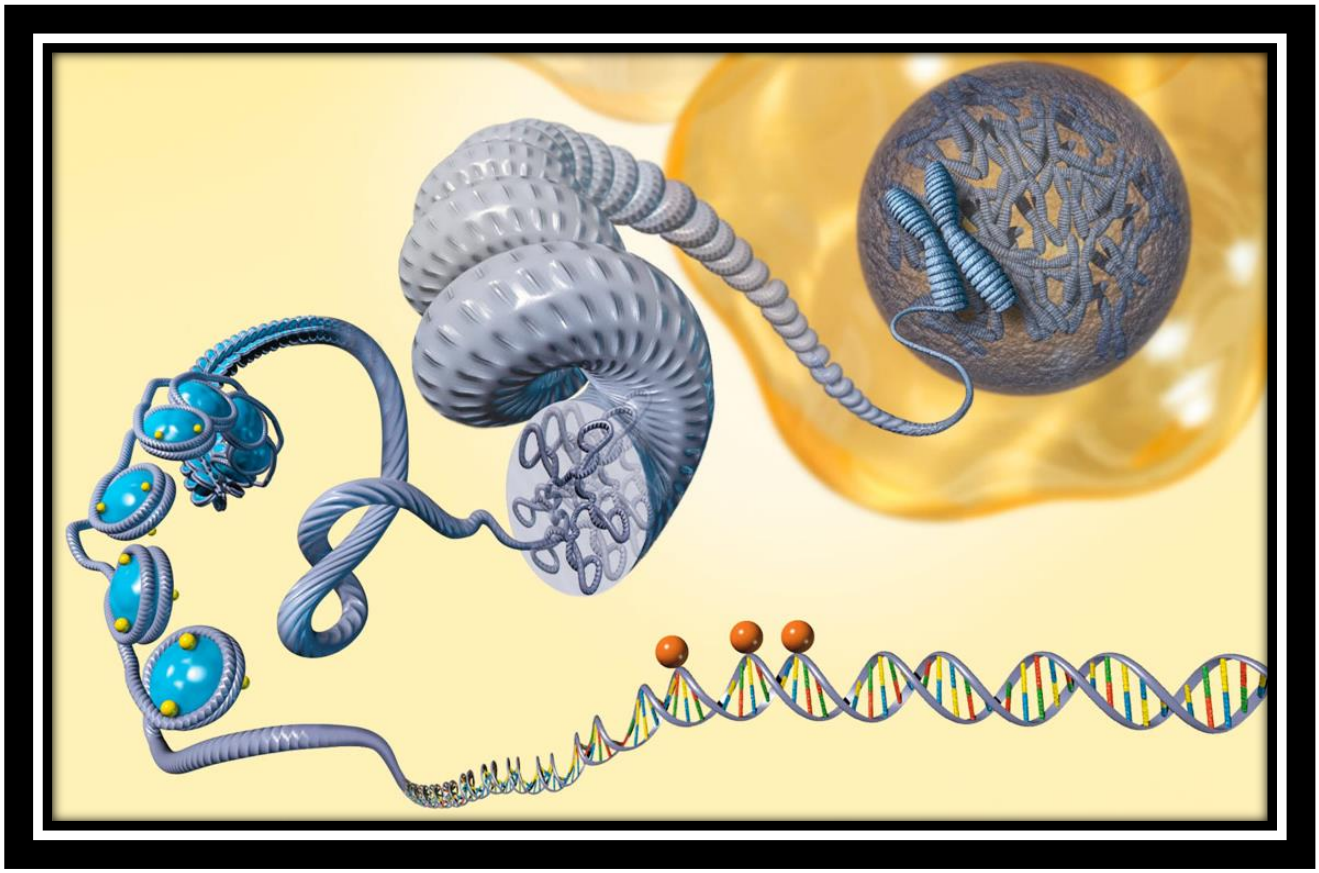
Epigenetics in Schizophrenia

Study of Epigenetic Regulation of Schizophrenia-relevant Genes in Phencyclidine Treated Rats



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PREFACE

Lene Lundgaard Donovan wrote the present master thesis during the 9th and 10th semester of Medicine with Industrial Specialization at Aalborg University, Denmark. The experimental work was carried out at the Laboratory of Neurobiology, Biomedicine, Aalborg University. A total of 9months was spend on this thesis, with completion on the 28th of May 2014.

In an attempt to uncover epigenetic mechanisms in schizophrenia, an introduction will first clarify the pathophysiology of schizophrenia, highlighting the need for investigation of molecular alterations in this obscure psychological disorder. Furthermore, the basic concepts of epigenetics will be presented, enabling the reader to grasp the concept of genetic regulation without changing the actual genetic sequence. The methodology will be clearly explained, followed by a presentation and discussion of the observed findings. References are denoted according to the Vancouver referencing style. Additional information of interest can be found in the appendices.

I would like to give thanks to my fellow master students, and a special thanks to PhD colleagues for invaluable advice and encouragement in times of despair. My outmost gratitude goes to my supervisor, Jacek Lichota, for excellent guidance, high standards, and encouragement to only accept absolute quality. Last, but not least, a loving thanks goes to my husband and family for bearing with me in times of need.

ABSTRACT

Administration of the NMDA receptor antagonist, Phencyclidine (PCP), is widely used as a model for schizophrenia. However, numerous molecular alterations following PCP exposure are still to be uncovered. Here, prefrontal cortices (PFCs) from rats exposed to a single acute injection of PCP (10mg/kg, sc.) were used to investigate long-term expression profile (8h, 24h, and 48h) of the neuronal activity marker, c-*fos*, and three schizophrenia-relevant genes, *parvalbumin, Gad67,* and *Chrna7*. Furthermore, histone methylation status was analyzed by native chromatin immunoprecipitation (NChIP), using antibodies directed against H3K4me3, H3K9me2, and H3K27me3. Bisulfite conversion of unmethylated cytosine residues was used for DNA methylation analysis of the *Chrna7* promoter.

The results indicate differential expression effects and histone alterations of PCP exposure on the investigated genes. c-*fos* was markedly upregulated after 8h, in spite of decreased H3K4me3 association. After 24h it was significantly decreased even though both H3K4me3 and H3K27me3 associations were decreased. *Parvalbumin* expression did not deviate from baseline at any of the investigated time-points, yet at 8h it was associated with less H3K4me3 and more H3K27me3. The α7-subunit gene, *Chrna7*, was downregulated 8h post-injection, correlating with the decreased association with H3K4me3. Furthermore, a tendency towards increased promoter DNA methylation was observed 8h post-injection. Exposure to PCP had a delayed effect on *Gad67* expression, as this gene was upregulated 24h after administration, yet at both 8h and 48h, baseline values were observed. The only histone mark of importance for *Gad67* regulation was H3K9me3, which was significantly more associated with the gene 24h after PCP exposure. Reports from post-mortem studies of schizophrenia-affected brains, consistently find decreased *parvalbumin* and *Gad67* expression, while no alterations are found in *Chrna7* expression. These discrepancies underline the difficulties encountered when modelling a complex human disorder in rodents.

TABLE OF CONTENT

ABBREVIATIONS

cDNA	Complementary DNA
Chrna7	α7-subunit encoding gene
CNS	Central nervous system
Ct	Cycle threshold
Dnmt	DNA methyltransferase
GABA	γ-aminobutyric acid
Gad67	67kDa isoform of glutamic acid decarboxylase
gDNA	Genomic DNA
H2/3/4	Histone 2/3/4
HDM	Histone demethylase
HMT	Histone methyltransferase
K	Lysine residue
me1/2/3	Mono-/di-/trimethylation
MeDIP	Methylated DNA immunoprecipitation
NChIP	Native chromatin immunoprecipitation
NMDA	N-methyl-D-aspartate
NTC	No template control
PCP	Phencyclidine
PFC	Prefrontal cortex
qPCR	Quantitative polymerase chain reaction
TSS	Transcription start site
UTR	Untranslated region
WHO	World Health Organization

1. INTRODUCTION

Over the past decades mental health has been subjected to much attention and research, rightfully so. According to World Health Organization (WHO), mental disorders are by far the greatest cause of chronic conditions in Europe. They also reveal that 27% of European adults had, within a year, experienced at least one mental disorder. As if one mental issue was not enough, 32% had another one on top, 18% had two more, and 14% had three or more disorders coinciding with the primary one. Women generally have a higher prevalence of mental health issues than men, and increased age increases the risk considerably. (1)

Schizophrenia is a severe and complex psychiatric disorder. The lifetime prevalence of this illness is about 1%, and WHO estimates 24 million people are affected worldwide. Schizophrenia often has a very early onset compared to other mental disorders, as it usually manifests during adolescence and early adulthood (age 15-35). Such early onset is one of the reasons why schizophrenia is the leading cause of years of life lost to disability, and the average life expectancy is reduced by 25 years. Symptoms of schizophrenia are divided into three categories: positive (psychotic), negative (anhedonic), and cognitive dysfunction. Positive symptoms include hallucinations and thought disorder, whereas negative symptoms can be inappropriate mood or apathy. Cognitive impairment can for example be working memory dysfunction. Although psychosis often represents the diagnostic time point, cognitive abnormalities may be present several years in advance. Furthermore, it is not the severity of the psychosis, but the degree of cognitive impairment, that is the best predictor of long-term functional outcome for the patient. (2–5)

Even though mental disorders, like schizophrenia, are old phenomena, the etiology and pathophysiology of the illnesses largely remain unclear; probably because of complex interactions between social, environmental, psychological, and biological factors. A hereditary component appears evident for schizophrenia, yet studies in monozygotic twins complicates this line of thought. If one twin is schizophrenic, the other twin only has a 53% chance of developing the disorder. The risk of developing schizophrenia in adopted entities is correlated to the presence of the disease in the biological parents, not the adoptive parents. However, the inheritance of schizophrenia does not conform to any typical type, such as autosomal dominant or sex-linked inheritance. Furthermore, a wide range of environmental exposures during development (both *in utero* and childhood), are risk factors for developing schizophrenia later in life. Evidently, many factors play a role in the etiology of schizophrenia, making the individual presentation vary greatly, and therefore the necessary treatment vary likewise. Three major types of treatment are available in the combat against mental illnesses: psychosocial, pharmaceuticals, and psychological. A major problem however, is the lack of effective treatment or the discovery of new treatment options, since many of the pharmaceuticals used today were discovered decades ago. The positive and negative symptoms can be managed by antipsychotic and antidepressive drugs, respectively, yet the cognitive impairments are largely left untreated. The fact that our understanding of the underlying causes of schizophrenia is still scarce, hinders the development of new treatment strategies. This leaves an increasing amount of patients with a seriously impairing condition but no effective treatment. (2,4–7)

2. NEURAL CIRCUITRY AFFECTED BY SCHIZOPHRENIA

After many years of research, schizophrenia is now considered a neurodevelopmental disorder, wherein many alterations in brain functionality occur. Psychosis is now known not to represent the onset of the illness, but to be a product of years of pathological processes. In fact, individuals diagnosed with schizophrenia have been found to have smaller whole brain volumes, especially the prefrontal region is affected (8). During adolescence the prefrontal grey matter volume normally declines, but in individuals with childhood-onset of schizophrenia, this decline is increased. It seems that the decreased prefrontal grey matter volume in schizophrenia is due to less cortical neuropil[1], and not because of fewer neurons or axon terminals. (4,8,9)

Advanced cognitive functions rely heavily on frontal lobe structures, especially important is the dorsolateral prefrontal cortex (PFC), correlating with the suggestion that frontal lobe dysfunction is a key player in schizophrenia. In fact, greater reduction in grey matter volume of the dorsolateral PFC is linked to more pronounced cognitive impairments. Furthermore, abnormalities in white matter of this region have also been found. However, the dorsolateral PFC does not show evidence of a diagnostic lesion, nor degeneration, but both cellular and neurochemical abnormalities have been suggested to be implicated in schizophrenia. (10–12)

As with many other psychiatric disorders, schizophrenia has been hypothesized to be caused by abnormalities in dopaminergic and glutamatergic neurotransmission.
The dopamine hypothesis is the longstanding sovereign of theories trying to explain schizophrenia. The initial hypothesis stated that schizophrenia was due to a hyperdopaminergic state in the brain. This conclusion was based on the observation that the effectiveness of antipsychotic drugs correlated with their affinity for the dopamine receptors. However, a revision of the classical theory was proposed in the early 1990s, since progress, especially in imaging studies, had revealed substantial new evidence. The main shift in the revised hypothesis was regional differentiation, specifically prefrontal hypodopaminergic and subcortical hyperdopaminergic states. Negative symptoms were proposed to result from the prefrontal hypodopaminergia, whereas positive symptoms were a result of striatal hyperdopaminergia. Although the 2nd version improved the hypothesis, a 3rd version was proposed in 2009, taking the surge of research from the past two decades into account. This hypothesis claims dopamine dysregulation is a result of multiple "hits", causing dysregulation at the presynaptic dopaminergic control level, which in turn is connected to psychosis and not schizophrenia itself. Even though the dopamine theory has proven very valuable in the search to uncover schizophrenic pathophysiology, many aspects of the disorder cannot be explained by dopaminergic dysregulation alone. (7,13–16)
The glutamate theory, on the other hand, stated that schizophrenia was the result of hypoglutamatergic signaling. It was based on the findings that N-methyl-D-aspartate (NMDA) receptor antagonists induce schizophrenia-like symptoms in healthy volunteers. One such antagonist, phencyclidine (PCP), induces positive, negative and cognitive symptoms similar to those of schizophrenia (7). Another important aspect of the glutamate hypothesis, is the wide distribution of glutamatergic neurons compared to dopaminergic neurons. Especially in regards to the cortex. All cortical efferents are glutamatergic, as are most of the cortical afferents and cortico-cortical connections. However, research now suggests that a hyper- rather than hypoglutamatergic state is important in schizophrenia. This hyperglutamatergic state is established through hypofunctionality of NMDA receptors on γ-aminobutyric acid (GABA) inhibitory interneurons. Less excitation of GABA neurons by NMDA receptors causes less inhibition of

[1] Neuropil is a region which has relatively few cell bodies but is synaptically dense

cortical neurons, thereby increasing their firing rate and stimulation of other non-NMDA glutamatergic receptors. Increased glutamate activity is especially pronounced in the PFC. (7,13)

The fact that both theories continue to exist decades after their conception, makes one wonder if they are both right in their own way. Each of the theories holds strong evidence that their neurotransmitter is essential to the pathophysiology of schizophrenia. Therefore, much research is currently trying to shed light on the interplay between dopamine and NMDA receptors. Both imaging and animal testing support a new hypothesis suggesting that both the hypodopaminergic cortical and hyperdopaminergic subcortical states are results of prolonged hypofunctionality of the NMDA receptor. (13,17)

3. PRINCIPLES OF EPIGENETICS

Epigenetics is a discipline that has received increased attention during the past decade,s since it is considered the missing link between the environment and the genome. With the mapping of the human genome at the time of the millennium, a lot of effort was put into finding pathophysiology related genes. However, this quest has proven more difficult than first anticipated, possibly because of the complex interaction between several factors, such as the environment and the genome, required for manifestation of many diseases. (18)

As the name signifies, epigenetic modifications are changes that occur "over" the genome, that is, new phenotypes are established without changing the DNA sequence. Epigenetic modifications regulate gene activity by altering protein-DNA interactions (e.g. the accessibility of the DNA sequence to transcriptional machinery) without affecting the sequence itself. Two main types of epigenetic targets exist, histone modifications and DNA methylation (Figure 1). (18–20)
In order to pack the entire genome into a nucleus, wrapping of the DNA helix around core histone proteins is essential. Histone proteins are highly conserved proteins divided into two categories: linker histone (H1) and core histones (H2A+B, H3, H4). Two copies of each core histone protein make up an octamer around which, 147 base pairs of DNA strand is wound 1.65 turns, forming a nucleosome, the basic unit of DNA packaging. The amino termini of the histone proteins protrude from the nucleosome, making them readily available to chemical modifications, which is what makes up histone modifications (18,20)
In regards to DNA methylation, it consists of the addition of a methyl group to a cytosine residue, usually occurring during DNA replication. However, as neurons are postmitotic cells, the term "neuroepigenetics" has emerged to address the epigenetic changes that occur in the central nervous system (CNS), despite the lack of cell division. Furthermore, many epigenetic marks are dynamic, rapidly changing and short lived, which has challenged the hereditable aspect of the general definition of epigenetics, so this is no longer essential. (19,21,22)

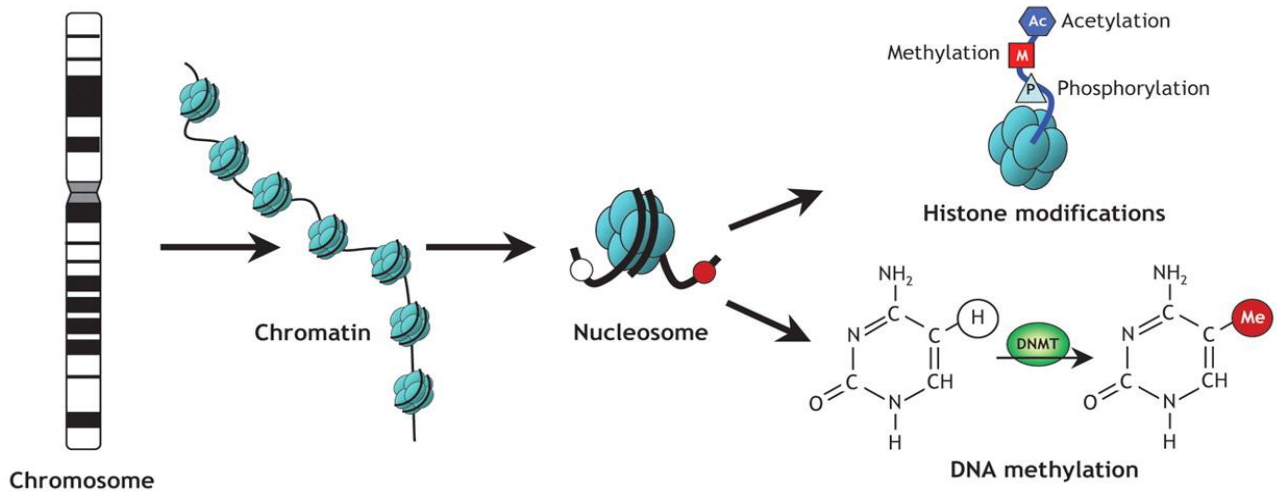

Figure 1: Chromosomes are made up of chromatin, which is a repeat of nucleosomes. Nucleosomes consist of core histones with DNA wrapped around it. Histone modifications most often occur on the histone tails, whereas the DNA can become methylated by DNA methyltransferases.
Me: methyl group, DNMT: DNA methyltransferase. (23)

3.1 DNA METHYLATION

DNA methylation refers to the covalent addition of a methyl group to the fifth carbon (C5) in the pyrimidine ring of a cytosine residue, within CpG dinucleotides. In mammals, 60-90% of CpG sites are methylated, and most of the residual unmethylated sites are found in CpG islands, in gene promoter regions. S-adenosylmethionine works as the methyl donor for the reaction, and the addition generally leads to repression of gene transcription (Figure 2) (18). However, recent evidence indicates a differential role of DNA methylation depending on the genetic region. Hypomethylation of promoter regions are associated with active gene transcription, yet exons can be densely methylated and still be transcribed (24). Since the covalent bond established between two carbon molecules is very strong, DNA methylation is usually regarded as an extremely stable epigenetic modification. However, compiling evidence is challenging this concept, as dynamic changes in DNA methylation status has been found in the CNS. (19–21,25)

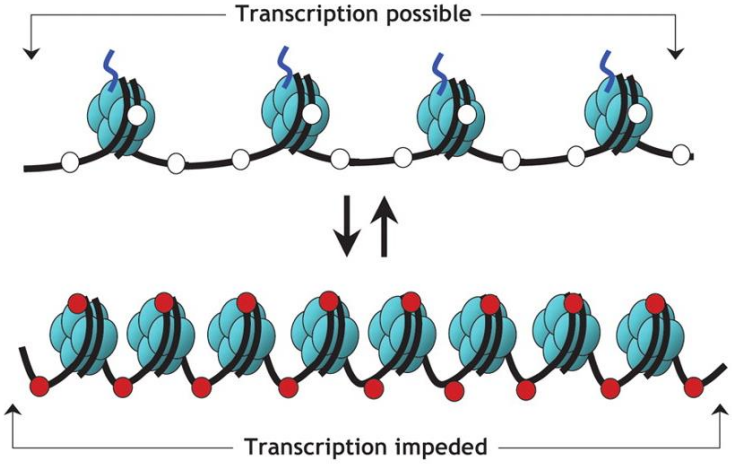

Figure 2: Dynamic modulation of the chromatin by DNA methylation and histone acetylation renders the genome accessible or not to transcriptional machinery.
White circle: unmethylated cytosine, red circle: methylated cytosine, blue line: acetylated histone tail. (23)

DNA methylation is established by DNA methyltransferase (Dnmt) enzymes, a family of proteins that, in eukaryotes, consists of four members: Dnmt1, Dnmt3a, Dnmt3b, Dnmt3*l*. (19)
Dnmt1 is often referred to as the maintenance Dnmt, as it preferentially methylates hemimethylated DNA strands, thereby ensuring the preservation of the epigenetic marks. Dnmt1 is the most abundant Dnmt, and it is situated at the replication fork, where it exerts its function by enzymatically binding a methyl group to the newly synthesized cytosine residue. The addition of a methyl group is highly specific, as Dnmt1 is dependent on allosteric activation by methylcytosine-containing hemimethylated DNA. Dnmt1 is extremely important in the embryonic stages, as targeted mutation of Dnmt1 leads to embryonic lethality before midgestation. In the CNS, similar importance of Dnmt1 is clear, as a complete loss of Dnmt1 in the brain causes death immediately after birth, demonstrating a crucial role for DNA methylation in neural cell survival. This is further emphasized by the discovery of continuous expression of Dnmt1 in neural tissue in adult mice, whereas most other organs display an insignificant expression of this gene. (19,26–29)
On the other hand, Dnmt3a and Dnmt3b are referred to as *de novo* Dnmts, since they methylate previously unmethylated cytosine residues. *De novo* Dnmts are especially important in germ cell development and embryogenesis, and even though they may have overlapping effect, the expression patterns of these two enzymes reveal their different importance. Dnmt3b is expressed in a short period of time in early neurogenesis of the mouse embryo, yet this developmental stage is highly dependent on the enzyme, as mutated Dnmt3b causes embryonic lethality, possibly because of multiple developmental defects. In contrast, Dnmt3a is expressed both during neurogenesis and in the adult mouse CNS, spiking in the first 3 weeks of postnatal life. This correlates with the fact that deletion of

Dnmt3a causes lethality in postnatal week 2-3, suggesting a crucial role of Dnmt3a in neural maturation. (30,31)
The last protein, Dnmt3*l* does not have enzymatic activity on its own, but potentiates the effect of the two *de novo* Dnmts (32).
Despite the in-depth knowledge about Dnmts, a demethylating enzyme is still to be discovered in vertebrates. The topic of active demethylation is subject to many controversies, however changes in DNA methylation are known to occur. (19,21)
Although rare, spontaneous demethylation happens, but the site is quickly re-methylated by Dnmts, from directions of the complementary strand. Even in the case of genomic damage, such as oxidative stress, the complementary strand directs re-methylation of the base excision repaired cytosine residue. (21)

As previously mentioned, DNA methylation patterns are often established during cell division, whereafter changes are rare. This is also evident by the low expression level of Dnmts in committed somatic cells, but the fact that postmitotic neurons hold substantial levels of Dnmts suggest that these enzymes, and thereby DNA methylation, are more dynamic and play critical roles in the nervous system. (19,31)

3.2 HISTONE MODIFICATIONS

Histones are highly conserved proteins, which enable the cell to compact vast amounts of DNA into the nucleus. These proteins are each made up of a globular domain and an amino terminus, also called the histone tail, which is the protein sequence that protrudes from the nucleosomal disc. These tails are generally charged and flexible, and they play an important role in the assembly of nucleosomes into chromatin. Their importance is demonstrated in yeast cells, where truncation of both tails in a histone pair (H2A/H2B or H3/H4) is lethal to the organism. Histone tails are extremely basic since they contain a high proportion of lysine and arginine residues. (33–35)

The modifications that occur on histone proteins consist of covalent addition of a small chemical group. There are at least eight different types of modifications known: acetylation, methylation, phosphorylation, ubiquitylation, SUMOylation, ADP ribosylation, deamination and proline isomerization; and so far, more than sixty different residues have been shown to be modified. Taken together, the many types and sites of modifications all orchestrate regulation of accessibility to the DNA, providing a mechanism for fine-tuned functional responses to stimuli. This is even more evident from the fact that the enzymes that produce the modifications are highly specific to a certain type of residue and its position, and that the modifications are dynamic and constantly changing. (18,22,33)

Histone modifications work in either of two ways: recruiting non-histone proteins to the site or unraveling the chromatin by breaking contacts between nucleosomes. These two ways are probably connected, as the modifications can either encourage or inhibit the binding of proteins to the chromatin, partly by adjusting the state of the chromatin. The two main states of chromatin, euchromatin and heterochromatin, are associated with different modification patterns, as initially proposed by Jenuwein & Allis. (22,33)
Heterochromatin is the “silent” or repressed state of transcription. This state is associated with high levels of methylation at specific residues, namely lysine 9 of H3 (H3K9), H3K27 and H4K20. Other modifications, such as ubiquitination, sumoylation and deamination, have also been implicated in the formation of heterochromatin. (22)
Euchromatin signifies a large part of the genome. In this state the chromatin is kept flexible and readily “unraveled” for transcription or repair. The major activating modification is acetylation, but also other

modifications are associated with actively transcribed euchromatin, namely tri-methylation of H3K4 (H3K4me3), phosphorylation and ubiquitination. (22)
As noticed from the above mentioned modifications, it is not possible to classify each type of modification as purely activating or repressing. Therefore, Kouzarides proposed that 'any given modification has the potential to activate or repress under different conditions'. In this light, context is the determining factor, exemplified by methylation of H3K36. If this modified residue is located in the coding region it has an activating effect, whereas if it is found in the promoter region it has a repressive effect. (22)

To complicate the picture even further, the possibility of interaction between modifications seems inevitable. First of all, many modifications take place on lysine residues, which must result in some degree of antagonism. Secondly, modifications of surrounding residues may affect the binding of modifying proteins. Thirdly, when epigenetic marks occur in combination, their effect is different than if they occur in isolation. Furthermore, the enzymes producing the modifications could be affected by another modification on their recognition site, or perhaps they could recognize their substrate better by "guidance" from another modification. Overall, histone modifications are likely to influence each other in a synergistic or antagonistic manner. (22,33,36)

As previously mentioned, many different types of histone modifications are known, but a full coverage of them all is beyond the scope of this thesis. Therefore, only methylation will be discussed in detail.

METHYLATION

Methylation is a reversible histone modification that can occur on all basic residues, namely lysine, arginine and histidine, however, only lysine methylation will be covered in this thesis. Methylation of histone tail residues play crucial roles in numerous biological processes, e.g. cell-cycle regulation, development and differentiation. (22,36,37)

Despite the importance of dynamic changes in response to stimuli, methylation is usually regarded as a rather stable modification, and it is believed that methyl groups have a slower turn over than other types of histone modifications, in fact, histone methylation was once considered to be irreversible. This theory was partly due to the missing discovery of a demethylating activity, which would provide methylation with the apparent dynamics of opposing enzymes, as was observed for other types of modifications (Figure 3). However, the recent discovery by Shi *et al.* (38) of the first demethylase undermines this way of thinking. (18,36,39)
The enzymes that establish the methylation modifications are collectively called histone methyl-transferases (HMTs), and S-adenosylmethionine serves as the methyl donor for the reaction. In regards to lysine methylation, HMTs are divided into two families depending on the conserved motif: SET-domain-containing or DOT1-like. Several different enzymes have been identified, and they all have in common that they are highly conserved over a range of species. Methylation does not affect the charge of the modified residue, so the exact functional mechanism of histone methylation is still unknown. However, it is known that the SET-domain-containing enzymes require adjacent cysteine-rich regions in order to display any catalytic effects. (36,39–42)
Since the discovery of the first demetylase in 2004, extensive research in this area has resulted in the identification of two families of histone demethylases (HDMs): the amine oxidases and the jumonji C enzymes (36). The amine oxidases demethylate lysine residues by an oxidation reaction that forms formaldehyde (38). In addition to formaldehyde generation, the jumonji C enzymes also form succinate, and they are dependent on the presence of iron and α-ketoglutarate (43).

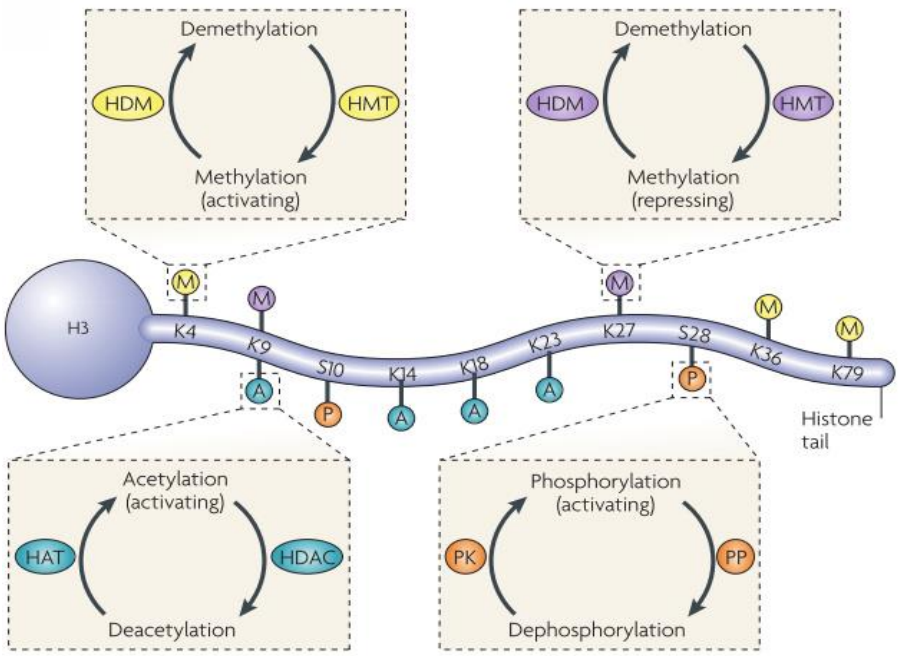

Figure 3: Overview of possible modifications on the residues of the tail of histone 3 by their respective modifying enzymes.
HDM: histone demethylase, HMT: histone methyltransferase, HAT: histone acetyltransferase, HDAC: histone deacetylase, PK: protein kinase, PP: protein phosphatase, M: methylation, A: acetylation, P: phosphorrylation, K: lysine residue, S: serine residue. (18)

The two opposing methylation modifying enzymes, HMT and HDM, are very substrate specific. Such high specificity is very important since methylation is associated with both gene activation and repression, depending on the modified residue (Figure 3). As if the residue specificity is not enough, the enzymes are also selective in their degree of methylation modification, since lysine residues can be either mono-, di- or trimethylated (me1/2/3). This multilayered precision is necessary to achieve the fine-tuned regulation that methylation exerts. (18,22,36,39)
Generally, three lysine residues are considered to be implicated in transcriptional activation: H3K4, H3K36, H3K79. H3K4 is a widely investigated modification that has been found to have differential influences depending on the degree of methylation. H3K4me1 is usually associated with enhancer function, whereas H3K4me2 is associated with both active and inactive euchromatic genes. Furthermore, H3K4me3 is yet another modification, which is associated with promoter activity of actively transcribed genes. However, H3K4me3 has also been found to be associated with transcriptional repression. (22,36,39,44)
Since there are three activating lysine residues, coincidentally there is also three residues that are associated with gene repression: H3K9, H3K27, H4K20. H3K9 methylation is a hallmark of heterochromatin, in fact, methylation at H3K9 has been linked to the inactivation of a whole X-chromosome in females. Afterwards, methylation of H3K27 is thought to be important for maintaining this inactivated state. However, as with H3K4, the degree of methylation of H3K9 and H3K27 is crucial for the functional effects of the modifications. Both H3K9me2 and H3K9me3 are more often present around transcription start site of silent genes than of active genes, correlating with the general silencing role. However, H3K9me1 is more often present around active promoters, suggesting an activating role of this modification. Similar patterns have been observed for methylation states of H3K27, as active genes often possess high levels of H3K27me1 downstream of transcription start site (TSS), whereas inactive genes have been found to contain high levels of H3K27me3 in their promoter and gene-body regions. However, H3K27me3 is not only found in association with inactive genes, in fact H3K27me3 and H3K4me3 are two modifications, which can illustrate the "cross-talk"-effect discussed above. Since these two modifications have opposing effects, their co-presence seems to keep genes in a balanced transcriptional state. (22,36,39,45,46)

3.3 INTERPLAY BETWEEN DNA METHYLATION AND HISTONE MODIFICATIONS

Through much research it has become apparent that DNA methylation and histone modifications are closely connected. This connection is a two-way relationship, since both histone modifications can direct DNA methylation, as well as DNA methylation can direct certain histone modifications (Figure 4). In fact, it has been suggested that histone methylation patterns correlate better to DNA methylation than to the genetic sequence itself. (18,47–49)

DNA methylation is regarded as a repressive modification, which adds up with the correlation found between it and methylation of H3K9, and the anti-correlation between it and H3K4 methylation, a repressive and an activating mark respectively. Actually, methylation of H3K4 is proposed to prevent *de novo* DNA methylation of promoter regions by disabling the Dnmt3*l* protein to bind to the nucleosome. This Dnmt does not have enzymatic function itself, as mentioned earlier, but it does contain a reading frame, which probes the methylation status of H3K4. If this residue is methylated, Dnmt3*l* cannot bind and recruit Dnmt3a to the nucleosome, which would otherwise induce *de novo* methylation of the DNA. It is also through this Dnmt3*l*-Dnmt3a complex that a H3K4-specific HDM correlates with increased DNA methylation, since this protein demethylates H3K4, making it possible for Dnmt3*l* to bind. Interestingly, Dnmt3*l* does not probe the methylation status of H3K9. (48–51)
Another family of proteins, the MLLs, are H3K4-specific HMTs, which also support the proposed protective effect of H3K4 methylation. They contain a SET-domain for methylating H3K4, but they also have DNA CpG-reading domains, which prevents DNA methylation or stabilizes unmethylated DNA, although the underlying mechanisms are still unknown. (49)

DNA methylation and H3K9me have a positive correlation, in fact, inhibiting DNA methylation leads to decreased H3K9 methylation levels. It is especially the H3K9-specific HMT G9a that plays a pivotal role in both DNA and histone methylation. G9a works as an interaction hub, and complexes that contain this protein recruit the two *de novo* DNA methylases, Dnmt3a+b. It has even been shown that fastening G9a to a random region of DNA causes both DNA and histone methylation of proximate sequences. (36,48,49,52,53)

A SET-domain containing HMT called EZH2, plays a pivotal role in the addition of methyl groups to H3K27. This enzyme has been found to interact with Dnmts, and if a promoter has reduced H3K27me3 or EZH2 occupancy, a profound decrease in Dnmt-binding was observed. Somehow the SET-domain is crucial to methylation of CpG dinucleotides in promoters, as mutant EZH2 lacking the SET-motif was unable to induce DNA methylation, indicating that methylation of H3K27 is important for the EZH2-mediated DNA methylation. (48,54)

These modifications, DNA methylation plus histone methylation, clearly show how the epigenome works together in concert to activate or repress the genome.

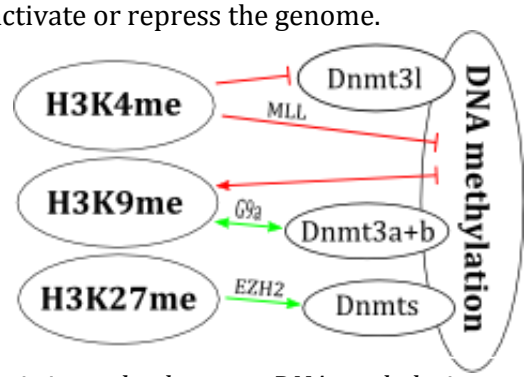

Figure 4: Overview of epigenetic interplay between DNA methylation and histone modifications. Green lines signifies increase, red lines indicates decrease. → leads to, ⊣ inhibition, Dnmt: DNA methyl-transferase.

4. MODELLING SCHIZOPHRENIA BY ADMINISTERING PHENCYCLIDINE

Phencyclidine (PCP) was initially used as a surgical anesthetic, but in the late 1950s it was recognized as psychotomimetic (55). Patients waking up from surgery experienced delirium, hallucinations, agitation, disordered speech and disoriented behavior, similar to symptoms observed in schizophrenic individuals. Following this discovery, healthy volunteers were used to show the full potential of PCP to induce schizophrenia-like symptoms, both positive/negative symptoms and cognitive dysfunction. Furthermore, if given to schizophrenic patients, their symptoms will exacerbate significantly. (17,56,57) Phencyclidine is a non-competitive antagonist of the NMDA-class of glutamate receptors. The effect of PCP is dependent on the use of the receptor, as PCP binds to a site within the pore, only accessible when the channel is open. Other receptor channels are also antagonized following binding of PCP, e.g. voltage-gated sodium and potassium channels, and nicotinic acetylcholine channels. However, the effects of PCP on these channels are less potent, returning to the main action of PCP exerted on the NMDA receptor. (56,57)

In the search of a valid animal model of schizophrenia, a deeply complex human disorder, many different PCP dosing-regimens has been investigated, e.g. chronic, sub-chronic, acute, and perinatal. However, only the acute single-dose approach will be covered in this thesis.

Acute administration of PCP to rodents result in increased firing of PFC neurons, persistent hyperlocomotive activity, and the function of the frontal cortex is impaired, as is the functionality of the temporal cortex. Furthermore, motivation and social behavior are both reduced. Except for the hyperlocomotion, all of these effects on rodents acutely exposed to PCP, are similar to findings from non-human primates exposed to a single-dose of PCP. In addition to these neurological properties of PCP exposure, endocrine function has also been found to be altered, e.g. insulin has been shown to transiently change its signaling after acute PCP, correlating with the discovery of changes in brain glucose metabolism after acute PCP administration. (17,56,57)

Even though PCP is mainly an antagonist of NMDA receptors, it has widespread effects on neurotransmitter systems in the brain. Seemingly contradictive, a dramatic increase in cortical glutamate efflux is seen after acute administration of PCP. However, significant decrease in GABAergic signaling of the PFC accompanies the increased glutamate efflux after acute PCP (58), in line with the glutamate hypothesis of schizophrenia (Chapter 2). In regards to dopamine on the other hand, acute exposure to PCP has been found to trigger profound activation of the dopaminergic system in the forebrain of rodents (59). This does not correlate with the revised prefrontal hypodopaminergic aspect of the dopamine theory of schizophrenia (Chapter 2), however, it is quite unlikely that a single rodent model can mirror all aspects of a complex human disorder. (17,57)

Despite the fact that PCP exerts its main function on glutamate receptors, it can still be used as a model to investigate several neurotransmitter systems in schizophrenia. It is, however, impossible to mimic all the human aspects of an illness as complex as schizophrenia in rodents. Nevertheless, PCP has a unique ability to simulate the symptomatology of schizophrenia, and can, in rodents, be used to investigate metabolic, behavioral and neurochemical alterations experienced by schizophrenics.

5. GENES OF INTEREST

5.1 C-FOS

The immediate early gene c-*fos* is also a proto-oncogene. As a member of the immediate early gene-family, transcription of c-*fos* is activated rapidly, but transiently, in response to external stimuli, without previous protein synthesis. The c-fos transcript has a very short half-life, only 10-15min. This has made scientists speculate that the c-fos protein has a regulatory role in cellular response to external stimuli, correlating with the role of c-fos in the transcription factor complex Activator Protein 1. (60–62)

In most cell types, c-*fos* expression is very low or undetectable under basal conditions, but it can be induced in generally all cell types (61). However, a constitutive expression of c-*fos* is found in the mature CNS (63). Several neurotransmitters can induce expression of c-*fos* in the brain, but an anatomical and temporal specificity seems to be in place. It has been found that c-*fos* expression plays a part in exitotoxicity, in fact, brain areas with a high density of NMDA receptors correlate with the expression pattern of c-*fos* after a generalized seizure. However, the expression of the c-fos protein is found to habituate to repetitive stimuli. (60–62)
Expression of c-*fos* is often used as a marker of neuronal activity, since it is rapidly induced by a multitude of stimuli. This is exploited in many aspects, e.g. in the discovery of distinct cellular activation in response to certain stimuli. However, it is proposed that c-*fos* expression does not necessarily increase neuronal activity, since an increase of c-fos in inhibitory interneurons can decrease neuronal excitability. (64,65)

5.2 ALPHA 7

One of the two most predominant nicotinic acetylcholine receptors in the brain is α7 . This receptor is homomeric, built up of five α7 subunits, with a binding site for its endogenous ligands (acetylcholine and choline) between each subunit, providing five binding sites per receptor (Figure 5). Alpha 7 belongs to the super family of ligand-gated ion channels, and has a profound preference for Ca^{2+}. The receptor is not limited to a single brain region nor a single cell type, and has been found to have widespread effects. Furthermore, α7 can be located both pre-synaptically, regulating neurotransmitter release, or post-synaptically, activating intracellular signaling cascades. (66–68)

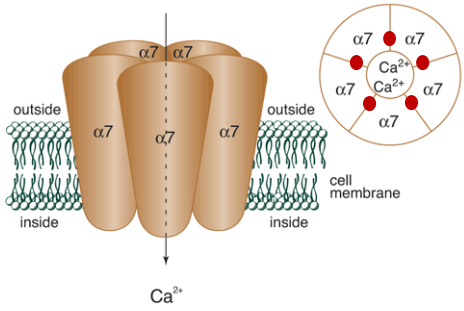

Figure 5: Graphical presentation of the α7 receptor. Red circles indicate ligand binding sites. Modified from (92)

It is well known that nicotine improves memory, attention and learning – cognitive functions that are severely impaired in schizophrenia. An exogenous ligand of the α7 receptor is nicotine, readily available in cigarettes. Since more than 65% of patients with schizophrenia are smokers, it has been proposed that they do so to self-medicate their cognitive deficits. The cholinergic system is essential to cognitive function, and high expression of α7-subunit gene, *Chrna7*, has been found in brain regions important to these functions, such as the PFC (69). Furthermore, enhanced attentional processes are achieved by stimulation of nicotinic acetylcholine receptors, e.g. by cigarette smoking. (66–68)

5.3 PARVALBUMIN

Parvalbumin is a calcium-binding protein, which is found on some inhibitory GABA interneurons. Parvalbumin acts as a calcium-buffer, accelerating the reduction of Ca^{2+} that usually accumulates in axon terminals, thereby enhancing GABA release during repetitive signaling. In the dorsolateral PFC, an important brain structure in the pathophysiology of schizophrenia (Chapter 2), approx. 25% of GABA interneurons are parvalbumin-positive. It does not appear that the number of parvalbumin-positive neurons is altered in schizophrenia, however, the expression of *parvalbumin* is found to be decreased in diagnosed individuals. (4,5)

Cortical parvalbumin-positive interneurons can be divided into two groups: chandelier cells and basket cells. The difference between the two cell-types is their synapsing site on the pyramidal neuron. Chandelier cells synapse on the axon initial segment of the pyramidal neuron, which is close to the site of action potential generation. Because of this close proximity, chandelier cells have been thought to exert very powerful, almost "veto power", inhibition on pyramidal cells. Decreased GABA from chandelier cells is thought to contribute to the pathophysiology of schizophrenia by impairing network activity of pyramidal neurons. (4,5,11)
Basket cells have also been suggested to be involved in schizophrenia. Cognitive impairments, a core symptom of schizophrenia (Chapter 1), seem to be a result of impaired prefrontal gamma oscillations[2]. Proper gamma oscillations are dependent on powerful inhibitory inputs from basket cells on to pyramidal neurons. Both pre- and postsynaptic alterations of basket cells are thought to weaken their inhibitory control of pyramidal neurons. It has been suggested that the decreased inhibition by basket cells observed in schizophrenia is a way of compensating for less pyramidal excitation occurring upstream. (4,11)
Although the definitive role of parvalbumin-positive inhibitory interneurons in schizophrenia is still to be uncovered, the alterations seen in both chandelier and basket cells seem to be specific to the disease process of schizophrenia, since no other psychiatric illness show similar abnormalities.(4)

5.4 GLUTAMIC ACID DECARBOXYLASE 67

The 67kDa isoform of glutamic acid decarboxylase (Gad67, also known as Gad1) is an enzyme involved in regulation of GABAergic signaling, by being responsible for most GABA synthesis. Deletion of the *Gad67* gene causes a 90% reduction of GABA levels in the brain and is embryonically lethal. (4,5,11)

In schizophrenia, decreased Gad67 mRNA and protein has consistently been found in the dorsolateral PFC (11). Furthermore, similar transcript findings have been done across other neocortical brain regions in schizophrenic individuals. Taking into account that *Gad67* is activity regulated, it has been suggested that the lower expression observed in schizophrenia is a reflection of decreased cortical activity. However, a significant variability in the *Gad67* expression pattern has been seen between diagnosed patients. (4,5,10,11)
Gad67 is a GABA-synthesizing enzyme, and ~25% of GABAergic interneurons in the dorsolateral PFC is parvalbumin-positive (see above). It is particularly in these parvalbumin-positive neurons that the lower Gad67 mRNA level is found, as up to 50% of these neurons have undetectable levels of Gad67 mRNA in schizophrenic subjects. Even lower protein levels of Gad67 have been found in the axon terminals of basket cells. Despite the variability of the decreased *Gad67* expression, it has been proposed that it is a core component of the schizophrenic disease process. (4,5,11)

[2] Oscillations are brain waves measurable by EEG, resulting from synchronized firing of neurons

6. METHODOLOGY

Schizophrenia is a severe and disabling disease, especially in regards to the cognitive impairments, as these deficits are mostly left untreated. The PFC has been shown to play a central part in the pathophysiology of schizophrenia, which is why this is the brain region investigated in the present thesis. Furthermore, since hereditability and environment seem to affect the development of schizophrenia, epigenetics is a prime candidate to play a part in the etiology. For this reason, the aim of this thesis is to investigate the epigenetic status of certain schizophrenia-relevant genes in detail. Rats treated with an acute single-dose of PCP were used to model schizophrenia. Firstly, an expression analysis was carried out on mRNA extracted from the left PFC, followed by an investigation of histone modifications on chromatin from the right PFC, of the same animals as the expression analysis was performed on (Figure 5). Because of the multitude of histone modifications, this thesis was limited to investigate H3K4me3, H3K9me2 and H3K27me3 modifications. Lastly, an analysis of the DNA methylation status of the α7-subunit gene, *Chrna7*, was carried out on DNA extracted from the same left PFC as used previously. In addition to these experiments, an optimization was tried on a methylated DNA immunoprecipitation (MeDIP) kit.

The following sections will address the experimental procedures, but for a detailed material list, please refer to Appendix 1.

6.1 ANIMALS AND STARTING MATERIAL

Prefrontal cortices were obtained from Copenhagen University (dr. Jens Mikkelsen), where all the animal handling was performed. Thirty-six male Wistar rats weighing 250-290g were used. The animals were divided into control or PCP-treated groups. The control group received a single dose of 0.9% saline (volume identical to PCP solution), whereas the PCP group was administered a 10mg/kg dose of PCP, both solutions were injected subcutaneously. Animals were sacrificed at three different time-points post-injection; 8h, 24h and 48h. At each time-point, six control and six PCP-treated animals were decapitated (Appendix 2). The brains were swiftly removed, the PFCs dissected out, frozen in liquid nitrogen, and stored at -80°C. The PFCs weighed 50-90mg each. As mentioned earlier, the right PFC was used for histone modification analysis, whereas the left PFC had its RNA, DNA and protein fractions extracted by means of Qiagen's Allprep DNA/RNA/Protein kit (Qiagen, Germany), according to manufacturer's protocol. The RNA fraction was used for expression analysis, the DNA fraction provided starting material for DNA methylation analysis, and the protein fraction was intended for qualitative investigation of histone modifying enzymes, however, this will not be included in the present thesis.

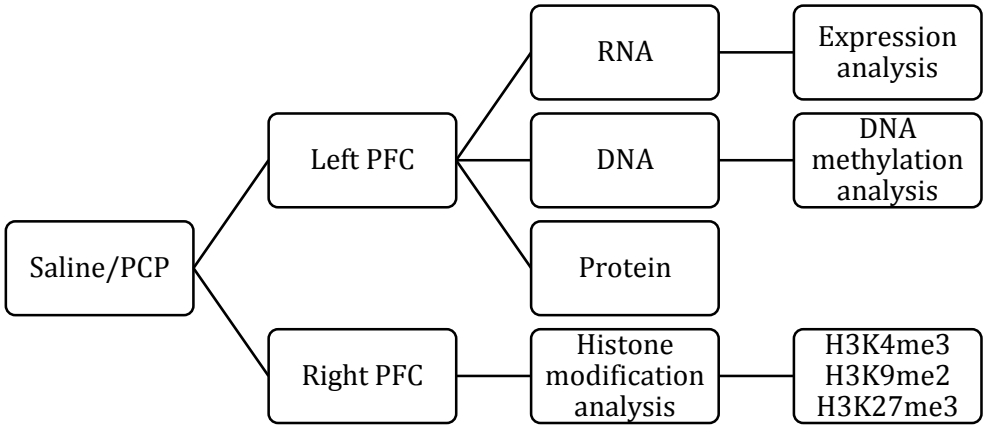

Figure 5: Workflow-chart of the experiments performed in the present thesis.

6.2 EXPRESSION ANALYSIS

DNase treatment:
The isolated total RNA from the left PFC was subjected to quantitative and qualitative assessment by spectrophotometry. For DNase treatment of the isolated RNA, RNase-free DNase I (Fermentas, Lithuania) was used, according to manufacturer's protocol. The treatment was performed for 30min at 37°C in a Veriti 96well Thermal Cycler (Applied Biosystems), followed by enzyme inactivation for 10min at 65°C after addition of 1µL 50mM EDTA.

cDNA synthesis:
The DNA-free RNA was subsequently used for complementary DNA (cDNA) synthesis on a Veriti 96well Thermal Cycler (Applied Biosystems) by Maxima H Minus First Strand Synthesis Kit (Thermo Scientific, USA), as according to manufacturer's protocol, although with slight changes in the thermo cycler program: 25°C for 10min, 50°C for 20min, 65°C for 10min, and 85°C for 5min. For each sample, a no reverse transcriptase (RT-) reaction was made, serving as negative control.

qPCR:
Quantitative polymerase chain reaction (qPCR) of the cDNA was performed on Mx3000 QPCR System (Stratagene) using the primers listed in Table 1 (TAG Copenhagen). 5µL template and 10µL Luminaris qPCR Master Mix (Thermo Scientific) with 10pmol of each primer was used in a final reaction volume of 20µL. The thermal profile used for amplification was as follows: 95°C for 10min, 40 cycles of 95°C for 30sec, 60°C for 30sec, and 72°C for 30sec. Amplification was followed by a melting curve program starting at 55°C and finishing at 95°C (Appendix 3). All samples were analyzed in triplicate, and for each gene, a no template control (NTC) was included as negative control.
The RT- samples were analyzed by qPCR in duplicates by the actin primers listed in Table 1, under the same conditions as mentioned above.

Table 1

Gene	Primers
Actin NM_031144	Fw 5' CCTCTGAACCCTAAGGCCAACCGTGAA Rv 5' AGTGGTACGACCAGAGGCATACAGGG
Chrna7 NM_012832.3	Fw 5' GGCATTGCCAGTATCTCCCTCCAGGC Rv 5' TGCAGGTCCAGTGACCACCCTCCAT
c-*fos* NM_022197.2	Fw 5' GGTCACAGAGCTGGAGCCCCTGTGC Rv 5' TCGTTGCTGCTGCTGCCCTTTCGGT
CycA NM_017101.1	Fw 5' TATCTGCACTGCCAAGACTGAGTG Rv 5' CTTCTTGCTGGTCTTGCCATTCC
Gad67 NM_017007.1	Fw 5' TGTCAATGCAACCGCAGGCACGACT Rv 5' GCGATGCTTCCGGGACATGAGCAGC
Parvalbumin NM_022499.2	Fw 5' TGCTGGAGACAAGGACGGGGATGGCA Rv 5' GAAACCCAGGAGGGCCGCGAGAAGG

Both actin and cycA were used as control genes for normalization, and the Pfaffl method (70) enabled calculation of fold changes of gene expression. Statistical analysis was performed using GraphPad Prism 5.0 software.

Laboratory of Neurobiology is currently undergoing a shift in master mix for qPCR, from using SYBR green (Agilent) to Luminaris, which has meant that a vast amount of primers need re-standadization. All the primers used for histone modification analysis have been verified on Luminaris, but some of the ones used for expression analysis still need this re-evaluation. However, all efficiencies obtained so far

are similar for SYBR green and Luminaris, which is why the results from the expression analysis is still valid despite using efficiencies obtained on SYBR green master mix.

6.3 HISTONE MODIFICATION ANALYSIS

Modifications of histones were investigated by means of native chromatin immunoprecipitation (NChIP). All solutions used were ice-cold and centrifugations were performed at 4°C. In order to minimize interaction between proteins and the eppendorf tube, Low protein binding tubes (Sarstedt) were used.

Preparation of dialysis tubes:
Dialysis tubing was cut into appropriate size, submerged in 0.5% SDS and boiled for 20min on magnetic stirrer (RCT basic (IKA)). Following a wash in demineralized water, the tubes were boiled for another 20min in 20mM EDTA. After two washes in clean MilliQ water, the material was stored at 4°C in 1mM EDTA until use.

Buffer-overview:

- *Extraction Buffer 1*: 0.4M Sucrose, 10mM Tris-HCl (pH 7.9-8), 5mM Sodium Butyrate, 5mM β-mercaptoethanol, protease inhibitor cocktail
- *Extraction Buffer 2*: 0.25M Sucrose, 10mM Tris-HCl (pH 7.9-8), 1% Triton X-100, 10mM $MgCl_2$, 5mM Sodium Butyrate, 5mM β-mercaptoethanol, protease inhibitor cocktail
- *Digestion Buffer*: 0.32M Sucrose, 50mM Tris-HCl (pH 7.5), 4mM $MgCl_2$, 1mM $CaCl_2$, 5mM Sodium Butyrate, protease inhibitor cocktail
- *Resuspension Buffer*: 10mM Tris-HCl (pH 7.9-8), 1mM EDTA, 5mM Sodium Butyrate
- *Dilution Buffer*: 1.1% Triton X-100, 1.2mM EDTA, 16.7mM Tris-HCl (pH 7.9-8), 167mM NaCl
- *Equilibration Buffer*: Sonicated λ DNA (0.3µg/µL), Bovine serum albumin (50mg/mL), Dilution Buffer
- *Low Salt Wash Buffer*: 150mM NaCl, 0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl (pH 7.9-8)
- *High Salt Wash Buffer*: 500mM NaCl, 0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl (pH 7.9-8)
- *LiCl Wash Buffer*: 0.25M LiCl, 1% Igepal (NP-40), 1% Sodium Deoxycholate, 1mM EDTA, 10mM Tris-HCl (pH 7.9-8)
- *TE Buffer*: 10mM Tris-HCl (pH 7.5), 1mM EDTA
- *Elution Buffer*: 1% SDS, 0.1M $NaHCO_3$

Chromatin isolation:
A right PFC was disrupted in 10mL Extraction Buffer 1 by Ultra Turrax (IKA) knife homogenizer in a 3 step manner: 10sec on each of the levels 3, 4, and 5. The homogenate was pelleted by 3000*g* centrifugation for 20min. The pellet was resuspended in 1mL Extraction Buffer 2, with subsequent centrifugation at 12,000*g* for 10min. The pellet was resuspended in 1mL Digestion Buffer, followed by quantification of DNA in the presence of 0.1% SDS by spectrophotometry (NanoPhotometer (IMPLEN)). To digest the chromatin, 50U MNase/0.5mg chromatin was incubated with the sample on Thermomixer Comfort (Eppendorf) at 37°C and 600rpm for 4min (Figure 6). In order to arrest the digestion, the reactions were chilled on ice and EDTA was added to a final concentration of 5mM, followed by 11,600*g* centrifugation for 5min. The first supernatant fraction (S1) was retained, and the pellet resuspended in

500µL Resuspension Buffer, which was dialyzed overnight in 4L Resuspension Buffer at 4°C on magnetic stirrer (RCT basic (IKA)). The dialyzed sample was transferred to an eppendorf tube and centrifuged at 2000*g* for 10min, providing the second supernatant fraction (S2).

Immunoprecipitation:

The two supernatant fractions (S1+S2) were pooled and used for immunoprecipitation. Reaction mixtures of 1 part sample and 4 parts Dilution Buffer were prepared, and incubated with antibody or rabbit serum (mock control) overnight on Intelli-mixer (Elmi) at 4°C and 20rpm. Coincided, Protein A coated magnetic beads (Invitrogen, USA) were incubated in 1mL Equilibration Buffer. To immunoprecipitate the antibody-histone complexes of interest, 30µL beads were added to the sample and incubated at 4°C, 20rpm for 3h on Intelli-mixer (Elmi). The beads were pelleted using DynaMag-2 magnetic rack (Dynal (Invitrogen)). The supernatant of mock control was retained as the input fraction, whereas the supernatant of antibody-incubated samples was discarded. The beads were subsequently washed on Intelli-mixer (Elmi) for 5x 10min at 4°C, 30rpm by the following buffers: Low Salt Wash Buffer, High Salt Wash Buffer, LiCl Wash Buffer, and TE Buffer (2x). To elute the chromatin, the beads were incubated twice at 65˚C for 15min with 250µL Elution Buffer and 1000rpm in a Thermomixer Comfort (Eppendorf). At the same time, 100µL input fraction was mixed with 400µL Elution Buffer and incubated for 30min at the same conditions as all samples, to ensure similar handling.

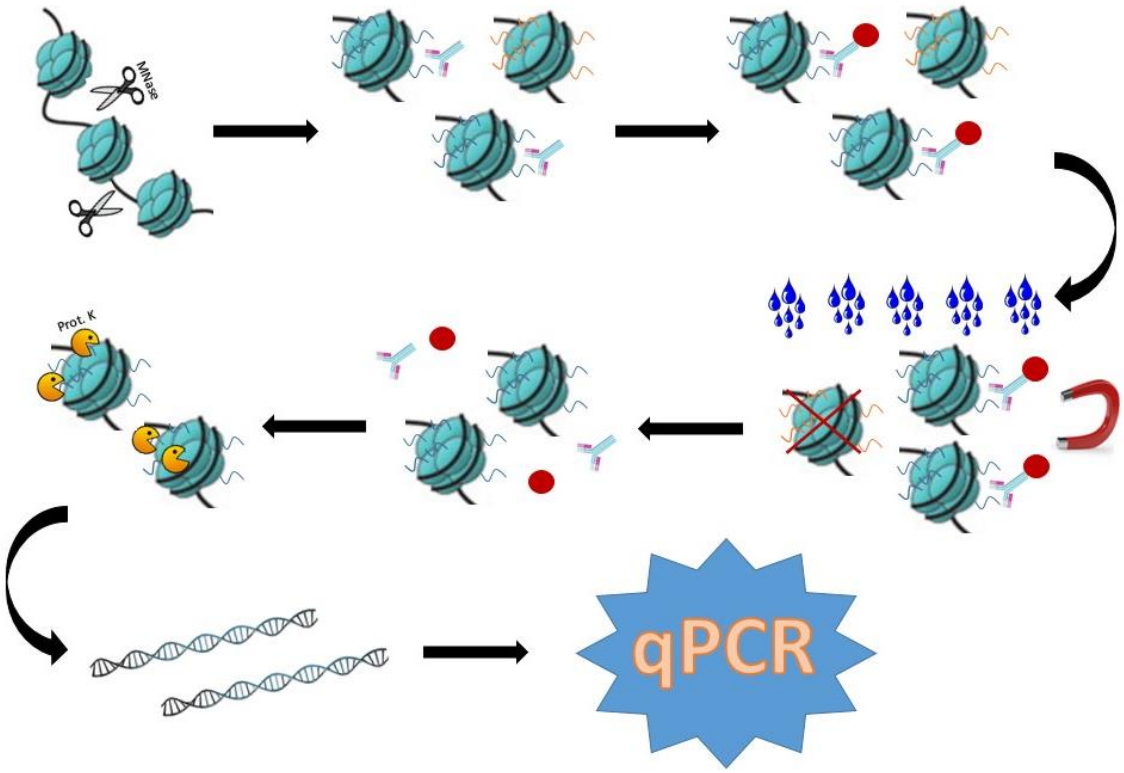

Figure 6: Step-by-step overview of the work-flow involved in native chromatin immunoprecipitation. Initial MNase fragmentation is followed by antibody binding to specific histone modifications. Protein A-coated magnetic beads binds to the antibody, allowing several washing steps to remove unbound and unspecifically bound nucleosomes. Elution from the beads and subsequent Proteinase K treatment enables DNA purification prior to qPCR.
Red circles indicate protein A-coated magnetic beads, blue histone tails signify histone modifications of interest, orange histone tails indicate histone modifications non-reactive with the used antibody. Modified elements from (23).

DNA purification:
To enable qPCR of the precipitated DNA fragments, the proteins of the eluted samples were digested by 20µg Proteinase K in the Elution Buffer (see above) supplemented with 10µL EDTA and 20µL Tris-HCl, for 3h at 45˚C and 600rpm in Thermomixer Comfort (Eppendorf). QIAquick PCR Purification kit (Qiagen) was subsequently used to purify DNA, as according to manufacturer's protocol.

qPCR:
The purified precipitated DNA was subjected to qPCR, by means of the primers listed in Table 2 (TAG Copenhagen), performed on a Mx3000 QPCR System (Stratagene). 5µl template and 10µL Luminaris qPCR Master Mix (Thermo Scientific) with 10pmol of each primer was used in a final reaction volume of 20µL. The thermal profile used for amplification was as follows: 95°C for 10min, 40 cycles of 95°C for 30sec, 60°C for 30sec, and 72°C for 30sec. Amplification was followed by a melting curve program starting at 55°C and finishing at 95°C. All samples were analyzed in triplicate, plus for each gene and animal, a no template control (NTC) was included as negative control.

Table 2

Gene	Primers
Actin NC_005111.3	Fw 5' GAGGCCGGTGAGTGAGCGAC Rv 5' GTTGCGCCGCCGGGTTTTAT
Chrna7 NC_005100.3	Fw 5' GGAACCAGCGAACGTCAATGT Rv 5' TGTAGCAAGAGCTTGGGAGGTG
c-*fos* NC_005105.3	Fw 5' TTTCCCCCCTCCAGT Rv 5' CTCAGTTGCTAGCTG
Gad67 NC_005102.3	Fw 5' TGCGTTCTGGATTACTCATGGGAC Rv 5' CTGACTGCCTCTGGCGCTTT
Gamma globin NC_005100.3	Fw 5' GTGTGAGGTCTAGAAGCTTGGAGATGA Rv 5' TGACCAATAGTCTCGGAGTCCTGGGGA
Parvalbumin NC_005106.3	Fw 5' GAGCCAGGATGGGGCTCAGATGCAC Rv 5' GGAGACAATGGCAGGGCAGGGTCAG

All results from one animal were normalized to its own input fraction. Furthermore, actin was used for normalization of DNA precipitated with antibody against H3K4me3, whereas gamma globin was used to normalize DNA precipitated with antibodies against H3K9me2 and H3K27me3. The Pfaffl method (70) enabled calculation of fold changes of gene association. Statistical analysis was performed using GraphPad Prism 5.0 software.

6.4 DNA METHYLATION ANALYSIS

Bisulfite conversion and PCR amplification:
The isolated genomic DNA (gDNA) from the left PFC was subjected to quantitative and qualitative assessment by spectrophotometry on NanoPhotometer (IMPLEN). 200ng of the isolated gDNA was used for bisulfite treatment by EpiTect Bilsulfite kit (Qiagen), according to manufacturer's protocol, in a Veriti 96well Thermal Cycler (Applied Biosystems). The bisulfite converted DNA was used for PCR, amplifying the *Chrna7* promoter by the primers in Table 3 (TAG Copenhagen). Amplification of 5µL template was performed by 0.1µL HotStarTaq DNA Polymerase (Qiagen) in a 20µL reaction, on Veriti 96well Thermal Cycler (Applied Biosystems) with the following program: 95°C for 15min, 40 cycles of 94°C for 30sec, 54°C for 2min, and 72°C for 1min, followed by 72°C for 10min. The PCR product was analyzed by 1.5% agarose gel containing 0.05% ethidium bromide for 15min at 100V in RunOne Electrophoresis Cell (Embi Tec). Afterwards, UV-exposure by TF-20M (Vilber Lourmat) enabled visualization and quick

excision of the amplified target bands, followed by gel extraction by QIAquick Gel Extraction kit (Qiagen), as according to manufacturer's protocol.

Table 3	
Gene	**Primers**
Chrna7 NC_005100.3	Fw 5' AGTAGTTTGGGGTTAGAGATT Rv 5' ACCAAAACCAACCAAATACC

Cloning:
The purified PCR product was used for ligation by the InsTAclone PCR Cloning kit (Thermo Scientific), according to manufacturer's protocol, however, scaling each reaction down to 1/3. Following ligation, the mixture was transformed by heat-shock into E.coli using Subcloning Efficiency DH5α Competent Cells (Invitrogen), according to manufacturer's protocol. The bacteria were incubated for 1h at 37°C, whereafter 100µL of each culture was spread onto LB-agar plates with 100mg/L ampicillin, and cultured overnight at 37°C to select for transformed bacteria. The following day, 10 distinctively separate colonies were picked for inoculation of 3mL LB-media with 100mg/L ampicillin and further overnight incubation at 37°C and 200rpm on KS 501 Digital (IKA).

Plasmid purification and sequencing:
Plasmids from each 3mL culture of bacteria were extracted and purified by GeneJET Plasmid Miniprep kit (Thermo Scientific), as according to manufacturer's protocol. For sequencing, 1.5µg plasmid DNA was freeze-dried and sent to Beckman Coulter Genomics (Essex, UK) for Sanger sequencing using M13F-20 primer (5' GTAAAACGACGGCCAGTG) (Appendix 4). Analysis of the chromatograms was performed using BiQ Analyzer software, and statistical tests were done by means of GraphPad Prism 5.0 software.

6.5 OPTIMIZATION OF METHYLATED DNA IMMUNOPRECIPITATION

EpiQuick MeDIP Ultra Kit (Epigentek, USA) was purchased with the intention of using it as an initial screening method for a full genomic DNA methylation analysis. First, manufacturer's protocol was followed precisely. Starting material was sonicated gDNA from cerebral endothelial cells, together with methylated DNA and unmethylated DNA as controls (both provided by the manufacturer). The subsequent qPCR was performed using primers for an active unmethylated gene promoter (actin) and an inactive methylated gene promoter (gamma globin) (Table 4, TAG Copenhagen), on a Mx3000 QPCR System (Stratagene). 10µL Luminaris qPCR Master Mix (Thermo Scientific) with 10pmol of each primer and 5µL of precipitated gDNA were mixed in a final reaction volume of 20µL, and the qPCR amplification was performed by the following program: 95°C for 10min, 40 cycles of 95°C for 30sec, 60°C for 30sec, and 72°C for 30sec, followed by a melting curve program. All samples were analyzed in duplicate, and water as a no template control (NTC) was included as negative control.

Afterwards the following modifications were made to the protocol: starting material (the provided methylated DNA and unmethylated DNA) was diluted 1:3 instead of 1:9, proteinase K treatment was performed for 10min instead of 20min, with a subsequent inactivation for 10min instead of 5min, and the DNA was used directly for qPCR without spin-column purification. Additionally, Low protein binding tubes (Sarstedt) were used. Furthermore, a sample from both input (before immunoprecipitation) and output (after immunoprecipitation) fractions were tested with qPCR, to which slight modifications in time profile were done: 95°C for 10min, 40 cycles of 95°C for 30sec, 55°C for 30sec, and 72°C for 30sec. qPCR amplification was performed on Mx3000 QPCR System (Stratagene) in a 20µL reaction, consisting of 10µL Luminaris qPCR Master Mix (Thermo Scientific) with 0.5µM of each primer (included in kit). Amplification was followed by a melting curve program starting at 55°C and finishing at 95°C. All

samples were analyzed in duplicate, and water as a no template control (NTC) was included as negative control.

In the article by R. Lee *et al.* 2011 (71), they found differential methylation status of the Pck1 gene in liver and cerebral cortex using the same method. Therefore, we extracted DNA from these two organs of rat by DNeasy blood & tissue kit (Qiagen), according to manufacturer's protocol, and used it for the MeDIP kit.
Another approach tried, was to use a fully methylated Pck1 sequence. This was produced by means of CpG Methyltransferase (Thermo Scientific), as according to manufacturer's protocol, in a Veriti 96well Thermal Cycler (Applied Biosystems). The methylated Pck1 sequence was used for immunoprecipitation together with an identical non-methylated sequence. The before-mentioned alterations to the kit protocol were applied. 5µL precipitated sample was anazyled by qPCR using primers amplifying the Pck1 gene (Table 4, TAG Copenhagen) in a Mx3000 QPCR System (Stratagene) and 10µL Luminaris qPCR Master Mix (Thermo Scientific) with 10pmol of each primer, in a final reaction volume of 20µL. Amplification was achieved by the following program: 95°C for 10min, 40 cycles of 95°C for 30sec, 60°C for 30sec, and 72°C for 30sec. Amplification was followed by a melting curve program starting at 55°C and finishing at 95°C. All samples were analyzed in duplicate, and a no template control (NTC) was included as negative control.

Table 4

Gene/use	Primers
Pck1 qPCR NC_005102.3	Fw 5' GGCCATCAACCCAGAAAACG Rv 5' GGCTCATCGATGCCTTCC
Pck1 PCR NC_005102.3	Fw 5' GGACGGGTAAGAGAAAGATG Rv 5' GCCTGAATAGAAGACTCACC
Actin NC_005111.3	Fw 5' GAGGCCGGTGAGTGAGCGAC Rv 5' GTTGCGCCGCCGGGTTTTAT
Gamma globin NC_005100.3	Fw 5' GTGTGAGGTCTAGAAGCTTGGAGATGA Rv 5' TGACCAATAGTCTCGGAGTCCTGGGGA

7. RESULTS

The temporal expression profile of three schizophrenia-relevant genes and a neuronal marker has been investigated 8h, 24h, and 48h post-PCP injection. Furthermore, epigenetic status has been assessed in regards to three histone modifications, namely H3K4me3, H3K9me2, H3K27me3, the two latter affecting expression in a repressive manner, whereas the first is an activating modification. Since neither of the genes displayed any significant expression alterations 48h after PCP exposure, histone modifications were only investigated at 8h and 24h post-PCP. All control groups are set to 100%, thereby only providing normalized values for the PCP-treated groups on graphical presentations.

7.1 C-FOS

Two different regions of the c-*fos* gene were investigated (Figure 7). The association of the gene with histone modifications was analyzed across transcription start site (TSS), whereas expression analysis was performed in the 4th exon.

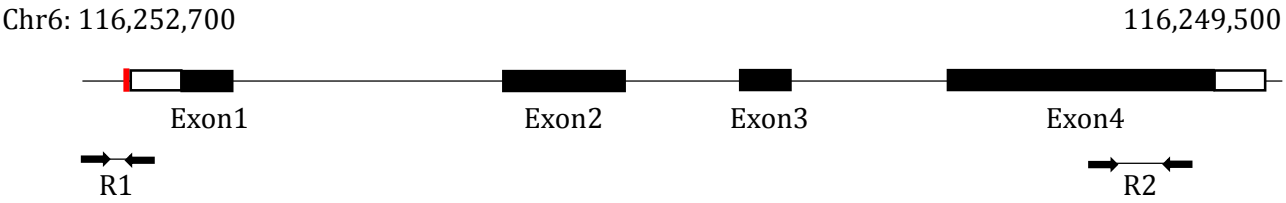

Figure 7: Schematic overview of rat c-fos gene on chromosome6, showing the regions amplified by primers used in the present study. Region1 (R1) was investigated for association with modified histones by native chromatin immunoprecipitation, whereas region2 was used for expression analysis. Red line indicates transcription start site (TSS), white boxes signifies untranslated region (UTR).

Although PCP is an NMDA receptor antagonist, both glutamatergic and dopaminergic signaling is increased following acute PCP (Chapter 4). A marked increase in c-*fos* expression was observed, as expected (Figure 8). However, a significant 250% increase 8h post-injection seems a long time for neuronal activity in response to a single acute dose. A substantial decrease in c-*fos* expression was observed 24h after PCP exposure (64%), initiating speculations whether the expression had been even higher closer to injection time, and the pronounced increase after 8h is in fact a glimpse of the decreasing expression, culminating around 24h post-exposure. After 48h, an insignificant decrease of 47% indicates a return to baseline expression.

Significant changes in histone methylation associated with the c-*fos* gene were observed, yet they do not appear to be very influential on the expression pattern. The only significant alteration 8h after PCP injection was a decrease of the activating H3K4me3 by 34%, yet a tendency towards slightly increased repressive H3K9me2 and H3K27me3 was observed. Overall, this would cause decreased gene expression, directly opposite to the profound increase in c-*fos* expression observed after 8h. After 24h, a significant decrease was observed for both the activating H3K4me3 and the repressive H3K27me3 modification, apparently cancelling each other out, but again, not correlating with the substantially decreased gene expression found 24h post-injection.

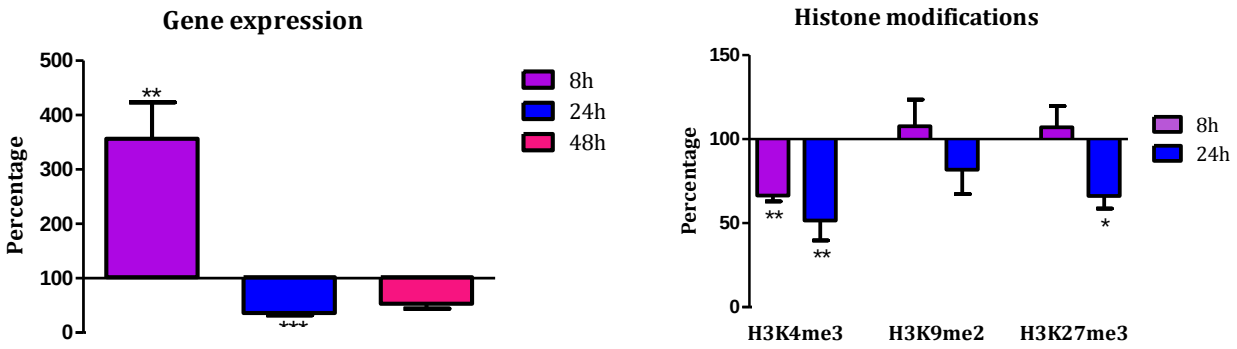

*Figure 8: Temporal profile of c-fos expression (left) and histone modifications (right) after single acute PCP injection (n=6). Statistical significance is based on unpaired t-test. * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001.*

7.2 ALPHA 7

The α7-subunit gene, *Chrna7*, was investigated in three ways: expression analysis, histone modification analysis, and DNA methylation analysis of its promoter. Figure 9 shows the different regions investigated by each analysis.

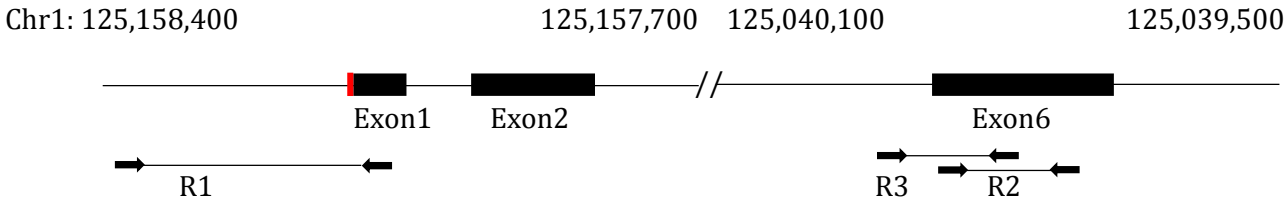

Figure 9: Schematic overview of rat Chrna7 gene on chromosome1, showing the regions amplified by primers used in the present study. Region1 (R1) was explored by DNA methylation analysis, region2 was used for expression analysis, region3 was investigated for association with modified histones by native chromatin immunoprecipitation. Red line indicates transcription start site (TSS).

The expression pattern of *Chrna7* decreased initially, but returned to baseline within 48h after PCP exposure (Figure 10). A significant decrease of *Chrna7* mRNA by 29% was observed 8h post-injection, followed by a return to baseline between 24h and 48h. The histone methylation modifications associated with the *Chrna7* gene, seemed to correlate with the expression profile. 8h after PCP exposure, the activating H3K4me3 was decreased significantly by 27%, and the repressive H3K9me2 and H3K27me3 were increased (13% and 18%, respectively), however, these results did not show significance. This decreased activation and increased repression correlate with the significantly decreased gene expression observed 8h post-PCP. 24h after PCP exposure, the picture is somewhat different, as both the H3K4me3 and H3K27me3 modifications had returned to baseline. Although insignificant, H3K9me2 still displayed 17% increase 24h after PCP injection. This is in agreement with the observed return to baseline of the gene expression around the 24h time-point.

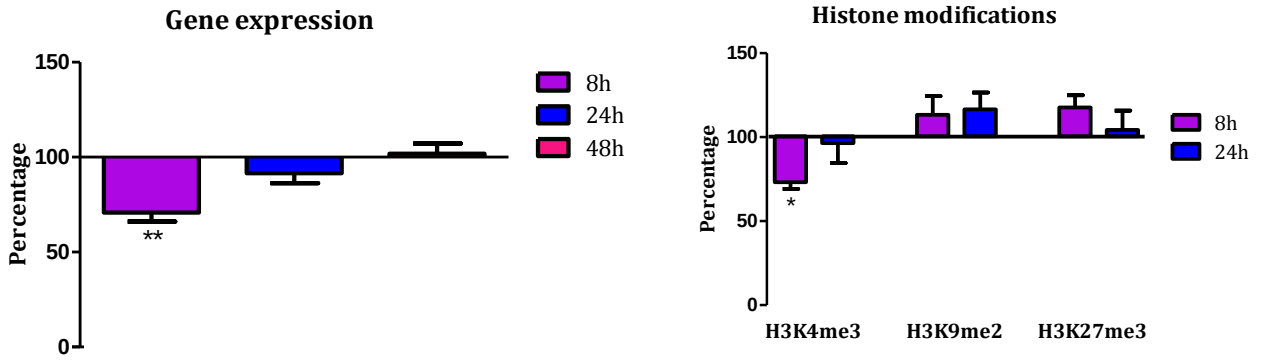

*Figure 10: Temporal profile of Chrna7 expression (left) and histone modifications (right) after single acute PCP injection (n=6). Statistical significance is based on unpaired t-test. * p ≤ 0.05, ** p ≤ 0.01.*

The DNA methylation analysis of the *Chrna7* promoter did not reveal any significant differences between saline or PCP treated animals 8h post-injection (Figure 11). However, a tendency towards increased promoter methylation was observed for the PCP group. In the present analysis, 39 CpG positions were investigated, and of these, 0.34% were methylated in the control group, whereas 0.51% were methylated 8h after PCP exposure. Based on the notion, that a single methylated position in the promoter region is enough to repress gene expression, the ratio between control and PCP exposure could be different. However, none of the investigated clones were methylated at more than one CpG position. Therefore, in the control group, 13% of clones had at least one methylated position, whereas it was the case for 20% of clones in the PCP-treated group.

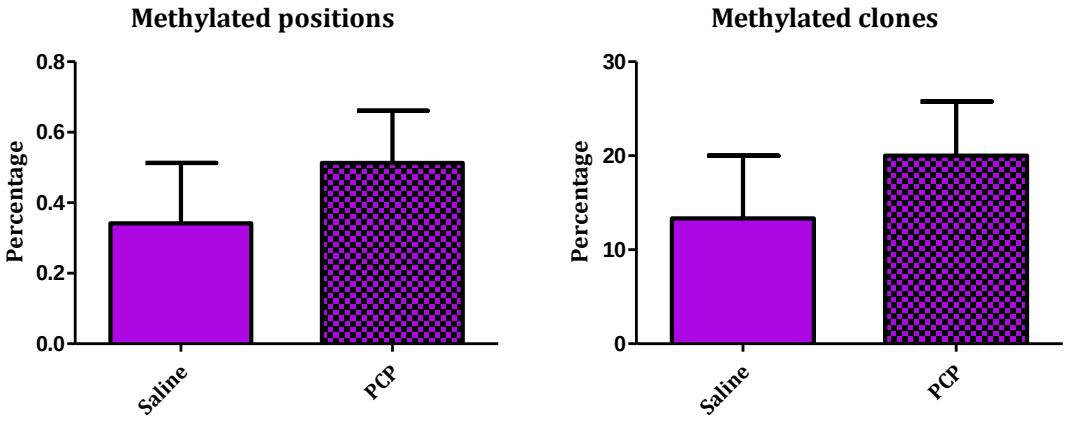

Figure 11: DNA methylation analysis of the Chrna7 promoter 8h after single acute PCP injection (n=3). Left: Percentage of the 39 CpG positions which are methylated. Right: Percentage of clones which contain at least one methylated CpG position.

7.3 PARVALBUMIN

The *parvalbumin* gene was investigated in two different regions (Figure 12). The association of the gene with histone modifications was analyzed in the first intron, whereas expression analysis was performed in the 3rd and 4th exon, spanning an intron, thereby omitting any false signal in case of contamination by gDNA.

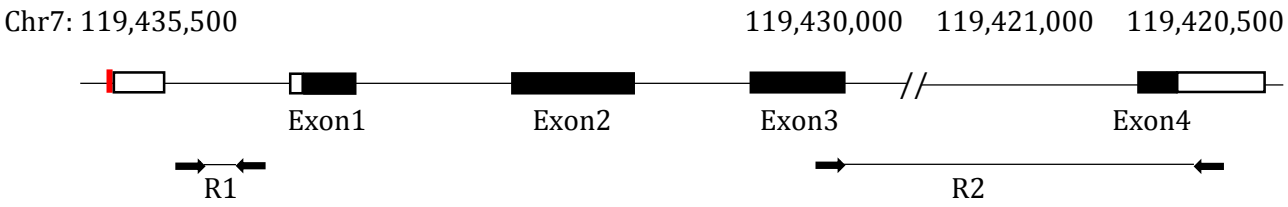

Figure 12: Schematic overview of rat parvalbumin gene on chromosome7, showing the regions amplified by primers used in the present study. Region1 (R1) was investigated for association with modified histones by native chromatin immunoprecipitation, whereas region2 was used for expression analysis. Red line indicates transcription start site (TSS), white boxes signifies untranslated region (UTR).

The temporal expression profile of the calcium-binding *parvalbumin* gene did not display any significant alterations, but minor fluctuations (±10%) were observed (Figure 13). At the intermediate time-point of 24h, *parvalbumin* expression was slightly increased, yet at both 8h and 48h a small decrease was observed. Although insignificant, the decreased expression 8h post-injection correlate with the histone methylation status associated with the *parvalbumin* gene at this time-point, since both decreased activation and increased repression was observed. We know from previous experiments, that *parvalbumin* is significantly downregulated by approx. 20% 2h after acute PCP administration (Jacek Lichota, personal communication). The activating modification H3K4me3 was decreased significantly by 24%, whereas the repressive H3K27me3 modification was increased by a significant 20%. Although not significant, the other repressive histone mark, H3K9me2, was also increased (34%). A somewhat similar tendency was observed 24h after PCP exposure. Although insignificant, decreased activation by H3K4me3 (22%) was observed, whereas the H3K9me2 and H3K27me3 were very similar to controls.

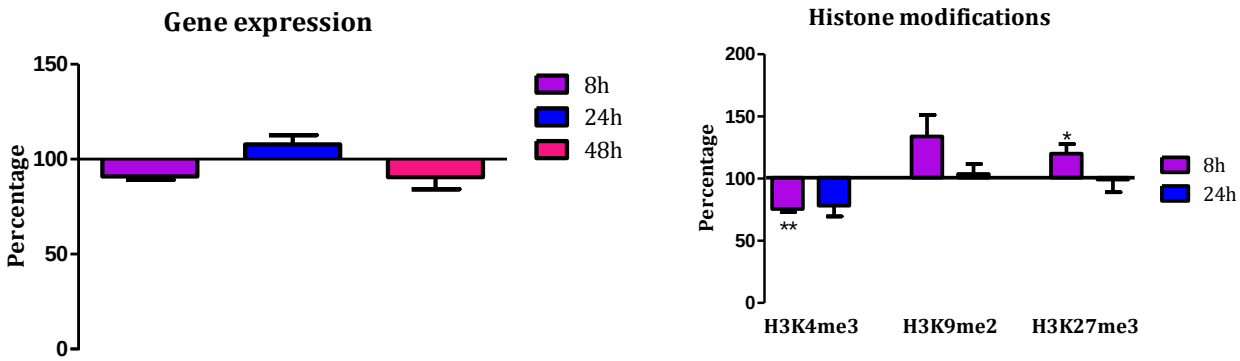

*Figure 13: Temporal profile of parvalbumin expression (left) and histone modifications (right) after single acute PCP injection (n=6). Statistical significance is based on unpaired t-test. * p ≤ 0.05, ** p ≤ 0.01.*

7.4 GLUTAMIC ACID DECARBOXYLASE 67

Figure 14 provides an overview of the investigated regions of the large *Gad67* gene. Histone modification analysis was performed in the promoter region upstream from TSS, and the expression analysis was done in exon 10 and 11. The investigated region spans an intron, hence any contamination by gDNA does not affect the obtained results.

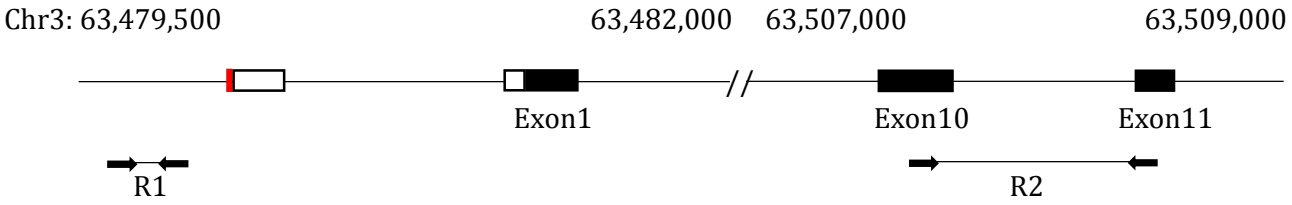

Figure 14: Schematic overview of rat Gad67 gene on chromosome3, showing the regions amplified by primers used in the present study. Region1 (R1) was investigated for association with modified histones by native chromatin immunoprecipitation, whereas region2 was used for expression analysis. Red line indicates transcription start site (TSS), white boxes signifies untranslated region (UTR).

The expression pattern of the GABA synthesizing enzyme gene, *Gad67*, only deviated from baseline 24h post PCP exposure (Figure 15). A significant increase of 13% was observed after 24h, whereas after 8h and 48h the expression was somewhat indifferent.

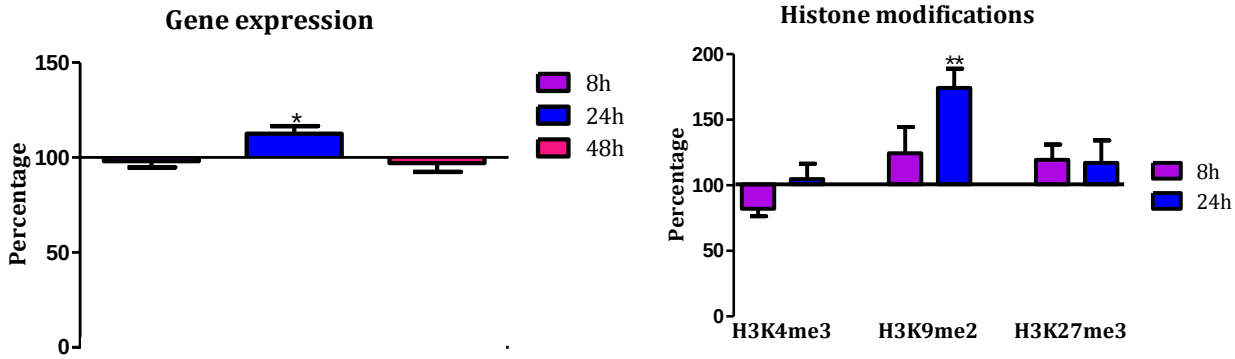

*Figure 15: Temporal profile of Gad67 expression (left) and histone modifications (right) after single acute PCP injection (n=6). Statistical significance is based on unpaired t-test. * $p \leq 0.05$, ** $p \leq 0.01$.*

The investigated histone modifications do not seem to play an important role in the regulation of *Gad67* expression, since inconsistencies were observed between the epigenetic alterations and the genetic expression. Although insignificant, the histone methylation pattern 8h after PCP injection, would indicate decreased expression, due to decreased activation by H3K4me3 (18%) and increased repression by H3K9me2 (24%) and H3K27me3 (19%). However, baseline expression was observed at this time-point. A significant increase of 74% of association between the *Gad67* gene and H3K9me2 was observed 24h after PCP exposure, yet at this time-point a significant increase in expression was also observed, further emphasizing that other factors play a part in the regulation of this gene. The other two investigated histone modifications displayed a slight insignificant increase 24h after PCP injection, possibly cancelling each other out.

7.5 METHYLATED DNA IMMUNOPRECIPITATION

The EpiQuick MeDIP Ultra Kit (Epigentek) did not at first glance seem to work well. Using the methylated DNA (positive control) and unmethylated DNA (negative control) provided by the manufacturer,

together with a sample (sonicated gDNA) of unknown methylation status, the expected order of appearance on the qPCR amplification plot would be methylated DNA – sample – unmethylated DNA. Since actin is an active gene, it is thought to be associated with little or no DNA methylation (72), and was therefore included as a negative control of the qPCR. On the other hand, the gamma globin gene is inactive in the brain (73); and therefore thought to be associated with high levels of DNA methylation, hence it was used as positive control of the qPCR. However, the actual results reveal a different picture (Figure 16). The only template that appeared on the amplification plot using the gamma globin primers, was the sample. Neither of the methylated DNA or unmethylated DNA provided a signal at all. Conversely, using the actin primers, all four templates were amplified, yet not in the desired order as mentioned above. From the amplification plot it seems that more unmethylated DNA was precipitated by the kit than the methylated DNA, which served as positive control. Furthermore, the mock control (serum used instead of antibody) provided a false positive signal, although this signal appeared several cycle thresholds (Cts) later.

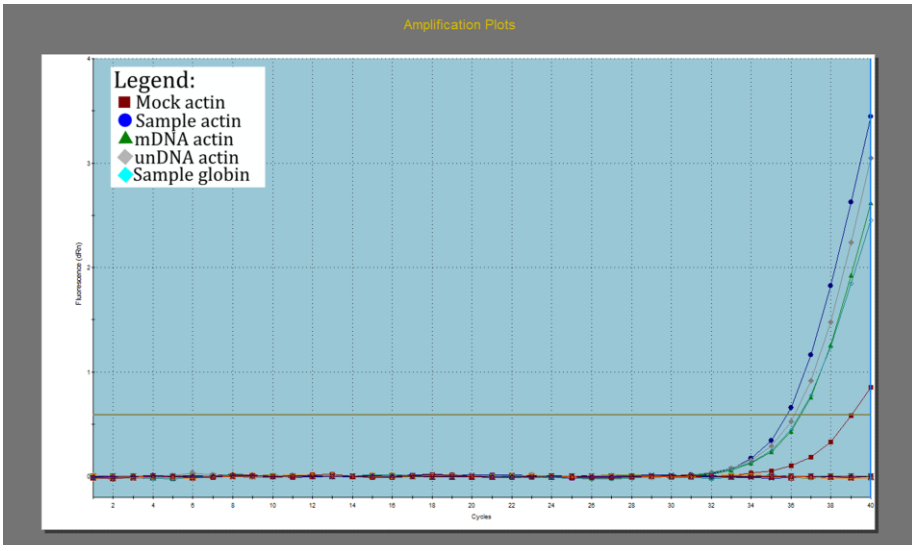

Figure 16: Amplification plot from qPCR of the first immunoprecipitation by the MeDIP kit.
In regards to the globin gene, only sample provided signal, whereas all four templates were present when amplified by actin primers.
mDNA: methylated DNA, unDNA: unmethylated DNA, sample: sonicated genomic DNA, mock: incubated with serum instead of antibody.

When the immunoprecipitation was performed only using the provided methylated DNA and unmethylated DNA, and more controls were added to the qPCR (input and output, the fractions prior to and after immunoprecipitation, respectively), which was performed by primers included in the kit, the picture was somewhat better, yet still not satisfying (Figure 17). First of all, the two input fractions display very different concentrations despite similar dilution, as stipulated by the manufacturer. In fact, based on the PCR results it was calculated that more than 17 times the amount of unmethylated DNA was used as input compared to the input of methylated DNA ($\Delta Ct = 4.16$). Secondly, the kit precipitated less than 3 times as much methylated DNA as unmethylated DNA ($\Delta Ct = 1.49$), and when taking into account that these samples were supposed to serve as positive and negative controls, the specificity of the immunoprecipitation is questionable.

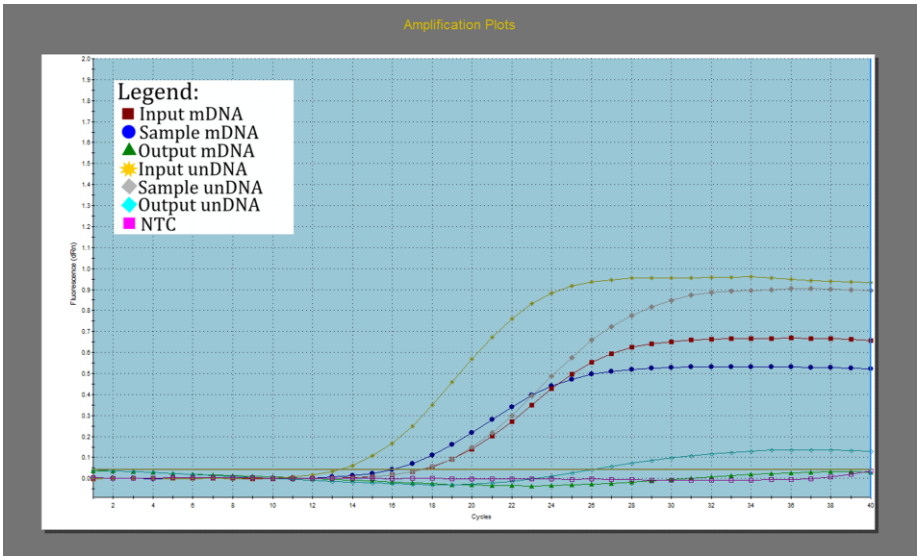

Figure 17: Amplification plot from qPCR of the second immunoprecipitation set-up. Considerable difference between input fractions is visible, whereas the specificity of the immunoprecipitation is questionable due to a very small difference in sample appearance. mDNA: methylated DNA, unDNA: unmethylated DNA.

Since R. Lee and colleagues (71) found significantly different methylation status of the Pck1 gene in liver and cerebral cortex, we subjected gDNA from these two organs to the MeDIP kit. Furthermore, we used a fully methylated copy of the gene, as positive control, together with a non-methylated copy of the gene. In contrast to R. Lee's findings, the MeDIP kit immunoprecipitated substantially more liver Pck1 than cortex Pck1 (~47 times more, ΔCt = 5.56) (Figure 18). However, the samples might not have been sonicated sufficiently, which would lower the efficiency of the precipitation. In regards to the fully methylated and the non-methylated Pck1, still very little difference between the precipitated fractions can be observed, only about 2.5 times more fully methylated Pck1 was precipitated than non-methylated Pck1 (ΔCt = 1.37), emphasizing the poor specificity of the kit.

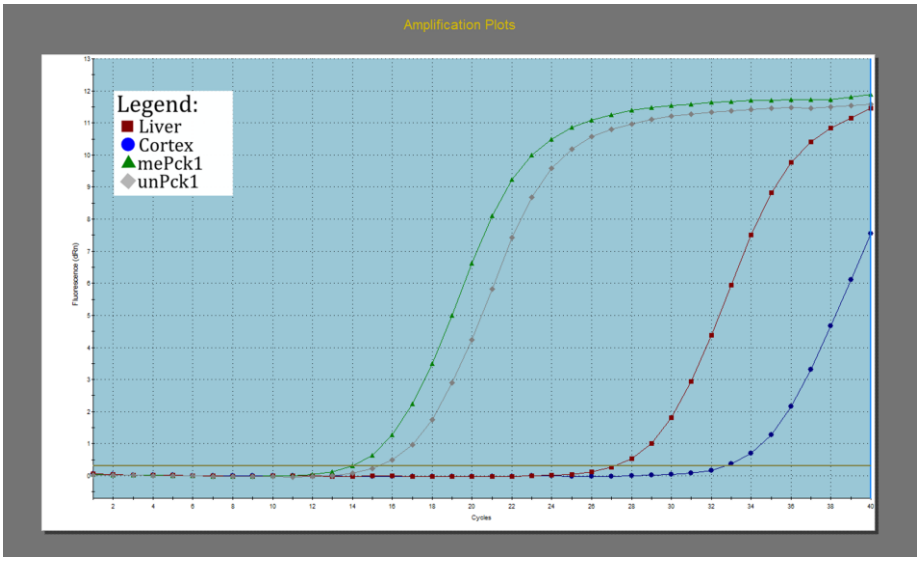

Figure 18: Amplification plot from qPCR of the second immunoprecipitation set-up. Liver and cortex precipitate very differently, whereas the non-methylated Pck1 display almost similar precipitation as the fully methylated Pck1. mePck1: methylated Pck1, unPck1: non-methylated Pck1.

8. DISCUSSION & PERSPECTIVATION

8.1 C-FOS

Similar to all immediate early genes, c-*fos* expression does not need preceding protein formation, therefore expression is rapidly increased in response to PCP, due to neuronal activation. Several researchers have investigated the temporal expression profile of c-*fos* immediately after administration of acute PCP, but only Gao *et al.* (74) has undertaken a prolonged study of the effect of acute PCP in the PFC of rats. They report a dramatic increase in c-fos mRNA 1h and 3h post-injection, but did not observe any changes at 6h, 24h and 48h after exposure. Although they used a quiet similar dose (8.6mg/kg) to that administered in the present study, significant discrepancies are observed between the two investigations.
Using in situ hybridization, F. Artigas's group (75,76) found that c-*fos* expression was markedly increased in the PFC 1h post acute PCP-injection (10mg/kg), scoring this region the highest relative c-fos mRNA content. Furthermore, they observed that this increased c-*fos* expression was limited to the pyramidal neurons exclusively, not the GABAergic neurons. Even with a lower dose of 5mg/kg PCP, Kalinichev *et al.* (77) found that c-*fos* expression in the PFC was 450% upregulated 4h after PCP exposure. These findings are in line with the suggestion that the presently observed 350% increased c-*fos* expression seen 8h post PCP-injection, is in fact a decreasing expression, which has been even higher closer to injection-time. However, when Savage *et al.* (64) used 2 and 3mg/kg PCP, they did not observe any c-fos response 4h after injection, indicating that the dose is important to the prolonged effects of PCP. Therefore, the 10mg/kg dose used in the present study would be expected to cause an even higher initial surge in c-*fos* expression than that observed by Kalinichev *et al.*

Gene expression is, at least for c-*fos*, an immediate response to external stimuli, whereas histone modifications have to be established, thereby delaying their regulatory effect. With that in mind, the epigenetic repressive state observed 8h after PCP exposure (decreased H3K4me3 and a tendency towards slightly increased H3K9me2 and H3K27me3), correlates with the decreased expression seen after 24h. However, if the increased gene expression observed 8h post-injection is in fact part of decreasing expression, the histone marks may already have been established and starting to take effect. Correlating with the notion that histone modifications are delayed compared to expression pattern, the histone methylation status after 24h seem to counteract each other, corresponding to the expression returning to baseline after 48h. However, it is important to bear in mind that the three modifications investigated in the present thesis are merely pieces of the epigenetic puzzle that regulate gene expression. Chandramohan *et al.* (78) showed that phospho-acetylation of H3 was important for the induction of c-*fos* in neurons of the dentate gyrus, since inhibition of this modification ablated the expression of c-*fos*. Therefore, other types of modifications than methylation may be more influential on the c-*fos* expression, and thereby responsible for the temporal profile observed here.

8.2 ALPHA 7

Although much effort has been put into investigating the effects of α7 agonists on behavior and cognition, no reports have been published on the expression profile of the *Chrna7* gene as a consequence of PCP administration. Hence, I hereby present the first temporal profile of *Chrna7* expression in the prefrontal cortex of rats subjected to 10mg/kg PCP. 8h post-injection was the only time-point displaying significant changes, namely a 29% decreased expression, which returned to baseline 24-48h after PCP exposure. However, several post-mortem investigations of brains from schizophrenic patients, did not observe any differences in mRNA levels of *Chrna7,* compared to healthy controls (79–82), yet decreased

α7 protein was reported by Martin-Ruiz *et al.* (81). Therefore, discrepancies between the human disorder and the rodent model seem evident. Furthermore, no differences in *Chrna7* expression were detected between the human post-mortem specimens from smoking and non-smoking individuals (79,82), indicating that nicotine, and thereby the use of the α7 receptor, does not affect the expression of the underlying *Chrna7* gene.

It is not only the genetic expression of *Chrna7* that has been overlooked by the scientific community, neither the genetic regulation has been explored much. The only investigation of epigenetic regulation of *Chrna7* expression came from Canastar *et al.* in 2012 (83), reporting hypermethylation of the gene promoter in several cell types. They found that promoter methylation correlated with gene expression in different tissues, indicative of DNA methylation playing a role in regulating *Chrna7* activity. In the present study, a slight tendency towards increased methylation of the *Chrna7* promoter in PCP-treated animals was observed, correlating with the decreased gene expression found at the same time-point. Furthermore, I hereby present the first evidence that histone modifications also seem to take part in regulating *Chrna7* expression, since decreased association between *Chrna7* and the activating H3K4me3 mark was observed 8h post PCP-injection, the same time as the decreased gene expression and the increased DNA methylation of the gene promoter. Although not reaching statistical significance, a tendency towards slightly increased association with the repressive H3K9me2 and H3K27me3 marks was observed, reinforcing the notion that epigenetic modifications are likely to orchestrate regulation of the *Chrna7* gene.

8.3 PARVALBUMIN

The expression pattern of the calcium-binding protein parvalbumin, does not seem to be affected by acute PCP exposure, since it does not deviate significantly from baseline at any of the three investigated time-points. Even though the dose used in the present study is rather high, the observed results are in agreement with findings from *in situ* hydbridization on a much lower dose of PCP. The group of J. Pratt (84,85) investigated the acute and long-term effect of a single PCP-injection (2.58mg/kg) on parvalbumin mRNA level in the PFC, and neither at 4h or 24h post-injection did they observe any significant changes. These results are in stark contrast to the consistently reported decreased parvalbumin level found in post-mortem brains of schizophrenic patients. However, sustained exposure to NMDA receptor antagonists are capable of producing such decreased *parvalbumin* expression in the PFC (86). This is in concordance with the notion, that parvalbumin reduction is a long-term adaptive alteration in response to upstream changes occurring in schizophrenia (Chapter 5.3). Therefore, it is important to keep in mind that, in rodents, different paradigms are capable of mimicking different aspects of a complex human disorder.

This is, to my knowledge, the first report of the association between the *parvalbumin* promoter and histone methylation at various lysine residues. No significant changes were observed 24h post PCP-injection, yet H3K4me3 and H3K27me3 were significantly decreased and increased, respectively, 8h after PCP exposure. Less activation and more repression would correlate with decreased expression levels, as observed in schizophrenic patients. Although the acute PCP paradigm is incapable of producing reduced *parvalbumin* expression, histone modifications might be the way that repetitive administration can do so. The altered epigenetic profile was observed after 8h, yet it returned to baseline 24h post-injection, initiating the thought, that chronic administration would reinforce and strengthen these epigenetic marks after each injection, thereby lowering the expression level after prolonged exposure, as it was observed by Amitai *et al.* (87).

8.4 GLUTAMIC ACID DECARBOXYLASE 67

The GABA-synthesizing enzyme, Gad67, only deviated from baseline by a slight increased expression 24h after PCP exposure. Not much effort has been put into investigating the effect of acute PCP on *Gad67* expression since 1983, when Peat & Gibb (88) reported that only sub-chronic PCP was capable of inducing altered *Gad67* activity. However, in regards to single-dose acute PCP they only investigated a short timespan (10min-120min), wherein they did not find any significant changes. Therefore, it is possible that the effect of acute PCP on *Gad67* expression is delayed, as observed in the present results. Although, for that to be true, a mechanism unique to PCP is at play, since Romón *et al.* (89) reported no significant *Gad67* expression alterations 24h after acute exposure to MK-801, another NMDA receptor antagonist. Furthermore, they also reported baseline Gad67 mRNA levels 4h post-injection, correlating with the unchanged expression 8h after PCP exposure, observed in the present study. All these unaltered Gad67 findings are in contrast to the consistently reported decrease in *Gad67* expression in post-mortem brains of schizophrenic patients (90). It is therefore likely that acute administration of NMDA receptor antagonists are poor models for studying the changes in Gad67 levels found in schizophrenic patients.

Correlating with the decreased expression of *Gad67* in schizophrenic patients, Huang *et al.* (90) found decreased H3K4me3 and increased H3K27me3 associated with *Gad67* in post mortem brains of individuals suffering from schizophrenia. In the present study, a slight tendency towards decreased H3K4me3 and increased H3K27me3 was observed, however, none of these results showed statistical significance. Huang *et al.* further report, that the H3K4-specific HMT, MLL, is responsible for the decreased methylation of K4 in association with *Gad67*. As mentioned in chapter 3, the MLL enzyme also prevents DNA methylation. However, DNA methylation seems important in the regulation of *Gad67* expression, since decreased Dnmt1 activity increased the *Gad67* expression in frontal cortex of mice, as investigated by Satta *et al.* (91). Therefore, a correlation seems evident between the decreased H3K4me3 caused by lack of MLL activity and increased DNA methylation caused by Dnmt1 in the regulation of *Gad67* expression. In the present study, *Gad67* was associated with significantly more H3K9me2 24h after PCP exposure, correlating with the reported association of *Gad67* with repressive epigenetic marks.
Schizophrenic patients often smoke heavily, as mentioned in chapter 5, which has been proposed to be a mode of self-medicating the cognitive impairments. Satta *et al.* (91) investigated the effects of nicotine on *Gad67* expression and DNA methylation of its promoter, and found that decreased Dnmt1 mRNA and protein increased the *Gad67* expression in mouse frontal cortex. Furthermore, the observed decrease in Dnmt1 expression was especially pronounced in GABAergic interneurons. Therefore, the positive effect of nicotine experienced by schizophrenic individuals, is possibly mediated by epigenetic changes of the *Gad67* gene in GABAergic neurons of the frontal cortex, improving cognitive functions through reversal of GABAergic deficits.

9. CONCLUSION

The present thesis provides evidence of histone methylation playing a part in epigenetic regulation of several schizophrenia-relevant genes. Acute exposure to the NMDA receptor antagonist, PCP, was used to mimic the primary psychotic episode experienced by schizophrenic patients. Both c-*fos* and the three schizophrenia-relevant genes, *Chrna7, parvalbumin, Gad67*, showed significant temporal alterations in methylation status of certain H3 lysine residues. The activating H3K4me3 histone modification took part in regulating the *Chrna7* gene, whereas *Gad67* was somewhat regulated by the repressive H3K9me2 histone mark. In regards to c-*fos* and *parvalbumin*, both the activating H3K4me3 and the repressive H3K27me3 modifications were involved in regulation of gene expression. These findings are the first report of how acute PCP alters epigenetic lysine methylation thereby regulating associated gene expression. Furthermore, a tendency towards increased methylation of the *Chrna7* promoter supports emerging evidence, that DNA methylation plays a role in regulating gene expression in the pathophysiology of schizophrenia.

I hereby present the first prolonged gene expression profile after acute PCP administration, providing evidence that even a single acute dose of PCP has long lasting effects on neuronal gene expression. However, several discrepancies were found between the presented results and observations from post-mortem studies of schizophrenia-affected brains, highlighting the fact that, rodent paradigms are not fully capable of modeling certain aspects of human disorders. Even though acute administration of PCP to rodents has been used for decades, several molecular aspects of the effect are still to be uncovered; especially in regards to epigenetic alterations, much work still awaits us.

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