Epigenetic dysregulation of mRNA expression in rat prefrontal cortex following acute PCP administration

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Josephine holm henriksen

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AALBORG UNIVERSITY

AALBORG UNIVERSITY

Department of Health Science and Technology
Aalborg University
Fredrik Bajers Vej 7D2
DK-9220 Aalborg
Denmark
+45 96 35 80 80
+45 98 15 40 08
http://hst.aau.dk

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Josephine Holm Henriksen

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Content

ABBREVIATIONS

Arc:	activity-regulated cytoskeleton-associated protein
CaMK:	calcium/calmodulin-regulated kinase
CREB:	cyclic adenosine monophosphate response element binding protein
DNMTs:	DNA methyltransferases
ERK:	extracellular-regulated kinase
FGAs:	first-generation antipsychotics
IEG:	immediate early gene
LTD:	long-term depression
LTP:	long-term potentiation
PCP:	phencyclidine
PFC:	prefrontal cortex
PKA:	protein kinase A
SGAs:	second-generation antipsychotics

1. Abstract

Schizophrenia is a serious mental disorder that affects up to 2% of the global population. Males are typically affected earlier, with onset in late adolescence or early adulthood, and more severely than females. Symptoms of schizophrenia are divided into three categories: Positive symptoms, negative symptoms, and cognitive symptoms. Schizophrenia is mainly treated with antipsychotics, which can be categorized as first-generation antipsychotics (FGAs) or second-generation antipsychotics (SGAs). The side-effects from FGAs include extrapyramidal symptoms, and due to these side-effects, SGAs are often preferred, as they have a milder side-effect profile.

Despite intense research, the etiology and pathophysiology of schizophrenia still remains unclear. However, it is generally accepted that schizophrenia is a multifactorial neurodevelopmental disorder influenced by genetic, epigenetic, and environmental factors. Suggested correlations between epigenetics and environmental factors include place and time of birth, parasitic infections, low IQ, maternal nutrition, and obstetric complication.

DNA methylation, gene expression, and histone modification are thought to play a role in the development of schizophrenia. Genes suggested to be involved in the pathophysiology of schizophrenia include activity-regulated cytoskeleton-associated protein (Arc), cfos, and brain-derived neurotrophic factor (BDNF).

The aims of this study were to (1) evaluate the effect of acute phencyclidine (PCP) administration (10 mg/kg) in a time study on mRNA expression of three schizophrenia-susceptible genes: Arc, cfos, and BDNF, in the PFC of adult rats, (2) evaluate the level of H4 acetylation of the promoters in rat PFC, following acute PCP administration, corresponding to each of the mRNA being investigated, (3) evaluate the level of H3 phospho-acetylation of the promoters in rat PFC, following acute PCP administration, corresponding to each of the mRNA being investigated , and (4) evaluate if a correlation between PCP-induced mRNA expression in Arc, cfos, and BDNF, and the H4 acetylation and H3 phospho-acetylation in their corresponding promoters, exist.

Gene expression analysis and chromatin immunoprecipitation (ChIP) assay was performed on prefrontal cortex (PFC) from 30 young adult, male Sprague-Dawley rats. The rats were divided into seven groups: Two control groups and five experimental groups, with acute administration of PCP in the experimental groups. Primers were designed based on mRNA sequences of Arc, cfos, and BDNF, and quantitative RT-PCR was performed in order to evaluate the PCP-induced mRNA expression of Arc, cfos, and BDNF in a time dependent manner. mRNA expression of Arc, cfos, and BDNF was evaluated 60, 120, 240, and 360 minutes as well as 24 hours after PCP administration. β-actin was used as housekeeping gene. Variations in histone H4 acetylation and histone H3 phospho-acetylation were examined on isolated chromatin.

Compared to the control groups, mRNA expression of Arc was significantly increased 240 minutes after PCP administration. Furthermore, mRNA expression of cfos was significantly increased 60 and 120 minutes after PCP administration compared to controls, while the mRNA expression of BDNF was significantly increased 240 and 360 minutes after PCP administration compared to controls. There was an increase in H4

acetylation of cfos and BDNF promoter 360 minutes after administration compared to controls. No differences in H3 phospho-acetylation of Arc, cfos, or BDNF was found when compared to controls.

In conclusion, acute administration of PCP in rat significantly increased the mRNA expression of Arc, cfos, and BDNF in the rat PFC, however at different time points. Increased H4 acetylation of cfos and BDNF promoter was also observed following PCP administration. This finding in BDNF mRNA correlated with the level of H4 acetylation of the corresponding BDNF promoter I, which also peaked in the PCP 360 min group. Thus, acute PCP administration might induce H4 acetylation in the BDNF promoter I, leading to an increase in BDNF mRNA expression, suggesting a transcriptional regulation of BDNF.

2. Introduction

Schizophrenia is a chronic and serious mental disorder affecting up to 2% of the global population (Marino, Knutsen & Williams 2008). Schizophrenia tends to manifest more severely and earlier in males, where the disease typically appears in late adolescence and early adulthood, while females have a later onset (Abel, Drake & Goldstein 2010, Szymanski et al. 1995, Häfner 1998). The disease is characterized by three symptom categories: Positive symptoms, negative symptoms, and cognitive dysfunction. Positive symptoms include delusions, auditory and visual hallucinations and otherwise bizarre behavior, whereas negative symptoms involve e.g. lack of motivation, alogia, and social withdrawal. Cognitive impairment usually presents before appearance of psychotic symptoms, and involves e.g. impaired executive functioning, working memory and attention, which remains throughout the course of the disease. (Marino, Knutsen & Williams 2008) According to the American Psychiatric Association's *Diagnostic and Statistical Manual of Mental Disorders IV* (American Psychiatric Association., American Psychiatric Association. Task Force on DSM-IV. 2005), four different subtypes of the disorder have been established on the basis of the symptomatology of the patients; (1) paranoid, (2) disorganized, (3) catatonic, and (4) undifferentiated. The worst prognosis is associated with the disorganized subtype (Walker et al. 2004) . Additional two types of schizophrenia exist; residual and schizophreniform disorder. The residual subtype, accounts for chronic patients (lifelong disability), experiencing disappearance of positive symptoms, and only experiencing negative symptoms for a period of time. Patients suffering from schizophreniform disorder do not meet the criteria for schizophrenia, and might account for people early in the course of the disease. (Walker et al. 2004) The progression of the disease varies among patients, many of which undergo prolonged periods of remission combined with relapses of psychotic episodes. Some experience full recovery, while others get chronic debilitation (Haro et al. 2008).

The pathophysiology of schizophrenia is complex and the initial understanding of pathogenesis was based on drugs discovered by clinical serendipity. One example is the dopamine receptor antagonist chlorpromazine, which was originally developed as an analeptic, but was coincidently discovered to be able to alleviate some of the symptoms of schizophrenia. Subsequently, the proposed molecular targets of various drugs have been used to develop theories of pathogenesis. (Marino, Knutsen & Williams 2008, Enna, Williams 2009) These theories include;

- The dopamine hypothesis of schizophrenia which has recently been reformulated. Substantial research (Papaleo et al. : Goldman-Rakic, 1999; Simpson et al., 2010; Snyder, 2006; Winterer and Weinberger, 2004) has found a hypodopaminergic function in the PFC and a hyperdopaminergic in the striatum of schizophrenic patients Dopamine is widely distributed in the brain and communicates with circuits linking subcortical and cortical brain regions (Jentsch, Roth 1999).
- The hypoglutamatergic state and hypofunction of the NMDA receptor in the brain has been hypothesized in schizophrenic patients. Glutamate is the major neurotransmitter in the nervous system and connects the hippocampus, PFC, and thalamus. Several of the positive, negative, and cognitive symptoms of schizophrenia are produced in healthy humans by a noncompetitive NMDA receptor antagonist. Also the positive and negative symptoms are exaggerated in schizophrenic patients (Kantrowitz, Javitt 2010, Coyle 2006).

- Hypofunctional GABAergic inhibitory control in neocortical circuits in schizophrenia (Gonzalez-Burgos, Hashimoto & Lewis 2010).
- Serotonine hypothesis of schizophrenia based on similarities between the symptoms of schizophrenix patients and LSD-induced hallucination.

2.1. Treatment of Schizophrenia

Based on evidence (Marino, Knutsen & Williams 2008, Miyamoto et al. 2004), the ideal treatment for schizophrenia is a combination of medication, psychological therapy, and community support, although most schizophrenic patients only receive treatment in the form of medication. Antipsychotic drugs form the basis for schizophrenia treatment, and can be categorized as *first-generation* antipsychotics (FGAs) or *second-generation* antipsychotics (SGAs). FGAs were introduced in the 1950s (Walker et al. 2004) and reduce dopamine activity by blocking dopamine receptors, especially the D2 subtype. FGAs have similar effect on the positive symptoms, but differ from each other in side effect profile. (Walker et al. 2004) The main side effects related to FGAs involve drug-induced movement abnormalities. These can be divided into early- and late-emerging motor side effects (Walker et al. 2004). Examples of early-emerging motor side effects are pseudoparkinsonism, dystonic reactions, and akathisia, while the most common late-emerging motor side effect is tardive dyskinesia. The cause of these extrapyramidal syndromes has not been clarified, but is thought to be associated with excessive dopamine D2 receptor blockade. Because of the side effect profiles, FGAs are often replaced by SGAs. (Walker et al. 2004) SGAs are a heterogeneous group of medications that occupy different neurotransmitter receptors, but all act as dopamine antagonists to some extent. Similar for this group of medications is the reduced risk of early- and late-emerging motor side effects. (Marder et al. 2002) Clozapine is a SGA, which due to its potentially serious side effects, such as agranulocytosis, is generally restricted to treatment of refractory patients (Alphs, Anand 1999). SGAs are antagonist to the dopamine D2 receptor as well as the serotonin 5-HT2A receptor (Walker et al. 2004). By blocking the serotonin receptor, the serotonergic function in the brain is reduced, and this has been suggested to account for the reduced risk of extrapyramidal side effects for SGAs (Richelson 1999). Another theory for reduced side effects is the lower affinity to the dopamine D2 receptor. FGAs bind to the dopamine D2 receptor with greater affinity than dopamine, while SGAs bind with lower affinity and are therefore rapidly released from the dopamine D2 receptor (Seeman 2002). FGAs and SGAs are effective in treating the positive symptoms, e.g. auditory and visual delusions and hallucinations, but FGAs may worsen the negative and cognitive symptoms (Marino, Knutsen & Williams 2008). Approximately 15% of the patients remain treatment resistant (American Psychiatric Association. 1989). People suffering from schizophrenia experience a significant impact on their economy and quality of life as do their families and the society (Bustillo et al. 2001). Furthermore it has been estimated that only 20–30% of all schizophrenics are capable of living independent lives. Unfortunately, in addition to a high rate of substance abuse (47%) among patients with schizophrenia (Regier et al. 1990), the leading cause of death is suicide. (Marino, Knutsen & Williams 2008, Schwartz, Cohen 2001, Wyatt et al. 1995)

Despite nearly a century of research, little is known about the etiology and pathophysiology of schizophrenia (Walker et al. 2004, Sawa, Snyder 2002). However, it is now generally accepted that the disease is a multifactorial neurodevelopmental disorder influenced by genetic, epigenetic, and environmental factors (Franzek, Beckmann 1998, Maier et al. 1993, Petronis 2004, Lewis, Lieberman 2000)

3. The Pathogenesis of Schizophrenia

A considerable amount of genetic research on the causality of schizophrenia has been conducted during the last century and the disease has been found to be highly heritable (Marino, Knutsen & Williams 2008, HUXLEY et al. 1964). So far, 13 genome-wide association studies (Athanasiu et al. 2010, International Schizophrenia Consortium et al. 2009, Lencz et al. 2007, Need et al. 2009, O'Donovan et al. 2008, Shi et al. 2009, Stefansson et al. 2009, Sullivan et al. 2008, Ikeda et al. 2011, Kirov et al. 2009, Shifman et al. 2008) of schizophrenia have been published. However, the sample sizes in each study were small and therefore not representable, and the results must therefore be interpreted with caution. A joint analysis of these studies was conducted by the Genome-Wide Association Consortium (International Schizophrenia Consortium et al. 2009), and common genetic variations were found. Unlike other diseases, schizophrenia is not caused by a single gene but rather a diversity of genetic and epigenetic variations, environmental risk factors, and their interaction (van Os, Rutten & Poulton 2008). One of the most reliable ways of assessing the genetic background of a particular disease is with the use of twin and adoption studies (Kaprio, Koskenvuo 2002, Cannon et al. 1998, Heston 1966, Higgins 1976, Wender et al. 1974, Tienari 1991, Tienari et al. 1994) This approach was used to establish the genetic role in the development of schizophrenia, and resulted in the estimation of a 50% and 10% risk of developing the disease in monozygotic and dizygotic twins, respectively (Kaprio, Koskenvuo 2002, Cannon et al. 1998). However, monozygotic twins do not always show the same disease susceptibility, which indicate the possibility of environmental and epigenetic contributions during ageing, in the development of the disease (Wong, Gottesman & Petronis 2005). In consistence with this observation it was found that the risk of schizophrenia is similar for offspring of both unaffected and affected monozygotic twins (Gottesman, Bertelsen 1989, Kringlen, Cramer 1989). This suggests that genetics, epigenetics, and environment factors play a role in the disease etiology, but despite the inherited component found in twin and adoption studies (Franzek, Beckmann 1998, Maier et al. 1993, Somnath, Janardhan Reddy & Jain 2002, Cardno et al. 1999, Lowing, Mirsky & Pereira 1983, Kety et al. 1994), the findings are inconsistent.

Correlations between epigenetics and environmental factors in the development of schizophrenia have been suggested, some of which include: place and time of birth (Krabbendam, van Os 2005), parasitic infections (Torrey et al. 2007), low IQ (Lewis, Levitt 2002), maternal nutrition (Weaver et al. 2004, Weaver et al. 2004, Eagles 1991), and obstetric complications (Dassa et al. 1996, Kinney et al. 1994, Maki et al. 2005, Patterson 2007). Furthermore, observations of socioeconomic groups have proposed an increased incidence of schizophrenia in those with a lower socioeconomic status (Aro, Aro & Keskimaki 1995). The role of epigenetic and environmental risk factors is consistent with the late onset of schizophrenia usually during or after adolescence. In addition to the environmental impact on epigenetics, a study (Gartner 1990) on inbred animals with low genetic variability demonstrated that other factors might influence the phenotypic variation (Wong, Gottesman & Petronis 2005).

It has been generally accepted that epigenetic modifications of gene expression are involved in phenotypic variations not determined by the DNA sequence (Bird 2007). Several studies (Petronis 2004, Fraga et al. 2005, Roth et al. 2009, Stone, Morrison & Pilowsky 2007, Abdolmaleky et al. 2005, Costa et al. 2009, Grayson et al. 2005, Mill et al. 2008, Pokholok et al. 2005, Boudreault et al. 2003, Kristjuhan et al. 2002, Ekwall 2005, Groc et al. 2007, Javitt, Zukin 1991, Vogelauer et al. 2000) have suggested the role of DNA

methylation and gene expression in schizophrenia and to a lesser extent, the contribution of histone modifications, which implicate that epigenetic alterations might participate in the molecular pathogenesis of schizophrenia.

3.1. Epigenetic Mechanisms of Gene Accessibility

The term epigenetics, introduced by Conrad Waddington in the 1950s (Morange 2009), is defined as molecular factors and processes around DNA, which are able to regulate gene expression patterns, independent of the DNA sequence. These changes are believed to be mitotically and/or meiotically stable and can therefore be passed on to subsequent generations. (Bird 2007, Skinner, Manikkam & Guerrero-Bosagna 2010). Stable epigenetic patterns are necessary for a tightly controlled regulation of gene expression required for e.g. prober cell differentiation during the developmental stages (Poetsch, Plass 2011) and X-chromosome inactivation in females (Heard 2004). Inappropriate developmental regulation can evolve into the formation of cancer (Poetsch, Plass 2011). Dysregulation of epigenetics has been found in several diseases, including psychiatric disorders such as schizophrenia (Tsankova et al. 2007). The mechanisms of which epigenetic modifications are inherited have been demonstrated for DNA methylation, but the heritability of histone tail modifications still needs to be elucidated. (Margueron, Reinberg 2010, Martin, Zhang 2007)

Histone Modifications regulate the Transcriptional State of Chromatin

Another mechanism for epigenetic control of gene expression involves posttranslational, covalent modifications of nucleosome histone tails. Chromatin can exist in any state between the condensed, inactive heterochromatin and the open, active euchromatin (Grewal, Jia 2007, Raisner, Madhani 2006). Regional compaction of chromatin and access of regulatory complexes to DNA is regulated by highly dynamic histone modifications, directing local events such as gene transcription and DNA repair, and genome wide functions such as DNA replication and chromo-some condensation (Kouzarides 2007) (Berger 2007). The posttranslational modifications of histones include: Acetylation at lysine residues, methylation at lysine or arginine residues, phosphorylation at serine or threonine residues, ubiquitylation at lysine residues, SUMOy-lation at lysine residues, ADP-ribosylation at glutamate residues, and isomerization at proline residues (Kouzarides 2007)(Tsankova et al. 2007). Specific enzymes catalyze each type of histone modification, and enzymes capable of reversing most modifications have been identified as well. The histone-modifying enzymes exist as part of large multiprotein complexes, which control chromatin organization and activity(Kouzarides 2007).

Observations of individual histone modifications correlating with distinct transcriptional effects have lead to formulation of the “histone code” hypothesis. This hypothesis suggests that specific modifications of one or more histone tail residues are “read” sequentially or summed up by protein complexes, which mediate the resulting effects (Jenuwein, Allis 2001). In general, acetylation is associated with DNA decondensation and increased transcriptional activity (Sterner, Berger 2000, Xu, Zhang & Grunstein 2005, Schneider et al. 2006), whereas methylation and phosphorylation can be found in DNA regions with either transcriptional activity or repression, de-pending on the specific residue that has been modified (Lee et al. 2005, Bannister, Kouzarides 2005, Oki, Aihara & Ito 2007, Lachner, Jenuwein 2002). The effects of ubiquitylation, SUMOylation, ADP-ribosylation, and proline isomerization are still poorly understood (Latham, Dent 2007, Zhou, Wang & Rosenfeld 2009, Gill 2004, Hassa et al. 2006).

Accumulating evidence suggests a role of histone modifications in the maintenance of expression patterns and differentiation status across cell generations. This requires transmission of general histone modification patterns during mitosis. Several theories have been proposed for how histone modifications are passed on to subsequent generations, including the recycling of old posttranslational modified histones, and recruitment of his-tone modifying enzymes to the replicated DNA. However, the exact mechanisms for inheritance of histone modifications still need to be investigated in detail. (Margueron, Reinberg 2010, Martin, Zhang 2007)

Histone Acetylation correlates with Active Gene Transcription

Acetylation of histone tails is associated with activity and unwinding of chromatin. This modification has primarily been observed in regions of active gene transcription, but also in regions of DNA re-pair, replication, and condensation. (Kouzarides 2007, Choi, Howe 2009) High levels of histone acetylation are found at promoters and at the 5' end of coding regions, with the degree of acetylation correlating to the rate of gene transcription (Pokholok et al. 2005, Wang et al. 2008, Liu et al. 2005). Moreover, low basal levels of histone acetylation have been demonstrated throughout the genome (Boudreault et al. 2003, Kristjuhan et al. 2002, Vogelauer et al. 2000).

Lysine acetylation is catalyzed by histone acetyltransferases (HATs), which transfer an acetyl group from acetyl-CoA to a lysine residue within the histone tails (Sterner, Berger 2000). In general, HATs catalyze acetylation at several lysine residues, but some enzymes have demonstrated higher specificity, only adding acetyl groups to specific lysine residues (Sterner, Berger 2000, Lee, Workman 2007). HATs are part of large multiprotein complexes that regulate HAT activity and target specific locations within the genome. (Lee, Workman 2007) Acetylations are highly dynamic modifications that can be reversed by histone deacetylases (HDACs), and thus the balance of acetylation and deacetylation fine-tunes gene expression (Ekwall 2005).

Two mechanisms describing how histone modifications control chromatin compaction and DNA activity have been suggested: 1) Directly through alterations of chromatin structure, and 2) indirect-ly through recruitment of effector proteins. Of the various histone modifications, acetylation has the largest potential to unwind chromatin through the direct-acting mechanism. Acetylation neutralizes the positive charge of the affected lysine residue, which is thought to stabilize the interactions of histones with DNA. (Kouzarides 2007, Choi, Howe 2009) Notably, acetylation of histone 4 (H4) at lysine 16 destabilizes internucleosomal contacts of the 30 nm fiber, leading to decondensation of chromatin (Shogren-Knaak et al. 2006). In addition, widespread histone acetylations weaken histone-DNA interactions, producing subtle changes in mononucleosome structure, such as increased sliding of nucleosomes or unwrapping of DNA (Ferreira, Flaus & Owen-Hughes 2007, Anderson, Lowary & Widom 2001). Decondensation of chromatin possibly allows access of DNA-binding proteins, such as transcriptions factors, required for DNA-based activities (Choi, Howe 2009). As is true for DNA methylation, the indirect-acting mechanism of histone modifications is thought to involve recruitment of specific protein complexes to modification sites, whereas the modifications can also sterically inhibit binding of proteins to chromatin (Kouzarides 2007). Histone acetylations are recognized by structurally conserved bromo-domains that have been identified within several chromatin-binding proteins (de la Cruz et al. 2005, Yang 2004). These pro-teins are most often part of larger protein complexes or recruit other proteins with histone-modifying enzymatic activities or

chromatin-remodeling effects, leading to further modification of chromatin. Overall this directs DNA-based activities such as transcription, replication, and repair (Choi, Howe 2009).

DNA methylation and histone modifications does not work as separate moieties in epigenetic control, but rather act in synergy to regulate gene expression. For example, it has been demonstrated that MBDs bind to methylated DNA. MBDs are associated with repressor complexes, having HDAC and histone methyltransferase (HMT) activity. (Klose, Bird 2006) HDACs remove acetyl modifications from histones, whereas HMTs add methyl groups, thereby resulting in gene silencing by chromosome condensation (Kouzarides 2007).

Histone H3 Phospho-Acetylation in Active Gene Transcription

Phosphorylation of histone H3 was initially thought to be involved in condensation of the chromosome during mitosis (Wei et al. 1998, GURLEY et al. 1978), however phosphorylation at the serine residue in histone H3 has lacently been implicated in transcriptional activation. H3 phosphorylation at serine 10 was found to phosphorylate in the pericentric heterochromatin during mitosis, from which it spread though out the genome. Also, this phosphorylation is necessary for initiating chromosome condensation. For recruitment of the condensin complex and assembly of the mitotic spindle, kinases are required (Giet, Glover 2001, Mahadevan, Willis & Barratt 1991).

The rapid response of phosphorylation of histone H3 and activation of cfos was observed by Mahadevan et al. (Mahadevan, Willis & Barratt 1991). The phosphorylation of histone H3 corresponded to the known expression profile of cfos, which indicated a link between the phosphorylation of histone H3 and transcriptional activation of cfos. This correlates with the changes in serine 10-histone H3 phosphorylation following neuronal activation. The residue of N-terminal tail in histone H3, in which serine 10 is located, proteins are able to bind covalently. In here, a relationship between acetylation and phosphorylation occur, which is associated with active transcription (Mahadevan, Willis & Barratt 1991).

3.2. Genes Susceptible to be involved in the Pathophysiology of Schizophrenia

Previous postmortem brain studies (Mill et al. 2008, Guidotti et al. 2000, Akbarian et al. 1995, Veldic et al. 2004) have investigated the differences in gene expression, comparing schizophrenic subjects and healthy controls. Several genes were found to be either up- or downregulated in the brains of schizophrenics, and for some genes the altered expression has been linked to epigenetic dysregulation at the promoters. However, results have been inconsistent and quantitatively the expression changes have only been subtle. (Roth et al. 2009, Stone, Morrison & Pilowsky 2007, Mill et al. 2008)

Activity-regulated Cytoskeleton-associated Protein

Activity-regulated cytoskeleton-associated protein (Arc) is an immediate early gene (IEG), initially found to be rapidly induced in the brain following seizure stimulation (Link et al. 1995, Lyford et al. 1995). The Arc protein is enriched in dendrites (Lyford et al. 1995) and is a marker of neuronal excitability (Li et al. 2009). It has been detected in e.g. the dentate gyrus (Nakahara et al. 2000), amygdala (Li et al. 2009, Lucas et al. 2008), striatum (Li et al. 2009, Nakahara et al. 2000), nucleus accumbens, and medial prefrontal cortex of rats (Li et al. 2009, Nakahara et al. 2000, Waltereit et al. 2001, Rapanelli et al. 2010, Kalinichev et al. 2008). In the prefrontal cortex (PFC), Arc is almost exclusively expressed in pyramidal cells and is therefore an indirect marker of pyramidal neuroexcitability (Vazdarjanova et al. 2006). Arc plays multiple roles in

synaptic regulation and has been found to be involved in long-term potentiation (LTP), long-term-depression (LTD) and homeostatic synaptic plasticity (Lyford et al. 1995, Messaoudi et al. 2007, Rial Verde et al. 2006, Plath et al. 2006), which are implicated in learning and memory formation (Plath et al. 2006). LTP results from rearrangement of the synapse and is thought to induce learning and memory storage by stabilizing changes in the synapse. LTP is initiated by calcium influx in the post-synaptic neuron and further formation of LTP requires IEG transcription, such as Arc. In addition to gene expression in the synapse, the formation of late phase LTP requires structural changes including enlargement of the post-synaptic dendritic spines, to sustain the synaptic changes. (Waltereit et al. 2001, Matsuzaki et al. 2004) In LTP the activation of the NMDA receptor and extracellular-regulated kinase (ERK) is necessary for transcription of numerous activity-regulated genes, including Arc. Arc mRNA is packed in kinesin-associated granules and transported into dendrites (Kanai, Dohmae & Hirokawa 2004), where it accumulates in the recently activated synapses, due to address markers generated by the activated NMDA receptors. (Steward, Worley 2001, Waung et al. 2008) The Arc mRNA is subsequently translated into Arc protein, which regulates the accumulated actin in the activated synapses, supporting LTP consolidation (Messaoudi et al. 2007, Giorgi et al. 2007). Persistent Arc transcription and translation is necessary to sustain the synaptic changes in late phase LTP, as Arc is rapidly degraded by nonsense-mediated RNA decay consolidation (Messaoudi et al. 2007, Giorgi et al. 2007).
Because of the regulatory mechanisms of Arc in the changes of synapses, altered Arc expression in the brains of schizophrenics might affect LTP consolidation, leading to cognitive symptoms such as learning and memory disabilities.

To my knowledge, no studies have examined the expression of Arc in the brains of schizophrenics. However, Arc expression in a rat model of schizophrenia has been investigated in few studies (Javitt, Zukin 1991, Thomsen, Hansen & Mikkelsen 2010, Thomsen et al. 2009). Phencyclidine (PCP) is a psychomimetic agent used to induce schizophrenia-like symptoms and neuropathological changes in animals (Javitt, Zukin 1991). Three studies observed a significant increase in Arc mRNA expression in the PFC and hippocampus following PCP treatment (Nakahara et al. 2000, Thomsen et al. 2009, Beraki et al. 2009). Furthermore, Nakahara et al. (Nakahara et al. 2000) also found increased Arc mRNA levels in nucleus accumbens and the cingulate cortex, but no changes were observed in the hippocampus or the striatum. Thomsen et al. (Thomsen, Hansen & Mikkelsen 2010) investigated the effect of PCP on Arc expression in the PFC, ventrolateral orbitofrontal cortex and nucleus accumbens of both juvenile and adult rats. They found a decreased expression in juvenile rats, while there was increased expression in the adult rats. In order to establish a possible connection between altered expression of Arc and the pathophysiology of schizophrenia, further investigation is needed.

Cfos

Cfos is an IEG that is widely expressed in the central nervous system. The level of cfos is normally low in quiescent cells, but the level increases following an extracellular stimulation, which leads to a rapid transient induction of cfos transcription. With a half-life of approximately 15 minutes, the cfos protein is believed to have a regulatory role in the cellular response to external stimuli. (Sheng, Greenberg 1990, Dragunow 1996). Transcriptional regulation of genes is suggested to be involved in neuronal plasticity, synaptic transmission, LTP, learning, and memory (Dragunow 1996, Todorova, Elbein & Kyosseva 2003). Cfos protein induction is believed to occur through multiple intracellular mechanisms, e.g. via the following pathways; the cAMP- and Ca^{2+}-pathways. Briefly, external stimuli elevate the amounts of intracellular cAMP

and Ca^{2+}, which in turn phosphorylate CREB via protein kinase A (PKA) and calcium/calmodulin-regulated kinase (CaMK), respectively. The activated CREB acts as a transcription factor of cfos protein. Following cfos protein induction cfos interacts with a cjun protein, which is also an IEG, and forms a heterodimeric transcription factor through "leucine-zipper" domains, present in both proteins. (O'Shea et al. 1989, Ransone et al. 1989, Schuermann et al. 1989, Turner, Tjian 1989) (Landschulz, Johnson & McKnight 1988, Kouzarides, Ziff 1988, Bos et al. 1989, Gentz et al. 1989). It has been suggested that the site of interaction is located among the side chains of the leucine-zipper domains between cfos and cjun proteins. The resulting transcription factor is able to bind to AP-1 containing DNA elements, stimulating or repressing the transcription of nearby genes, depending on the type of cjun it interacts with. (Curran, Franza 1988, Angel et al. 1987, Lee, Mitchell & Tjian 1987)
The cfos protein, together with the cjun protein, acts as a transcription factor for genes involved in e.g. neuronal plasticity and LTP, and alterations in cfos expression might therefore be involved in altered LTP formation and memory deficits in the brains of schizophrenic subjects.

In the brains of schizophrenic patients, Kyosseva et al. (Kyosseva et al. 2000) showed an upregulation of transcription factors within the promoter of cfos in the cerebellar vermis. In a later study (Kyosseva 2004) Kyosseva et al. also revealed an increase in cfos protein in the thalamus. In addition to post mortem brain studies, acute PCP administration significantly increased cfos expression in rat PFC (Kalinichev et al. 2008) and in the parietal cortex of mice (Sugita et al. 1996). An increase in cfos expression in the thalamus following acute PCP administration in rats has been found (Kargieman et al. 2007) and as the thalamus contains projecting neurons to the PFC, the PFC is suggested to be implicated in the PCP-mediated cognitive disturbances (Groenewegen et al. 1990) 1990). Furthermore, abnormalities in this region has also been implicated in schizophrenia (Konick, Friedman 2001) Thus, several studies points to the involvement of expressional alterations of cfos in the brains of schizophrenic patients and an upregulation might be involved in some of the cognitive impairments found in schizophrenics. However, this still needs to be elucidated, as it has only been investigated in few post mortem brains of schizophrenics (Kyosseva et al. 2000, Kyosseva 2004).

Brain-Derived Neurotrophic Factor

Brain-derived neurotrophic factor (BDNF) is a member of the protein family of neurotrophic factors. During development, BDNF is highly expressed in the nervous system compared to other tissues and is important in regulating the development of the peripheral and central nervous system by growth, differentiation and survival of the neurons. (Bibel, Barde 2000) In the more mature brain and in the adult brain, BDNF is highly expressed in the hippocampus and PFC (Baquet, Gorski & Jones 2004, Conner et al. 1997, Ernfors et al. 1990, Timmusk, Persson & Metsis 1994) and regulates synaptic transmission and plasticity (Pezet, McMahon 2006). Pruunsild et al. showed that the human BDNF gene contains one coding exon (exon IX) and ten non-coding exons (exons I-VIII and X-XI), in which nine are defined as 5' exons. (Aid et al. 2007) Of the 11 human exons, only exons I, VII, VIII, and IX contain in-frame ATG that can be used for translation start sites resulting in production of the prepro-BDNF proteins. For the remaining exons, except exon VIII, the ATG region in the 3'exon IX is needed for translation. Moreover, promoters of exons I, II, III, IV, V, Vh, VI, VII, and IXabcd were found to act separately. (Pruunsild et al. 2007) Additionally, the rat BDNF gene has eight non-coding 5' exons with separate promoters and one 3' exon, which encode the mature BDNF protein. Four 5' exons in the rat BDNF are homologous to four in the human BDNF gene. Of the non-coding exons in the rat BDNF gene, exon I is the only one in which an initial translational code has been identified.

(Pruunsild et al. 2007) BDNF is believed to exert its action through neuronal activity, which leads to BDNF transcription, transport, and finally translation into the dendrites (Lu 2003). BDNF promotes neuronal differentiation, growth, and survival through interaction with the receptor tyrosine kinase TrkB, triggering cross tyrosine phosphorylations in the intracellular Trk domain. Thereby three different TrkB-intracellular signalling pathways are activated: (1) the Ras-mitogen-activated protein kinase (MAPK) signaling cascade, (2) phosphatidylinositol 3-kinase (PI3K) cascade, and (3) the PLCγ–Ca^{2+} pathway. (Minichiello 2009, Yoshii, Constantine-Paton 2010) In the PFC, BDNF and its receptor, TrkB, regulate the development of GABA interneurons. Dysfunction of the inhibitory interneurons has been found in the PFC of schizophrenic subjects. (Ventimiglia et al. 1995, Arenas et al. 1996, Rutherford, Nelson & Turrigiano 1998, Vicario-Abejón et al. 1998, Bolton, Pittman & Lo 2000, Marty, Wehrlé & Sotelo 2000, Yamada et al. 2002, Kohara et al. 2003) Therefore, a possible connection between impaired BDNF and/or TrkB and altered GABA-related genes in the PFC, might be involved in working memory impairments. (Weickert et al. 2003, Hashimoto et al. 2005) Overexpression of BDNF levels in the PFC of transgenic mice also revealed memory deficits in e.g. the passive voidance task and reduced performance in a water maze. Thus, increasing BDNF levels in the PFC might obstruct memory processes. (Cunha et al. 2009, Elvevåg, Goldberg 2000)

As BDNF is involved in the formation of cognitive functions within the PFC, alterations in BDNF expression might lead to the dysfunctional cognitive impairments, such as working memory deficits, which have been observed in the brains of schizophrenic subjects. (Cunha et al. 2009, Elvevåg, Goldberg 2000) In the PFC of schizophrenic patients, a decreased mRNA expression of the BDNF receptor, TrkB, has been reported by Weickert et al. (Weickert et al. 2005). Furthermore, a single nucleotide polymorphism of the pro-BDNF sequence (Val66met) was discovered by Egan et al. (Egan et al. 2003), which affects the activity-dependent secretion of BDNF that can lead to impaired LTP and LTD. Another study (Hashimoto et al. 2005) found a decrease of BDNF and TrkB in the PFC in the brains of schizophrenic patients, which also supports a possible role of BDNF alteration in working memory deficits. Acute PCP administration increased BDNF levels in corticolimbic regions in the rat (Kalinichev et al. 2008, Grottick et al. 2005) which is consistent with the post mortem findings in schizophrenic brains conducted by Durany et al. (Durany et al. 2001). Various studies (Hashimoto et al. 2005, Durany et al. 2001) have investigated the possible alterations in BDNF expression as a potential risk for developing schizophrenia, even though the concrete mechanisms for this needs to be further elucidated.

4. The Phencyclidine Animal Model of Schizophrenia

To understand the pathophysiology of schizophrenia and for hastening the generation of new treatments, the development of reliable animal models is necessary. To date, several animal models of schizophrenia are available but because of the diverse nature of the disease, generating a model with respect to all of the pathophysiological findings is difficult. The current animal models have been developed based on the hypothesis of neurotransmitter dysfunctions (Marino, Knutsen & Williams 2008) found in schizophrenic patients. The models in general can be divided into four different categories: environmental, genetic, pharmacological, and lesion-based models (Carpenter, Koenig 2008). The models are generally selected for research with emphasis on the specific brain region and the mechanisms being investigated. Based on the first neurochemical hypothesis of dopaminergic hyperfunction and glutamatergic hypofunction in

schizophrenia (Marino, Knutsen & Williams 2008) diverse animal models have been created by altering the dopamine and glutamate function to induce the behavioral and neurotransmitter changes seen in schizophrenic subjects (Bubenikova-Valesova et al. 2008)(Carpenter, Koenig 2008)(Marcotte, Pearson & Srivastava 2001).

Phencyclidine (PCP), a non-competitive NMDA receptor antagonist, was originally developed as an anesthetic agent. However, its administration was found to be associated with a number of adverse effects with the development of psychotic episodes (Baldridge, Bessen 1990). In healthy subjects, PCP, induces positive symptoms including delusions and hallucinations (Krystal et al. 1994, LUBY et al. 1959), and negative symptoms such as social isolation and poverty of speech (LUBY et al. 1959). Cognitive impairments have also been demonstrated expressed as prepulse inhibition (PPI), which resembles the findings of startle-gating deficits of schizophrenic patients (Javitt, Zukin 1991, Geyer, Braff 1987, Braff, Grillon & Geyer 1992, Jones, Watson & Fone 2011). PCP profoundly exacerbates both the positive and negative symptoms in schizophrenic patients (Javitt, Zukin 1991). In addition, hypofrontality in frontal cortex has been observed in chronic PCP abusers (Pratt et al. 2008) and is believed to be associated with cognitive dysfunction in schizophrenics (Ingvar, Franzen 1974, Weinberger, Berman 1996).

Subsequently, PCP has been used in an animal model of schizophrenia and the model has been shown to be capable of inducing profound symptoms of schizophrenia compared to other pharmacological models (LUBY et al. 1959)(Javitt 1987)(Sams-Dodd 1996)(Javitt, Zukin 1991) Functionally, it is believed that NMDA receptor antagonists decrease excitatory pyramidal input to GABAergic interneurons in the PFC. Subsequently, this decreases inhibition of pyramidal neurons (Homayoun, Moghaddam 2007), causing pyramidal hyperactivity which may lead to permanent neuronal damage and hypofrontality (Newcomer et al. 1999). This decreases cognitive function, whereas impaired gamma oscillations in the hippocampus (Uhlhaas, Singer 2010, Lewis, Hashimoto & Volk 2005) and other brain regions disrupts working memory. (Thomsen, Hansen & Mikkelsen 2010, Jenkins et al. 2008, Jenkins, Harte & Reynolds 2010, Abdul-Monim, Neill & Reynolds 2007, Cochran et al. 2003) In addition, it has been proposed that the acute blockage of NMDA receptors increase in glutamatergic efferent projections, leading to increase in dopamine release in the nucleus accumbens (Jentsch, Taylor & Roth 1998, Takahata, Moghaddam 2003).

Different dose and time regimens of PCP administration, including acute and chronic PCP administration, have been used with varying effects of inducing schizophrenia-like symptoms (Jones, Watson & Fone 2011, Li, He & Chen 2011). Acute PCP administration in rat produces a number of schizophrenia-like behaviors including hyperlocomotion (Kalinichev et al. 2008, Jones, Watson & Fone 2011, Castellani, Adams 1981, Redmond et al. 1999) social withdrawal (Sams-Dodd 1996, Steinpreis, Salamone 1993) impaired working memory (Jentsch, Roth 1999, Linn et al. 2007, Zajaczkowski, Czyrak & Wedzony 2003, Sams-Dodd 1995), and cognitive impairment (Jones, Watson & Fone 2011, Egerton et al. 2005, Adams, Moghaddam 1998, Geyer, Segal & Greenberg 1984). Also, a decrease in PPI was found in rats (Jentsch, Roth 1999, Sams-Dodd 1995). An increase in extracellular levels of glutamate (Amitai et al. 2011), dopamine (Steinpreis et al. 1994), and serotonine in the frontal cortex has been demonstrated following acute PCP administration in rats (Jentsch, Roth 1999, Moghaddam et al. 1997). These altered metabolic findings are comparable to a study by Soyka et al. (Soyka et al. 2005), which demonstrated a hypermetabolic pattern in schizophrenic patients exhibiting positive acute psychotic state. A number of studies have investigated the acute effect of PCP administration in gene expressions. A significant increase in cfos (Kalinichev et al. 2008) and Arc

(Thomsen et al. 2009, Beraki et al. 2009) expression in the rat PFC was observed, and also in the thalamus (Kargieman et al. 2007, Jodo et al. 2010), which might lead to cognitive disturbances.
Thus, acute PCP treatment can be used as a model for schizophrenia as it is able to induce a variety of schizophrenia-like symptoms in the rat.

5. Aims

The overall purpose of this study is to investigate the expressional and epigenetic effects of acute PCP administration in rat PFC. The aims are to:

- Evaluate the effect of acute PCP administration in a time study on mRNA expression of three schizophrenia-susceptible genes, Arc, cfos, and BDNF, in the PFC of adult rats.
- Evaluate the level of H4 acetylation following acute PCP administration of the promoters, corresponding to each of the mRNA being investigated, in rat PFC.
- Evaluate the level of H3 phospho-acetylation following acute PCP administration of the promoters, corresponding to each of the mRNA being investigated, in rat PFC.
- Evaluate if a correlation between PCP-induced mRNA expression in Arc, cfos, and BDNF, and the H4 acetylation and H3 phospho-acetylation following acute PCP administration of the promoters, corresponding to each of the mRNA being investigated, in rat PFC.
- Evaluate if a correlation between PCP-induced mRNA expression in Arc, cfos, and BDNF, and the H4 acetylation and H3 phospho-acetylation in their corresponding promoters, exist.

6. Materials and Methods

6.1. Materials

Standard chemicals were obtained from Sigma-Aldrich (Germany), AppliChem (Denmark) or Merck (Germany), unless otherwise stated.

6.2. Animals

Gene expression analysis and chromatin immunoprecipitation (ChIP) assay was performed on prefrontal cortex (PFC) from 30 young adult, male Sprague-Dawley rats (weighing 210-250 g) provided by Adjunct Professor Jens D. Mikkelsen (Aalborg University). All experiments were conducted in accordance with guidelines of the National Institute of Health and the Animal Experimentation Inspectorate, Ministry of Justice, Denmark.

6.3. Drug Treatment

The animals were divided into seven groups (n = 2-4 per group); two control groups and five experimental groups, see appendix I. Animals in the control and experimental groups, each received a single subcutaneous saline-water injection (0.9% saline) or PCP injection (10 mg/kg), respectively. PCP hydrochloride (Tocris Bioscience, United Kingdom, #2557) was used based on previous studies (Sams-Dodd 1996, Castellani, Adams 1981, Redmond et al. 1999, Adams, Moghaddam 1998, Geyer, Segal & Greenberg 1984), in which the effect of PCP was found to imitate some of the symptoms observed in schizophrenic subjects, which included hyperlocomotion (Castellani, Adams 1981, Redmond et al. 1999), social withdrawal (Sams-Dodd 1996), and cognitive impairment (Adams, Moghaddam 1998, Geyer, Segal & Greenberg 1984). The animals were decapitated at different time points following PCP and saline-water injections. The brains were collected and PFC dissected out for further investigation. The experimental results from the control groups were pooled before statistical analysis for both the expression profiling study and for ChIP.

6.4. Primer Design

Primers for expression analysis and ChIP assay were designed based on the National Center for Biotechnology Information (NCBI) (Anonymous). The primer parameters selected for primer design can be found in Table 1. Primers for expression analysis were designed on the basis of mRNA sequence of the gene of interest, found in the NCBI database. All targeted sequences were separated by at least one intron at the corresponding genomic DNA. Primers for ChIP assay were designed based on the genomic sequence of the gene of interest also found in the NCBI database. All genomic primers lie close to transcription start site. A full list of primer sequences can be found in appendix II.

	Minimum	Optimal	Maximum
PCR product size (bp)	120		150
Primer melting temperature (°C)	63	65	67
Primer size (bp)	22	25	28
Primer GC content (%)	20		70

Table 1: Parameters for mRNA and genomic primers for the evaluation of expression profiling and chromatine immune precipitation.

Primer Efficiencies

With the use of 5x two-fold dilution series, primer efficiencies were determined with the use of cDNA or DNA from rat cerebellum and hippocampus. To measure the amplification of the different samples, a mastermix consisting of Brilliant© II SYBR© Green (Agilent Technologies, USA, #600828), which fluoresces upon binding to dsDNA after each PCR cycle, was used. Furthermore, forward and reverse primers were added to the mastermix together with 30 nm ROX Dye (Agilent Technologies, USA, #600530-53), which normalize for inconsistent well-to-well volumes. The samples were run in duplicates in 96-well plates using a Stratagene Mx3000P™ QPCR System (Agilent Technologies, AH Diagnostics Denmark) programmed to initiate the Taq DNA polymerase at 95°C for 10 minutes, followed by 40 cycles of: denaturation at 95°C for 30 s followed by annealing and extension at 60°C for 30 s. To determine whether the increased fluorescence in the samples was due to true amplification and not nonspecific amplification e.g. formation of primer-dimers, a dissociation profile was generated as follows: 95°C for 1 minute, 55°C for 30 s and 95°C for additional 30 s. At the final rise in temperature, fluorescence data was continuously collected.

6.5. mRNA Expression Analysis

To examine possible changes in gene expression in a time study of PCP, quantitative Real Time PCR (Quantitative RT-PCR) was performed on isolated RNA from PFC.

Optimizing RNA Template for Quantitative RT-PCR

To evaluate and optimize the amount of RNA template in cDNA synthesis and subsequently qRT-PCR, cDNA synthesized from different RNA templates (10 ng, 100 ng, or 1000 ng) was used per reaction for qRT-PCR. Arc UTR primers were used for amplification of the synthesized cDNA. Briefly, a mastermix consisting of Brilliant© II SYBR© Green, forward and reverse primers, and 30 nm ROX Dye was added. The samples were run in duplicates in 96-well plates using a Stratagene Mx3000P™ QPCR System programmed to 95°C for 10 minutes, followed by 40 cycles of: 95°C for 30 s and 60°C for 30 s. Additionally, a dissociation profile was generated as follows: 95°C for 1 minutes, 55°C for 30 s and 95°C for additional 30 s. β-actin was used as housekeeping gene.

Tissue Preparation

For the mRNA expression analysis, tissue from left PFC hemisphere of 23 animals was snap-frozen in liquid nitrogen and then stored at -80°C. The tissue preparations were carried out at Rigshospitalet (Denmark) by Adjunct Professor Jens D. Mikkelsen (Aalborg University) and MS. Majbrit Myrup Jensen (Neurobiology Research Unit, Rigshospitalet, Denmark), and subsequently sent to Aalborg University.

RNA Isolation

RNA was extracted from PFC using NucleoSpin® RNA II Total RNA Isolation Kit (Macherey-Nagel, Germany), with the protocol for cultured cells and tissue and the support protocol for difficult tissues, according to the manufacturer's instructions. The procedure was carried out at room temperature (RT). Briefly, the samples were homogenized with an Ultra-Turrax T10 basic (IKA®, Germany) with an 8 mm pistil knife in the presence of β-mercaptoethanol and a guanidine thiocyanate-containing lysis buffer, lysing the cell membrane and inactivating RNases present in the sample. Cell debris was removed using NuceloSpin® Filters and 70% ethanol was added to precipitate the RNA. The RNA was bound onto a silica membrane and contaminated DNA was removed by rDNase. Three washing steps with different buffers were conducted, inactivating rDNase, removing salts, metabolites, and cellular components. RNA was finally eluted in 60 µl RNase-free H_2O and DNase treatment of the isolated RNA was performed. The RNA concentration of each sample was determined by spectrophotometry (NanoPhotometer™, Implen, Germany) and the RNA was subsequently stored at -20°C until further use.

cDNA Synthesis

PFC isolated RNA was used for first strand cDNA synthesis with the use of both RevertAid™ H Minus First Strand Synthesis Kit (Fermentas, Germany, #K1632) and Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Fermentas, Germany, #1641), according to the manufacturer's instructions. The first kit that was used was RevertAid™ H Minus First Strand Synthesis Kit. Briefly, RNA template ranging from 100 to 1000 ng and 1 µl random hexamer primer were brought to a volume of 12 µl. Additional components were added and mixed in the following order; 5X Reaction Buffer, 20 µ/µl Ribolock™ RNase Inhibitor, 10mM dNTP Mix, and 200 µ/µl RevertAid™ Minus M-MulV Reverse Transcriptase to a total volume of 20 µl. The samples were incubated in a Veriti™ 96-well Thermal Cycler (Applied Biosystems, Denmark) at 25°C for 5 minutes and 42°C for 60 minutes. The reaction was terminated at 70°C for 5 minutes. For the second kit, Maxima First Strand cDNA Synthesis Kit for RT-qPCR, RNA template at 10 or 100 ng was used. Following components were added in the indicated order: 5X Reaction Mix containing reaction buffer, dNTPs, oligo $(dT)_{18}$ and random hexamer primers, Maxima™ Enzyme Mix containing RNase inhibitors and reverse transcriptase, and RNA in a total volume of 20 µl. The samples were incubated in the Thermal Cycler at 25°C for 10 minutes and 50°C for 15 minutes. The reaction was terminated at 85°C for 5 minutes.

Quantitative RT-PCR for Examination of Housekeeping gene (β-actin) Stability

In order to ensure that the housekeeping gene, β-actin, keeps stable regardless of experimental conditions, cDNA from two rats from three different groups (control 60 min, PCP 120 min, PCP 24h) were run in a Stratagene Mx3000P™ QPCR System for quantitative RT-PCR analysis, as described in . Each sample was run in duplicates with 10 µl of DNA eluate per reaction. A mastermix consisting of Brilliant© II SYBR© Green, forward and reverse genomic primers, and ROX Dye was added to each well and the program was set to 95°C for 10 minutes, followed by 40 cycles of: 95°C for 30 seconds and 60°C for 30 seconds. This was followed by a dissociation program.

Quantitative RT-PCR for mRNA Expression Profiling

Following optimization of qRT-PCR, cDNA synthesized from 100 ng RNA template per reaction was used. To measure the amplification of the different samples, a mastermix consisting of e.g. Brilliant© II SYBR© Green, forward and reverse primers, and 30 nm ROX Dye was added. The samples were run in duplicates in 96-well plates using a Stratagene Mx3000P™ QPCR System programmed to 95°C for 10 minutes, followed by 40

cycles of: 95°C for 30 seconds and 60°C for 30 seconds. Furthermore, a dissociation profile was generated as follows: 95°C for 1 minutes, 55°C for 30 seconds and 95°C for an additional 30 seconds. β-actin was used as housekeeping gene.

6.6. Chromatin Immunoprecipitation (ChIP) Assay

To examine possible connections between altered mRNA expression following PCP injections and specific histone modifications, ChIP assay was performed. Variations in histone H4 acetylation and histone H3 phospho-acetylation were examined on isolated chromatin from right PFC hemisphere.

Tissue Preparation

For ChIP assay, the right hemisphere of 20 animals was chopped into smaller pieces whilst being immersed in oxygenated phosphate buffered saline (PBS) containing protease inhibitors. For chromatin cross-linking, the tissue was incubated on ice for 15 minutes in 1% paraformaldehyde (PFA). The tissue was subsequently incubated for at least five minutes with 130 mM glycine to stop the cross-linking reaction. The PFA-glycine buffer was aspirated and the tissue was washed four times in PBS containing protease inhibitors. The remaining liquid was aspirated and the tissue was snap-frozen in liquid nitrogen and then stored at -80°C. The tissue preparations for ChIP were carried out at Rigshospitalet (Denmark) by Associate Professor Jens D. Mikkelsen and MS. Majbrit Myrup Jensen (Neurobiology Research Unit, Rigshospitalet, Denmark), and subsequently sent to Aalborg University.

Preparation of Cross-Linked Chromatin for Antibody Incubation

Frozen rat PFC was immersed into 10 mL of protecting cold extraction buffer 1 (0.4 M sucrose, 10 mM Tris-Cl pH 7.9-8, 5 mM β-mercaptoethanol, protease inhibitors) and homogenized immediately 1x30 seconds, level 3 followed by 1x30 seconds, level 4 with an Ultra Turrax T25 with an 8 mm pistil knife. The homogenate was centrifuged at 4°C, 3000 g for 20 minutes in a centrifuge and the pellet was subsequently resuspended in 1 mL of cold extraction buffer 2 (0.25 M sucrose, 10 mM Tris-Cl pH 7.9-8, 1% Triton X-100, 10 mM $MgCl_2$, 5 mM β-mercaptoethanol, protease inhibitors) to facilitate permeability of cell membranes, and centrifuged at 4°C, 12000 g for 10 minutes. The pellet was resuspended in 300 µl lysis buffer (50 mM Tris-Cl pH 7.9-8, 10 mM EDTA, 1% SDS, protease inhibitors), vortexed and incubated on ice for 30 minutes. The lysate was diluted 10x with dilution buffer (1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-Cl pH 7.9-8, 167 mM NaCl) and subsequently sonicated with the use of a high intensity ultrasonic Vibra-Cell™ VCX130 (Sonics, USA) processor in an ice and ethanol mixture. The sonicator was set to 5x30 seconds, 25% output power and 20 seconds breaks in between each sonication pulse with an expected average fragment size of 500 bp (energy input of 220 J). The sonicated chromatin was split into two eppendorfs and spinned down at 4°C, 20000 g for 10 minutes. The supernatants were subsequently combined and 30 µl from each sample was extracted for size determination of chromatin fragments following sonication.

Immunoprecipitation of Cross-Linked Chromatin

For immunoprecipitation, aliquots were prepared each with 500 µl of supernatant. 5 µl of the following polyclonal antibodies was added: anti-acetyl-histone H4 (Millipore, #06-866) and anti-phospho (Ser10)-acetyl (Lys14)-Histone H3 (Millipore, #07-081). For non-immunoprecipitated samples (mock), rabbit serum (Invitrogen, #10510) was added to test for non-specific binding. Dynabeads® protein A (Invitrogen, Norway, #100-02D) was washed with dilution buffer and afterwards resuspended in equilibration buffer (dilution buffer, 1 µg λ DNA, 2 mg bovine serum albumin) to block for unspecific binding. All samples, including

Dynabeads Protein A, were incubated over night at 4°C at 20 rpm on an Intelli-Mixer RM-2 rotator (Elmi, Latvia).
The following day, 50 µl of beads were distributed to each tube and incubated at 4°C for 3 hours on a rotator to bind immune complexes in the samples. The beads were subsequently collected using a bead separation magnet (Invitrogen, Denmark) and 500 µl of supernatant from mock sample was used as positive control (input). Remaining supernatants were discarded and the immune complexes were washed at 4°C for 10 minutes at 30 rpm on the rotator with the following salt buffers: Low salt buffer (150 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Cl pH 7.9-8), high salt buffer (500 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Cl pH 7.9-8), and LiCl buffer (0.25 mM LiCl, 1% Igepal, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-Cl pH 7.9-8) to remove non-specific chromatin interactions and prepare the chromatin for precipitation. Finally, immune complexes were washed with 2x TE buffer (10 mM Tris-HCL pH 7.5, 1 mM EDTA). Immune complexes were eluted twice with freshly made elution buffer (1% SDS, 0.1 M $NaHCO_3$), in order to denature proteins present in the samples, in an Eppendorf Thermomixer Comfort (Eppendorf, Germany) at 65°C, 1000 rpm for 15 minutes. The combined eluates were incubated with 190 mM NaCl at 65°C, 1000 rpm over night to reverse chromatin cross-linking. Also, the samples extracted for size determination of chromatin fragments following sonication, were incubated with NaCl overnight at 65°C in the thermomixer. Protein degradation was carried out by incubating the samples in a thermomixer at 45°C, 1000 rpm for 3 hours with 20.1 mg/mL Proteinase K (Fermentas, Germany), 9 mM EDTA, and 36.3 mM Tris-Cl (pH 6.5-6.8).

Recovering DNA from the Eluates

DNA extraction was performed using ChIP DNA Clean and Concentrator Kit™ (Zymo Research,USA, #D5205) according to the manufacturer's instructions. Briefly, 5x volumes of ChIP DNA Binding Buffer were added and mixed with each sample (5:1) and subsequently transferred to a column and centrifuged at RT, 10000 g for 1 minute, binding the DNA to the columns. Afterwards, the columns were washed twice with wash buffer and centrifuged at RT, 10000 g for 1 minute. The DNA was finally eluted by centrifuging the samples at RT, 10000 g for 1 minute with 100 µl elution buffer. The samples were stored at 4°C. DNA from the sonicated samples was only eluted in 15 µl elution buffer and directly run in on a 2% agarose gel containing ethidium bromide, using a RunOne-Electrophoresis Cell (Embi Tec, USA). The gel was processed on a Kodak Image Station 4000MM PRO (Carestream Health, Inc.) with an excitation filter of 530 nm and an emission filter of 600 nm.

Quantitative Real Time PCR of Immunoprecipitated DNA

To measure the amount of immunoprecipitated DNA obtained from the ChIP assay, qRT-PCR was run using the Stratagene Mx3000P™ QPCR System. Each sample was run in duplicates with 10 µl of DNA eluate per reaction. A mastermix consisting of Brilliant© II SYBR© Green, forward and reverse genomic primers, and ROX Dye were added to each well and the program was set to 95°C for 10 minutes, followed by 40 cycles of: 95°C for 30 seconds and 60°C for 30 seconds. This was followed by a dissociation program. The results were subsequently normalized to β-actin.

6.7. Data Analysis

For the expression analysis, fold change of cDNA starting amounts was calculated for each animal with the use of primer efficiencies in the following equation:

$$\frac{(1+E_{gene\ of\ interest})^{Ct_{gene\ of\ interest}}}{(1+E_{housekeeping\ gene})^{Ct_{housekeeping\ gene}}}.$$

To determine the degree of histone H4 acetylation and histone H3 phospho-acetylation of ChIP DNA for each animal, the following equation was used with alternating data of H4Ac and H3S10K14:

$$\frac{(1+E_{gene\ of\ interest})^{-Ct\ Ip(gene\ of\ interest)-Ct\ input(gene\ of\ interest)}}{(1+E_{housekeeping\ gene})^{-Ct\ Ip(ghousekeeping\ gene)-Ct\ input(housekeeping\ gene)}}$$

β-actin was used as housekeeping gene in all calculations for both the mRNA expression analysis and ChIP assay. Furthermore, statistical analyses for mRNA expression and histone H4 and H3 evaluation were performed in GraphPad Prism 5 (GraphPad Software) using Kruskal-Wallis and Dunn's post test and One-Way ANOVA and Tukey post test.

7. Results

7.1. β-actin - a Stable Housekeeping Gene following PCP Administration

Throughout this study, β-actin has been used as a housekeeping gene. To examine the stability of β-actin during the experimental settings, including PCP treatment, qRT-PCR was used. With a total of six animals divided in three groups (control, PCP 120 and PCP 24h) the level of β-actin from six different quantitative RT-PCR analyses was examined. In addition, the stability of β-actin was also observed with different amounts of RNA template for cDNA synthesis, prior to quantitative RT-PCR. The obtained Ct-values are plotted in Figure 1.

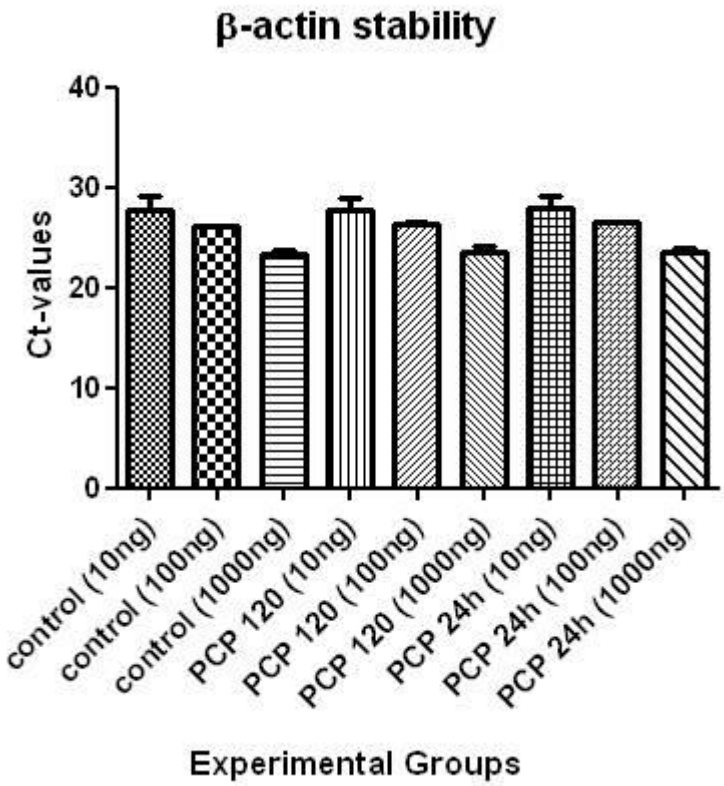

Figure 1: The stability of β-actin regardless of RNA template concentration and acute phencyclidine (PCP) treatment in rat PFC. Data from six different quantitative RT-PCR analysis was examined in three different experimental groups (control, PCP 120, and PCP 24h). Also, varying amounts of RNA template (10 ng, 100 ng, and 1000 ng) was investigated. The data are plotted directly with Ct-values obtained in quantitative RT-PCR.

7.2. Optimization of Quantitative RT-PCR

In order to ensure that the cDNA amplifications observed in qRT-PCR were true, and that sufficient amount of reagents was used for the right amount of cDNA, different quantities of template RNA was used for cDNA synthesis (10, 100 and 1000ng per reaction). The cDNA was subsequently analyzed with the use of qRT-PCR. With a total of six animals divided in three groups (control, PCP 120 and PCP 24h), the level of Arc

mRNA with different amounts of RNA template was investigated. The expression of Arc, relative to β-actin is presented in Figure 2. When comparing the graphs, the same tendency of increased mRNA in the PCP 120 group is obtained, regardless of RNA template.

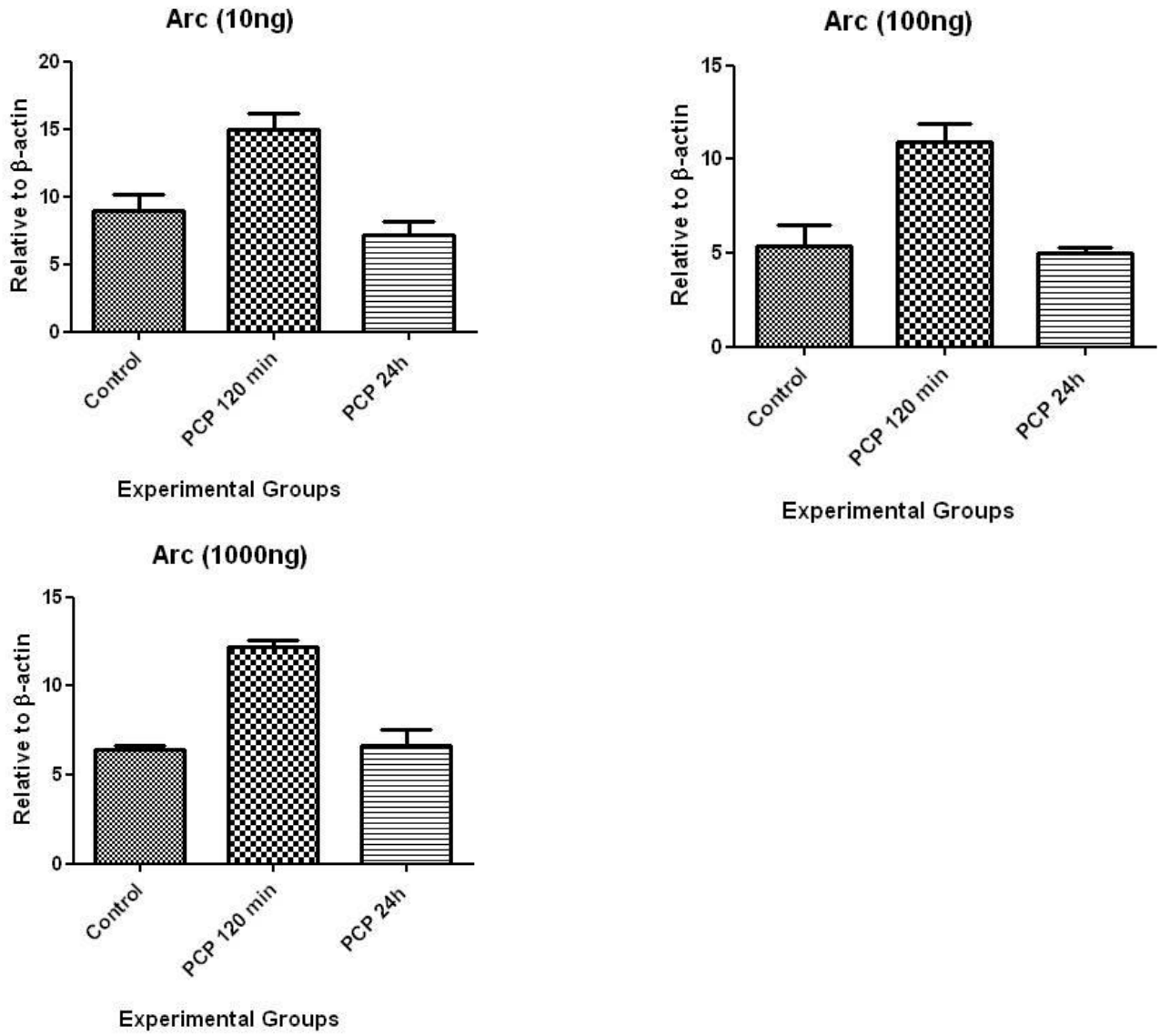

Figure 2: Optimization of RNA template for quantitative RT-PCR. The experimental groups each represent a group of male Sprague-Dawley rats, decapitated at different time points following a single dose injection of saline water (0.9%) or phencyclidine (PCP) (10mg/kg). Six rats from three different groups (control, PCP 120 and PCP 24h) were used. Quantitative RT-PCR was performed with different amounts of RNA template for cDNA synthesis (10 ng, 100 ng and 1000 ng) with primers detecting the Arc mRNA expression. The mRNA levels are illustrated as relative quantity normalized to β-actin.

7.3. Gene Expression Profiling in the Prefrontal Cortex of PCP-Treated Rats

To examine the mRNA expression of schizophrenia-related genes (Arc, cfos, and BDNF) in rat PFC following acute PCP administration, qRT-PCR was used. mRNA was analyzed for five groups of rats with different decapitation times to assess the effect of acute PCP administration in rat PFC over time. mRNA expression

was also investigated in the control group. For the entire gene expression analysis it is important to notice that each of the PCP 360 and PCP 24h groups only contained two animals. The results for mRNA expression are presented in Figure 3**Fejl! Henvisningskilde ikke fundet.**Figure 4 as relative quantity normalized to β-actin and the control group was set to 100%.

Acute Exposure of PCP Alters the Expression of Arc mRNA in the Rat PFC

Kruskal-Wallis with Dunn's post test was firstly used for the analysis of Arc mRNA expression in the PFC. Significant decrease (*p-value* 0.01 to 0.05) in Arc mRNA was found in the PCP 360 group compared to the PCP 240 group. See Figure 3A. Furthermore, statistical analysis was performed using One-Way ANOVA and Tukeys post test which revealed significant differences in Arc mRNA expression between three groups, following acute PCP-administration. See Figure 3B. A very significant (*p-value* 0.001 to 0.01) increase of approximately 78% in mRNA in the PCP 240 group relative to control was observed. Also, a significant difference (*p-value* 0.001 to 0.01) was found between the PCP 240 and 360 groups. However, the SEM of both the PCP 240 and 360 groups, are somewhat large. Finally, Arc mRNA in the PCP 24h group was found to be significantly (*p-value* 0.01 to 0.05) decreased compared to the PCP 240 group. The observed changes in Arc mRNA at different time points following acute PCP treatment can be seen in Figure 3.

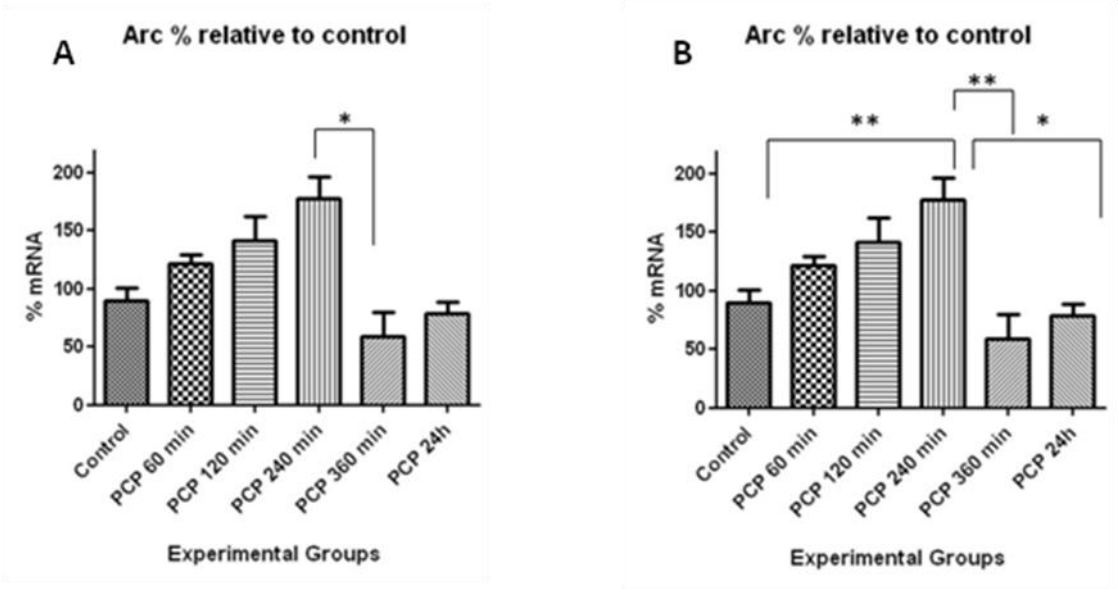

Figure 3: The expression of Arc mRNA in the rat PFC following acute phencyclidine (PCP) administration. The experimental groups each represent a group of male Sprague Dawley rats, decapitated at different time points following a single dose injection of saline water (0.9%) or PCP (10mg/kg). The mRNA levels are illustrated as relative quantity normalized to β-actin, and the gene expression of the control group is set to 100%. A: Results from the Kruskal-Wallis with Dunn's post test, B: Results from One-Way ANOVA with Tukey post test. All data are represented as mean ± SEM. Asterisk denotes significant differences in gene expression between different groups. *p-value 0.01 to 0.05, **p-value 0.001 to 0.01.

An Increase in Cfos mRNA Expression Following Acute PCP Treatment

Kruskal-Wallis test with Dunn's post test revealed a significant increase (*p-value* 0.01 to 0.05) in cfos mRNA in the PCP 120 group when compared to control. See Figure 4A. When using One-Way ANOVA with Tukeys post test alterations in cfos mRNA expression revealed significant differences following the administration of acute PCP. Nearly all groups showed significant differences when comparing columns, except between the control group and the PCP 360 and 24h group, PCP 60 and 120, nor between PCP 360 and 24h. As illustrated in Figure 4B, significant (*p-value* < 0.001) increases of approximately 160% and 250% were found

when comparing the control group with the PCP 60 and 120 groups, respectively. Furthermore, significant decrease in cfos mRNA expression between the PCP 120 group and both the PCP 240 (*p-value* 0.01 to 0.05) and PCP 24h (*p-value* < 0.001) were found. In the PCP 120 group, a larger SEM was observed.

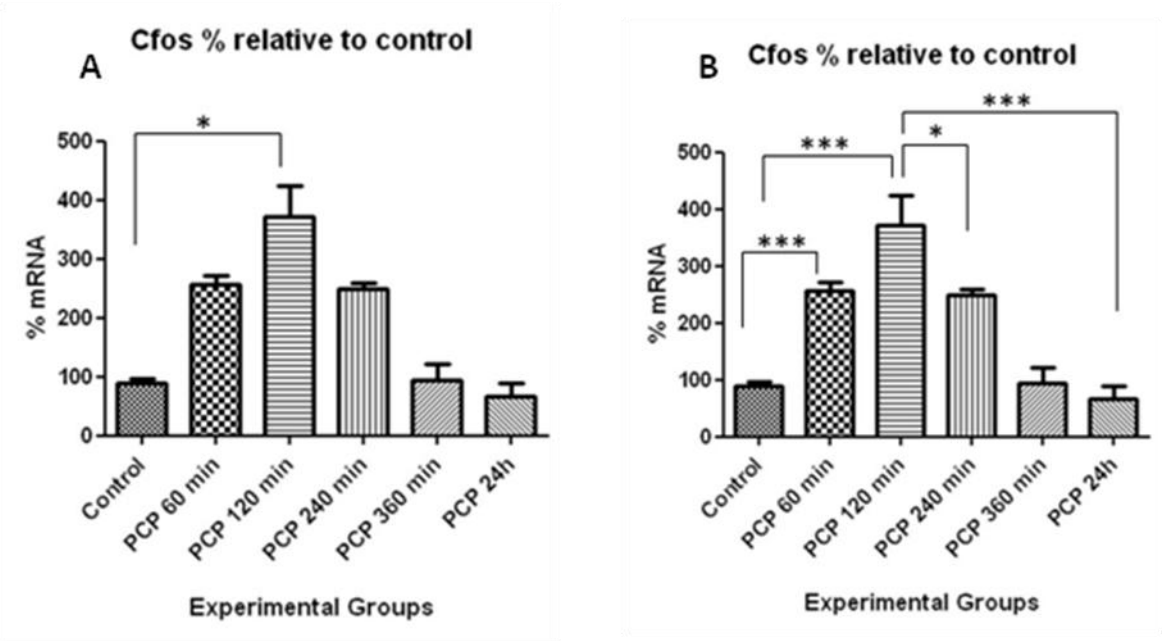

Figure 4: The expression of cfos mRNA in the rat PFC following acute phencyclidine (PCP) administration. The experimental groups each represent a group of male Sprague Dawley rats, decapitated at different time points following a single dose injection of saline water (0.9%) or PCP (10mg/kg). The mRNA levels are illustrated as relative quantity normalized to β-actin, and the gene expression of the control group is set to 100%. A: Results from the Kruskal-Wallis with Dunn's post test, B: Results from One-Way ANOVA with Tukey post test. All data are represented as mean ± SEM. Asterisk denotes significant differences in gene expression between different groups. ****p-value* 0.01 to 0.05, ******p-value* <0.001.

BDNF mRNA Expression is Highly Upregulated Following Acute PCP Administration in Rat PFC

A single rat in the PCP 240 group was excluded from statistical analysis as the data showed substantial differentiations from the rest of the group. Moreover, Kruskal-Wallis with Dunn's post test was used for analysis and a significant increase (*p-value* 0.01 to 0.05) was found in the PCP 360 group compared to control. See Figure 5A. One-Way ANOVA with Tukeys post test was also used for statistical analysis. Several statistical significant differences between the different groups following acute PCP were found, when examining the BDNF mRNA expression. Highly significant (*p-value* < 0.001) increases of approximately 400% and 840% were found between the control and PCP 240 group, and the control and PCP 360 group, respectively. See Figure 5B. Furthermore, a significant increase (*p-value* 0.01 to 0.05) was found between the control group and PCP 120, PCP 120 and 240, as well as between PCP 240 and 360. Also, significant values (*p-value* < 0.001) were obtained between the PCP 60 group and both PCP 240 and 360, PCP 120 and 360, and finally between the PCP 240 and 24h groups. Overall, the SEM is very small, however some tendencies for larger SEM can be found in the PCP 240 and 360 groups.

For the sake of clarity, not all significant differences have been included with an asterisk on the figure.

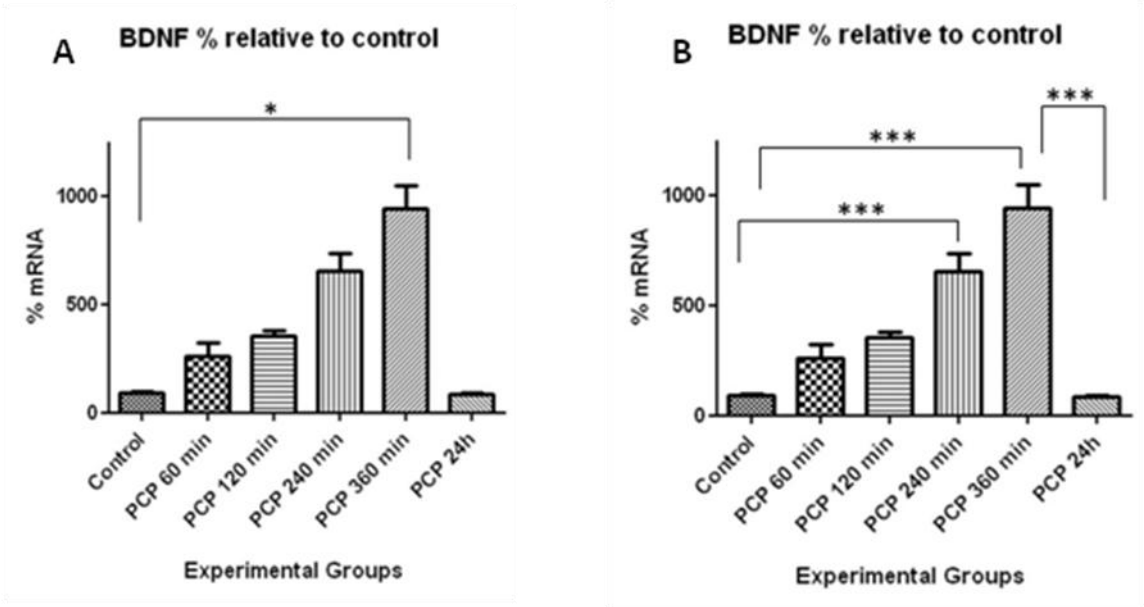

Figure 5: The expression of BDNF mRNA in the rat PFC following acute phencyclidine (PCP) administration. The experimental groups each represent a group of male Sprague Dawley rats, decapitated at different time points following a single dose injection of saline water (0.9%) or PCP (10mg/kg). The mRNA levels are illustrated as relative quantity normalized to β-actin, and the gene expression of the control group is set to 100%. A: Results from the Kruskal-Wallis with Dunn's post test, B: Results from One-Way ANOVA with Tukey post test. All data are represented as mean ± SEM. Asterisk denotes significant differences in gene expression between different groups and are illustrated in the graph. ****p-value* 0.01 to 0.05, ******p-value* <0.001.

7.4. Histone H4 Acetylation of Genes in the PFC of PCP-Treated Rats

Another objective of this study was to investigate the amount of H4 acetylation associated with the promoters of Arc, cfos and BDNF using anti-H4Ac in ChIP analysis. Furthermore, I wanted to investigate whether this epigenetic mechanism might be involved in the observed PCP-induced alterations in gene expression, as seen in Figure 3Figure 4Figure 5. DNA from ChIP assay was measured in the PCP and control groups using qRT-PCR. For each sample the amount of immunoprecipitated DNA was normalized to input, to correct for variation in starting chromatin quantity. However, the results for Arc DNA were excluded do to unsuccessful run of qRT-PCR. The amount of DNA associated with H4 acetylation of the promoters of BDNF and cfos is represented in Figure 6Figure 7, as relative quantity normalized to β-actin, and control group DNA quantities set to 100%.

Histone H4 Acetylation at the Promoter of Cfos following Acute PCP Administration

ChIP assay was unsuccessful for one animal in the PCP 240 group and was subsequently excluded from statistical analysis, reducing the group number from four to three. ChIP revealed a significant (*p-value* 0.001 to 0.01) increase in H4 acetylation of cfos promoter of approximately 110% in the PCP 360 group when compared to control. Furthermore, a significant (*p-value* 0.001 to 0.01) decrease in H4 acetylation was found between the PCP 360 and 24h group, however, an enlarged SEM was present in the PCP 360 group. See Figure 6. No significant changes were detected in any of the other groups, but some trends of PCP-induced alterations were observed. For instance, non-significant increases of approximately 37% and 50% in H4 acetylation were found in the PCP 120 and 240 groups, respectively, compared to control group.

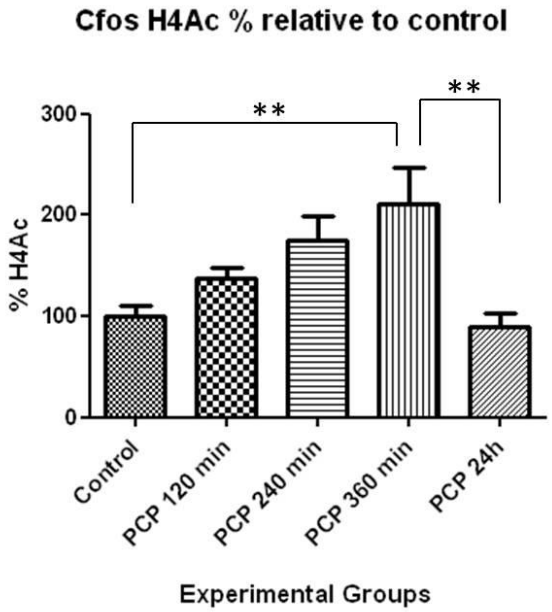

Figure 6: Amounts of DNA associated with H4 acetylation of cfos promoter in the rat PFC following acute phencyclidine (PCP) administration. The experimental groups each represent a group of male Sprague-Dawley rats, decapitated at different time points following a single dose injection of saline water (0.9%) or PCP (10mg/kg). The amounts of DNA associated with H4 acetylation for each gene is presented as relative quantity normalized to β-actin, and the DNA quantities of the control group is set to 100%. One-Way ANOVA with Tukey post test was used. All data are represented as mean ± SEM. Asterisk denotes significant differences in gene expression between different groups. **_p-value_ 0.001 to 0.01.

Changes in H4 Acetylation at the site of BDNF Promoter following PCP Treatment

Two animals from the PCP 120 and 240 groups were excluded from BDNF promoter I analysis due to differences in the Ct duplicates obtained from qRT-PCR. As a consequence, the number of animals was reduced to three in both groups. ChIP analysis of the BDNF promoter I, revealed several significant differences in H4 acetylation following acute PCP administration. Compared to the control group, the level of H4 acetylation increased by 90% in the PCP 360 group and in addition, significant (*p-value* 0.01 to 0.05) increases were also found in the PCP 360 group, when comparing to PCP 120 and 240 groups. Finally, by comparing the PCP 360 and 24h groups, a significant (*p-value* 0.01 to 0.05) decrease in H4 acetylation was revealed. It should be noticed that the SEM of the PCP 360 group is large.

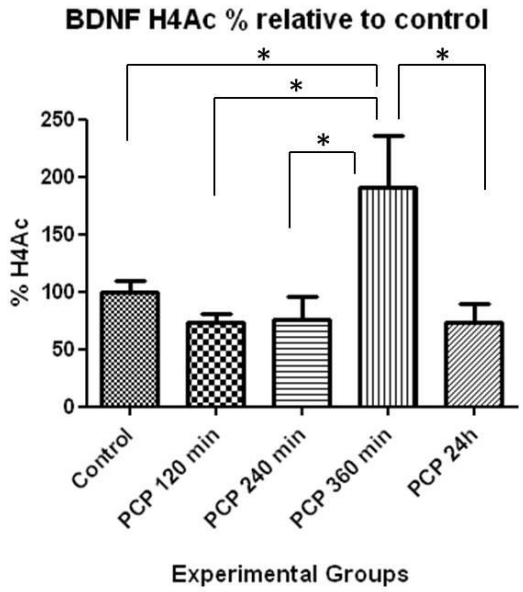

Figure 7: Amounts of DNA associated with H4 acetylation with the use of BDNF promoter I, in the rat PFC following acute phencyclidine (PCP) administration. The experimental groups each represent a group of male Sprague-Dawley rats, decapitated at different time points following a single dose injection of saline water (0.9%) or PCP (10mg/kg). By the use of ChIP assay and RT-PCR, the amounts of DNA associated with H4 acetylation is presented as relative quantity normalized to β-actin, and the DNA quantities of the control group is set to 100%. One-Way ANOVA with Tukey post test was used. All data are represented as mean ± SEM. Asterisk denotes significant differences in gene expression between different groups. ****p-value* 0.01to 0.05.

7.5. Histone H3 Phospho-acetylation of Genes in the PFC of PCP-Treated Rats

The last objective of this study was to examine the amount of H3 phospho-acetylation at the site of the promoters for Arc, cfos and BDNF with the use of anti-H3S10K14 in ChIP analysis. In addition, the involvement of this specific epigenetic modification in the observed PCP-induced alterations in mRNA expression was investigated, see Figure 3, 4 and 5. DNA from ChIP assay was measured in the control and PCP groups using qRT-PCR. For each sample, the amount of immunoprecipitated DNA was normalized to input, in order to correct for variation in starting chromatin quantity. The amount of DNA associated with H3 phospho-acetylation of Arc, BDNF I and cfos promoters is represented in Figure 8, Figure 9Figure 10 as relative quantity normalized to β-actin, and control group DNA quantities set to 100%.

Evaluating a Possible Association between H3 Phospho-acetylation and Arc Promoter Resulting from Acute PCP Treatment

To examine the degree of H3 phospho-acetylation and a possible correlation with Arc mRNA expression following PCP treatment, ChIP assay was performed. Due to unsuccessful qRT-PCR, two animals were excluded from the study, one from the control group and the other from the PCP 360 group. Therefore, the PCP 360 group only holds two animals. No significant differences were found in H3 phospho-acetylation between any of the experimental groups and a large SEM in both the PCP 120 and 24h groups was observed.

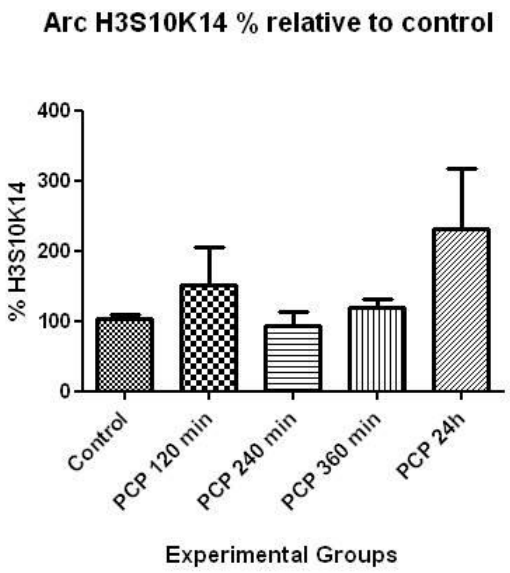

Figure 8: Amounts of DNA associated with H3 phospho-acetylation with the use of Arc promoter in the rat PFC following acute phencyclidine (PCP) administration. The experimental groups each represent a group of male Sprague Dawley rats, decapitated at different time points following a single dose injection of saline water (0.9%) or PCP (10mg/kg). By the use of ChIP assay and RT-PCR, the amounts of DNA associated with H3 phospho-acetylation is presented as relative quantity normalized to β-actin, and the DNA quantities of the control group is set to 100%. One-Way ANOVA with Tukey post test was used. All data are represented as mean ± SEM. No significant differences in DNA quantity were found between the different groups.

Acute PCP Administration revealed no Differences in H3 Phospho-acetylation Levels at the Site of Cfos Promoter

A total of five animals were excluded from the statistical analysis of ChIP, two of which were due to unsuccessful Ct values obtained from qRT-PCR. Each of these animals belonged to either the control or the PCP 120 groups. In addition, the remaining three excluded animals belonged to the PCP 360 group. As a consequence, the entire PCP 360 group was omitted as the H3 phospho-acetylation ratios differed more than 1.5 standard deviations from the mean. Statistical analysis of the degree of H3 phospho-acetylation from the remaining animals was performed. See Figure 9. No significant differences compared to the control group or in-between the groups were found. However, there was a trend toward an increase of H3 phospho-acetylation in the PCP 120 group compared to the control group.

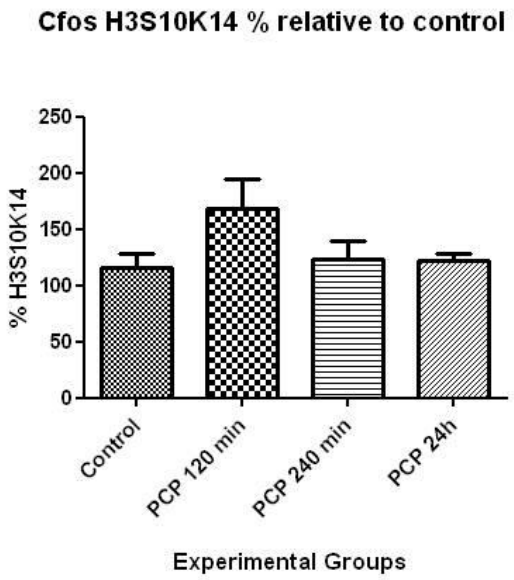

Figure 9: Amounts of DNA associated with H3 phospho-acetylation with the use of cfos promoter in the rat PFC following acute phencyclidine (PCP) administration. The experimental groups each represent a group of male Sprague Dawley rats, decapitated at different time points following a single dose injection of saline water (0.9%) or PCP (10mg/kg). By the use of ChIP assay and RT-PCR, the amounts of DNA associated with H3 phospho-acetylation is presented as relative quantity normalized to β-actin, and the DNA quantities of the control group is set to 100%. One-Way ANOVA with Tukey post test was used. All data are represented as mean ± SEM. No significant differences in DNA quantity were found between the different groups.

No Differences in H3 Phospho-acetylation Levels at the Site of BDNF Promoter 1 was Observed following Acute PCP Administration

Following acute PCP administration, the level of H3 phospho-acetylation associated with BDNF promoter I was evaluated. Again, some animals were not included in the statistical analysis. Ct values obtained from an animal in the PCP 240 group were insufficient for evaluation and the animal was subsequently excluded from the analysis. Additionally, the H3 phospho-acetylation ratio of an animal in the control group differed more than 1.5 standard deviations from the mean. No statistically significant differences were found in the PCP groups compared to control, nor between the PCP groups.

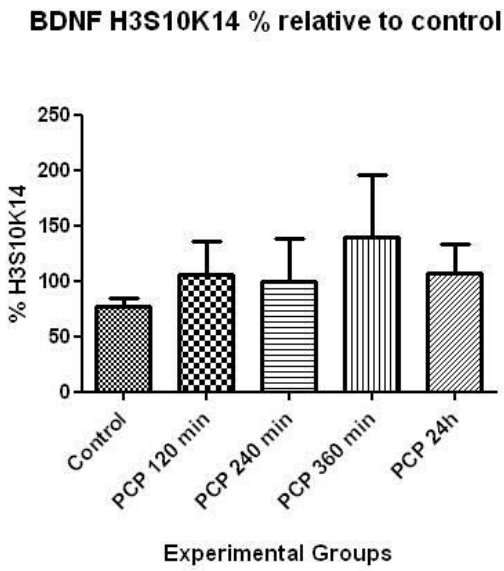

Figure 10: Amounts of DNA associated with H3 phospho-acetylation with the use of BDNF promoter I in the rat PFC following acute phencyclidine (PCP) administration. The experimental groups each represent a group of male Sprague-Dawley rats, decapitated at different time points following a single dose injection of saline water (0.9%) or PCP (10mg/kg). By the use of ChIP assay and RT-PCR, the amounts of DNA associated with H3 phospho-acetylation is presented as relative quantity normalized to β-actin, and the DNA quantities of the control group is set to 100%. One-Way ANOVA with Tukey post test was used. All data are represented as mean ± SEM. No significant differences in DNA quantity were found between the different groups.

8. Discussion

To date, studies (Kalinichev et al. 2008, Kargieman et al. 2007, Hashimoto et al. 2005) have investigated possible alterations in gene expression in the PFC of post mortem brain tissue from schizophrenic patients as well as in the PFC of acute PCP administered rats. Durany et al. 2001 found BDNF protein to be increased in the cortical areas, including the PFC of the brains of schizophrenic subjects. Additionally, Kalinichev et al. 2011 found a significant upregulation of Arc, cfos, and BDNF mRNA in the rat PFC following acute PCP administration. However, the number of studies investigating altered Arc, cfos, and BDNF in the PFC of both rats and schizophrenic subjects is low and more studies are needed to clarify the exact mechanisms of the development of schizophrenia.

In the current study, the mRNA expression of three genes has been investigated using qRT-PCR in a rat model for schizophrenia following acute PCP treatment. Significant differences between the groups were found for all three genes: Arc, cfos, and BDNF. Additionally, H4 acetylation and H3 phospho-acetylation of the corresponding promoters of Arc, cfos, and BDNF mRNA were investigated with the use of ChIP assay and qRT-PCR. The data was subsequently compared with the expressional findings in order to clarify if the altered mRNA expression following acute PCP treatment could be linked to altered histone modifications in the PFC. As the experimental groups each contained a low number of animals (n = 2-6 per group) none of the results obtained from the expression and ChIP analysis followed the Gaussian distribution. Consequently, a nonparametric test, Kruskal-Wallis test was used for statistical analysis. However, expression profiling was repeated more than once with the same outcome. Therefore, I expect the same findings in the different groups in a larger population of subjects and thus I also included the results following One-Way ANOVA, a parametric test, which has more power than a nonparametric.

8.1. β-actin – A Stable Housekeeping Gene in PCP-Treated Rats

A prober housekeeping gene, which is unaffected by any experimental setting, is essential when normalizing the data from both the expression and ChIP assays. In this study, acute PCP injections were administered to rats and therefore the housekeeping gene should be unaffected by this treatment. β-actin has been widely used as a reference gene, but there are contradictive findings regarding its suitability in pathological conditions (Radonic et al. 2004, Hansen et al. 2004). To my knowledge, no studies have investigated the stability of β-actin following PCP treatment. Therefore, I investigated the expression level of β-actin in three different groups: Control, PCP 120 and 24h. In addition, the levels of β-actin were also examined with various amounts of RNA template to see if any differences could be found. As can be seen in Figure 1, the Ct-values between the groups are consistent with low SEM. However, it should be noted that the groups are only represented with two animals in each, but as I have consistently compared the Ct-values of β-actin between various runs in qRT-PCR, I find beta-actin to be a stable housekeeping gene which can be used in PCP experiments.

8.2. Quantitative RT-PCR – Optimizing RNA Template Starting Amount

Normally, when performing gene expression analysis, RNA of approximately 1000ng is used for each first strand synthesis. The newly synthesized cDNA is then used as template for qRT-PCR. In the current study, different amounts of RNA template was investigated to ensure the presence of sufficient amounts of mastermix components in the qRT-PCR analysis, including Brilliant SYBR green, and forward and reverse primers. This was performed to prevent saturation during qRT-PCR ensuring true results of amplifications. Again, this was performed with the use of six animals, two in each of the three groups: Control, PCP 120 and 24h. Primers for Arc UTR were used, as the expression analysis consistently revealed the same tendencies in these three groups when using 1000ng. qRT-PCR was performed with varying amounts of RNA template (10ng, 100ng, and 1000ng) without altering the amount of the mastermix components. The results were subsequently calculated relative to β-actin and presented in Figure 2. Regardless of starting amount of RNA template, the results revealed the same tendency in Arc UTR expression. However, a difference was observed when comparing 10ng with 100 and 1000ng. This suggests that there are sufficient amounts of reagents and suitable amounts of cDNA with reactions consisting of 100 and 1000ng. Consequently, with 100ng being adequate for qRT-PCR, this was used for the following mRNA expression analysis.

8.3. Gene Expression Profiling and a Possible Correlation to Histone H4 Acetylation or Histone H3 Phospho-acetylation levels, in the PFC of acute PCP-Treated Rats

To examine the levels of mRNA expression in the PFC following acute PCP administration, qRT-PCR was performed using primers to detect the expression levels of three genes; Arc, cfos and BDNF. The alterations in gene expression were measured at different time points to assess the acute effect of PCP. As previously mentioned, Kruskal-Wallis should be used for statistical analysis as the data did not follow a Gaussian distribution. However, as the same outcome was observed when repeating the expression analysis, I expect that the same findings would be found in a larger population of rats. Therefore, I used both the Kruskal-Wallis test and One-Way ANOVA with the post-hoc tests Dunn's and Tukey, respectively.

Arc

Arc, an IEG, is considered a marker for neuronal excitability, and has been found to be involved in long term memory formation (Plath et al. 2006). Analyzing the Arc mRNA results with Kruskal-Wallis test, comparing columns with Dunn's post test, a significant decrease (p-*value* < 0.05) in Arc mRNA was found in the PCP 360 group compared to PCP 240 group. Although no other significant differences could be found using the nonparametric test, insignificant increases in Arc mRNA was present, when comparing the control group with the PCP 60, 120, and 240 groups. Thus, acute PCP treatment reveals an alteration in gene expression for up to 24 hours following treatment. At 24 hours, the mRNA levels had decreased to the level of the control group. With the use of One-Way ANOVA and Tukeys post test, three significant differences were observed; significant increase in Arc mRNA in the PCP 240 group compared to control and significant downregulations in both PCP 360 and 24h, compared to the PCP 240 group. These findings suggest an acute effect of PCP in Arc mRNA induction in the rat PFC; however, it would be interesting to see if this decrease in mRNA expression is maintained after 24 hours. Only few studies (Nakahara et al. 2000, Kalinichev et al.

2008, Thomsen, Hansen & Mikkelsen 2010) have investigated the expression of Arc mRNA in the brains of PCP-treated animals. The observed increase in Arc mRNA following PCP is consistent with findings of Kalinichev et al. (Kalinichev et al. 2008) and Nakahara et al. (Nakahara et al. 2000) who also observed a significant increase in Arc mRNA in PFC following acute PCP treatment. In addition, Thomsen et al. 2010 (Thomsen, Hansen & Mikkelsen 2010) also found Arc mRNA increase in the PFC, but instead of acute PCP treatment, the effect was observed following sub-chronic PCP treatment.

To my knowledge, no studies have examined the H3 phospho-acetylation of the Arc promoter. In order to investigate whether the altered Arc mRNA could be linked to epigenetic changes, H3 phospho-acetylation was investigated with ChIP assay. No significant changes were observed following acute PCP treatment between the groups, see Figure 8. As H3S10K14 correlates with transcriptional activation, a possible link between the expressional findings in Arc mRNA and H3 phospho-acetylation would be shown by a correlation between the two figures (Figure 3Figure 8). However, when comparing the findings of Arc mRNA in Figure 3 and the H3 phospho-acetylation in Figure 8, no correlation can be found. For instance, in Figure 3B, an increase in Arc mRNA was observed between the control group and the PCP 240 group but no changes were observed between the same groups in the H3 phospho-acetylation study. Thus, no assumptions of a possible correlation between the expression of Arc gene and H3 phospho-acetylation can be made.

As Arc has been found in pyramidal neurons of the PFC, Arc is suggested to be an indirect measure of pyramidal cell activity, which is coupled to cognitive function (Vazdarjanova et al. 2006). Thus, when administering a NMDA receptor antagonist, synaptic activation of GABAergic interneurons by NMDA receptors is decreased and it has subsequently been suggested that a decrease in controlling the GABAergic output to pyramidal neurons, results in impaired synchronization of pyramidal cell firing, leading to the cognitive dysfunctions observed in schizophrenic patients. (Lewis, Hashimoto & Volk 2005, Goldberg, Yuste & Tamas 2003, Olney, Farber 1995, Cardin et al. 2009, Sohal et al. 2009) The findings in the current study regarding Arc mRNA alterations in the PFC following PCP administration, support the theory of impaired cognitive function in which Arc is suggested to be involved in synaptic plasticity.

Cfos

In this study, the mRNA expression of the IEG, cfos, has been investigated. Cfos has been suggested to be involved in the cognitive impairments observed in schizophrenia (Groenewegen et al. 1990, Groenewegen et al. 1990, Jodo et al. 2010). Following qRT-PCR, the data was analyzed using Kruskal-Wallis test including the Dunn's post test. A significant increase (p-*value* < 0.05) of cfos mRNA in the PCP 120 group compared to control was found. However, there was large SEM in the PCP 120 group. KRuskal-Wallis did not reveal any significant differences between the different groups following acute PCP treatment. However, an insignificant increase was found between the control group and the PCP 60 group, and furthermore an insignificant tendency of decreased cfos mRNA was observed in the PCP 240, 360 and 24h groups, compared to the control group. The level of cfos mRNA was increased following PCP administration, as was observed with Arc mRNA. However, cfos mRNA expression peaked after 120 minutes, unlike Arc mRNA, which peaked after 240 minutes. With the use of One-Way ANOVA and Tukeys post test, several significant differences were found in cfos mRNA expression when comparing the groups. An upward increase in cfos mRNA was observed from the PCP 60 and 120 groups, after which the expression decreased in the following PCP group. Therefore, acute PCP treatment was found to induce cfos mRNA upregulation in the

rat PFC until the effect of PCP on cfos expression presumably declined four hours after the single injection. This correlates with cfos being an IEG, only being transiently expressed following external stimulation. In this case, acute PCP administration is regarded as the external stimulus. However, cfos has a half-life of approximately 15 minutes (Sheng, Greenberg 1990, Sheng, Greenberg 1990, Dragunow 1996, Todorova, Elbein & Kyosseva 2003), but the increase in cfos mRNA continues until two hours after the PCP injection. The reason for this might be the high dose of PCP being administered at once but this has not been investigated. However, when taking into account that cfos is transiently upregulated following a stimulus, the decrease in cfos mRNA in the PCP 240, 360 and 24h groups fits. Consistent with the findinds in the current study, Kargieman et al. (Kargieman et al. 2007) also found cfos mRNA to be increased following acute PCP administration (10mg/kg) in the PFC with the use of in situ hybridization. In addition, Kalinichev et al. (Kalinichev et al. 2008) also observed an increase in cfos mRNA in the PFC following acute PCP treatment. The increased cfos mRNA in the PFC following acute PCP treatment corresponds to the glutamatergic hyperfunctional findings in other studies (Ventimiglia et al. 1995, Arenas et al. 1996, Adams, Moghaddam 1998), which might be the result of disinhibition of excitatory neurons projecting to the PFC, leading to stimulation of cfos containing neurons, thus increasing the cfos mRNA expression. This effect might correlate with the finding that BDNF overexpression in the PFC altered the cognitive functions in mice (Cunha et al. 2009, Elvevåg, Goldberg 2000)

The level of H4 acetylation and H3 phospho-acetylation in the cfos promoter was also investigated in this study. No significant differences between the groups were observed when analyzing the data using the Kruskal-Wallis test. However, One-Way ANOVA and Tukeys post test, revealed two significant differences in the H4 acetylation study; a significant increase (*p-value* 0.001 to 0.01) in H4 acetylation in the PCP 360 group compared to control, and a significant decrease (*p-value* 0.001 to 0.01) in the PCP 24h compared to the PCP 360 group. No significant differences were found in H3 phospho-acetylation of the cfos promoter but a small tendency of increased H3 phospho-acetylation in the PCP 120 group compared to control could be suggested. However, the SEM of the PCP 120 group was large and five animals had been excluded from statistical analysis. Therefore, no assumptions or conclusions can be made based on these findings. H4 acetylation has normally been correlated with transcriptional activation, thus I have compared the results obtained from the cfos mRNA study and the H4 acetylation study. A continued increase of H4 acetylation of the cfos promoter until reaching the PCP 360 group, does not correlate with the findings in cfos mRNA, where an increase in cfos mRNA peaks in the PCP 120 group and decreases in the PCP 240, 360 and 24h groups. This might indicate that other factors influence the transcription of cfos mRNA than H4 acetylation. Also, it is important to mention that d, neither of the ChIP assays investigating the cfos promoter H4 acetylation and H3 phosho-acetylation was repeated unlike the mRNA study. Therefore, no conclusions can be made upon these findings following ChIP and no valid correlation between the mRNA findings and H4 acetylation and H3 phospho-acetylation can be made.

BDNF

BDNF, a neurotrophic factor, is highly expressed in both the developmental (Bibel, Barde 2000) and the adult nervous system, especially in the PFC and hippocampus of the adult brain (Baquet, Gorski & Jones 2004, Conner et al. 1997, Ernfors et al. 1990, Timmusk, Persson & Metsis 1994). BDNF is involved in numerous activities in the neurons by participating in e.g. the differentiation and survival of neurons (Bibel, Barde 2000) as well as regulation of synaptic transmission and plasticity. (Pezet, McMahon 2006) The results obtained from the BDNF mRNA expression analysis following acute PCP treatment, were

investigated using Kruskal-Wallis test and Dunn's post test. A significant increase (*p-value* 0.01 to 0.05) in BDNF mRNA was found in the PCP 360 group when compared to control. See Figure 5A. No other statistical significant differences were found between the experimental groups, however, several trends toward BDNF mRNA alteration between the groups were observed. An upward increase in BDNF mRNA was found in the PCP 60, 120, 240, and 360 groups, after which the expression decreased in the PCP 24h group until reaching the mRNA level of the control group. Using the parametric One-Way ANOVA and Tukeys post test for BDNF mRNA analysis, numerous significant alterations in mRNA expression between the groups were found. See figure 5B. A significant increase (*p-value* 0.01 to 0.05) of approximately 400% in the PCP 240 group compared to control was found, and also a significant increase exceeding 800% in the PCP 360 group compared to control was found. Six hours following acute PCP administration the level of BDNF mRNA decreased significantly (*p-value* 0.01 to 0.05), thus the PCP induced effect on BDNF mRNA subside at this point. In accordance to the current study, increase in mRNA BDNF following acute PCP administration in corticolimbic regions in the rat has been found in a study by Grottick et al. (Grottick et al. 2005). These findings are consistent with that observed in the cortical areas of schizophrenic patients (Durany et al. 2001). However, Weickert et al. (Weickert et al. 2003) found decreased BDNF expression in the PFC of schizophrenic patients. Kaiser et al. (Kaiser et al. 2004) observed the effect of acute PCP administration in BDNF mRNA in the rat anterior portion of frontal cortex. A peak in BDNF mRNA was found 2-4 hours after PCP treatment however, a persistent expression change in BDNF lasted for at least eight hours. This finding is inconsistent with the current study where BDNF mRNA peaked in the rat PFC six hours following acute PCP administration. The increased BDNF mRNA then returned to the level of the control group 24 hours after PCP treatment. As the expression level of BDNF mRNA was not measured in the present study, a possible persistency in increased expression levels following eight hours after PCP cannot be rejected.

In addition to the examination of expression levels of the BDNF mRNA, the level of H4 acetylation and H3 phospho-acetylation in the BDNF promoter I was investigated. With the use of Kruskal-Wallis test and Dunn's post test, no significant differences were observed in the H4 acetylation level or the H3 phospho-acetylation level in the BDNF promoter I. However, using One-Way ANOVA and Tukeys post test, revealed significant decreases (*p-value* 0.01 to 0.05) in H4 acetylation in the control, PCP 120, 240, and 24h groups, compared to the PCP 360 group. H3 phospho-acetylation revealed no significant differences between any of the groups. Due to large SEM in the PCP 360 group in the H4 acetylation analysis and exclusion of two animals in the statistical analysis, caution should be made before making any assumptions between the levels of histone modifications and the gene expression. This was also observed for the H3 phospho-acetylation analysis. When comparing the level of BDNF mRNA expression with the results of H4 acetylation and H3 phospho-acetylation of the correlating promoter I, a possible connection between H4 acetylation and BDNF mRNA expression exist. As can be seen in Figure 5A and 7, a correlation between an increase in BDNF mRNA six hours following acute PCP administration, and H4 acetylation was found. Also, a link between the decreased BDNF mRNA in the PCP 24h groups can be found for H4 acetylation. Thus, H4 acetylation might be implicated in the expression of BDNF in the rat PFC following acute PCP administration. Due to large SEM in the H3 phospho-acetylated findings in BDNF promoter I, no correlation to the expressional findings of BDNF mRNA can be made. See Figure 10. A possible connection between the altered expression levels of BDNF in the PFC following acute PCP treatment, might be involved in disturbed neural plasticity leading to cognitive impairments as observed in schizophrenic patients (Cunha et al. 2009, Elvevåg, Goldberg 2000) In order to evaluate the regulational manner of BDNF mRNA, further investigations

of the H4 acetylation and H3 phospho-acetylation in BDNF promoter I have to be conducted as this study only was performed once due to a lack of time.

The examination of H4 acetylation and H3 phospho-acetylation in the promoters of the three genes, Arc, cfos, and BDNF, were only conducted once. Therefore, the results have not been repeated and no conclusion can be made based on the findings in the different promoters. Also, several animals from each study was excluded because of failure in the qRT-PCR or due to large standard deviations in the groups.

8.4. The interaction of Arc, cfos, and BDNF mRNA expression in the PFC of Acute PCP Treated Rats

All three genes investigated in this study are somewhat interconnected. As described in *Activity-regulated Cytoskeleton-associated Protein,* page 12, Arc is an IEG, which is widely expressed in pyramidal neurons of the PFC (Vazdarjanova et al. 2006) and is a marker for neuronal excitability. As with BDNF, Arc has also been implicated in the formation of synaptic plasticity in the PFC (Bramham et al. 2010). BDNF regulates the development of GABAergic interneurons via pyramidal neuronal excitability in which Arc is represented (Hashimoto et al. 2005). Thus, BDNF is able to elicit the activation of Arc-induced synaptic plasticity. This correlates with the findings in both Arc and BDNF mRNA expression following acute PCP, where both Arc and BDNF is increased in the PFC. See Figure 3 and 5. Administration of acute PCP could have lead to the following actions in the PFC; An increase in BDNF mRNA in turn upregulates Arc mRNA via excitation of pyramidal neurons. Again, the pyramidal neurons activate GABAergic interneurons of the PFC which leads to an increased inhibitory effect on excitatory neurons. In schizophrenic patients, dysfunctional activities in the PFC might lead to cognitive impairments, such as working memory. (Hashimoto et al. 2005)

A connection between BDNF and cfos has also been implicated in LTP formation as a study using a non-competitive NMDA receptor antagonist (Hughes, Dragunow 1993) found a delayed induction of BDNF mRNA compared to cfos. Even though the study did not include the role of PCP in BDNF and cfos induction, the delayed induction of BDNF mRNA following cfos might be the case in the present study. A peak in cfos mRNA levels two hours following acute PCP administration was found. See Figure 4. In contrast, a delayed BDNF mRNA induction was observed six hours following PCP treatment. See Figure 5. Thus, possible alterations in both cfos and BDNF mRNA expression might lead to impairments in synaptic plasticity in the brains of schizophrenics.

8.5. Conclusion

In conclusion to the findings of this study, acute PCP administration induced significant alterations in Arc, cfos, and BDNF mRNA at different time points, in the PFC of rats. However, the increases in mRNA peaked at different time points, for each gene. Thus, the administration of acute PCP might alter mRNA levels of the three genes differently or at different time points. In addition to the findings in mRNA alterations, the levels of DNA associated with H4 acetylation and H3 phospho-acetylation were determined. DNA associated with H4 acetylation was significantly increased at the cfos promoter, however, a correlation between the PCP-induced cfos mRNA and the H4 acetylation was not found. In contrast to these findings, a significant increase in BDNF mRNA was found following acute PCP administration. This finding correlated

with the level of H4 acetylation of the corresponding BDNF promoter I, which also peaked in the PCP 360 min group. Thus, acute PCP administration might induce H4 acetylation in the BDNF promoter I, leading to an increase in BDNF mRNA expression, suggesting a transcriptional regulation of BDNF.

9. References

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10. Appendices

Appendix I	Groups of Animals
Appendix II	Primer Sequences
Appendix III	Calculations

Appendix I

Groups of Animals

Rat number	Drug	Dose (mg/kg)	Time of decapitation following drug administration (min)	Included in following experiment(s)
1	saline	0.9 %	60	Gene expression
2	saline	0.9 %	60	Gene expression
3	saline	0.9 %	60	Gene expression
4	saline	0.9 %	120	Gene expression
5	saline	0.9 %	120	Gene expression
6	saline	0.9 %	120	Gene expression
7	saline	0.9 %	120	ChIP
8	saline	0.9 %	120	ChIP
9	saline	0.9 %	120	ChIP
10	saline	0.9 %	120	ChIP
11	saline	0.9 %	120	ChIP
12	saline	0.9 %	120	ChIP
13	Phencyclidine	10	60	Gene expression, ChIP
14	Phencyclidine	10	60	Gene expression, ChIP
15	Phencyclidine	10	60	Gene expression, ChIP
16	Phencyclidine	10	60	Gene expression, ChIP
17	Phencyclidine	10	120	Gene expression, ChIP
18	Phencyclidine	10	120	Gene expression, ChIP
19	Phencyclidine	10	120	Gene expression, ChIP
20	Phencyclidine	10	120	Gene expression, ChIP
21	Phencyclidine	10	240	Gene expression, ChIP
22	Phencyclidine	10	240	Gene expression, ChIP
23	Phencyclidine	10	240	Gene expression, ChIP
24	Phencyclidine	10	240	Gene expression, ChIP
25	Phencyclidine	10	360	Gene expression, ChIP
26	Phencyclidine	10	360	Gene expression, ChIP
27	Phencyclidine	10	360	Gene expression, ChIP
28	Phencyclidine	10	1440	Gene expression, ChIP
29	Phencyclidine	10	1440	Gene expression, ChIP
30	Phencyclidine	10	1440	ChIP

Appendix II

Primer Sequences

mRNA primers for gene expression analysis		
Gene	Forward/Reverse	Primer sequence
Arc 5UTR	Forward	GGCTCTGAATCCTTCTGGCTT
Arc 5UTR	Reverse	TCATATGGTCCAGCTCCATCTG
Cfos	Forward	GGTCACAGAGCTGGAGCCCCTGTGC
Cfos	Reverse	TCGTTGCTGCTGCTGCCCTTTCGGT
BDNF exon I	Forward	ACCTTCCAGCATCTGTTGGGGAGACG
BDNF exon I	Reverse	GCGCAGCCTTCATGCAACCGAAGTA
Actin 3UTR	Forward	AAGGGACACCGTAGAGGGGTGGAGC
Actin 3UTR	Reverse	CAGGAGCGTGCCCACGAGTGTCTAC

Genomic primers for chromatin immunoprecipitation assay		
Gene	Forward/Reverse	Primer sequence
Arc P	Forward	ACCCAGCATGGGGACTCAAGGGCCT
Arc P	Reverse	GCTGCTCACCTGGGGGTGACTGCTC
Cfos P	Forward	TTCTCTGTTCCGCTCATGACGT
Cfos P	Reverse	CTTCTCAGTTGCTAGCTGCAATCG
BDNF P I	Forward	AGGGAAAGTTGTGGGGCTGATGCGCT
BDNF P I	Reverse	GGGTCTCCAGCGGACGTGGTCGTGA

Appendix III

CALCULATIONS

Gene expression results (Arc, BDNF, Cfos)

All 23 rats included

Normalized to beta-actin

The control group was pooled and set to 100 %.

Primer efficiencies

Actin UTR	98%	0,98
Arc	1%	1
Cfos	83,80%	0,838
BDNF	124,40%	1,244

Rat no #	Drug	Actin	Arc	Actin	BDNF	Actin	Cfos
1	control	26,93	23,74	26,46	30,81	25,78	26,95
2	control	27,3	23,63	26,86	30,63	25,95	26,77
3	control	26,9	22,43	26,48	29,85	25,74	25,86
4	control	26,7	22,51	26	29,85	25,19	25,87
5	control	27,3	23,73	26,73	30,9	26,14	27,87
6	control	26,4	22,54	26,02	29,81	25,23	26,48
7	PCP 60	27,03	23,04	26,73	29,77	25,95	25,49
8	PCP 60	27,43	23,29	27,18	30,17	26,25	25,72
9	PCP 60	27,74	23,61	27,15	29,81	26,43	25,96
10	PCP 60	26,73	22,32	26,36	28,38	25,45	24,48
12	PCP 120	26,94	22,73	26,5	28,77	25,63	24,32
13	PCP 120	26,8	22,71	26,51	28,92	25,61	24,81
14	PCP 120	28,3	23,52	27,87	29,77	26,83	25,35
15	PCP 240	27,7	23,21	26,91	27,58	26,06	25,39
16	PCP 240	27,97	23,07	27,49	28,65	26,69	26,08
17	PCP 240	29,69	24,63	28,91	30,38	28,12	27,91
18	PCP 360	27,39	22,98	26,83	28,2	26,01	25,59
20	PCP 360	27,68	24,11	27,14	28,24	26,43	27,1
21	PCP 360	27,39	24,85	26,96	27,81	26,2	27,84
22	PCP 24h	27,33	23,96	26,82	30,9	26,09	28,29
23	PCP 24h	27,21	23,49	26,91	30,75	26,1	27,23

Normalized to actin

Rat no #	Arc	BDNF	Cfos
1	6,962119553	0,001083295	3,341313227
2	9,67432998	0,001646634	4,187284443
3	16,91183821	0,002385954	6,312293517
4	13,95646329	0,001718948	4,309013704
5	9,026469043	0,001211307	2,440724676
6	11,13640254	0,001799853	3,054876964
7	12,10957755	0,003019327	9,126299407
8	13,38250979	0,002971669	9,738561317
9	13,24872788	0,003894652	9,515938259
10	16,25066107	0,007212168	11,99364195
12	14,11719534	0,005790287	14,95016876
13	13,00875988	0,005164322	10,94418669
14	20,67280397	0,006578222	18,12877467
15	17,01059759	0,020047028	10,45592439
16	22,54049098	0,012546464	10,56484438
17	24,75259327	0,008175539	9,211747993
18	16,14322355	0,011499597	8,946642286
20	8,992061551	0,013759666	4,754422117
21	4,416356962	0,017224555	2,589697685
22	7,855628801	0,001288114	1,826671359
23	10,02455791	0,001546349	3,50617274
	11,2779371	0,001640999	3,940917755
	8,866869809	60938,50275	25,37480004

Means that it differs more/less than 1.5 STD. From the mean and is therefore excluded from analysis.

St. Dev

Arc	Control	PCP 60	PCP 120	PCP 240	PCP 360	PCP 24h
Sum	67,66762	54,99148	47,79876	80,44691	13,40842	17,88019
Gn. Snit	11,27794	13,74787	15,93292	20,11173	6,704209	8,94
St.Dev	3,611158	1,763576	4,142105	4,195337	3,235512	1,53
Plus 1.5 st. dev	16,69467	16,39323	22,14608	26,40473	11,55748	11,24059
Minus 1.5 st. dev	5,8612	11,10251	9,719762	13,81872	1,850942	6,639597

BDNF	Control	PCP 60	PCP 120	PCP 240	PCP 360	PCP 24h
Sum	0,009846	0,017098	0,017533	0,052269	0,030984	0,002834
Gn. Snit	0,001641	0,004274	0,005844	0,013067	0,015492	0,00
St.Dev	0,000465	0,002004	0,000708	0,005012	0,00245	0,000183
Plus 1.5 st. dev	0,002339	0,00728	0,006907	0,020586	0,019167	0,001691
Minus 1.5 st. dev	0,000943	0,001269	0,004782	0,005548	0,011817	0,001143

Cfos	Control	PCP 60	PCP 120	PCP 240	PCP 360	PCP 24h
Sum	23,64551	40,37444	44,02313	39,17916	7,34412	5,332844
Gn. Snit	3,940918	10,09361	14,67438	9,79479	3,67206	2,67
St.Dev	1,357556	1,291714	3,600225	0,834541	1,530691	1,19
Plus 1.5 st. dev	5,977251	12,03118	20,07471	11,0466	5,968097	4,447802
Minus 1.5 st. dev	1,904584	8,156039	9,274039	8,542979	1,376023	0,885042

Procent to control

Rat no #	Arc	BDNF	Cfos
1	61,73	66,01	84,79
2	85,78	100,34	106,25
3	149,96	145,40	160,17
4	123,75	104,75	109,34
5	80,04	73,82	61,93
6	98,75	109,68	77,52
7	107,37	183,99	231,58
8	118,66	181,09	247,11
9	117,47	237,33	241,47
10	144,09	439,50	304,34
12	125,18	352,85	379,36
13	115,35	314,71	277,71
14	183,30	400,87	460,01
15	150,83	1221,64	265,32
16	199,86	764,56	268,08
17	219,48	498,21	233,75
18	143,14	700,77	227,02
20	79,73	838,49	120,64
21	39,16	1049,64	65,71
22	69,65	78,50	46,35
23	88,89	94,23	88,97

Means that it differs more/less than 1.5 STD. From the mean and is therefore excluded from analysis.

Chip results for Arc, cfos and BDNF

Normalized to actin

		Cfos		Arc	BDNF	
	Rat no #	H4Ac	H3S10K14	H3S10K14	H4Ac	H3S10K14
PCP 120 min	11	0,480365	1,161375	3,090012	0,096509339	1,991022
	12	0,382629	0,749123	1,473042	0,109393364	1,342817
	13	0,55522	0,885699	0,623774	0,090898828	0,711675
	14	0,474629	0,668686	0,893127	0,076865542	0,618651
PCP 240 min	15	0,453069	0,658319	0,643916	0,138779446	0,468111
	16	0,23865	0,895943	1,336363	0,049354358	1,90494
	17	0,61818	0,613696	1,237888	0,104328573	0,921697
	18	0,742999	0,490593	0,547292	0,072675741	0,470243
PCP 360 min	19	0,873107	0,923131	1,961666	0,361572139	2,769831
	20	0,480742	0,914933	1,32881	0,183858249	1,001512
	21	0,835118	0,764682	1,066192	0,193950584	0,835056
PCP 24h	22	0,209217	0,611955	1,319764	0,086845461	0,621332
	23	0,354387	0,624045	1,62748	0,062942139	1,316669
	24	0,360954	0,724196	4,079869	0,13509565	1,581136
Kontrol 120 min	30	0,3057	0,541594	0,831411	0,113464352	0,686339
	31	0,298298	0,582615	0,947322	0,14512004	0,739263
	32	0,271679	0,114499	1,216838	0,072706009	2,337447
	33	0,340398	0,711525	1,205105	0,144127694	1,106583
	34	0,347634	0,449168	0,948746	0,134043128	0,768554
	35	0,507362	0,822574	0,915602	0,164112032	0,948732
		0,345178	**0,536996**	**1,010837**	**0,128928876**	**1,09782**
		289,7052	186,2212	98,92787	775,6214368	91,08963

Procent to control

		Cfos		Arc	BDNF	
	Rat no #	H4Ac	H3S10K14	H3S10K14	H4Ac	H3S10K14
PCP 120 min	11	139,1642	216,2726	305,6883	74,85471198	181,3614
	12	110,8497	139,5027	145,7249	84,84783795	122,3167
	13	160,8502	164,936	61,70859	70,5030795	64,82622
	14	137,5026	124,5236	88,35513	59,61856219	56,35266
PCP 240 min	15	131,2565	122,5931	63,70125	107,640313	42,6401
	16	69,13821	166,8437	132,2036	38,28029825	173,5202
	17	179,09	114,2833	122,4616	80,91947741	83,95707
	18	215,2506	91,35876	54,14239	56,36886229	42,83429
PCP 360 min	19	252,9436	171,9066	194,0634	280,4431017	252,3029
	20	139,2733	170,3799	131,4563	142,6043994	91,22738
	21	241,938	142,4	105,4761	150,4322306	76,06493
PCP 24h	22	60,61121	113,959	130,5615	67,35920124	56,5969
	23	102,6678	116,2104	161,0032	48,81927197	119,9349
	24	104,5704	134,8606	403,6127	104,7830823	144,0251
Kontrol 120 min	30	88,56297	100,8563	82,24974	88,00538389	62,51841
	31	86,41835	108,4953	93,71659	112,5582142	67,33922
	32	78,70672	21,32216	120,3792	56,39233896	212,9172
	33	98,61499	132,5011	119,2185	111,7885293	100,7982
	34	100,7115	83,64456	93,85744	103,9667233	70,00727
	35	146,9855	153,1807	90,57853	127,2888104	86,41969

Primer efficiencies	
Arc	0,942
Cfos	0,933
BDNF 1	0,955
Pvalb	0,955
Actin	0,98

St. Dev

Cfos H4	**Control**	**PCP 120**	**PCP 240**	**PCP 360**	**PCP 24h**
Sum	2,071071	1,892844	2,052898	2,188966	0,924558
Gn. Snit	0,345178	0,473211	0,513225	0,729655	0,308186
St.Dev	0,084251	0,070672	0,218191	0,216401	0,085773
Plus 1.5 st. dev	0,471555	0,57922	0,840511	1,054257	0,436845
Minus 1.5 st. dev	0,218802	0,367202	0,185938	0,405054	0,179527

BDNF H4	**Control**	**PCP 120**	**PCP 240**	**PCP 360**	**PCP 24h**
Sum	0,773573	0,373667	0,365138	0,739381	0,284883
Gn. Snit	0,128929	0,093417	0,091285	0,24646	0,094961
St.Dev	0,032116	0,01348	0,03886	0,099817	0,036755
Plus 1.5 st. dev	0,177104	0,113636	0,149575	0,396186	0,150094
Minus 1.5 st. dev	0,080754	0,073197	0,032994	0,096734	0,039829

Cfos H3	**Control**	**PCP 120**	**PCP 240**	**PCP 360**	**PCP 24h**
Sum	3,221974	3,464884	2,658552	4,356668	1,960195
Gn. Snit	0,536996	0,866221	0,664638	1,452223	0,653398
St.Dev	0,245086	0,2162	0,169734	0,089208	0,085773
Plus 1.5 st. dev	0,904624	1,19052	0,919239	1,586035	0,782058
Minus 1.5 st. dev	0,169367	0,541922	0,410037	1,31841	0,524739

BDNF H3	**Control**	**PCP 120**	**PCP 240**	**PCP 360**	**PCP 24h**
Sum	6,586919	4,664165	3,764992	4,6064	3,519137
Gn. Snit	1,09782	1,166041	0,941248	1,535467	1,173046
St.Dev	0,626941	0,637162	0,676951	1,072226	0,495759
Plus 1.5 st. dev	2,038231	2,121785	1,956674	3,143806	1,916683
Minus 1.5 st. dev	0,157408	0,210298	-0,07418	-0,07287	0,429408

Arc H3	Control	PCP 120	PCP 240	PCP 360	PCP 24h
Sum	6,065025	6,079955	3,765458	4,356668	7,027113
Gn. Snit	1,010837	1,519989	0,941365	1,452223	2,342371
St.Dev	0,160829	1,105039	0,403203	0,460316	1,512563
Plus 1.5 st. dev	1,252081	3,177548	1,54617	2,142697	4,611215
Minus 1.5 st. dev	0,769594	-0,13757	0,33656	0,761748	0,073527