevent binding of CTCF, which has been found to result in pausing of RNA polymerase II, thereby potentially influencing splicing (Shukla et al. 2011).

### 1.1.2. HISTONE MODIFICATIONS

As described in the previous section, chromatin exists in many activity-states. The basic building block of chromatin is the nucleosome formed by an octamer of histone proteins and 147 bp DNA wrapped around the histone core. The general variants of histone proteins are linker histone H1 and H2A, H2B, H3, and H4. The nucleosome is composed of a H3-H4 tetramer flanked on both sides by a H2A-H2B dimer (Finch et al. 1977). Amino acid residues on amino (N)-terminal tails of

histones can be extensively modified by more than 100 covalent modifications including acetylation (Wade et al. 1997), methylation (Jenuwein 2001), sumoylation (Shiio & Eisenman 2003), phosphorylation (Oki et al. 2007) and ubiquitination (Shilatifard 2006). Enzymes that work bidirectionally adds or removes these marks and can be modulated by pharmaceuticals (Szyf 2009). The patterns of modifications are importantly involved in defining accessibility of the DNA to the transcription machinery. Histone acetylation is a mark of gene activity and chromatin decondensation, whereas methylation and phosphorylation can correlate with both transcriptional activation and repression, depending on the modified residues (Tsankova et al. 2007). With the diversity of modifications and their simultaneous influences, chromatin exists in a continuum of many structural states ranging from active euchromatin to condensed heterochromatin (Figure 3). Enzymes capable of catalysing and reversing most types of histone modifications have been identified. These are part of large multi protein complexes that control chromatin organisation and activity (Kouzarides 2007). Most histone acetyltransferases (HATs) can catalyse acetylation of several lysine residues whereas some have higher specificity and target specific residues (Lee & Workman 2007). By being part of large multi protein complexes it is believed that specific locations in the genome become targeted (Lee & Workman 2007).

It is believed that histone modifications control chromatin compaction by two primary mechanisms: Either by recruiting effector proteins or by directly altering chromatin structure. It has been found that acetylation of H4K16 neutralises the positive charge of the lysine and destabilises internucleosomal contacts of the 30 nm fiber which leads to decondensation (Shogren-Knaak et al. 2006). This can allow access of DNA binding proteins including transcription factors. The indirect mechanism of histone modifications is to recruit specific protein complexes to modification sites. Histone acetylation is recognised by bromodomains whereas chromodomains recognise histone methylation and these domains are part of larger protein complexes that further modify chromatin (Yang 2004; Choi & Howe 2009). In addition, the histone modifications may also sterically inhibit binding of proteins to chromatin (Kouzarides 2007).

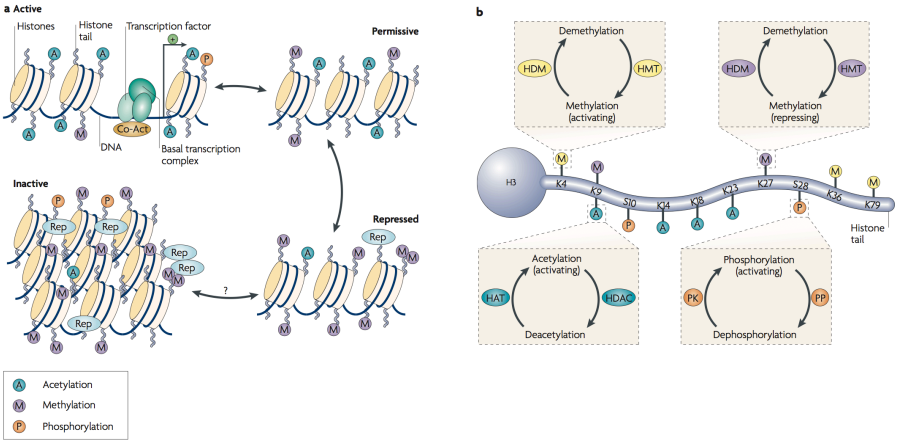


**Figure 3. Modifications responsible for the structural states of chromatin and enzymes involved in remodelling.** DNA is wrapped around a histone octamer composed of two copies each of the histones H2A, H2B, H3, and H4 with modifiable amino (N) termini of the histones facing outward from the nucleosome complex. a) Chromatin exists in a continuum of many structural states ranging from active euchromatin to condensed heterochromatin. Acetylation (A), methylation (M), and phosphorylation (P) of histone tails and corresponding binding of transcription factors and co-activators (co-Act) or repressors (Rep) modulate structural states of the nucleosome. Active chromatin (top left) is characterised by histone acetylation that opens the nucleosome and allow binding of transcription factors and the basal transcriptional complex. The chromatin also occurs in permissive states (top right) and repressed states (bottom right). The inactivated condensed state (bottom left) occurs when gene activity is permanently silenced. b) Common covalent modifications of H3, including acetylation, methylation, and phosphorylation of amino acid residues. Acetylation and phosphorylation are activating whereas methylation can be either activating or repressing depending on the modified amino acid residue. Modifications are catalysed by groups of enzymes: Acetylation is catalysed by histone acetyltransferases (HATs) and reversed by histone deacetylases (HDACs); lysine methylation is catalysed by histone methyltransferases (HMTs) and reversed by histone demethylase (HDMs); phosphorylation is catalysed by protein kinases (PK) and may potentially be reversed by protein phosphatases (PP). Modified from (Tsankova et al. 2007).

As described above DNA methylation binding proteins may recruit repressor complexes with histone deacetylase (HDAC) and histone methyltransferase (HMT)

activity. In addition, HDAC inhibition reduces global DNA methylation, DNMT1 protein levels, and its interaction with chromatin (Arzenani et al. 2011), which further emphasises that these mechanisms are highly interconnected.

### 1.1.3. NEUROEPIGENETICS: EPIGENETICS ARE IMPORTANT FOR NEURAL PLASTICITY

The action of DNMTs is generally restricted to dividing cells and their mRNA expression is very high during development. However, in the adult brain which consists primarily of postmitotic neurons and glial cells the expression of DNMT1 and DNMT3a mRNA is surprisingly high (Feng et al. 2010a). In the brain, neurotransmitters constitute the signalling mechanisms by which neurons communicate and the resulting synaptic changes may be importantly regulated by DNA methylation and histone modifications (Sharma & Chase 2012). Current evidence shows that DNA methylation is dynamically and bi-directionally regulated in the adult CNS (Miller & Sweatt 2007; Levenson et al. 2006; Feng et al. 2010a). Particularly, inhibition of DNMTs in the mammalian brain results in rapid and dramatic changes in DNA methylation of genes involved in synaptic plasticity (Levenson et al. 2006).

For several years it has been known that epigenetic mechanisms play a key role in memory formation in the adult brain (Day & Sweatt 2010). The first studies focused on the hippocampus, a region important for establishment of long-term spatial and episodic memory, and found that behavioural learning triggered changes in DNA methylation (Lubin et al. 2008; Miller & Sweatt 2007). Contextual fear conditioning results in transcriptional regulation of BDNF and changes in promoter DNA methylation status (Lubin et al. 2008). NMDA receptor blockade prevents both memory associated DNA methylation changes, transcriptional changes, and memory formation (Lubin et al. 2008). Memory formation involves both increased and decreased methylation as fear conditioning results in rapid methylation and transcriptional repression of the memory-suppressor gene *Pp1* and demethylation and transcriptional activation of the plasticity gene *Reelin* (Miller & Sweatt 2007). A subsequent study demonstrated that knockout of *Dnmt1* and *Dnmt3a* results in

abnormal long-term plasticity in the hippocampal CA1 region together with deficits in learning and memory (Feng et al. 2010a). In a more recent study it was found that 25 minutes after a 5 minutes spatial exploration, *Arc* gene expression increased in CA1 while methylation at the promoter decreased and methylation at the intragenic region increased (Penner et al. 2011).

### 1.1.4. PHARMACOLOGICAL INTERVENTION

Evidence is emerging that several diseases including psychiatric disorders result from defects in gene function (Szyf 2009). These defects may result from DNA mutations or from change in epigenetic programming. With pharmaceuticals targeting epigenetic mechanisms it is potentially possible to reverse aberrant gene expression profiles associated with these disease states (Szyf 2009). In this dissertation particular focus is put on schizophrenia and side effects resulting from electroconvulsive seizures (ECS). The pharmaceuticals that are described here have been selected because of their relevance for these conditions but the background will be elaborated in the relevant chapters and not here. Thus, the descriptions below are merely functional.

#### 1.1.4.1. DNMT inhibitors

The tree most common catalytic inhibitors of DNMTs are the nucleoside analogs 5-azaC, 5-azaCdR, and zebularine (Szyf 2009). The mechanism by which they inhibit DNMTs is common. They are first phosphorylated to a triphosphate nucleotide and are subsequently incorporated in DNA during DNA synthesis. During normal DNA replication and maintenance methylation of the unmethylated strand, DNMT1 forms a covalent bond with the 6' carbon position of the cytosine ring. DNMT1 then transfers a methyl group from the methyl donor S-adenosyl methionine (SAM) to the 5' carbon position of the cytosine ring, whereby the enzyme is released from its covalent bond. If 5-azaC has replaced cytosine and been incorporated in DNA, the methyl transfer cannot take place and the DNMT is trapped on the DNA (Wu & Santi 1985). Replication continues in the absence of DNMT1 and DNA methylation patterns are not copied.

The progress described above necessitates that DNA replication takes place. Therefore, it was very recently considered surprising that DNMT inhibition was also functional in the adult brain (Szyf 2009). However, as described in the sections above, the expression of DNMT1 and DNMT3a mRNA is surprisingly high in the brain and methylation and demethylation occur rapidly. As the demethylation process most likely involves base excision repair this provides a mechanism by which 5-azaC may become integrated in DNA allowing subsequent DNMT capture.

#### 1.1.4.2. HDAC inhibitors

As described in the previous sections HATs acetylate histones whereas HDACs remove acetylation. HDAC inhibitors (HDACi) are considered promising potential therapeutics for treatment of psychiatric disorders (Szyf 2009). The commonly used mood stabiliser valproate has widespread effects besides functioning as a nonspecific class I and II HDAC inhibitor (Göttlicher et al. 2001; Tsankova et al. 2007).

The putative mechanism of HDACi is to tilt the balance of acetylation-deacetylation resulting in an increase in acetylation. This leads to hyperacetylation of histone tails and induction of genes that have previously been repressed (Szyf 2009). All identified HDACi block one or several classes of HDACs, which should consequently influence gene expression globally. However, microarray gene expression experiments have revealed that only a part of the transcriptome is activated or supressed by HDACi (Dannenberg & Edenberg 2006; Chiba et al. 2004; Lee et al. 2004). This is likely because the HDACs and HATs are targeted to specific genes and HDACi will only influence genes that are associated with HDACs and also targeted by HATs (Szyf 2009). As described in the sections above an increase in acetylation can neutralise the positive charge of the lysine and destabilises internucleosomal contacts which leads to chromatin decondensation (Shogren-Knaak et al. 2006). In addition, HDAC inhibition reduces global DNA methylation, DNMT1 protein levels, and its interaction with chromatin (Arzenani et al. 2011). The specific mechanisms are unknown. However, HDAC inhibition may lead to nucleosome-depleted regions, which prevents DNMT binding and specific

histone marks may also directly prevent binding of DNMTs. As transcription from a promoter increases this could lead to active promoter demethylation as described in the section above.

## 1.2. SCHIZOPHRENIA

Schizophrenia is a debilitating mental disorder with a lifetime risk of approximately 1% (Sullivan et al. 2012). Morbidity and mortality are high and the condition is associated with considerable costs for both affected individuals and society. Schizophrenia is not itself fatal but associated with increased risk for suicide as well as conditions that adversely affect health such as cardiovascular disease, metabolic syndrome, and insulin resistance (Pompili et al. 2008). Schizophrenia most commonly presents with delusions and auditory hallucinations late in adolescence or in early adulthood (Tandon et al. 2008). Sustained recovery occurs for less than 14% within five years following a psychotic episode (Robinson et al. 2004), while the long-term outcome is slightly better with an additional 16% with late phase recovery within 25 years (Harrison et al. 2001). In Europe less than 20% of individuals diagnosed with schizophrenia are employed (Marwaha et al. 2007). These outcomes can be improved by early diagnosis and medical intervention (Reser 2007). However, our basic understanding of the pathophysiology of schizophrenia is still limited and consequently there are no means for curative treatment or prevention (Insel 2010).

### 1.2.1. SYMPTOMATOLOGY

Schizophrenia is defined as a syndrome and characterised by a broad and heterogeneous symptom spectrum (WHO 1993). Patients typically suffer from three categories of symptoms: positive, negative, and cognitive (Nishioka et al. 2012). Positive symptoms include illusions, hallucinations, and paranoia. These symptoms are believed to result from malfunctional mesolimbic circuits involving nucleus accumbens, the main reward and motivation centre of the brain. Negative symptoms include anhedonia, lack of motivation, and social withdrawal and have been attributed to the prefrontal cortex (Stahl 2013). Cognitive symptoms include a broad

variety of deficits, affecting e.g. attention, association, learning, and memory (Coren et al. 1984). Consequently cognitive deficits involve a broad range of neuroanatomical regions (Henke 2010). As no biomarkers for diagnostic laboratory testing exist, the diagnosis is based on clinical assessment of symptoms according to the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) (A.P.A. 2013).

### 1.2.2. NEUROPATHOLOGY AND HYPOTHESIS OF SCHIZOPHRENIA

Despite intensive research the etiology of schizophrenia remains poorly understood. Occurrence of schizophrenia before puberty is rare and incidence increases in early adulthood (Tandon et al. 2008). The brain continues to develop until this stage and may be particularly vulnerable during this period. It is believed that schizophrenia may be partly explained by deficits in the course of neurodevelopment and that the deficits result from genetic and environmental risk factors (Rapoport et al. 2005; Sullivan et al. 2003; Insel 2010). Three major etiologic hypotheses have been proposed: the dopamine, the glutamate-gaba, and the neurodevelopmental hypothesis:

*The dopamine hypothesis* was based on observations of the psychosis-inducing effects of dopamine releasing drugs such as amphetamine, and the anti-psychotic efficacy of drugs that block the dopamine D2 receptor (Carlsson 1988). The early neuroleptic medications such as chlorpromazine and haloperidol have been replaced by “atypical” antipsychotics with fewer extra-pyramidal side effects but treatment efficacy has not improved (Lieberman et al. 2005). These antipsychotics reduce delusions and hallucinations but have not improved functional recovery, including the ability to work (Insel 2010).

*The glutamate-gaba hypothesis* originated from focus on cognitive symptoms. This was based on observations that low doses of NMDA receptor antagonists resulted in attention and memory problems in healthy individuals (Coyle 2006). The theory is that schizophrenia and especially the cognitive symptoms may result from

low NMDA receptor activity on GABAergic interneurons in the prefrontal cortex, which leads to disinhibition and hyperactivity of cortical pyramidal neurons.

*The neurodevelopmental hypothesis* proposes that developmental insults resulting from genetic and environmental risks, results in the emergence of psychosis in late adolescence or early adulthood (Insel 2010). More than two decades ago it was proposed that schizophrenia is a neurodevelopmental disorder or several disorders that involve alterations in brain circuits (Feinberg 1982; Weinberger 1987; Murray et al. 1991) and evidence supporting this hypothesis is growing. In most cases psychosis occurs between 18 and 25 years of age when the prefrontal cortex is still developing. However, our understanding on the normal and abnormal cortical development occurring during this period is limited. Neuroimaging studies have revealed decreases in grey matter density until mid-twenties and the prefrontal cortex is the last to mature (Paus et al. 2008). The cellular basis it not clear although post-mortem studies indicate that both synaptic elimination and increased myelination continues until this stage (Huttenlocher 1984; Insel 2010). Studies on non-human primate brains have revealed that refinement of circuits during early adulthood includes pruning of excitatory synapses, and proliferation of inhibitory circuits (Rakic et al. 1986; Hashimoto et al. 2009; Lewis & Gonzalez-Burgos 2008). Altogether these observations indicate that late stage brain maturation includes a calibration of excitatory-inhibitory balance in the cortex (Figure 4).

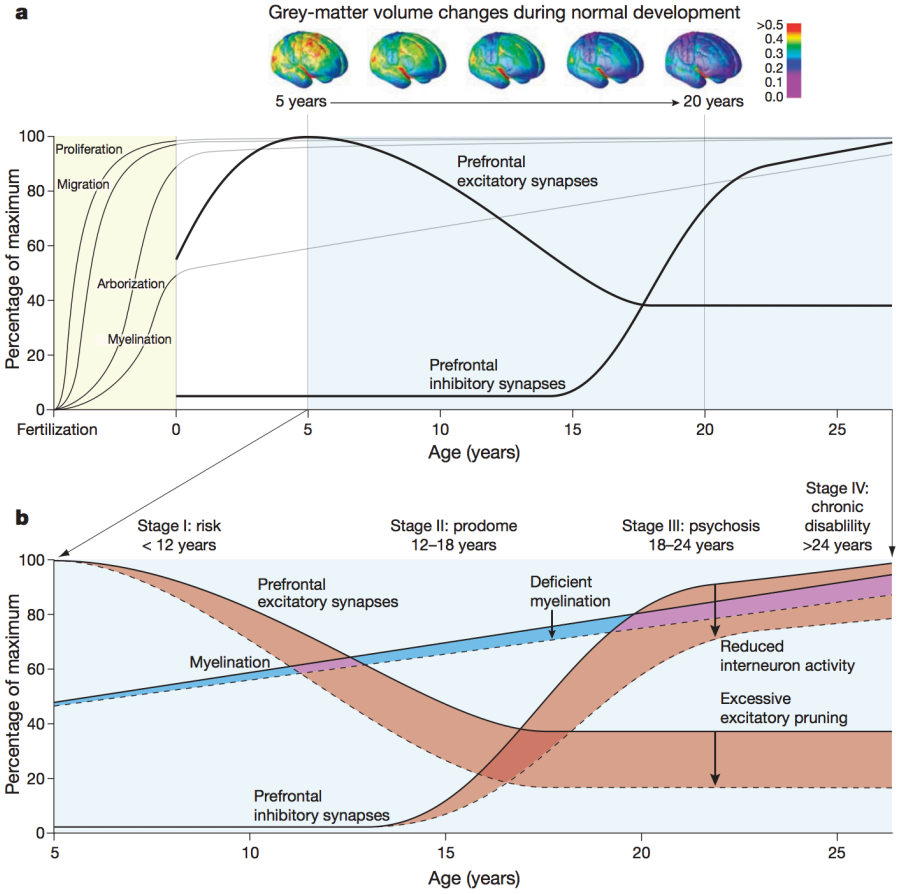


**Figure 4. Neurodevelopmental model of schizophrenia. a**, normal cortical development involves proliferation, migration, arborisation (circuit formation), and myelination. Proliferation and migration occur mostly prenatally whereas arborisation and myelination continues through the first two postnatal decades. The combined effects are thought to account for the progressive reduction of grey-matter volume. Data from human and non-human primate brain indicate increases in inhibitory and decreases in excitatory synaptic strength occurring in prefrontal cortex until early adulthood. These changes occur during the period of prodrome and at the time psychosis emergeces. **b**, the trajectory in children developing schizophrenia could include reduction of synapses from inhibitory interneurons in the prefrontal cortex and excessive pruning of excitatory pathways in the prefrontal cortex. A reduction of myelination could alter connectivity. Modified from (Insel 2010).

In support of the neurodevelopmental hypothesis, longitudinal population based studies have revealed that prodromal symptoms are evident long before onset of psychosis. It has been reported that adults with schizophrenia have delayed developmental milestones within the first year (Sørensen et al. 2010) and that children who later developed schizophrenia have reduced IQ (Woodberry et al. 2008; Reichenberg et al. 2010). Post-mortem studies have consistently reported a loss of GABA and reductions in enzymes responsible for glutamate biosynthesis, but these changes may represent the consequences of chronic illness or treatment, rather than the cause of schizophrenia (Coyle 2006). The challenges of separating cause and effect can be overcome by genetic analysis.

### 1.2.3. GENETIC RISK FACTORS

The strongest single predictor for risk of developing schizophrenia is familial history (Hallmayer 2000). Based on twin, family, and adoption studies the heritability of schizophrenia has been estimated to be 64% and 81%, respectively in two separate meta-analyses (Lichtenstein et al. 2009; Sullivan et al. 2003). The risk for schizophrenia has been estimated to be 46% when both parents have schizophrenia and the concordance rate for monozygotic twins is 48% (Gottesman 1990). These results are consistent with the view of schizophrenia as a complex trait that results from genetic and environmental etiological influences. The genetic studies initially included linkage studies, candidate gene association studies and assessments of larger structural variants. More recently, GWAS was introduced and accounts for an increasing number of studies. Family, linkage, and case-control studies have revealed genes including *COMT*, *DTNBp1*, *NRG1*, *RGS4*, *GRM3*, *G72*, *PP3CC*, *CHRNA7*, and *PRODH* as being risk factors for schizophrenia (Badner & Gershon 2002; Harrison & Weinberger 2005; Lewis et al. 2003; Munafò et al. 2006; Ng et al. 2009). GWAS have identified genes such as *ZNF804A*, *TCF4*, *NRGN*, and MIR137 (O'Donovan et al. 2008; Purcell et al. 2009; Ripke et al. 2011; Shi et al. 2009; Stefansson et al. 2009).

It has been debated whether genetic risk for schizophrenia is caused by interaction of several common variants each of very small effect (common disease – common variant model, (Purcell et al. 2009)) or by rare but highly penetrant genetic variants (common disease – rare variant model, (Stone et al. 2008)). In a recent GWAS analysis it was estimated that approximately 8300 independent SNPs contribute to risk (Ripke et al. 2013). However, such studies only include relatively common SNPs, and future next generation sequencing studies could potentially reveal rare SNPs, insertions or deletions of higher effect sizes.

Recently, a combined meta-analysis of 18 GWAS ensued by a family-based replication study of schizophrenia risk genes, identified the *BRD1* promoter SNP rs138880 as the variant showing the most significant association with the disease (Aberg et al. 2013). This augments previous studies linking rs138880 with both schizophrenia and bipolar disorders in large Caucasian case-control samples (Nyegaard et al. 2010; Severinsen et al. 2006) as well as the previous identification of a susceptibility locus containing *BRD1* in the Faroese population (Jorgensen et al. 2002). The function of *BRD1* and its possible implications in schizophrenia will be further described in upcoming sections.

Genetic linkage to the *CHRNA7* gene was first found to an endophenotype in schizophrenia, the P50 deficit (Freedman et al. 1997) and then to the disease itself. The *CHRNA7* gene, encoding the α7 nAChR, is located at chromosome 15q13.3, a region that has been identified as a candidate risk loci for schizophrenia (Leonard & Freedman 2006). SNPs in the promoter region of *CHRNA7* show significant association with schizophrenia (Stephens et al. 2009). The function of *CHRNA7* and its possible implications in schizophrenia will be further described in upcoming sections.

### 1.2.4. ENVIRONMENTAL RISK FACTORS

Epidemiological studies have established that certain environmental factors are associated with increased risk of schizophrenia. These risk factors are generally divided as early (prenatal until gestation) and late (childhood and early adulthood). The early risk factors for later development of schizophrenia include: Pre-natal virus infections, greater parental age, reduced nutrition, low socioeconomic class, urban birth, winter birth, premature birth, and delivery-related hypoxia (Brown et al. 2010; Perrin et al. 2007; Torrey et al. 1997; Nishioka et al. 2012). The late risk factors include: Social stress, childhood abuse, urbanicity, migration, and cannabis abuse (van Winkel et al. 2008; Fisher et al. 2014; Pedersen & Mortensen 2001; Cantor-Graae & Selten 2005; Moore et al. 2007). The early phase risk factors are very likely to affect normal neurodevelopment whereas the late factors may be particularly important for predisposed individuals.

### 1.2.5. EPIGENETIC REGULATION IN PSYCHIATRIC DISORDERS

A possible role of DNA methylation in the pathogenesis of schizophrenia was suggested decades ago after clinical studies revealed that treatment with the methyl-donor SAM elicited psychotic episodes in some patients with schizophrenia (Antun et al. 1971). Postmortem studies have revealed that DNA methylation changes are present in brains of schizophrenics. Studies have reported hypermethylation of the *Reelin* promoter and downregulation of expression in several brain regions (Abdolmaleky et al. 2005; Grayson et al. 2005). Reelin is a glycoprotein that is expressed during development and in adult GABAergic neurons and is important for proper neural positioning during brain development. In addition, methylation changes have been reported for *COMT* (Abdolmaleky et al. 2006) and *SOX10* (Iwamoto et al. 2005). One of the challenges with post-mortem studies is that the changes may represent the consequences of chronic illness or treatment, rather than the cause of schizophrenia (Coyle 2006). Nevertheless, reversal of such changes could be important for relieving symptoms and could be possible with pharmaceuticals targeting epigenetic mechanisms, as described above.

Epigenetic changes may also result from genetic alterations and as described above the number of schizophrenia-associated genomic loci is growing. The majority of these GWAS-identified markers do not directly alter protein sequence but seem more important for regulating gene expression, as disease-associated variants affect transcription factor recognition sequences and frequently alter allelic chromatin states (Maurano et al. 2012). In human brain samples it has been established that methylation of a high number of CpG sites show significant cis associations with SNPs and a lower number show significant trans associations (Zhang et al. 2010a). For the schizophrenia associated *DRD4* gene four SNPs showed significant associations with DNA methylation (Docherty et al. 2012).

In a recent study, methylomic variation was profiled in prefrontal cortex from schizophrenia patients and controls (Pidsley et al. 2014). Disease-associated differential DNA methylation was identified at multiple loci. Importantly, the loci co-localize with genes important for neurodevelopment and genes that have been genetically linked with schizophrenia. In addition, the schizophrenia-associated differentially methylated positions were enriched for loci at which DNA methylation is dynamically regulated during human fetal brain development (days 23-184 post-conception). Thus, genetic variants could potentially lead to altered gene expression in a mechanism involving epigenetic regulation.

### 1.2.6. CANDIDATE GENES

Much effort is put into identification of risk factors for schizophrenia and twin and adoption studies have demonstrated a clear inherited component (Ripke et al. 2014). The SNPs associated with schizophrenia that have been identified in GWAS are statistically significantly associated with the disorder but mechanistic studies are needed to reveal their functional contribution to pathogenesis. Generally, the identified markers seem to be more important for regulating gene expression rather than direct alteration of protein sequence (Maurano et al. 2012). In this dissertation particular attention is given to *BRD1* and *CHRNA7*, two genes that are associated with the disease but whose mechanisms are very different.

### *1.2.6.1. BRD1*

*BRD1* encodes the bromodomain-containing protein 1 (BRD1), a transcription factor widely expressed in human tissues including the brain, that has been identified in protein complexes possessing acetyltransferase activity specific for histone H3 (Doyon et al. 2006; Mishima et al. 2011). BRD1 has been found to bind in several genomic regions including promoter regions and to regulate expression of a large number of genes (Fryland et al., manuscript submitted). Importantly, a high number of these genes are schizophrenia risk genes and the majority are part of signalling pathways important for neurodevelopment, emphasising the possible involvement of *BRD1* in a polygenic disease such as schizophrenia. BRD1 is important in embryogenesis as knockout of *Brd1* in mice leads to impaired eye development and neural tube closure and ultimately a lethal maturation defect in embryonic hematopoiesis (Mishima et al. 2011). Adult *Brd1*$^{+/-}$ mice display dysregulated cerebral gene expression, behavioural abnormalities translating to the major hallmarks of schizophrenia, and neurochemical alterations involving dopamine levels and glutamate and GABAergic signalling (Qvist et al., manuscript submitted). In the developing fetal pig brain *BRD1* is highly expressed at early embryonic stages and expression then declines in the later stages (Severinsen et al. 2006). An important role for BRD1 in the adult brain is suggested by regulation after both ECS (Fryland et al. 2012) and chronic restraint stress (Christensen et al. 2012). Notably, *Brd1* mRNA and protein is upregulated in the hippocampus following 21 days of chronic restraint stress in rats, suggesting involvement in regulatory processes underlying adaptation to stress in the mature CNS. Thus, with the overall functions of *BRD1*, dysregulation of the gene could result in neurodevelopmental deficits.

### *1.2.6.2. CHRNA7*

The *CHRNA7* gene, encoding the α7 nAChR, is located at chromosome 15q13.3, a region that has been identified as a candidate risk loci for schizophrenia (Leonard & Freedman 2006). SNPs in the promoter region of *CHRNA7* show significant association with schizophrenia (Stephens et al. 2009) and several rare promoter SNPs decrease promoter activity (Leonard et al. 2002). Notably, these promoter

SNPs are associated with failure to inhibit the P50 auditory evoked potential response, a deficit that is found in most schizophrenics and 50% of their first-degree relatives (Leonard et al. 2002). The α7 nAChR is a homopentameric ligand-gated channel with high permeability for $Ca^{2+}$ that presynaptically increases neurotransmitter release from specific terminals and postsynaptically affects gene expression (Albuquerque et al. 2009; Araud et al. 2011). The receptor is widely distributed in human tissues including the brain (Albuquerque et al. 2009; Dani & Bertrand 2007).

Estimated 80% of schizophrenics are smokers and an idea of using the α7 nAChR as a target for treatment of cognitive dysfunction arose when smoking was discovered to normalise sensory processing deficits in schizophrenic patients (Adler et al. 1993; Olincy et al. 1998; Leonard et al. 2007) and improve cognitive deficits (Levin et al. 2006; Rezvani & Levin 2001). The α7 nAChR is now considered a promising drug target for treatment of cognitive dysfunction (Thomsen et al. 2010; Wallace & Porter 2011) and α7 nAChR agonists have been reported to produce improvements in memory and executive functions in both schizophrenics and healthy volunteers (Preskorn et al. 2014; Kitagawa et al. 2003; Freedman et al. 2008; Olincy et al. 2006).

## 1.3. DEPRESSION

Major depressive disorder is characterised by one or more major depressive episodes and has a lifetime prevalence of more than 16% for the general population (Kessler et al. 2003). The disease accounts for 12.1% of total years lived with disability and remains a major health problem worldwide (Ustün et al. 2004). Patients present with rather variable symptoms such as: depressed mood, anhedonia, feelings of guilt or low self-worth, poor concentration, and disturbances in sleep and appetite (Nestler et al. 2002). Accordingly, depression should be considered a heterogeneous syndrome comprised of numerous diseases of distinct causes and pathophysiologies.

While our understanding of pathophysiology is still limited, it is well established that genetic and environmental factors are important players in vulnerability for

depression (Nestler et al. 2002). The genetic contribution has been estimated to be 37% (Sullivan et al. 2000). Common environmental influences have very small effects, while individual-specific environmental influences was estimated to be 63% (Sullivan et al. 2000). Although there have been several reports of genetic variants associated with major depression (López-León et al. 2008; van Rossum et al. 2006; Wray et al. 2012), the findings have been difficult to replicate. In a recent mega-analysis of GWAS for major depressive disorder there were no robust and replicable findings (Ripke et al. 2013). This is most likely because the high prevalence of major depressive disorder causes the sample to be underpowered to detect genetic effects typical for complex traits (Ripke et al. 2013).

Both genetic variation and environmental factors are known to influence epigenetic mechanisms (Klengel et al. 2014). By investigating epigenetic changes it may be possible to get an integrated view of how genetic and environmental factors alter risk (Menke & Binder 2014). There have been several findings of epigenetic changes related to depression and particularly exposure to stressful early life events have been studied. Early life stress is associated with increased DNA methylation levels in the promoter region of *NR3C1*, encoding the glucocorticoid receptor (Perroud et al. 2011; McGowan et al. 2009; Melas et al. 2013). Normally glucocorticoid receptors on neurons in the hippocampus and paraventricular nucleus of the hypothalamus exert negative feedback to the hypothalamic–pituitary–adrenal axis and it has been suggested that hypermethylation and reduced gene expression could lead to increased cortisol levels in stressful situations (Perroud et al. 2011). The serotonin transporter gene *SLC6A4* has also been extensively studied. It has been found that stressful job situations are associated with decreased promoter methylation (Alasaari et al. 2012) whereas in depressed patients increased promoter methylation is associated with childhood adverse events and worse clinical presentation (Kang et al. 2013a). In addition, a study of monozygotic twins revealed that promoter methylation was positively correlated with depressive symptoms (Zhao et al. 2013). *BDNF* has also been extensively studied. BDNF is a neurotrophin and is involved in processes such as proliferation, migration, differentiation, and cell survival (Huang & Reichardt 2001). *BDNF* is particularly interesting due to its

strong association with depression and its induction by antidepressants (Murphy et al. 2013). Increased methylation of the *BNDF* promoter has been associated with suicidal attempt history (Kang et al. 2013b) and the same has been observed in post-mortem brain samples (Keller et al. 2010).

Current pharmaceutical treatments are only effective in approximately 60-65% patients and require long-term administration to achieve therapeutic effects (Schloss & Henn 2004). The clinical efficacy of ECT ranges from 85-90%, and generally also requires repeated administration (Schloss & Henn 2004). The slow onset of therapeutic effects suggests that long term adaptions are required and these might involve epigenetic regulation. The high therapeutic efficacy of ECT has triggered a large number of studies attempting to identify new drug targets. These studies have not resulted in identification of new therapeutic mechanisms and consequently the treatment is still widely used. Unfortunately, ECT is also associated with adverse effects and these are also being intensively studied.

### 1.3.1. ECS: NEURONAL DEPOLARISATION AND GENE EXPRESSION

Electroconvulsive therapy (ECT) remains one of the most effective treatments of severe depression (Berton & Nestler 2006). Clinical efficacy ranges from 85-90%, whereas for pharmaceutical antidepressants it is only 60–65% (Schloss & Henn 2004). Electroconvulsive stimulation (ECS), an animal model of ECT, causes a widespread release of neurotransmitters, which affects a variety of transporters and receptors in the brain (Schloss & Henn 2004). The therapeutic mechanism likely involves cellular changes, including increased neurogenesis, increased cell proliferation, fiber sprouting, and enhanced synaptic signalling all of which have been observed after ECS (Schloss & Henn 2004). Such changes have been suggested to be orchestrated at the level of gene expression and the first studies of ECS-induced gene expression were published decades ago (Leviel et al. 1990; Winston et al. 1990; Xie et al. 1989; Zawia & Bondy 1990). It has been speculated that chromatin remodelling at gene promoter regions can mediate acute and chronic effects on gene activity (Tsankova et al. 2004).

Unfortunately, adverse effects such as cognitive and memory deficits have been reported after ECT, making it the last option only when pharmaceutical antidepressants are ineffective (Fava & Kendler 2000). Expression of immediate early genes (IEGs), including *Arc* is important in regulating neuronal plasticity during memory formation and consolidation (Abraham et al. 1994; Guzowski et al. 2000; Bramham et al. 2009). In cortical neurons the transcription of *Arc* is tightly coupled to stimuli triggering neuronal depolarisation (Link et al. 1995; Lyford et al. 1995). *Arc* mRNA accumulates at the dendritic arbor of stimulated neurons and translation of ARC protein occurs specifically at active synapses (Link et al. 1995; Lyford et al. 1995; Wallace et al. 1998; Bagni et al. 2000).

In mice, ECS causes rapid active DNA demethylation or *de novo* methylation at a high number of CpG sites in dentate granule neurons (Guo et al. 2011b). Pre-treatment of the mice with a highly selective NMDA receptor antagonist abolished ECS-induced changes, which confirm that these epigenetic changes are neuronal activity-dependent. In addition, infusion of DNMT inhibitors abolishes activity-induced *de novo* methylation with no obvious effect on demethylation. Combined with the knowledge described above about the importance of DNA methylation in memory formation, this observation emphasises that DNA methylation could cause memory deficits following ECT.

# 2. OBJECTIVES

This research aimed to investigate the involvement of DNA methylation in etiology and treatment of psychiatric disorders.

Study I aimed to evaluate,

- The potential role of DNA methylation in regulating *BRD1* transcription.
- Possibility of modulating transcription by DNMT inhibition.
- The influence of SNP rs138880 on transcriptional regulation.
- The effect of common mood stabilisers on *BRD1* transcription.

Study II aimed to evaluate,

- The potential role of DNA methylation in regulating *CHRNA7* transcription.
- Possibility of modulating transcription by HDAC inhibition
- Involvement of DNA methylation in *CHRNA7* regulation in-vivo

Study III aimed to evaluate,

- Spatiotemporal profiles of Arc and c-Fos protein expression
- Epigenetic regulation of *Arc* and *c-Fos*

Study IV aimed to evaluate,

- The temporal gene expression profiles for several target genes after acute electroconvulsive stimulation
- (This study was primarily set up for investing *Arc*, *Dnmt1* and *Dnmt3a* expression for use in study V).

Study V aimed to evaluate,

- The temporal gene expression profiles for *Arc*, *Dnmt1*, and *Dnmt3a* after acute electroconvulsive stimulation
- The potential of blocking DNMT activity in order to prevent *de novo* methylation of *Arc*.
- DNA methylation of the *Arc* promoter and intragenic region at a timepoint later than previously observed (48 hours instead of 24 hours) after acute electroconvulsive stimulation

# 4. RESULTS

## STUDY I

**DNA methylation regulates *BRD1* and is increased by the schizophrenia associated SNP rs138880**

Mads Dyrvig, Per Qvist, Jacek Lichota, Knud Erik Larsen, Mette Nyegaard, Anders D. Børglum, and Jane H. Christensen

This manuscript is in preparation

# STUDY II

**DNA methylation regulates *CHRNA7* transcription in human cortical tissue and can be modulated by HDAC inhibitor valproate in human cell lines**

Mads Dyrvig, Jens D. Mikkelsen, and Jacek Lichota

This manuscript is in preparation

## STUDY III

**Epigenetic regulation of *Arc* and *c-Fos* in the hippocampus after acute electroconvulsive stimulation in the rat**

Mads Dyrvig, Henrik H. Hansen, Søren H. Christiansen, David P.D. Woldbye, Jens D. Mikkelsen, Jacek Lichota

This manuscript is published in: Brain Research Bulletin 88 (2012) 507–513

Brain Research Bulletin 88 (2012) 507–513

Contents lists available at SciVerse ScienceDirect

Brain Research Bulletin

journal homepage: www.elsevier.com/locate/brainresbull

Research report

# Epigenetic regulation of *Arc* and *c-Fos* in the hippocampus after acute electroconvulsive stimulation in the rat

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ARTICLE INFO

*Article history:*
Received 15 March 2012
Received in revised form 4 May 2012
Accepted 4 May 2012
Available online 18 May 2012

*Keywords:*
Histone acetylation
DNA methylation
Depression
Electroconvulsive seizure

ABSTRACT

Electroconvulsive stimulation (ECS) remains one of the most effective treatments of major depression. However, the underlying molecular changes still remain to be elucidated. Since ECS causes rapid and significant changes in gene expression we have looked at epigenetic regulation of two important immediate early genes that are both induced after ECS: *c-Fos* and *Arc*. We examined *Arc* and *c-Fos* protein expression and found *Arc* present over 4 h, in contrast to *c-Fos* presence lasting only 1 h. Both genes had returned to baseline expression at 24 h post-ECS. Histone H4 acetylation (H4Ac) is one of the important epigenetic marks associated with gene activation. We show increased H4Ac at the *c-Fos* promoter at 1 h post-ECS. Surprisingly, we also observed a significant increase in DNA methylation of the *Arc* gene promoter at 24 h post-ECS. DNA methylation, which is responsible for gene silencing, is a rather stable covalent modification. This suggests that *Arc* expression has been repressed and may consequently remain inhibited for a prolonged period post-ECS. *Arc* plays a critical role in the maintenance phase of long-term potentiation (LTP) and consolidation of memory in the rat brain. Thus, this study is one of the first to demonstrate DNA methylation as a regulator of ECS-induced gene expression and it provides a molecular link to the memory deficits observed after ECS.



## 1. Introduction

Electroconvulsive therapy (ECT) represents one of the most effective treatments against severe depression (Berton and Nestler, 2006). The mechanism of action may involve gene expression and it has been speculated that chromatin remodeling at gene promoter regions mediate acute and chronic effects on gene activity after electroconvulsive stimulation (ECS) (Tsankova et al., 2004), an experimental model of ECT. Some of the affected genes and their associated epigenetic modifications may be beneficially involved in recovery whereas others may be adverse. So far little attention has been paid to the identification and alleviation of adverse effects associated with the treatment, the most prominent of which are negative effects on memory and cognitive functions (Fava and Kendler, 2000).

DNA methylation and histone modifications play an important role in setting up either permissive or repressive environments for transcriptional machinery (Dulac, 2010). The histone code hypothesis suggests that specific modifications of one or more histone tail residues at particular promoter regions are read sequentially or summed up, thereby defining the epigenetic state of the gene (Jenuwein and Allis, 2001). Histone acetylation, especially histone H4 (H4Ac), is a hallmark of an open, transcriptionally active chromatin (Renthal et al., 2007). In addition, H4Ac has previously been found to correlate well with the expression of *c-Fos*, *Bdnf*, and *Creb* mRNA at several time points after acute and chronic ECS (Tsankova et al., 2004). While histone acetylation is a modification that can be dynamically regulated in the CNS (Levenson et al., 2004), DNA methylation has been thought to be involved in setting up transcriptional landscapes only in mitotically active cells. However, there is growing evidence that DNA methylation is a very dynamic modification in the CNS (Feng et al., 2010; Levenson et al., 2006; Martinowich et al., 2003; Miller and Sweatt, 2007). Unlike other post-mitotic cells, neurons express DNA methyltransferase 1 (DNMT1) and this enzyme is involved in DNA repair, neurodegeneration (Brooks et al., 1996; Endres et al., 2000, 2001; Fan et al., 2001) and cognitive disorders such as schizophrenia, Rett syndrome and

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http://dx.doi.org/10.1016/j.brainresbull.2012.05.004

Fragile X mental retardation (Amir et al., 1999; Sutcliffe et al., 1992; Veldic et al., 2004).

Expression of immediate early genes (IEGs), including activity-regulated cytoskeleton-associated protein (*Arc* also known as *Arg3.1*) and *c-Fos* play important roles in regulating neuronal plasticity during memory formation and consolidation (Abraham et al., 1994; Bramham et al., 2009; Kaczmarek, 1992). *Arc* was initially identified in cortical neurons as an IEG with its transcription being tightly coupled to stimuli triggering neuronal depolarization (Link et al., 1995; Lyford et al., 1995). While many IEGs serve specifically as transcription factors, *Arc* mRNA is accumulated at the dendritic arbor of stimulated neurons (Link et al., 1995; Lyford et al., 1995; Wallace et al., 1998). Thus, translation of the *Arc* protein is specifically targeted to occur at active synapses (Bagni et al., 2000; Wallace et al., 1998), which is considered the molecular basis for *Arc*-mediated long-term synapse-specific modifications (Rodríguez et al., 2005). Notably, *Arc* mRNA is also induced in hippocampal and neocortical neurons after behavioral learning paradigms and *Arc* protein expression plays a critical role in the maintenance phase of long-term potentiation (LTP) and consolidation of memory in the rat brain (Guzowski et al., 2000). Similarly, *c-Fos* knockout mice display defects in long-term memory (Fleischmann et al., 2003).

Both *Arc* and *c-Fos* expression are increased in the hippocampal formation following ECS (Larsen et al., 2005; Lyford et al., 1995; Steward and Worley, 2001; Wallace et al., 1998; Woldbye et al., 1996). Interestingly, we previously found reduced *Arc* gene expression in the dentate gyrus 24 h after acute ECS and in the CA1 24 h after chronic ECS (Larsen et al., 2005), brain regions that are centrally involved in memory consolidation (Ramirez-Amaya, 2005). Considering the importance of *Arc* mRNA and protein in networks that underlie information processing and memory, this decrease might represent a molecular cause of memory and learning deficits, observed in rats as a consequence of ECS (Andrade et al., 2011; Luo et al., 2011; Yao et al., 2010). Similarly, deficits in memory are seen in depressed patients undergoing ECT. In this report, we explored epigenetic regulation of hippocampal *Arc* and *c-Fos* as well as spatiotemporal profile changes of both proteins following acute ECS.

## 2. Materials and methods

### 2.1. Animals and treatment

Adult male Sprague-Dawley rats (270–290 g, Charles River Laboratories, Hamburg, Germany) were used. All experiments were conducted in accordance with guidelines of the National Institute of Health (NIH Publications No. 85-23, 1985) and the Animal Experimentation Inspectorate, Ministry of Justice, Denmark. Animals were given a single ECS via ear clip electrodes (50 mA, 0.5 s, unidirectional square wave pulses; (Woldbye et al., 1996)). Sham animals were handled similarly but without the passing of current. The experiment was terminated following a described post-ECS period for in situ hybridization and histochemical analyses (1 h, 4 h, 24 h), for ChIP and DNA methylation analyses (1 h and 24 h), and for immunohistochemical analyses (10 min, 1 h, 4 h, 8 h, 24 h).

### 2.2. In situ hybridization

In situ hybridization was performed as described previously (Larsen et al., 2005) using synthetic oligonucleotide DNA probes complimentary to the rat *Arc* (targeting bases 789–839 (Pei et al., 2004)) and *c-Fos* (targeting bases 133–180 (Madsen et al., 1999)) (DNA Technology, Aarhus, Denmark). The sections were dried and exposed together with $^{14}C$ standards to a Kodak BiomaxMR film (Sigma Aldrich) for 2 weeks. Optical densities were quantified in the granular cell layer and molecular layer using a computer image analysis system (QuantityOne v.4.5.2, BioRad, Hercules, CA). The definition of these areas was based on well-defined landmarks in the same section, in particular in sections with low level of expression in animals 24 h post-ECS. The individual value for each animal was the average of three individual sections measured bilaterally within the areas of interest. Data were analyzed using one-way ANOVA followed by Newman Keul's post hoc test.

### 2.3. Chromatin immunoprecipitation (ChIP)

Dissected hippocampi were chopped into smaller pieces, transferred to an Eppendorf tube and subsequently incubated 15 min on ice in 1.5 mL 1% paraformaldehyde/PBS. The crosslinking procedure was terminated by addition of 125 mM glycine for a minimum of 5 min, then followed by 4 washes in 750 μL PBS with protease inhibitors (Complete Protease Inhibitor Cocktail (Roche, Switzerland)). The ChIP assay was performed as described previously (Tsankova et al., 2004) with minor modifications. The samples were split into 600 μL chromatin portions. 6 μL antibody directed against H4 acetylated at Lys5, Lys8, Lys12, and Lys16 (Upstate #06-866) was added. For mock control, rabbit serum was used. The samples were incubated overnight at 4 °C with gentle agitation. The immune complexes were collected using 50 μL blocked Dynabeads® Protein A by incubating for 3 h at 4 °C with gentle agitation. The beads were collected by magnetic force, washed once with 1 mL for 10 min with each of these buffers: low salt buffer (150 mM NaCl, 1% SDS, 1% triton X-100, 2 mM EDTA, 20 mM HEPES pH 8), 1 mL high salt buffer (like low-salt but 500 mM NaCl), 1 mL LiCl wash buffer (0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM HEPES pH 8) and twice with 1 mL TE buffer (10 mM Tris–HCl pH 8, 1 mM EDTA). The immune complexes were eluted twice with 250 μL elution buffer (1% SDS, 0.1 M $NaHCO_3$) for 15 min, 65 °C. The cross linking was reversed by addition of 20 μL 5 M NaCl, 12 h at 65 °C. DNA was purified by ChIP DNA Clean & Concentrator™ (Zymo Research, USA) according to manufacturer's protocol. All PCR amplifications were performed in 20 μL reaction volume using SYBR-Green Brilliant II master mix (Stratagene, USA). Following program was used: 95 °C 10 min, 40 cycles: 95 °C 30 s, 60 °C 30 s. The relative quantities of DNA in the analyzed samples were calculated by the Pfaffl method (Pfaffl, 2001). The primers to amplify the *Arc* promoter, *c-Fos* promoter and the control housekeeping gene *Beta-actin* were: *Arc* fwd GACAAGGCAGAGGAGAGTGTC, *Arc* rev CCGGAGTGACTAATGTGCTCT, *c-Fos* fwd TTCTCTGTTCCGCTCATGACGT, *c-Fos* rev CTTCTCAGTTGCTAGCTGCAATCG, Beta-actin fwd GTGGCACCACCATGTACCCAGGCAT, Beta-actin rev ACTACAGGGCTGACCACACCCCACT for each primer set applied, the immunoprecipitated samples were amplified on the same plate and all determination was carried out in triplicates in one qPCR run. Data were normalized with the house-keeping gene *Beta-actin* and analyzed by GraphPad Prism software using unpaired *t*-test.

### 2.4. DNA methylation analysis

Hippocampal DNA was extracted with a DNeasyBlood & Tissue kit (Qiagen, Germany), following manufacturer's instructions for purification of total DNA from animal tissues (Spin-Column Protocol). All DNA samples were bisulfite converted with an EpiTect® Bisulfite kit (Qiagen), according to the manufacturer's protocol for sodium bisulfite conversion of unmethylated cytosines. 10 μL of bisulfite converted DNA was used as template in a 20 μL PCR reaction mixture with the following primers: *c-Fos* fwd TAATTGTGAATATTTATAGGTGAAAGTTAT, *c-Fos* rev ACTCTATCCAATCTTCTCAATTACTAA, *Arc* fwd GGAGAGTGTTTTTGGTTTTTAAGATT, *Arc* rev CTCAAACTAAAAAAACCCAAAAACTA. TrueStart polymerase (Fermentas, Lithuania) was used in the following PCR program: 95 °C for 5 min, followed by 35 cycles of 95 °C for 1 min, 55 °C for 2:30 min, 68 °C for 1 min, and a final step of 68 °C for 5 min. PCR products were isolated from agarose gel with a NucleoSpin® Extract II Kit (Macherey-Nagel, Germany) and cloned using the InsTAclone™ PCR Cloning Kit (Fermentas, Lithuania). Selected plasmid DNA were extracted with a NucleoSpin® Plasmid kit (Macherey-Nagel, Germany) and 1.5 μg of each prep was freeze-dried and sent to Beckman Coulter Genomics (United Kingdom) for sequencing. Data were analyzed by GraphPad Prism software using one-way ANOVA with Newman Keul's post hoc test.

### 2.5. Western blotting

The polyclonal *Arc* antibody (Thomsen et al., 2008) was validated on western blotting. Samples were homogenized in a lysis buffer (137 mM NaCl, 20 mM Tris, 1% NP-40, 10% glycerol, 48 mM NaF, $H_2O$, 2× complete inhibitor cocktail (Roche, Indianapolis, IN) and 2 mM $Na_2VO_3$), incubated on ice (>15 min), centrifuged (16,100 × *g*, 15 min) and the supernatants collected. Samples (50 μg total protein) were separated by 4–12% gradient SDS-PAGE and western blotting was performed using *Arc* antisera (1:1000–2000). Specifically bound antibody was detected by incubation with either alkaline phosphatase or horseradish peroxidase-conjugated (1:50,000; GE Healthcare, Piscataway, NJ) anti-rabbit antibody, respectively.

### 2.6. Immunohistochemical analysis

Rats were deeply anaesthetized with pentobarbital (mebumal 50 mg/mL, 3.0 mL/kg, SAD, Copenhagen, Denmark) and perfused transcardially with PBS followed by 4% paraformaldehyde in 0.1 M phosphate buffer for 10 min. The forebrains were immersed in fixative overnight and subsequently submerged in 30% sucrose in PBS at 4 °C for three days. 40 μm serial coronal sections were cut through the forebrain in series of five on a freezing microtome and representative sections were processed for *c-Fos* immunoreactivity. The sections were rinsed for 3× 10 min in 0.01 M PBS, incubated for 10 min in 1% $H_2O_2$–PBS to block endogenous peroxidase activity, and for a minimum of 20 min in 0.01 M PBS with 0.3% Triton X-100 (TX), 5% swine serum, and 1% bovine serum albumin (BSA) to block non-specific binding sites.

The sections were then incubated at 4 °C for 24 h in the primary antiserum diluted 1:1000 (*Arc*; #8541 bleed 3) or 1:4000 (*c-Fos*), respectively, in 0.01 M PBS with 0.3% Triton X-100 and 1% BSA. After incubation in primary antibody, immunoreactivity was detected by the avidin–biotin method using diaminobenzidine as chromagen. The sections were washed in 0.01 M PBS with 0.1% TX (PBS-TX), and incubated for 60 min in biotinylated donkey anti-rabbit (Jackson Laboratories, Ben Harbor, ME) diluted 1:2000 (for *Arc*) or 1:4000 (for *c-Fos*), respectively, in PBS-TX with 0.3% BSA, washed again and transferred to the avidin–biotin ABC complex (Vector Laboratories, Burlingame, CA) diluted 1:250 in PBS-TX and 0.3% BSA. After a careful wash in PBS-TX, and in Tris–HCl (pH 7.6) for 2× 10 min, the sections were incubated in 0.05% diaminobenzidine (Sigma, St. Louis, MI, USA) with 0.05% $H_2O_2$ in Tris–HCl buffer for 10 min and then washed twice in PBS buffer. The sections were mounted on gelatinized glass slides, dried, and coverslipped in Pertex.

## 3. Results

To confirm our previous findings (Larsen et al., 2005; Woldbye et al., 1996) we initially used in situ hybridization histochemical analysis of *Arc* and *c-Fos* mRNA expression. For *Arc* this confirmed expression in the granule cell layer seen up to 4 h after the strong stimulus. As reported earlier (Link et al., 1995), *Arc* mRNA was detected not only over the soma of the granule cell layers, but also in the molecular layer reflecting the translocation to the dendrites of these cells. For *c-Fos*, the expression of mRNA peaked at 1 h and declined to sham levels at 4 h (Sup. Fig. 1).

### 3.1. Histone H4 acetylation (H4Ac) at Arc and c-Fos promoters

The ChIP assay was performed on chromatin isolated from hippocampus 1 h and 24 h post-ECS and compared to chromatin extracted from hippocampus from rat under sham conditions. Regulation of H4Ac levels of the specific DNA fragments were calculated after qPCR as the ratio of normalized mean quantities for the ECS samples to the normalized mean quantity of the sham samples from each set of animals. As for the *c-Fos* promoter, a significant increase in H4Ac was found 1 h post-ECS, with a return to sham levels 24 h after ECS (Fig. 1A). In contrast, the *Arc* promoter showed no significant changes in H4Ac at any time point investigated, suggesting that this modification is not involved in transcriptional activation of *Arc* (Fig. 1B).

### 3.2. DNA methylation of Arc and c-Fos Promoters

Promoter DNA methylation, another epigenetic factor influencing gene expression, was investigated. DNA isolated from the same set of animals (1 h and 24 h post-ECS and corresponding sham) was subjected to bisulfite conversion. The proximal promoter region of *c-Fos* ranging from nucleotides −163 until +16 comprised 12 CpG sites that can potentially be methylated. Investigation of the *c-Fos* promoter revealed hypomethylation at these sites for sham controls as well as for ECS-treated animals at both 1 h and 24 h post-ECS (data not shown).

The promoter region of the *Arc* gene, ranging from −242 to +31, comprising 30 CpG sites (Fig. 2A), was likewise analyzed for DNA methylation in all groups. We analyzed 60 clones for each groups of animals and the percentage of methylated clones in each group was compared. Clones with one or more methylated sites were counted as "methylated," and each value was divided by the total number of clones. Interestingly, at 24 h post-ECS a strong significant promoter methylation was observed compared to sham controls while a slight tendency toward an increase was observed at 1 h post-ECS. The *Arc* promoter was not heavily methylated in sham rats showing only about 22% methylated clones whereas methylation reached more than 51% methylated clones at 24 h post ECS (Fig. 2B). The distribution of the methylation on the individual CpG sites in the analyzed promoter sequence is depicted in Fig. 2C. The bisulfite conversion efficiency was calculated based on conversion rates of non-CpG cytosines and was higher than 98% for both genes.

### 3.3. Raising novel antiserum against recombinant Arc

To confirm that the epigenetic modifications did not only influence gene transcription but also expression of the proteins we decided to investigate spatiotemporal changes in hippocampal *Arc* and *c-Fos* protein.

Firstly, the novel antiserum raised against recombinant *Arc* was characterized. When applying antiserum from rabbits immunized with recombinant *Arc* protein to Western blotting procedures, the selected antiserum stained a single band of approximately 55 kDa in a protein lysate of hippocampal crude synaptic vesicles (Fig. 3A). The same antiserum recognized a single band with similar molecular weight in PC12 cells and the band density increased following incubation with NGF (Fig. 3B), Also, hippocampus extracts from animals treated with ECS contained high levels of *Arc*-immunoreactivity compared to sham as revealed by Western blots (Fig. 3B). Pre-absorption with the immunizing recombinant protein completely blocked the immunoreactivity (Fig. 3B).

### 3.4. The temporal profiles of Arc and c-Fos gene expression after ECS

Under sham conditions, only the granular layer of the dentate gyrus contained single *Arc*-expressing cells, while no immunoreactivity could be detected in the molecular layer, hilus, stratum lacunosum-moleculare, and stratum radiatum. Staining of sections from animals exposed to ECS revealed a strong immunoreaction in the dentate granule cells at both 1 and 4 h after the stimulation and a lower level of staining was observed 8 h after the stimulation (Fig. 4; left panel). No difference from sham was observed 10 min after ECS. There were no positive cells detected in the dentate gyrus under sham conditions (24 h). In comparison, ECS-induced *c-Fos* protein expression followed a different time course as the stimulatory effect on *c-Fos* expression peaked at 1 h post-ECS, gradually declining at 4 h and returning to basal levels at 8 h (Fig. 4; right panel).

The robust staining intensity in hippocampal sections was observed after both immunohistochemistry (Fig. 4) and in situ hybridization (Sup. Fig. 1). The time profile in *Arc* mRNA levels was comparable to the protein profile with expression in the granule cell layer seen up to 4 h after the strong stimulus (Fig. 4; left panel and Sup. Fig. 1). Similarly, the expression of *c-Fos* mRNA peaked at 1 h and declined to sham levels at 4 h (Sup. Fig. 1). As reported earlier (Link et al., 1995), *Arc* mRNA was detected not only over the soma of the granule cell layers, but also in the molecular layer reflecting the translocation to the dendrites of these cells (Sup. Fig. 1).

### 3.5. Differences in neuronal expression of Arc and c-Fos

While acute ECS did not induce *Arc* expression in the hilus at any time point, protein expression of *c-Fos* in the hilus was observed 4 h post-ECS (Fig. 4). The temporal profiles of expression were only analyzed over the dentate granular layer, where the two markers are strongly induced. Notably, in the same hilar cells *c-Fos* but not *Arc* was expressed. Another difference was that the time profile in *Arc* revealed the strongest labeling was seen after 4 h, where the expression in the granular layer was highly reduced (Fig. 4). The same delay was detected in mRNA expression with some neurons highly expressing *c-Fos* mRNA in the hilus 4 h after ECS (Sup. Fig. 1).

## 4. Discussion

In this study, we explored epigenetic regulation, mRNA expression and localization of protein products of *Arc* and *c-Fos* in the

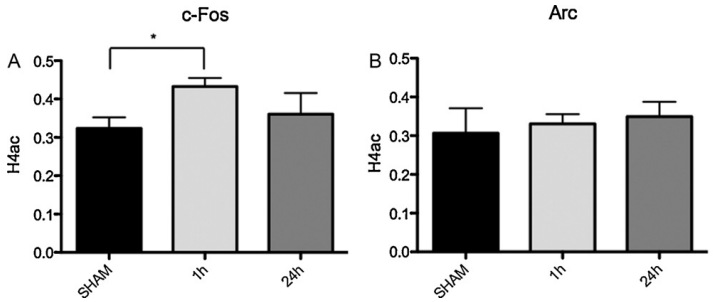


**Fig. 1.** Regulation of H4Ac at the *c-Fos* (A) and *Arc* (B) promoter after acute ECS as compared to sham animals. Data are expressed as mean ± SEM relative to *Beta-actin*, a gene not regulated by ECS $n = 6$/group; $p < 0.05$, unpaired $t$-test.

hippocampus of rats treated with acute ECS. We used ChIP assaying and bisulfite conversion of DNA to show that acetylation of histone H4 occurs during increased expression of the *c-Fos* gene, whereas DNA methylation is strongly induced on the *Arc* promoter 24 h post-ECS.

A number of reports have shown that *Arc* mRNA is rapidly induced in the dentate granule cells after ECS (Donai et al., 2003; Fujimoto et al., 2004; Guzowski et al., 1999; Larsen et al., 2005; Lyford et al., 1995; Steward and Worley, 2001). *Arc* mRNA expression signifies targeting of mRNA to dendritic localization in the granule cells of the dentate gyrus, where it is translated into protein (Tzingounis and Nicoll, 2006). The very strong inductions of *Arc* after ECS may have important consequences to hippocampal function. Thus *Arc* binds to endophilin 3 and dynamin and this process leads to internalization of AMPA receptors (Chowdhury et al., 2006; Rial Verde et al., 2006). However, the concentration of *Arc* in

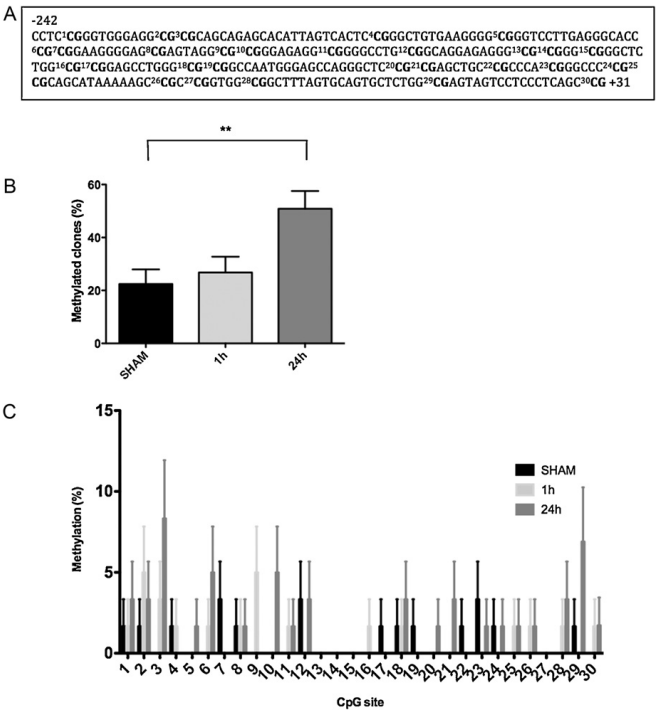


**Fig. 2.** DNA methylation of the *Arc* promoter. (A) Sequence of *Arc* promoter with each CpG numbered and marked bold. (B) Mean ± SEM percentage of cloned *Arc* promoter sequences revealing one or more methylated CpG sites. (C) Mean ± SEM percentage of methylation for each CpG dinucleotide within the *Arc* promoter. 10 clones per sample, with $n = 6$/group; $^{**}p < 0.01$, one-way ANOVA with Newman Keul's post hoc test.

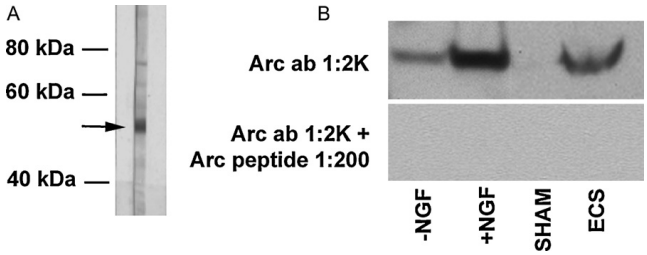


**Fig. 3.** Characterization of the polyclonal antiserum raised in rabbit against the recombinant murine *Arc* protein domain (1–396). The antiserum recognizes a single band of approximately 55 kDa in enriched synaptic vesicles from rat hippocampus (panel A). Both treatments produce a strong induction of *Arc*. Detection of *Arc* immunoreactivity in PC12 cells stimulated with NGF or hippocampal protein lysates from rats exposed to either acute ECS or sham treatment reveal a single band of the same size (panel B). Pre-incubation with the recombinant *Arc* completely abolished immunoreactivity (panel B).

the dendrite is probably critical for optimal memory storage. Lack of *Arc* function, as revealed by *Arc* knockout mice, leads to severe problems in memory consolidation (Plath et al., 2006) and over-accumulation is seen in patients with severe cognitive impairments (Greer et al., 2010). In this perspective, the very large accumulation of *Arc* in the dendrites and other compartments of neurons seen up to 4 h after ECS as well as a transcriptional repression resulting from DNA methylation could impair memory consolidation and lead to memory loss, which are major early negative side effects after ECT (Semkovska and McLoughlin, 2010).

Here we show for the first time, that very strong transcriptional up-regulation of *Arc* is followed by promoter DNA methylation 24 h after acute ECS. This may lead to a transient downregulation of *Arc* mRNA and protein expression. At 1 h post-ECS we also observed a small non-significant increase in DNA methylation. We used whole hippocampi for DNA methylation analysis and hence the increase may be explained by differences in the temporal profile of *Arc* gene expression in different subregions of the hippocampus. Maximal induction of *Arc* mRNA levels are seen in dentate granule cells 4 h after the stimulus whereas in the CA1 and parietal cortex the expression peaks within 1 h and returns to baseline levels within 2 h (Larsen et al., 2005). This indicates that gene expression and possibly DNA methylation patterns have different temporal profiles depending on the subregion.

This is not the first study to report changes in DNA methylation following ECS. A genome-wide study revealed that approximately 1.4% of 219,991 CpGs measured showed rapid active demethylation or de novo methylation in dentate granule neurons following ECS (Guo et al., 2011). A number of recent studies have highlighted the importance of DNA methylation in synaptic plasticity. A contextual fear conditioning study revealed very dynamic changes in hippocampal DNA methylation of the promoters of two genes, *Pp1* and *Reelin* (Miller and Sweatt, 2007). In another study it was found that *Reelin* and *Bdnf* exhibited rapid and dramatic changes in cytosine methylation when DNMT activity was inhibited (Levenson et al., 2006). Furthermore, double knockout of *Dnmt1* and *Dnmt3a* results in abnormal long-term plasticity in the hippocampal CA1 region together with deficits in learning and memory (Feng et al., 2010)

We also examined H4Ac association with the promoters of *Arc* and *c-Fos*. We found a significant increase of histone acetylation at 1 h post-ECS at the *c-Fos* but not *Arc* promoter. An increase in H4Ac at the promoter of *c-Fos* has previously been reported 30 min and 2 h after acute ECS (Tsankova et al., 2004). Furthermore, it has recently been shown that spatial memory deficits induced by ECS could be reduced by pre-treatment with the HDAC inhibitor phenylbutyric acid (PBA) (Yao et al., 2010). It was found that acute ECS decreased expression of *c-Fos*, but not *Arc*, at 24 h post ECS. Pre-treatment with PBA prevented the decrease in *c-Fos* expression emphasizing that H4Ac is likely involved in transcriptional regulation of *c-Fos*. In addition, it should be considered that HDAC inhibition has been found to reduce global DNA methylation, DNMT1 protein levels, and its interactions with chromatin (Arzenani et al., 2011).

To further characterize expression of hippocampal *Arc* and *c-Fos* we carried out a detailed analysis of their spatiotemporal profiles of expression. *Arc* mRNA levels reached a plateau at 1–4 h, while *c-Fos* mRNA levels peaked significantly earlier at 1 h post-ECS. This is also reflected by the observation that the intensity of *Arc* and *c-Fos* immunostaining peaked in the dentate granular at 4 h and 1 h, respectively. Furthermore, 8 h after the stimulation *Arc* protein remained elevated whereas *c-Fos* protein was not detected. The strong induction of both genes in the dentate granule cells suggests that they are induced through the same or partly overlapping signaling pathways. However, despite the shared increase in mRNA abundance after ECS the composition of transcription factors mediating the activation of the two genes is likely different. It has been shown that *Arc* is induced by MEK-dependent pathways (Waltereit et al., 2001; Yasuda et al., 2007). Also, the BDNF-induced increase of *Arc* mRNA is rapidly mediated by MEK, whereas the long-term changes are $Ca^{2+}$-dependent (Yasuda et al., 2007).

We also investigated *Arc*-immunoreactivity that was found present throughout the neuronal cytoplasm and in the nucleus of sham animals. A strong accumulation of *Arc*-immunoreactivity was also observed in the nuclei of neurons after ECS. The morphological data suggest that *Arc* is transported into the nucleus, but the possible functional significance of *Arc* in the nucleus is unknown. It is of interest that neurons found to contain cytoplasmic and nuclear *Arc* were virtually absent 24 h after ECS, suggesting that also *Arc* located in other intracellular compartments than the dendrites is regulated by neuronal activity. The precise localization of the two gene products revealed that with one exception they were expressed in the same hippocampal structures. It has been shown that only a maximum of 5% of the neurons containing *Arc* after ECS are GABAergic, the vast majority are glutamatergic (Vazdarjanova et al., 2006). However, we find that a population of hilar neurons that are likely inhibitory express *c-Fos* and not *Arc*. The time course study revealed that these neurons are not only different in their content of IEGs but also display a slower reduction of *c-Fos* expression upon acute ECS than the dentate granule cells. The activated neurons are interneurons many of which contain neuropeptide Y (NPY) (Woldbye et al., 1996). NPYergic interneurons are likely involved in inhibition of excitation in the granule cells (Madsen et al., 1999; Woldbye et al., 1997). This raises interesting perspectives about differences in the regulation of gene expression in hilar interneurons and granule cells.

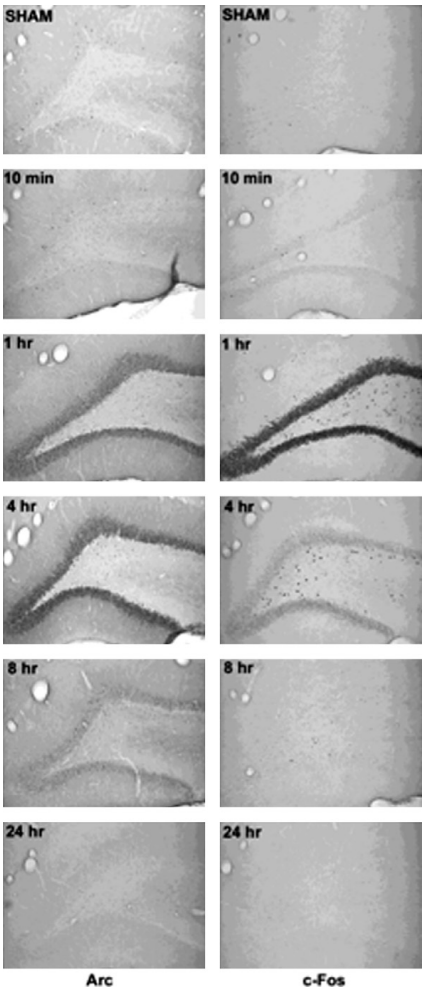


**Fig. 4.** Comparison of *Arc* and *c-Fos* protein expression in the granular cell layer of the same animals after a single ECS. ECS-induced *Arc* and *c-Fos* protein expression displays spatiotemporal differences, because whereas acute ECS triggers massive protein expression of both *Arc* and *c-Fos* expression in the granula layer of the dentate gyrus, the stimulated expression of *Arc* declines at a lower rate than *c-Fos*. Note that ECS triggers expression of *c-Fos* while leaving *Arc* expression almost undetectable in the hilus.

The results of this study will contribute to a better understanding of the molecular mechanisms governing gene expression after ECS. DNA methylation is particularly interesting as it might explain memory deficits experienced by patients after ECT. However, further studies are needed to reveal the mechanisms by which *Arc* becomes methylated. This should involve signaling pathways and catalyzing proteins such as the DNMTs. In addition, other memory-related genes including N-methyl-D-aspartate receptor 2A/B (*Grin2A/2B*), postsynaptic density 95 (*Psd95*), and *Creb* are obvious candidates for investigation of DNA methylation. A comprehensive understanding of the molecular mechanisms induced by ECS may enable pharmaceutical relieving of side effects and ultimately development of novel pharmaceuticals mimicking the positive effects of ECT.

## Acknowledgments

We would like to thank Merete Fredsgaard and Ditte Bech Kristensen for an outstanding technical assistance. This work was supported by the Danish Medical Research Council, NeuroSearch A/S, A.P. Møller Foundation and the NOVO Nordisk Foundation. The authors declare no conflict of interest.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.brainresbull.2012.05.004.

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# STUDY IV

**Temporal gene expression profile after acute electroconvulsive stimulation in the rat**

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This manuscript is published in: Gene 539(1) (2014) 8–14

Gene 539 (2014) 8–14

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

# Temporal gene expression profile after acute electroconvulsive stimulation in the rat

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ARTICLE INFO

*Article history:*
Received 2 August 2013
Received in revised form 17 January 2014
Accepted 25 January 2014
Available online 8 February 2014

*Keywords:*
Hippocampus
qPCR
Immediate early genes
Neuropeptides
Synaptic vesicle proteins
Depression

ABSTRACT

Electroconvulsive therapy (ECT) remains one of the most effective treatments of major depression. It has been suggested that the mechanisms of action involve gene expression. In recent decades there have been several investigations of gene expression following both acute and chronic electroconvulsive stimulation (ECS). These studies have focused on several distinct gene targets but have generally included only few time points after ECS for measuring gene expression. Here we measured gene expression of three types of genes: Immediate early genes, synaptic proteins, and neuropeptides at six time points following an acute ECS. We find significant increases for *c-Fos*, *Egr1*, *Neuritin 1* (*Nrn 1*), *Bdnf*, *Snap29*, *Synaptotagmin III* (*Syt 3*), *Synapsin I* (*Syn 1*), and *Psd95* at differing time points after ECS. For some genes these changes are prolonged whereas for others they are transient. *Npy* expression significantly increases whereas the gene expression of its receptors *Npy1r*, *Npy2r*, and *Npy5r* initially decreases. These decreases are followed by a significant increase for *Npy2r*, suggesting anticonvulsive adaptations following seizures. In summary, we find distinct changes in mRNA quantities that are characteristic for each gene. Considering the observed transitory and inverse changes in expression patterns, these data underline the importance of conducting measurements at several time points post-ECS.



## 1. Introduction

Despite many years of research, electroconvulsive therapy (ECT) is still the most effective treatment for major depression. The clinical efficacy ranges from 85 to 90%, whereas for pharmaceutical antidepressants it is only 60–65% (Schloss and Henn, 2004). The majority of patients undergoing ECT has previously been administered at least one antidepressant without success, further emphasizing the efficacy of the treatment (Moksnes and Ilner, 2010). ECT requires repeated administration for clinical efficacy but has a faster onset than antidepressant therapy (Pagnin et al., 2004). The first signs of improvements can often be observed after the first ECT and occurs in 65% of patients (Moksnes and Ilner, 2010). Unfortunately, adverse effects such as cognitive and memory deficits have been reported after ECT, making it the last choice of treatment (Fava and Kendler, 2000).

For many years, research has been focused on elucidating the antidepressant mechanisms of ECT. Electroconvulsive stimulation (ECS), an animal model of ECT, causes a widespread release of neurotransmitters, which affects a variety of transporters and receptors in the brain (Schloss and Henn, 2004). The therapeutic mechanism likely involves cellular changes, including increased neurogenesis, increased cell proliferation, fiber sprouting, and enhanced synaptic signaling all of which have been observed after ECS (Schloss and Henn, 2004). Such changes have been suggested to be orchestrated at the level of gene expression and the first studies of ECS-induced gene expression were published decades ago (Leviel et al., 1990; Winston et al., 1990; Xie et al., 1989; Zawia and Bondy, 1990). Further studies have revealed regulation of distinct neurotrophic signaling pathways (Altar et al., 2004), synaptic vesicle proteins (Elfving et al., 2008; Yamada et al., 2002), and immediate early genes (IEGs) (Dyrvig et al., 2012; Tsankova et al., 2004).

IEGs are rapidly induced by extracellular stimuli and act as transcription factors on downstream targets or as effector proteins. FBJ osteosarcoma oncogene (c-Fos) and early growth response 1 (EGR1) upregulation leads to long-term adaptions in neuronal gene regulation and may affect synaptic plasticity (Abraham et al., 1994; Kaczmarek, 1992). NRN 1 is an activity-dependent protein involved in neuronal plasticity by promoting neuronal migration (Naeve et al., 1997). These IEGs have all previously been found to be increased in the hippocampal formation following ECS (Newton et al., 2003; O'Donovan et al., 1998; Woldbye et al., 1996). Neuropeptides have received particular interest as part of the neurotrophic hypothesis of depression. Brain-derived neurotrophic factor (*Bdnf*) has been the leading candidate as acute and chronic stress decreases its expression whereas antidepressants have the opposite effect (Berton and Nestler, 2006). Neuropeptides are of particular interest due to their neuroprotective effects. BDNF activates TrkB influencing its associated pathways that have been

*Abbreviations:* ECS, electroconvulsive seizures; ECT, electroconvulsive therapy; IEG, immediate early gene.

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http://dx.doi.org/10.1016/j.gene.2014.01.072

implicated in neuronal differentiation, neurite outgrowth, cell survival, and synaptic plasticity (Boulle et al., 2012). Neuropeptide Y (NPY) has been found to inhibit seizures and has been linked to anxiety and depression (Kask et al., 2002; Thorsell et al., 2000; Vezzani et al., 1999; Woldbye et al., 1997). In the brain, NPY primarily bind neuropeptide Y receptor Y1 (NPY1R), neuropeptide Y receptor Y2 (NPY2R), and neuropeptide Y receptor Y5 (NPY5R) and exerts inhibitory effects on neuronal hyperexcitability in hippocampal seizure models (Vezzani et al., 1999; Woldbye and Kokaia, 2004). In the hippocampus, NPY2R plays a role in the seizure suppressing properties whereas NPY1R may facilitate seizures (Bahh et al., 2005; Lin et al., 2006a).

The widespread release of neurotransmitters following ECS affects both transporters and receptors (Schloss and Henn, 2004). Synaptic vesicle proteins are required for vesicle fusion and neurotransmitter release. Expression of synaptosomal-associated protein, 29 kDa (*Snap29*), *Syt 3*, and *Syn 1* was recently investigated in the hippocampus after acute and chronic ECS and regulation of such genes has been suggested to be a part of the therapeutic response (Elfving et al., 2008). Postsynaptic density protein 95 (PSD95) is a scaffold protein that maintains normal function of the NMDA receptor (Lin et al., 2006b) and PSD95 protein levels are reduced after ECS (Yao et al., 2010). Such reduction may impair learning and memory (Sultana et al., 2010).

The genes selected for the present study have previously been found to be regulated at the expression and/or protein level. Most of these studies have investigated changes in expression level only at one or two time points. However, the genes that have been investigated belong to diverse functional classes and expression changes may be short lasting or prolonged due to direct regulation via transcription factors or epigenetic modifications. Thus, it may be important to include several time points to make correct conclusions. To test this hypothesis, we decided to investigate the temporal gene expression profile up to 48 h following an acute ECS. To our knowledge this is the first study presenting the expression of several gene classes at numerous time points following an acute ECS. We decided to focus on the hippocampus due to its recognized role in depression and because gene expression is heavily affected by ECS in this region (Altar et al., 2004).

## 2. Materials and methods

### 2.1. Animals and treatment

Adult male Sprague–Dawley rats (270–350 g, Charles River Laboratories, Hamburg, Germany) were used. All experiments were conducted in accordance with guidelines of the National Institute of Health (NIH Publications No. 85-23, 1985) and the Animal Experimentation Inspectorate, Ministry of Justice, Denmark. Rats received a single transauricular ECS using a metal forceps (50 mA, 0.5 s, unidirectional square wave pulses) as previously described (Woldbye et al., 1996). All animals developed tonic-clonic seizure activity lasting 20–30 s. Animals were sacrificed 1 h, 4 h, 8 h, 16 h, 24 h or 48 h after ECS. Sham animals were handled similarly but without the passing of current. Three sham animals were sacrificed after 1 h and three animals after 24 h. After decapitation, the hippocampi were quickly removed, dissected and frozen in liquid nitrogen. The samples were stored at −80 °C until use.

### 2.2. Tissue homogenization and RNA extraction

Total RNA was extracted with an AllPrep DNA/RNA Mini Kit (Qiagen, Germany), following manufacturer's instructions for simultaneous purification of genomic DNA and total RNA from Animal Tissues. The tissue was disrupted and homogenized for 30 s. with a T10 basic ULTRA TURRAX Homogenizer (IKA, Germany). The RNA was stored at −80 °C until further use.

### 2.3. RNA quality assessment and cDNA synthesis

RNA purity and concentration were determined with a NanoPhotometer™ (IMPLEN, Germany). All samples were gel electrophorized on a 1.2% agarose gel stained with ethidium bromide to ensure integrity of the RNA. All samples displayed sharp 18 s and 28 s rRNA bands. To prevent DNA carryover, 1 μg of RNA was DNase digested with DNase I (Thermo Scientific, Germany), according to manufacturer's instructions. 100 ng of RNA was reverse transcribed with a RevertAid™ Premium First Strand cDNA Synthesis Kit (Thermo Scientific, Germany), using the protocol for RT-qPCR. No reverse transcriptase control reactions were prepared identically but without addition of Revertaid Premium Enzyme Mix. The reactions were incubated at 25 °C for 10 min, 50 °C for 20 min, 65 °C for 10 min, and 85 °C for 5 min. cDNA and no reverse transcriptase reactions were diluted 1:40 with DEPC water and used directly as qPCR template.

### 2.4. Real-time PCR

The real-time PCR reactions were performed in a Mx3000P Real-Time PCR System (Stratagene, USA), using 96-well PCR plates sealed with heat seal film. The reaction mixtures consisted of 1× Maxima SYBR Green qPCR Master Mix (2×), ROX solution provided (Thermo Scientific, Germany) with a final ROX concentration of 10 nM, 0.5 μM of each primer, and 10 μl diluted cDNA in a total volume of 20 μl. The cycling protocol started with one cycle of 95 °C for 10 min for enzyme activation, followed by 40 cycles of: 95 °C for 30 s., 60 °C for 30 s, and 72 °C for 30 s. All samples were run in duplex and reactions were repeated if any deviation was observed. After finished amplification, a melting curve program was performed starting at 60 °C and ending at 95 °C to investigate for presence of primer-dimers or non-specific amplicons.

Eight housekeeping genes have previously been tested in hippocampal tissue following ECS (Fryland et al., 2012). Using Normfinder software it was found that Actb and CycA were the best-suited combination of reference genes for normalization. CycA primers have been published previously (Peinnequin et al., 2004) and used in several publications. Sequences for the remaining transcripts were obtained from NCBI (http://www.ncbi.nlm.nih.gov/gene/) and primers were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The primers were designed to have amplicon sizes of 100–150 bp and to be intron spanning whenever possible (see Table 1 for primer sequences). PCR products were gel electrophorized on a 2% agarose gel stained with ethidium bromide to test for the presence of a specific band of the correct size. Standard curves were generated for each primer pair to determine efficiencies and all were in the range of 90–110%. Gene expression was calculated using the efficiency corrected method of Pfaffl (2001). For each animal gene expression was normalized using the geometric mean of Actb and CycA. Target gene expression in ECS groups was compared to expression in SHAM animals decapitated after 1 h and 24 h. No statistically significant differences in target gene expression were observed between these SHAM groups (unpaired t-test) except for EGR1 (13% difference). Statistical data analysis was performed using one-way ANOVA with Dunnett's post hoc test. All analyses were conducted using GraphPad Prism 5.0 (GraphPad software, San Diego, CA, USA). p values $< 0.05$ were considered to be statistically significant.

## 3. Results

### 3.1. Immediate early genes

IEGs *c-Fos*, *Egr1* and *Nrn 1* have previously been found to be highly upregulated after ECS and this is also evident in this study (Fig. 1). For *c-Fos* we observe a more than 20-fold increase at 1 h post-ECS ($p < 0.001$) after which the expression gradually declines (Fig. 1a). At

**Table 1**
Characteristics of gene-specific qPCR primers.

| Gene name | Accession no. | Primer sequence | Amplicon size (bp) | Spanning Intron |
|---|---|---|---|---|
| *Actb* | NM_031144.3 | (+) CCTCTGAACCCTAAGGCCAACCGTGAA<br>(−) AGTGGTACGACCAGAGGCATACAGGG | 123 | + |
| *Cyca* | NM_017101.1 | (+) TATCTGCACTGCCAAGACTGAGTG<br>(−) CTTCTTGCTGGTCTTGCCATTCC | 127 | − |
| *c-Fos* | NM_022197.2 | (+) GGTCACAGAGCTGGAGCCCCTGTGC<br>(−) TCGTTGCTGCTGCTGCCCTTTCGGT | 150 | − |
| *Egr1* | NM_012551.2 | (+) CAACCCTACGAGCACCTGACCACAG<br>(−) TCCAGGGAGAAGCGGCCAGTATAGG | 137 | + |
| *Nrn 1* | NM_053346.1 | (+) AGGTCTGCGGTCGCAAATGGTTTGA<br>(−) AAAGACTGCATCGCACTTGCCTGCT | 141 | + |
| *Bdnf* (exon IX) | NM_001270638.1 | (+) AGTCCCGGTATCAAAAGGCCAACTGAA<br>(−) AGGGCCCGAACATACGATTGGGTAGT | 147 | − |
| *Npy* | NM_012614.2 | (+) CATGGCCAGATACTACTCCGCTCTGCGA<br>(−) AGCCTTGTTCTGGGGGCATTTTCTGTGC | 141 | + |
| *Npy1r* | NM_001113357.1 | (+) TGGTGCTGCAGTATTTTGGCCCACT<br>(−) GCAGCATGACGTTGATTCGTTTGGTCT | 150 | + |
| *Npy2r* | NM_023968.1 | (+) TGTTCCATCATCTTGCTGGGCGTAGT<br>(−) AAGAGTGAATGGCAGGCACAGGGTG | 153 | − |
| *Npy5r* | NM_012869.1 | (+) ACGGCAAACCATGGCTACTTCC<br>(−) GCAGTGCTGAGCCAAAGGTCT | 121 | − |
| *Snap29* | NM_053810.3 | (+) CCTCCACCTGAGCAGAATGGCA<br>(−) GGGTGGCTGGCTTGGTACTTG | 107 | + |
| *Synapto-tagmin III* | NM_019122.1 | (+) TCCTGCTGCCTCCTAGTGGTG<br>(−) TGGGTCTGCTTGGGTGGTCA | 137 | + |
| *Syn 1* | NM_001110782.2/NM_019133.2 | (+) GTCATCGACGAGCCGCACAC<br>(−) CCACGGAGAATCCACCATTGGC | 139 | + |
| *Psd95* | NM_019621.1 | (+) AGCTTCCGCTTCGGGGATGT<br>(−) ACCTTGACCACTCTCGTCGCT | 148 | + |

4 h, the expression is still more than 2-fold increased compared to sham but thereafter the expression continues to drop, reaching 59% compared to sham. Similar to *c-Fos*, the expression of *Egr1* is dramatically increased at 1 h post-ECS with a near 5-fold increase as compared to sham ($p < 0.001$; Fig. 1b). Expression then quickly declines and remains at levels comparable to sham for the remaining time points. The expression of *Nrn 1* (Fig. 1c) increases more slowly after ECS as compared to *c-Fos* and *Egr1*. A minor 12% increase is observed at 1 h after which expression reaches a maximum 75% increase at 4 h ($p < 0.001$). Expression then starts to drop but remains significant with a 44% increase above sham at 8 h ($p < 0.001$). At later time points, expression returns to sham levels. *Bdnf* expression (Fig. 1d) is also included here, as the gene has several promoters, some of which can be induced as an IEG (Lauterborn et al., 1996). At 1 h and 4 h post-ECS, the expression of *Bdnf* increases significantly approximately 3.4-fold compared to sham ($p < 0.001$). At 8 h the *Bdnf* expression decreases but remains 55%

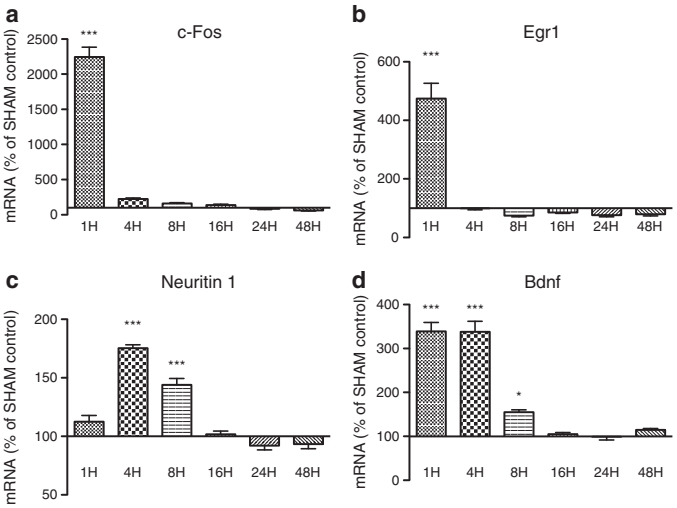


**Fig. 1.** Expression of immediate early genes following acute ECS. mRNA expression in the hippocampus of sham rats or rats exposed to acute ECS at: 1 h, 4 h, 8 h, 16 h, 24 h, and 48 h (n = 6/group). *c-Fos* (a), *Egr1* (b), *Nrn 1* (c), and *Bdnf* (d). Data are presented as mean value of the group with SEM. *$p < 0.05$, ***$p < 0.001$, one-way ANOVA with Dunnett's post-hoc test.

higher than sham ($p < 0.05$). Thereafter expression decreases to sham levels for the remaining time points.

### 3.2. Npy system

At the first hours following an acute ECS, *Npy* expression (Fig. 2a) does not change significantly. At 4 h we observe a small increase after which expression increases significantly 3.3-fold compared to sham at 8 h ($p < 0.001$) and remains at 2.7-fold at 16 h ($p < 0.001$). The expression then declines to sham levels at 24 h and 48 h. The expression of *Npy* receptors initially declines for all investigated subtypes (Figs. 2b–d): *Npy1r* is at 45% ($p < 0.001$), *Npy2r* at 78% ($p < 0.05$), and *Npy5r* at 58% ($p < 0.001$) compared to sham after 4 h. The expression of *Npy1r* then increases but remains significantly decreased at 8 h ($p < 0.01$), 16 h ($p < 0.001$), and 24 h ($p < 0.05$) compared to sham. At 48 h the expression is near sham levels. The expression of *Npy2r* continues to decrease and reaches 63% of sham at 8 h ($p < 0.001$). Expression then increases significantly, being 23% higher than sham at 48 h ($p < 0.05$). After an initial decrease at 1 h and 4 h, the expression of *Npy5r* increases to near sham levels at 8 h and 16 h. At 24 h expression is significantly decreased ($p < 0.05$) before returning to sham levels at 48 h.

### 3.3. Synaptic proteins

As opposed to the expression changes observed for IEGs and the *Npy* system only small, though significant, changes are observed for synaptic proteins. *Snap29* (Fig. 3a) reaches a maximal 17% increase in expression at 8 h ($p < 0.001$). Expression remains elevated at 16 h ($p < 0.01$) and then gradually declines to sham levels. *Syt 3* (Fig. 3b) remains stable for the first period following ECS and then significantly increases at 16 h ($p < 0.01$) by 12% compared to sham. A similar level is observable at 24 h though not statistically significant before expression declines at 48 h. The expression of *Syn 1* (Fig. 3c) follows a different pattern with a gradual increase after ECS, which reaches significance at 4 h ($p < 0.05$). Expression further increases at 8 h ($p < 0.001$) and reaches a maximum 37% increase at 16 h ($p < 0.001$). Expression then starts decreasing but is still significantly increased at 24 h ($p < 0.05$), before returning to sham levels at 48 h. The expression of *Psd95* (Fig. 3d) does not initially increase following ECS but gradually increases after 4 h ($p < 0.01$) and reaches a maximum 21–23% increase at 8 h and 16 h ($p < 0.001$). Expression then declines but remains significantly increased with 16% at 24 h ($p < 0.001$) and 14% at 48 h ($p < 0.01$) as compared to sham.

## 4. Discussion

Here we investigate the temporal gene expression profile of several genes in the hippocampus following an acute ECS. We observe that changes in gene expression following an acute ECS are often relatively transient. *c-Fos*, *Egr1*, and *Syt 3* are significantly regulated only at a single of our selected time points. Thus, selecting only one time point for measurements may be clearly insufficient. As seen with *Npy2r* mRNA measurements, gene expression may even follow an inverse pattern leading to spurious conclusions if only time points immediately post-ECS are selected. This observation may explain why the data presented here both augments and show disagreements with other studies. For instance, Elfving et al. (2008) previously investigated the expression of synaptic vesicle proteins in the hippocampus and frontal cortex 6 h after acute and chronic ECS. In their study *Snap29* expression was found to be significantly increased and we confirm that expression is significantly upregulated at 8–16 h after an acute ECS. For *Syn 1* they found a non-significant upregulation whereas we find a significant upregulation 4–24 h after ECS. They also found *Syt 3* to be significantly downregulated which is in contrast to a significant upregulation at 16 h in the present study. At 4 h we do in fact observe a non-significant decrease for *Syt 3* which emphasizes the likeliness of decreased expression at 6 h as they observe. Elfving et al. (2008) suggest that *Snap29* upregulation and *Syt 3* downregulation may be early specific markers for the effect of ECS. However, our data emphasize that the expression changes are transient and that a single time point is not enough to make conclusions regarding therapeutic mechanisms.

In the clinical setting, ECT is administered repeatedly to achieve a full therapeutic effect (Pagnin et al., 2004). However, in many patients the first improvements can be observed already after the first ECT (Moksnes and Ilner, 2010) which suggests involvement of both acute

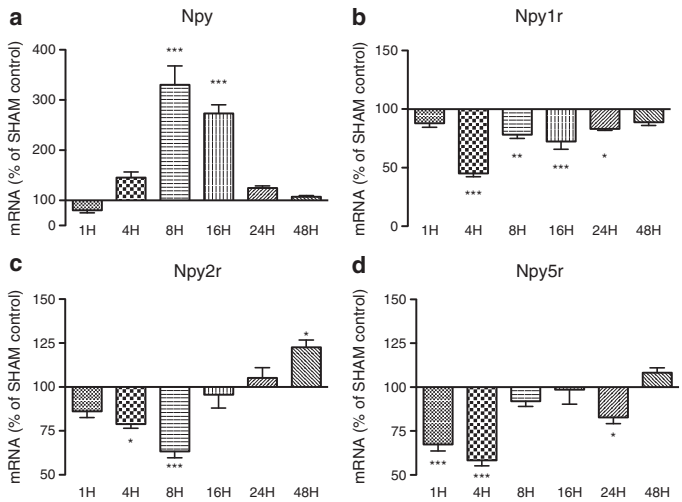


**Fig. 2.** Expression of *Npy* and *Npy* receptor genes following acute ECS. mRNA expression in the hippocampus of sham rats or rats exposed to acute ECS at: 1 h, 4 h, 8 h, 16 h, 24 h, and 48 h ($n = 6$/group). *Npy* (a), *Npy1r* (b), *Npy2r* (c), and *Npy5r* (d). Data are presented as mean value of the group with SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, one-way ANOVA with Dunnett's post-hoc test.

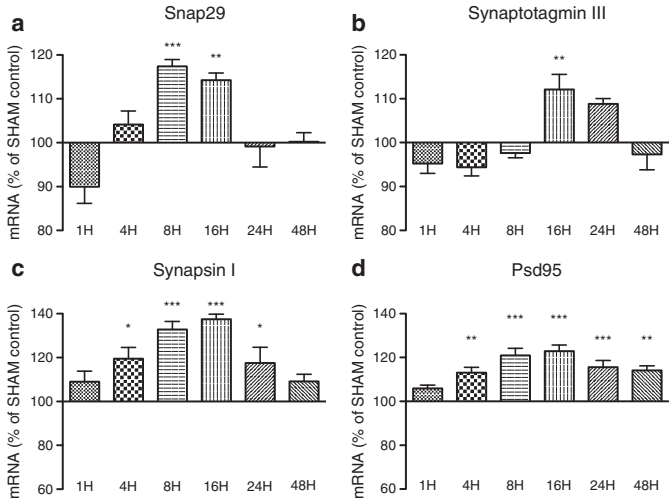


**Fig. 3.** Expression of genes coding for synaptic proteins following acute ECS. mRNA expression in the hippocampus of sham rats or rats exposed to acute ECS at: 1 h, 4 h, 8 h, 16 h, 24 h, and 48 h (n = 6/group). *Snap29* (a), *Syt 3* (b), *Syn 1* (c), and *Psd95* (d). Data are presented as mean value of the group with SEM. *p < 0.05, **p < 0.01, ***p < 0.001, one-way ANOVA with Dunnett's post-hoc test.

mechanisms and long-term adaptions. As evident in the current study, expression of distinct gene classes is affected differently and exhibits various delays before transcription is affected. As expected, IEGs are affected rapidly. c-Fos and EGR1 function as transcription factors and likely induce regulation of downstream targets. The transient increases in *c-Fos*, *Egr1*, and *Nrn 1* expression is consistent with previous findings (Newton et al., 2003; Wallace et al., 1998; Zawia and Bondy, 1990). Similar to the apparent decrease in *c-Fos* expression at 24 h and 48 h, a decrease has also been observed 24 h after chronic ECS (Tsankova et al., 2004; Winston et al., 1990). More recently Calais et al. (2013) investigated the expression of IEGs *c-Fos*, *Egr1* and Arc after acute and chronic ECS in the hippocampus for up to 90 days after the last seizure. As observed in the current study, acute ECS induced a transient increase in expression of *c-Fos* and *Egr1* after which a significant decrease was observed. The induction of IEGs is significantly lower in animals that receive chronic ECS as compared to animals receiving only one shock (Calais et al., 2013; Jung et al., 1996) and it has been found that chronic treatment desensitizes subsequent ECS-induced IEG expression (Tzingounis and Nicoll, 2006; Winston et al., 1990). Thus, IEGs are not likely to be related to the antidepressant properties of chronic ECS (Larsen et al., 2005). However, as observed here, IEGs may be involved in an immediate acute response that leads to gene regulation of downstream targets (Fig. 4).

In the current study, gene expression is presented relative to SHAM animals sacrificed after 1 h and 24 h. At these time points we observe no or very limited differences between the SHAM animals. However, as we measure gene expression at several time points after ECS it should be noted that expression of some genes involved in synaptic plasticity and memory formation are affected by circadian fluctuations in the rat hippocampus (Eckel-Mahan, 2012). To our knowledge such observations has only been made for *BDNF* and *c-Fos*. Using in situ hybridization it was found that *BDNF* show up to 20% of circadian fluctuations (Schaaf et al., 2000). In dentate gyrus the levels peak during the inactive period and are decreased at the start of the active period. In CA3, a minor increase is observed during the active period. As these changes are partly opposing and we use whole hippocampus, circadian influences should be limited. For *c-Fos* it was found that expression gradually increases to an approximate 3-fold increase when animals are active during the dark-cycle (Grassi-Zucconi et al., 1993). This could potentially affect the measurement after 16 h, the only performed during the dark-cycle. However, we do not observe that expression is increased at this

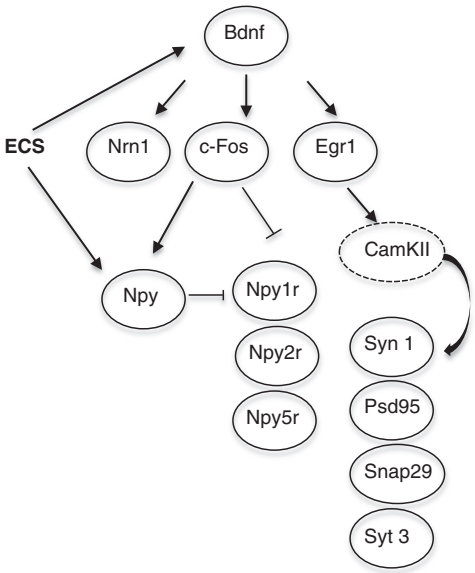


**Fig. 4.** Diagram showing proposed sequence of events based on the presented data and the literature. Dashed line depicts gene not included in the analysis in this article.

time point. This may be explained by the fact that *c-Fos* expression is blocked for a prolonged period after ECS (Winston et al., 1990).

*Bdnf* has a complex structure with eight 5′-untranslated exons and one protein coding 3′-exon (Aid et al., 2007). Activity dependent increases in *Bdnf* expression has been reported to occur within 20 min of stimulation (Gall, 1993; Isackson et al., 1991), which is suggestive of an IEG response. This was investigated by Lauterborn et al. (1996) who found that *Bdnf* exon IV and VI (according to the new nomenclature (Aid et al., 2007)) was transcribed in the presence of the protein synthesis inhibitor cycloheximide. Here we investigated expression of the total *Bdnf* transcript (exon IX). It is apparent that *Bdnf* expression increases at 1 h as is the case for *c-Fos* and *Egr1*. It is also evident that *Bdnf* expression remains increased substantially longer than *c-Fos* and *Egr1*, which may be a result of expression driven by non-IEG response promoters. Indeed, it has been found that *Bdnf* mRNA is significantly increased in the dentate gyrus 6 h after an acute ECS, but expression decreases to sham levels at 24 h, whereas chronic ECS causes significant increases up to 48 h post-ECS (Zetterström et al., 1998). Thus, it is likely that IEG response promoters primarily drive *Bdnf* expression induced by acute ECS, whereas transcription is driven at non-IEG response promoters after chronic ECS. A similar observation was made for *Npy* where it was found that *Npy* gradually increased with the number of ECS (Mikkelsen and Woldbye, 2006). Some patients show improvements after the first ECT. However, repeated stimulation delivered consecutively over several days (6–14 days) is required to produce long-term beneficial effects (Silverstone and Silverstone, 2004). Genes such as *Bdnf* and *Npy* that we find to be highly expressed after an acute ECS, and gradually increase or remain expressed for prolonged periods after chronic ECS, could offer an explanation for these clinical observations. However, it should be noted that ECT-induced gene expression differ between regions and these changes may even cause opposing behavioral outcomes depending on the region. The study by Elfving et al. (2008) support that transcriptional changes differ between brain regions. Even more importantly, in a study by Taliaz et al. (2013) it was found that differences in the expression of BDNF in the hippocampus and ventral tegmental area causes opposite outcomes. It was found that chronic ECS induced BDNF upregulation in the hippocampus and downregulation in the ventral tegmental area. An antidepressant like-effect was not prevented by blocking hippocampal BDNF induction in the hippocampus but achieved by BDNF knockdown in the ventral tegmental area.

The *Npy* system is regulated after both ECS and seizures (Husum et al., 1998; Mikkelsen et al., 1994). The antidepressant properties of NPY have been suggested to involve anticonvulsive adaptions (Sackeim et al., 1983). However, the anticonvulsant and anxiolytic effects of NPY likely involve activation of different receptors and take place in distinct brain regions, namely the hippocampus and amygdala (Mikkelsen and Woldbye, 2006). Expression of *Npy1r*, *Npy2r* (Madsen et al., 2000), and *Npy5r* (Christensen et al., 2006) are affected by ECS. Here we find that *Npy2r* is significantly upregulated 48 h after an acute ECS. Considering the anticonvulsive properties of NPY2R in the hippocampus this corresponds well with the fact that seizure thresholds increases as ECT treatment regimen advances (Post et al., 1996).

In conclusion, the results of this study will contribute to an improved understanding of the temporal gene expression profile after an acute ECS. The patterns presented highlight the widespread actions of ECS and the diversity of expression changes for each gene type. More importantly, this study emphasizes the importance of including several time points for investigations of ECS-induced gene expression, particularly when investigating expression immediately following acute ECS. As this study only investigates transcriptional changes, future experiments should be performed to investigate how these correlates with protein expression. We have previously (Dyrvig et al., 2012) measured *c-Fos* protein expression 10 min, 1 h, 4 h, 8 h, and 24 h after ECS and for this gene we observe the expected correlation. However, for the remaining genes it should be established if minor or inverse transcriptional changes are also evident at the protein level.

## Conflict of interest

The authors declare no conflict of interest.

## Acknowledgments

This work was supported by The Obel Family Foundation and Fonden til Lægevidenskabens Fremme.

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# STUDY V

**Decitabine attenuates *Dnmt3a* upregulation after electroconvulsive stimulation but does not prevent expression and epigenetic changes for the *Arc* gene**

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This manuscript has been submitted to Neuropharmacology

# 4. DISCUSSION

The objective of this dissertation has been to study DNA methylation in relation to the etiology and treatment of psychiatric disorders. In the presented manuscripts we established that DNA methylation is important for transcriptional regulation of *BRD1* and that the SNP rs138880, located in the *BRD1* promoter region, is associated with increased methylation. We then established that the *CHRNA7* gene is regulated by DNA methylation in the adult brain and that HDAC inhibition increases its expression and reduces promoter methylation. Finally, we focused on methylation changes at the *Arc* promoter following ECS. Thus, the overall focus of the dissertation was on changes in DNA methylation resulting from both endogenous (genetic) and exogenous (ECS and treatment) sources.

## 4.1. RELEVANCE OF BLOOD AS A BIOMARKER

It is difficult to study psychiatric diseases as brain tissue can only be obtained post mortem or from animal models (Smith et al. 2014). Creating valid animal models of neuropsychiatric disorders is extremely challenging both because of the subjective nature of symptoms and particularly because of our limited understanding of disease mechanisms (Nestler & Hyman 2010). Even if we had better understanding, it would be difficult to create a model with good construct validity because of the highly complex genetic architecture of psychiatric disorders. The use of post mortem brain tissue is informative but it is particularly limited by the capacity to study on-going disease stages. Therefore, blood is often used as a biomarker that may reflect mechanisms occurring in the brain (Tylee et al. 2013). In study 1 we also use blood, but a relevant question is how suitable blood really is as a biomarker for the brain.

It is known that DNA methylation patterns show large variations across the genome by developmental stage and by tissue type (Liang et al. 2011). However, it has been reported that early life adverse environment leaves a long lasting epigenetic footprint in DNA methylation that can be observed in both blood and brain (Menke & Binder 2014). A review of the epigenomic literature revealed that CpG-island methylation levels are highly correlated between blood and brain (Tylee et al. 2013).

DNA methylation levels may be affected by environmental insults as mentioned above, but also by genetic variations. In a very recent study metQTLs were compared across ancestry, developmental stage, and tissue type (Smith et al. 2014) and it was found that metQTLs were consistently detected across these groups. Comparisons of tissue types included four brain regions (frontal cortex, temporal cortex, cerebellum, and pons) and peripheral blood. Comparisons between blood samples and brain tissue among individuals of European ancestry revealed an overlap of metQTLs of 18.5-31.6%, whereas the overlap between brain regions was 35.8-71.7%. Thus, there is overlap of detected metQTLs, but the size emphasise that additional examinations in relevant tissues are needed to make thorough conclusions. In study 1 we observe that methylation of CpG sites cg15145965 and cg06057569 in the *BRD1* promoter region are correlated with rs138880 alleles in both adipose tissue and blood. In addition, promoter constructs carrying the rs138880 risk allele (C-allele) causes decreased transcriptional drive in a neuronal cell line (Qvist et al., manuscript submitted), which provides evidence that the SNP is also relevant in brain tissue.

## 4.2. RATIONALE FOR STUDYING EPIGENETIC ALTERATIONS CAUSED BY GENETIC VARIANTS

In study 1 we find that the rs138880 SNP located in the *BRD1* promoter region is associated with increased DNA methylation. Current evidence suggests that DNA methylation is a secondary event following decreased promoter activity (Jones 2012). MatInspector software analysis suggest that the transcriptional repressor HES1 binds specifically to the risk C-allele as was reported previously (Severinsen et al. 2006) and that BPTF and RBP2 bind specifically to the A-allele. Thus, most likely DNA methylation is a consequence of and not the cause of reduced *BRD1* expression.

In study 2 we find that DNA methylation at Region 2+3 is significantly correlated with *CHRNA7* expression in the human temporal cortex. A large number of SNPs in the core promoter of *CHRNA7* are associated with decreased transcription (Leonard et al. 2002). We did not genotype the samples but did metQTL analysis and found

that *CHRNA7* promoter SNPs rs6494165, rs1514246, and rs883473 showed significant correlation with methylation levels of CpG sites located in Region 2 and 3. This suggests that as for the *BRD1* promoter, decreased transcriptional activity of the *CHRNA7* promoter could increase the chance of *de novo* methylation and lead to higher methylation levels as is observed for two of the biopsies.

So what is the importance of linking methylation to reduced *BRD1* and *CHRNA7* expression? If it should become a therapeutic goal to normalise *BRD1* expression, or increase *CHRNA7* expression as an adjuvant to potentiate α7 nAChR agonists, there are a number of options. 1) Change the genotype of the SNP. 2) Target the transcription factor binding to the SNP or increase transcription via other transcription factors. 3) Reverse DNA methylation by pharmacological intervention, which would leave the region accessible to other transcription factors. Only option 2 and 3 currently seem realistic and for the latter option there are already therapeutic drugs, including valproate that have been approved for therapeutic interventions of other conditions.

## 4.3. FUNCTIONAL CORRELATION OF *BRD1* SCHIZOPHRENIA RISK ALLELES

Genetic schizophrenia risk variants in and around the *BRD1* gene have repeatedly been identified (Aberg et al. 2013; Nyegaard et al. 2010; Severinsen et al. 2006; Jorgensen et al. 2002). The schizophrenia associated SNPs in or near the *BRD1* gene are part of a haploblock that spans both *BRD1* and the nearby ZBED4 gene (Qvist et al., manuscript submitted). Cis-eQTL analysis using expression phenotypes of HapMap3 individuals revealed that the schizophrenia risk variants correlate with reduced *BRD1* and not *ZBED4* mRNA (Qvist et al., manuscript submitted). In study 1, a metQTL analysis was performed using the MuTHER resource, a public available profiling dataset including Ilumina 450K adipose methylome data from 648 twins (Grundberg et al. 2013). The 15 other SNPs in high linkage disequilibrium with rs138880 also revealed significant effects on the same probes that appeared affected by rs138880.

As described above, the rs138880 C-allele is predicted to harbour a binding site for the transcriptional repressor HES1 (Severinsen et al. 2006), whereas the A-allele may harbour binding sites for BPTF and RBP2. Thus, we believe that differences in transcription factor binding lead to increased methylation associated with the C-allele and this is supported by the decreased transcriptional drive of promoter constructs carrying the rs138880 risk allele (Qvist et al., manuscript submitted). In Study 1 we find that rs138880 is associated with increased methylation at Region 2 and 3 of the *BRD1* promoter in blood, and these regions are important for regulating the minor transcript variants Exon 1C and 1B. This is in agreement with the limited approximate 3% reduction of *BRD1* mRNA in carriers of the rs138880 risk allele in a B lymphoblastoid cell line established from HapMap3 individuals (Qvist et al, manuscript submitted). Collectively these observations strongly indicate that the rs138880 risk allele is the causative variant and that increased DNA methylation of the *BRD1* promoter and reduced *BRD1* expression on a well-founded basis can be considered an etiopathologic risk factor in schizophrenia. However, it should be noted that with the presumably limited decrease in expression of *BRD1* in carriers of the rs138880 risk allele, and the high frequency of the risk allele (minor allele frequency of 0.16 in controls and 0.25 in schizophrenic individuals of European ancestry (Severinsen et al. 2006)), it is obvious that the risk allele is not alone sufficient to cause schizophrenia.

## 4.4. FUNCTIONAL CONSEQUENCES OF *BRD1* AND *CHRNA7* DEFICIENCY

In the developing fetal pig brain *BRD1* is highly expressed at early embryonic stages and its expression declines in the later stages (Severinsen et al. 2006). Interestingly, the two regions (Region 2 and 3) exhibiting increased DNA methylation in carriers of the rs138880 risk allele co-localise with CpG sites undergoing changes in DNA methylation levels during fetal brain development. Based on the increasing methylation at the Exon 1C promoter and concomitant decreasing methylation at the Exon 1B promoter it seems likely that expression of Exon 1C is higher in early fetal

brain development and gradually decreases whereas for Exon 1B it is the opposite. Importantly, the data also indicate that methylation changes in carriers of the rs138880 risk allele may adversely affect *BRD1* expression in both the fetal and adult brain. But what is the functional consequence of reduced *BRD1* expression? And is *BRD1* a relevant target for treatment? The *BRD1* gene is highly expressed in embryogenesis and inactivation of both alleles leads to impaired eye development, neural tube closure, and a lethal maturation defect in hematopoiesis (Mishima et al. 2011). BRD1 regulates several genes acting in signalling pathways important during neurodevelopment (Fryland et al., manuscript submitted) and that helps explain the widespread deficits. Indeed, in *Brd1*$^{+/-}$ mice these genes are dysregulated and the mice display region-specific changes in dopamine levels and disturbed NMDA and GABA mediated signalling (Qvist et al., manuscript submitted). Overall the findings fit with the neurodevelopmental hypothesis of schizophrenia which suggest that disruption of brain development during early life followed by progressive neurobiological processes underlies emergence of psychosis (McGrath et al. 2003). *BRD1* may however be equally important for the adult brain as it is regulated by both electroconvulsive seizures (Fryland et al. 2012) and chronic restraint stress in the hippocampus (Christensen et al. 2012). BRD1 is important for acetylation of histone H3K14 and binds in the promoter of several schizophrenia risk genes (Fryland et al., manuscript submitted). Combined with the knowledge that HDAC inhibitors exert hippocampal-dependent antidepressant like activities (Covington et al. 2011) this suggests that BRD1 may be involved in regulatory processes underlying stress adaptations (Christensen et al. 2012). Importantly, the stress induced *BRD1* upregulation may be blocked or attenuated by increased DNA methylation of Region 2 and 3 in carriers of the rs138880 risk allele, which could prevent stress adaptions. This will depend on whether stress induced *BRD1* upregulation is mediated by transcription factors that are able to bind to methylated DNA as has been observed in some cases (Hsieh 2000) or if transcription factor binding is inhibited by methylation as has also been observed (Prendergast & Ziff 1991; You et al. 2011).

SNPs in the promoter region of *CHRNA7* show significant association with schizophrenia (Stephens et al. 2009) and several rare promoter SNPs decrease promoter activity (Leonard et al. 2002). This corresponds well with observations of reduced α7 nAChR expression in hippocampus (Freedman et al. 1995) and cortex (Guan et al., 1999; Marutle et al., 2001) of schizophrenic individuals. Notably, the promoter SNPs are associated with failure to inhibit the P50 auditory evoked potential response, a deficit that is found in most schizophrenics and 50% of their first-degree relatives (Leonard et al. 2002).

## 4.5. SMALL CHANGES IN METHYLATION LEVELS CAN IMPACT GENE EXPRESSION

In study 1, 2, and 5 we examined changes in methylation and expression of target genes resulting from DNMT or HDAC inhibition. In study 1, focusing on *BRD1*, exposure of HeLa cells to zebularine decreased average methylation at Region 2 from 92.2% to 68.7% whereas at Region 3 the average methylation decreased from 15.2% to 10.5%. This was associated with upregulation of Exon 1C to 285% of control levels and upregulation of Exon 1B to 231% of control levels. In study 2, focusing on *CHRNA7*, HeLa cells were exposed to valproate and this resulted in a decrease in methylation at Region 1 from 19.9% to 18.8% and at Region 3 from 22.6% to 20.3%. This was associated with 8.5-fold upregulation of *CHRNA7*. This emphasises that transcriptional activity depends on both epigenetic regulation and transcriptional drive. In general it seems like the methylation changes observed in psychiatric disorders are of modest size. For example in a recent study, by Pidsley et al. the 100 top-ranked schizophrenia associated differently methylated positions, revealed methylation levels 1-12% above or below control individuals with the majority showing differences around 5% (Pidsley et al. 2014). However, as described above although the methylation changes are rather small, the effects on transcription may be large.

## 4.6. IMPORTANCE OF LOW DEGREE METHYLATION

In study 2, 3, and 5 we focused on regions that are methylated at very low levels. Low degree methylation has previously been reported to correlate with transcriptional repression of *Arc* and *Gad1* in rats (Penner et al. 2011; Zhang et al. 2010b). In study 2 we showed that methylation of Region 2+3 in the *CHRNA7* promoter in brain biopsies is very low with an average CpG methylation of 1.75% in the two biopsies with the highest methylation and an average of 0.65% for the reaming nine biopsies. Hence, there is approximately 3-fold difference in methylation levels with a corresponding 4-fold negative correlation in expression. Interestingly, the study clearly demonstrated the negative correlation between methylation levels at Region 2+3 and *CHRNA7* expression. In study 3 and 5 we focused on methylation of the *Arc* promoter that is also methylated at a low degree. In study 5 we discuss how increasing HDAC activity could be involved in *Arc* repression and it has been established that HDAC2 and histone acetylation of H3 is also important for regulating *Arc* transcription (Moonat et al. 2013). However, we also find that DNMT inhibitor decitabine induces a significant upregulation of *Arc* expression, which suggests that even though methylation is low, the mechanism is essential for maintaining a transcriptionally inactive/low activity state.

## 4.7. INTERACTION BETWEEN HISTONE ACETYLATION AND DNA METHYLATION

In Study 2 we treated HeLa cells with valproate and this caused a decrease in methylation at Region 3 of the *CHRNA7* promoter from 22.6% to 20.3%. This observation supports the interaction between histone modifications and DNA methylation. The process by which demethylation occurs could be similar to the process described in the introduction. Under normal circumstances the promoter is inactive and marked with repressive modifications e.g. H3K9me3 (Lin et al. 2007). Methylated DNA is recognised by methyl binding domain proteins (MBDs), such as MeCP2, that are part of large protein complexes containing HDACs and histone methyltransferases (HMTs) resulting in further transcriptional repression (Tsankova et al. 2007). Treatment of cells with HDAC inhibitor valproate would disrupt this

cascade by increasing histone acetylation, which could neutralise the positive charge of the lysine and destabilise internucleosomal contacts and lead to chromatin decondensation (Shogren-Knaak et al. 2006). As transcription from the promoter increases this could lead to active promoter demethylation involving TET enzymes as described in the introduction.

Although not included in study 2, methylation levels at Region 3 in HeLa cells treated with valproate was also examined by bisulfite sequencing. The analysis revealed the same tendency for decreased methylation as observed by pyrosequencing in the study. In addition, it revealed that even in this homogenous cell culture, the methylation patterns were very different in each cell, with the number of methylated CpG sites ranging from 1-6 out of the total 16 CpG sites in Region 3. Notably, treatment with valproate caused the percentage of clones being methylated at 0 or 1 CpG sites to increase from 17% in untreated cells to 37% after valproate treatment. This indicates that at least part of the increase in expression results from an increasing proportion of cells becoming transcriptionally active.

## 4.8. PHARMACOLOGICAL PREVENTION OF SIDE EFFECTS INDUCED BY ECS

ECT is one of the most effective treatments of major depression, but it is associated with both anterograde and retrograde memory impairments (Squire 1986; Lisanby et al. 2000). Anterograde memory impairments are particularly severe during the first few hours after ECT (Squire 1986; Sackeim et al. 2007; Smith et al. 2010) and capacity for learning usually takes up to 4 weeks to restore (Sackeim et al. 2007; Smith et al. 2010). In mice ECS causes rapid active DNA demethylation or *de novo* methylation at a high number of CpG sites in dentate granule neurons (Guo et al. 2011b). These changes are accompanied by upregulation of *de novo* methyltransferase *Dnmt3a* and pre-infusion of DNMT inhibitor decitabine before ECS abolishes *de novo* methylation (Guo et al. 2011b). In study 5 we attempted to use the same strategy for preventing *de novo* methylation of the *Arc* promoter. It is known that regulation of *Arc* expression needs to be balanced as lack of *Arc* function

in knock-out mice leads to severe problems in memory consolidation (Plath et al. 2006) whereas over-accumulation correlates with severe memory impairments (Greer et al. 2010). For rather obvious reasons the experiment was primarily thought as a proof-of-concept study. First of all learning and memory processes have been linked to rapid and dynamic regulation of DNA methylation (Miller & Sweatt 2007; Lubin et al. 2008; Penner et al. 2011). In addition, knockout of *Dnmt1* and *Dnmt3a* results in abnormal long-term plasticity in the hippocampal CA1 region together with deficits in learning and memory (Feng et al. 2010a). Consequently, decitabine would undoubtedly by itself affect memory formation until being washed out. As discussed in study 5, an alternative strategy could be to inhibit HDAC activity. Indeed, it has been demonstrated that pre-treatment with the HDAC inhibitor phenylbutyric acid (PBA) reduced spatial memory deficits induced by ECS (Yao et al. 2010). It has been established that HDAC2 and histone acetylation of H3 are important for regulating *Arc* transcription (Moonat et al. 2013) but further studies are needed to reveal its potential in relation to ECS.

## 4.9. HYDROXYMETHYLATION IS ABUNDANT IN THE MAMMALIAN BRAIN

In recent years an additional DNA modification at the 5' position of cytosine, 5-hydroxymethylcytosine (5hmc) has attracted increasing attention (Wen & Tang 2014). As described in section 1.1.1.1 in the introduction, 5hmc is an intermediate in the demethylation pathway, in a process involving oxidation of 5mc by TET enzymes. However, 5hmc is both abundant and stabile and evidence has emerged that 5hmc is also involved in regulating chromatin structure (Mellén et al. 2012). 5hmc has been detected in all tissue and cell types investigated to date (Wen & Tang 2014). Normal bisulfite conversion techniques do not distinguish 5hmc from 5mc and it has been estimated that 5hmc constitutes 10-20% of all methylated cytosines in mammalian brain tissues (Münzel et al. 2010). In a study mapping 5hmc and comparing the patterns to 5mc in the human brain it was found that 5hmc is more selectively targeted to genes than 5mc (Jin et al. 2011). Particularly, 5hmc is enriched at gene bodies and promoters and are largely absent from non-gene

regions. In promoters with high or intermediate CpG density 5hmc did not correlate with gene expression as opposed to presence of 5mc. However, in gene promoters with low CpG density there was a positive correlation between 5hmc and expression. In study 1, 2, 3, and 5 we used conventional bisulfite conversion and can therefore not distinguish 5hmc from 5mc. Studying 5hmc in all the settings could be informative, but particularly study 3 and 5, focusing on methylation of *Arc* could benefit from such studies. It has been found that neuronal activity decreases *Tet1* expression in CA1 of mice 3 hours after flurothyl-induced seizures (Kaas et al. 2013). In the same setting it was found, that 24 hours post-seizures the total levels of both 5mc and 5hmc levels decreased significantly. This highlights that levels of 5hmc are also affected by seizures. Furthermore, it was found that overexpression of TET1 induced increases in expression of several IEGs including *Arc*. However, it should be noted that expression of these genes was also significantly elevated in response to the catalytically inactive TET1m, suggesting that TET1 regulates the expression of these genes, at least partly, independent of 5mC to 5hmC conversion. Nevertheless, the abovementioned study highlights that 5hmc is highly relevant to study in relation to regulation of the *Arc* gene after ECS.

# 5. CONCLUDING REMARKS

Over recent years an increasing amount of research have established that epigenetic mechanisms are importantly involved in etiology and treatment of psychiatric disorders. However, much work is needed to unravel the interplay between genetics, environment, and epigenetics. The studies included in this dissertation have provided important new knowledge regarding the epigenetic regulation of the genes *BRD1* and *CHRNA7*. Regulation of *BRD1* is particularly interesting in relation to neurodevelopment and mental disorders since BRD1 regulates several risk genes. Regulation of *CHRNA7* on the other hand is particularly interesting as the gene is associated with the P50 deficit found in most schizophrenics, and as it encodes the α7 nAChR, which is considered a promising target for treatment of cognitive dysfunction. These genes are only two out of an abundant number of genes that have been associated with schizophrenia. The mechanisms associated with such genetic risk variants remain warranted. By studying gene expression of *Dnmt3a* and methylation and expression of *Arc* after ECS, we also tested the potential of using DNMT inhibition as a strategy to prevent ECS-induced side effects. Although the study did not reveal the desired effect, it revealed that decitabine attenuates ECS-induced *Dnmt3a* upregulation and that *Arc* is most likely regulated by DNA methylation. In addition, the studies revealed that three different drugs, zebularine, decitabine, and valproate all affected DNA methylation in the applied settings. Future studies will shed light on the importance of epigenetic mechanisms in relation to psychiatric disorders and reveal the therapeutic potential of epigenetic modulation.

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This declaration concerns the following article/manuscript

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| Authors | Mads Dyrvig, Per Qvist, Jacek Lichota, Knud Erik Larsen, Mette Nyegaard, Anders D. Børglum, and Jane H. Christensen |

The article is: In preparation
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| 2. Planning of the experiments and methodology design and development | E |
| 3. Involvement in the experimental work | E |
| 4. Interpretation of the results | E |
| 5. Writing of the first draft of the manuscript | E |
| 6. Finalization of the manuscript and submission | D |

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| Authors | Mads Dyrvig, Casper René Gøtzsche, David P.D. Woldbye, Jacek Lichota |

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