Diploma work

Administration of morphine and/or cocaine modulates the expression levels of genes related to plasticity mechanisms in rat hippocampus

Thérés Larsson
Performed at the Department of Neuroroscience, Uppsala University
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Supervisor
Madeleine Le Grevés

Examiner
Lars-Göran Mårtensson

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Abstract

Drugs of abuse regulate the expression of several genes involved in receptor regulation and signalling, transcription factors and cytoskeleton proteins. Due to its implication in long-term memory consolidation, rat hippocampus was selected to identify genes that are differently regulated by morphine and/or cocaine administration. The animals were randomly separated into four groups given saline or morphine for five days followed by an acute dose of saline or cocaine the second day after last saline/morphine injection. Quantitative real-time polymerase chain reaction was used to analyze differentiation in the mRNA level. Significant alteration in mRNA expression was observed in six genes; growth associated protein 43 (Gap43), brain-derived neurotrophic factor (Bdnf), neurotrophic tyrosine kinase receptor (trkB), fms-like tyrosine kinase 1 (Flt1), microtubule-associated protein 2 (Map2) and Ca²⁺/calmodulin-dependent protein kinas II (CaMK-II). These data demonstrate that administration of morphine and cocaine severally or in combination alter the expression of genes related with hippocampal structural plasticity.
Missbruk av droger orsakar förändringar i uttrycket av gener involverade i receptor reglering och signalering, transkriptions faktorer och cytoskeleton relaterade proteiner. För att ta reda på hur morfin och kokain påverkar gener relaterade till minnet, studerades (hos råttor) den del av hjärnan som kallas hippocampus. Försöksdjuren delades slumpmässigt in i fyra olika grupper. Under de fem första dagarna fick råttorna salin eller morfin, följt av en akut dos av salin eller kokain två dagar efter den senaste salin/morfin injektionen. Kvantitativ realltids polymerase chain reaction användes för att studera förändringar i mRNA expressionen. Signifikanta förändringar påvisades i sex gener; growth associated protein 43 (Gap43), brain-derived neurotrophic factor (Bdnf), neurotrophic tyrosine kinase receptor (trkB), fms-like tyrosine kinase 1 (Flt1), microtubule-associated protein 2 (Map2) and Ca\(^{2+}\)/calmodulin-dependent protein kinas II (CaMK-II). Resultaten från denna studie visar att intag av morfin och kokain, var för sig eller i kombination, leder till förändringar av gener involverade i strukturell plasticitet.
Abbreviations

Bdnf  | brain-derived neurotrophic factor
CaMK-II | Ca$^{2+}$/calmodulin-dependent protein kinase II
Flt1  | fms-like tyrosine kinase 1
Gap43 | growth associated protein 43
HKG   | house keeping genes
LTP   | long-term potentiation
Map2  | microtubule-associated protein 2
MTs   | microtubules
m/s   | morphine-cocaine treated rats
m/c   | morphine-saline treated rats
NAc   | nucleus accumbens
PCR   | polymerase chain reaction
qRT-PCR | quantitative real-time RT-PCR
s/c   | saline-cocaine treated rats
s/s   | saline-saline treated rats, control group
VEGF  | vascular endothelial growth factor
VTA   | ventral tegmental area
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1 Introduction

Abuse and addiction to drugs has become a major problem around the world. Statistical data shows that over 35 million (14.5%) of the human population of United States, age 12 or older has abused some form of drug in 2006 [1]. Cocaine and morphine are both highly abused substrates, which upon administration produce long-lasting changes in the nervous system [2].

Cocaine is an effective psychostimulant drug that produces a euphoria feeling upon consumption. Administration of cocaine results in increased level of extracellular monoamine neurotransmitters thereby increased synaptic activity [3]. Repeated abuse of cocaine is often associated with elevated locomotor activity in experimental animals a phenomenon referred to as behavioural sensitization [4]. Morphine and other opiates, exerts its effect mainly through G-coupled µ-opioid receptors [3]. Morphine is clinical used for its analgesic effects and act directly on the central nervous system (CNS) to relieve pain. Repeated administration of both cocaine and morphine can result in tolerance, withdrawal, sensitization and eventually addiction [5].

Because of its rewarding properties, the brain reward system has been of primary focus for investigating neuroadaptations associated with drugs of abuse [5, 6]. Several studies have shown that drugs of abuse produces changes in this brain area in a similar way as the neuroadaptations in hippocampus associated with learning and memory. Dendritic spines are the major site of excitatory inputs in the brain and enlargement of these spines has been shown to be involved in consolidation of long-term potentiation (LTP) and memory [7]. Increased spine density has been observed after cocaine administration [8], while morphine has shown to have an opposite effect, decreasing the density of the dendritic spine [9]. Further chronic treatment with morphine has shown to reduce hippocampal LTP [10] as well as alternation of neural and glial cytoskeleton components in rat striatum [11] and hippocampus [12].
1.1 Plasticity

Structural plasticity is the result of persistent changes, such as reorganization or strengthening of synaptic connections, in specific neural circuits due to experiences. A lot of research has focused on the molecular mechanism involved in the structural alternation of neuronal circuits associated with learning and memory (reviewed in [13]). Changes in morphology of dendrites and dendritic spines due to memory consolidation have also been evaluated by several researchers [7, 14]. Synaptic plasticity is however also involved in addiction and studies implicate that drugs of abuse are involved in many of the same cellular and molecular mechanisms in other forms of synaptic plasticity and may, in some cases, usurp the role of these mechanisms [2, 5].

1.2 Hippocampus

The hippocampus (figure 1) is part of the temporal lobe of the brain and has long been known to be involved in learning and memory. It has been shown that the hippocampus is especially important for converting short-term memory into long-term or permanently encoded memories. The hippocampus does not generally store memory, but is involved in the maintaining of the memories until they are transferred to more permanent storage in different areas of cerebral cortex [15].

Information is projected to hippocampus through neurons from the brain stem with common neurotransmitters such as norepinephrine (NE), acetylcholine (ACh) and serotonin (5HT, 5-hydroxytryptamine). The main excitatory projection from the hippocampus is, however, the glutamateric neurons, which connect the hippocampus with structures such as the cortex and the amygdala [15]. Glutamateric neurons are also projected to the nucleus accumbens (NAc) an important structure for the rewarding and reinforcing properties associated with drug consumption [6].

![Figure 1. Location of hippocampus (shown in blue) in the human brain [16].](image-url)
1.3 Structural plasticity and memory

Memories can broadly be divided in two systems; memories available to the conscious mind, declarative memory, and memories not available to consciousness, procedural memory. Studies indicate that the midline diencephalic and medial temporal lobes, in particular the hippocampus, are important for the establishment of new declarative memories [17].

Learning and memory are associated with synaptic changes, in which temporary changes are referred to as short-term memory and persistent synaptic changes as long-term memory [7]. Short-term memory lasts seconds to minutes after the present moment has passed, but with long-term memory, information can be stored for weeks, years or even for a lifetime [17]. The ability to store memory requires stabilization or consolidation of these synaptic changes and studies indicate that gene expression and protein synthesis are necessary for persistent long-term memory (reviewed in [18]).

In addition to learning, structural changes can also be induced through long-term potentiation, (LTP) and several years of studies suggest the possibility of LTP as a neural mechanism underlying learning and memory (reviewed in [13]).

1.3.1 Long-term potentiation

Long-term potentiation (LTP) can be defined as a long-lasting increase in synaptic strength. LTP is both input-specific and associative. It is an input specific process in the sense that when LTP is induced by the stimulation of one synapse, inactive synapses in the rest of the cell are not affected [19]. The property of associativity ensures that a weak stimulus, not itself suitable to trigger a response, can undergo LTP if an independent pathway originating from the same cell is strongly activated [20].

LTP occurs at many synapses which are dependent on N-methyl D-aspartate (NMDA) receptors (reviewed in [13]), a subtype of glutamate receptor highly permeable to Ca\(^{2+}\) and other divalent cations [21]. At negative potential, however, the ion channel is blocked by extracellular Mg\(^{2+}\) [22, 23] and dissociation of the Mg\(^{2+}\) is necessary for Ca\(^{2+}\) to enter the cell. Dissociation of Mg\(^{2+}\) and activation of the NMDA receptor requires both glutamate binding and depolarization of the membrane [22, 23]. It has also been suggested that glycine, acting as a co-agonist, is necessary for activation [24, 25].

Influx of calcium into the postsynaptic cell will, if the Ca\(^{2+}\) concentration is sufficiently elevated, lead to different transduction cascades (figure 2), resulting in modulation of synaptic strength, LTP (discussed in [13]). A key component for LTP induction and synaptic efficacy is Ca\(^{2+}/\)calmodulin-dependent protein kinase II, CaMK-II (discussed in [26, 27]). During LTP, CaMK-II is activated by Ca\(^{2+}/\)calmodulin (CaM). Once activated, CaMK-II can autophosphorylate Thr286. The autophosphorylated form of the enzyme no longer needs activation from CaM and can remain activated long after the Ca\(^{2+}\) level has returned to baseline levels. Modulation of cytoskeletal proteins and gene transcription are some effects of the activation. Elevated intracellular Ca\(^{2+}\) can also result in activation of several other protein kinases, such as protein kinases A (PKA) and mitogen-activated protein kinase (MAPK) which have been shown to be important for synaptic plasticity and memory storage (discussed in [18]).
Figure 2. Schematic overview of some intracellular signalling pathways involved in synaptic plasticity. Synaptic plasticity is often dependent on Ca2+ influx through NMDA receptors (NMDAR). The influx of Ca2+ activates several signalling pathways that eventually strengthen the synapse. Activation of Ca2+/calmodulin-dependent protein kinase II (CaMK-II) through Ca2+/calmodulin (CaM) results in modulation of cytoskeleton proteins and/or gene transcription and thus alternation in dendritic spine morphology. Synaptic activity also promotes CaMK-II translocation to the postsynaptic density (PSD). Activation of the cycle adenosine 3',5'-monophosphate (cAMP) and mitogen-activated protein kinase (MAPK) pathway results in phosphorylation and activation of cAMP response-element-binding protein (CREB) and further gene transcription. CREB can also be activated by CaM through Ca2+/calmodulin-dependent protein kinase type IV (CaMK-IV). (Map2, microtubule associated protein 2; AMPAR, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionil acid receptors; trkB, neurotrophic tyrosine kinase receptor; Bdnf, brain-derived neurotrophic factor; AC, adenyl cyclase; RSK, ribosomal s6 kinase).

1.3.2 Morphology changes of dendrites and dendritic spines.
Dendritic spines, small perturbations along the dendritic shaft, are localized on the postsynaptic side of the neuron, providing the essential link between an axon and dendrite. Dendritic spines comprises a postsynaptic density, PSD, (for review see [28]) containing receptors, channels and signalling molecules, which have a function to convert the synaptic activity into proper biochemical responses (discussed in [29]). The cytoskeleton of dendritic spines are composed mostly of actin filaments [30, 31], an important component for the morphological properties of the spine [32]. Filamentous actin, F-actin, forms bundles and networks by which the structure of the cell, as well as the motile force of the cell, can be regulated (reviewed in [33]). Due to its morphology properties, actin may have a fundamental role in the structural changes that lead to plastic changes during learning and experiences (reviewed in [7]). LTP, for example, is associated with the production of new dendritic spines. Several studies have shown that induction of LTP results in new spines and that blockade of LTP prevents these morphological changes [34, 35]. Another cytoskeleton component of dendrites as well as axons is microtubules (MTs). MTs promote the growth of axon and dendrites and its dynamics are controlled by several regulatory factors [36].
1.4 Plasticity and addiction

Addiction is defined as a compulsive pattern of drug-seeking and drug-taking behaviour, despite negative consequences. Addictive drugs are known to be both rewarding and reinforcing, because of their action on the brain reward system [6]. The key component in the brain rewarding system is the mesolimbic pathway, in which dopaminergic neurons are projected from the ventral tegmental area (VTA) to NAc (figure 3). Natural stimuli such as food, water and sex, affect this system by the release of dopamine in NAc, causing appropriate responses, evolutionarily important for survival, reproduction and fitness [37]. The same effect is achieved by drug use. In principle all addictive drugs, despite mechanism of action, act on the mesolimbic pathway [2] enhancing the dopamine release in the NAc [38]. The effects of addictive drugs are however, both stronger and more prolonged compared to a natural stimuli [6].

Addiction is a life-long condition and the molecular mechanism underlying this persistence has been discussed in several papers [2, 5]. Addiction is the result of many complex neural adaptations that develops over a period of time. Changes in synaptic strength [5], regulation of gene transcription and structural changes in specific neurons [2] are all possible candidate mechanisms underlying addiction.

Figure 3. Key neural circuits in addiction. Dopamine projections (red lines) from the ventral tegmental area (VTA) to nucleus accumbens (NAc) and prefrontal cortex (PFC) constitute the mesolimbic- and mesocortical pathway respectively, also referred to as the mesocorticolimbic system. The mesocorticolimbic brain area is involved in the locomotor stimuli and positive reinforcement properties, associated with repeated drug abuse. Enhanced dopamine efflux in NAc upon drug abuse, is associated both with increased locomotor activity and greater rewarding properties. Dotted red, lines indicate limbic afferent projections to NAc. Reprinted by permission from Macmillan Publishers Ltd: Nature [2], copyright 2000.
1.4.1 Sensitization

Sensitization, i.e. the escalating effects of the same drug upon repeated drug administration, is known to be induced by several drugs of abuse. Sensitization is associated with enhanced positive reinforcing properties, as well as increased locomotor activity (for review see [4]). The increase in locomotor activity behaviours are thought to be dependent on an increase in dopamine transmission in the NAc (discussed in [39]), just as the rewarding properties are. The primary focus for investigating sensitization has therefore been the mesocorticolimbic brain region (figure 3), especially NAc. Cocaine and morphine both enhance the dopamine efflux in NAc [3]. Cocaine acts by blocking the dopamine transporters, thus inhibiting dopamine re-uptake from the synaptic cleft. Morphine, on the other hand, interacts with µ-opioid receptors and inhibits GABAergic neurons which normally inhibit dopamine transmission. However, research of the posterior region has also underscored reorganization of glutamateric and GABAergic projections as a potential role in sensitization (for review see [40]).

Sensitization is most effectively induced upon intermittent drug exposure [41] but studies have shown that psychostimulants such as amphetamine and cocaine can produce long-lasting sensitization after just a single injection [42]. Several studies have also proven that drugs, even drugs of different pharmacology classes, can produce cross-sensitization to one another [43-45].

1.5 Genes of interest

For this study, 26 genes known to be involved in LTP, cytoskeleton regulation and/or synaptic plasticity were analyzed (see table 1). Of these genes, following six are of interest as they showed to be regulated by morphine and/or cocaine administration.

Map2

Microtubule associated protein 2 (Map2) has shown to be important for neurite outgrowth [46] and neuronal plasticity (reviewed in [47]). Studies have shown that Map2 mainly is localized in dendrites where it interacts with actin, MTs and neurofilament to regulate the structural properties of the cell (reviewed in [48]). Most attention for the regulation has however been on the interaction with MTs. The ability to bind and stabilize MTs has shown to depend on the state of phosphorylation of Map2 and it seems that both phosphorylation and dephosphorylation of the protein is involved in the structural alteration triggered by neuronal activity [48].

Gap43

Growth associated protein 43 (Gap43) is abundant in the axons of neurons, where it participates in synaptic membrane reorganization and synaptic function (reviewed in [49]). Gap43 interacts with several cytoskeletal and signalling molecules such as calmodulin, actin and G-proteins and there is a theory that Gap43, by regulation of actin, may be involved in neurotransmitter release [50]. The possibility that Gap43 is involved in synaptic plasticity is further supported by evidence that Gap43 is important for hippocampal LTP [51].
Bdnf and trkB
Brain-derived neurotrophic factor (Bdnf) belongs to the neurotrophin family, secretory proteins involved in the regulation of survival and differentiation of neurons during development (reviewed in [52]). Bdnf exerts its biological action through the neurotrophic tyrosine kinase receptor, trkB. Both Bdnf and trkB are widely distributed throughout the CNS [53, 54]. Studies have shown that Bdnf and trkB are important for hippocampal LTP as well as learning and memory [55-57] but also have important structural properties [58].

Fli1
Fms-like tyrosine kinase 1 (Flt1) is a receptor to the vascular endothelial growth factor (VEGF). VEGF is thought to be involved in hippocampal-dependent neurogenesis and an increase in VEGF mRNA level as well as protein concentration has been observed during learning [59]. The role of Flt1 in neurogenesis is unclear, but it has been suggested that VEGF is regulated by negative feedback of Flt1 [59].

CaMK-II-δ
Ca$^{2+}$/calmodulin kinase, type II (CaMK-II) are multiple functional protein kinases that are involved in several cellular processes, such as neurotransmitter synthesis, gene transcription and ion channel function [60]. CaMK-II comprises several subunits, composed from four separate genes (α, β, γ and δ). In this study the δ-subunit was investigated as it has been suggested that this particular subunit may be involved in neurogenesis [61].

<table>
<thead>
<tr>
<th>Accession nr</th>
<th>Protein name</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_019361.1</td>
<td>Activity-regulated cytoskeleton-</td>
<td>May be involved in neuroplasticity, long-term potentiation and memory</td>
</tr>
<tr>
<td></td>
<td>associated protein, Arc</td>
<td>consolidation</td>
</tr>
<tr>
<td>XM_238167.4</td>
<td>Filamin, alpha, Flna</td>
<td>Cytoskeletal organization</td>
</tr>
<tr>
<td>NM_017195.1</td>
<td>Growth associated protein 43, Gap-43</td>
<td>Involved in neurite formation, regeneration and plasticity</td>
</tr>
<tr>
<td>NM_017009.2</td>
<td>Glial fibrillary acidic protein, Gfap</td>
<td>Astrocyte specific intermediate filament</td>
</tr>
<tr>
<td>NM_019169.2</td>
<td>Synuclein, alpha, Snca</td>
<td>Considered role in synaptic vesicle organization</td>
</tr>
<tr>
<td>NM_017166.1</td>
<td>Stathmin, Stmn1</td>
<td>Promotes microtubule depolarization</td>
</tr>
<tr>
<td>NM_080689.4</td>
<td>Dynamin 1, Dnm1</td>
<td>Plays a role in synaptic vesicle recycling</td>
</tr>
<tr>
<td>NM_001108527.1</td>
<td>Sideroflexin 4, Sfxn4</td>
<td>Involved in iron ion transport</td>
</tr>
<tr>
<td>NM_019306.1</td>
<td>FMS-like tyrosine kinase 1, Flt1</td>
<td>Receptor for the vascular endothelial growth factor, VEGF may be involved in cell proliferation and neurogenesis</td>
</tr>
<tr>
<td>Accession nr</td>
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<td>Function</td>
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<td>XM_224429.4</td>
<td>Protocadherin 9, <em>Pcdh9</em></td>
<td>Involved in cell adhesion</td>
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<td>NM_019299.1</td>
<td>Clathrin heavy polypeptide, <em>Cltc HC</em></td>
<td>Involved in clathrine-coated pit formation</td>
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<td>X54100.1</td>
<td>Microtubule-associated protein 2, <em>Map2</em></td>
<td>Regulates and organizes microtubules; essential for dendritic growth</td>
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<tr>
<td>NM_017212.1</td>
<td>Microtubule-associated protein tau, <em>Mapt</em></td>
<td>Interact with tubulin to stabilize microtubules</td>
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<td>NM_017066.2</td>
<td>Pleiotrophin, <em>PTN</em></td>
<td>Growth factor; binds heparin</td>
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<td>X70496.1</td>
<td>RAB interacting factor, <em>Mss4</em></td>
<td>Intracellular vesicular transport</td>
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<td>NM_147138.2</td>
<td>ZW10 interactor, <em>Zwint-I</em></td>
<td>May play a role in exocytosis of synaptic vesicles in neurotransmission</td>
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<td>NM_001004082</td>
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<td>Chaperon activity; may be involved in the regulation of synaptic transmission</td>
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<td>NM_080885.1</td>
<td>Cyclin-dependent kinase; <em>Cdk5</em></td>
<td>Synaptic regulation and neural development</td>
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<tr>
<td>NM_012513</td>
<td>Brain derived neurotrophic factor, <em>Bdnf</em></td>
<td>Plays a role in the development of long-term potentiation and regulation of synaptic plasticity</td>
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<tr>
<td>M55291</td>
<td>Neurotrophic tyrosine kinase receptor, <em>trkB</em></td>
<td>Receptor for the brain derived neurotrophic factor</td>
</tr>
<tr>
<td>RNU11418</td>
<td>Glutamate receptor, ionotropic, N-methyl D-aspartate 1, <em>NR1</em></td>
<td>Subunit of the NMDA receptor; important for LTP</td>
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<tr>
<td>M91561</td>
<td>Glutamate receptor, ionotropic N-methyl D-aspartate 2A, <em>NR2A</em></td>
<td>NMDA receptor subunit, binding site for glutamate; important for LTP</td>
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<tr>
<td>NM_012574</td>
<td>Glutamate receptor, ionotropic, N-methyl D-aspartate 2B, <em>NR2B</em></td>
<td>Subunit of the NMDA receptor, regulates the Ca(^{2+}) influx important for LTP; binding site for glutamate</td>
</tr>
<tr>
<td>NM_012519.2</td>
<td>Calcium/calmodulin-dependent protein kinas II, <em>CamK-II</em></td>
<td>δ-subunit, may be important for neurogenesis</td>
</tr>
<tr>
<td>NM_031017.1</td>
<td>c-AMP-response-element-binding protein; <em>CREB</em></td>
<td>Transcription factor involved in learning and memory</td>
</tr>
</tbody>
</table>
1.6 Aim of study
The aim of this study was to clarify the influence of morphine and/or cocaine (psychostimulants) on gene expression levels encoding proteins associated with hippocampal structural plasticity. The hippocampus, which is essential for memory acquisition, is in many ways under the influence of the reward system but its role in developing drug dependence and behavioral sensitization is still poorly understood. The results of this study may elucidate the mechanisms behind drug addiction.
2 Methods and experimental procedure

2.1 Techniques

2.1.1 Polymerase Chain Reaction
Polymerase chain reaction (PCR) is a molecular biology technique that allows amplification of short DNA sequences (100-500bp) within a longer DNA molecule [62]. Any region of a DNA molecule can be chosen, as long as the ends of the particular sequence are known. PCR uses primers, short oligonucleotides that are complementary to the defined sequences to delimit the region for amplification. The sequence and the length of the primers are important for the specificity of the reaction. PCR proceeds throughout amplification cycles, in which a series of different temperatures for varying time are repeated. In the first step of the cycle, the denaturation step, the DNA strands are separated from each other. This requires a high temperature - around 94°C. The denaturation step is followed by the annealing step, in which hybridization of the primers to the DNA template occurs. The annealing temperature depends on which primer being used. The extension step is the last temperature dependent step of the cycle. In this step DNA is synthesized and the temperature depends on the DNA polymerase used.

2.1.2 Reverse transcription polymerase Chain Reaction and Real-time RT-PCR
Reverse transcriptase (RT)- PCR enables RNA to be used as template for PCR [62, 63]. The first step is to convert RNA to complementary DNA (cDNA) using the enzyme reverse transcriptase. The cDNA is then amplified using PCR. Any RNA can be used, as long it is free from contaminated genomic DNA.

In real-time PCR or quantitative real-time RT-PCR (qRT-PCR), measurements are made at each amplification cycle [63]. To be able to measure the DNA product, a fluorescent dye or probe is needed. A commonly used fluorescent dye is SYBR Green, which produces a fluorescent signal when it binds to double stranded DNA. The more PCR product, the more light emission increases. A disadvantage with SYBR Green is that it binds to any double stranded DNA molecule in the sample, which may lead to an overestimation of the product.

Before running the genes of interest, the samples are tested on a number of so called housekeeping genes (HKG), genes that are expressed in almost every cell of the body. Amplification of the housekeeping genes verify that the target nucleic acid and reaction components are of acceptable quality [62]. Data from the HKG are used to calculate a normalization factor to identify and remove variations of the measurement process, samples etc. necessary to achieve a valid estimation of the gene expression [63].

In real time PCR reactions the relative ratio of products and reagents vary [63]. At the beginning of the process, the initial phase, the reagents are in excess and the template and product in very low concentrations. There will be no competition for the primer binding and the amplification will proceed at constant rate. The initial phase is followed by a linear phase, due to competition between the reagent and product for primer binding. The amplification finally reaches a plateau phase in which no more products are accumulated. Quantitative data from the real time PCR are collected during the exponential phase of amplification (figure 4). The point at which the fluorescence crosses the threshold is called cycle threshold (Ct) which is the cycle of the reaction corresponding to the beginning of the exponential phase of amplification. Ct-values from each duplicate are used for further analysis.
Figure 4. Quantitative data from qRT-PCR
Data from qRT-PCR are collected during the exponential phase of amplification. The cycle threshold is the cycle of the reaction corresponding to the beginning of the exponential phase of amplification. A) Normal view. B) Logarithmic, log, view
Figure 5. Melt curve after qRT-PCR amplification
The melting curve is studied after each completed cycle, to confirm that only a single product has been amplified. The melting temperature of the negative control should preferably differ from the melting points of the samples.

2.2 Animals
Thirtytwo male Spargue-Dawley rats, with an initial body weight of 330-380 gram were housed four per cage on a 12h dark/light cycle. Animals received food and water ad libitum. The animals were randomly separated into four groups and treated as follows:

S/S: Daily injections of saline (1 ml/kg) for five days. A challenge injection of saline (1 ml/kg) was given to the animals on the second day after the last saline injection.
S/C: Daily injections of saline (1 ml/kg) for five days. A challenge injection of cocaine (10 mg/kg) was given to the animals on the second day after the last saline injection.
M/C: Daily injections of morphine (10 mg/kg) for five days. A challenge injection of cocaine (10 mg/kg) was given on the second day after the last morphine injection.
M/S: Daily injection of morphine (10mg/kg) for five days. A challenge injection of saline (1 ml/kg) was given on the second day after the last morphine injection.

Morphine and cocaine were dissolved in sterile saline. All injections were given intra-peritoneally. Detailed procedure protocol is shown in Appendix A.

2.3 RNA isolation
Tissues from the left side of hippocampus were homogenized in TRIZOL reagent (Invitrogen, Sweden), to lyse the cell and its components, using Ultra-sonication and incubated at 15-30°C for 5 minutes in room temperature. The RNA was extracted using chloroform, for separation into organic and aqueous phases, followed by incubation at 15-30°C for 2-3 minutes, room temperature and subsequently centrifuged, 13 000 rpm, 20 minutes at 4°C. After centrifugation the upper aqueous phase, containing RNA, was transferred to fresh tubes. Samples were incubated in isopropanol for two hours at -20°C or left over night to precipitate the RNA. The samples were centrifuged, 13 000 rpm, 20 minutes at 4°C, and the supernatant was removed. The pellet was washed twice in ethanol, first 75% and then ice-cold 80%, air-dried for 30 minutes and dissolved in 1xDNAase buffer.
2.4 DNAase treatment

Since RNA often is contaminated with DNA, RNAse free DNAse was added to each sample to eliminate any genomic DNA. Samples were then incubated at 37°C for 1.5 hour in a heat block, followed by inactivation of the enzyme at 75°C for 15 minutes. The samples were immediately put on ice.

2.5 Polymerase Chain Reaction

0.5 µl template was mixed in a final volume of 10 µl containing 1xPCR reaction mix (1 µl 10xBuffer Mg-free, 0.3 µl 50 mM MgCl2, 0.25 µl 1% Tween (W), 0.1 µl 20 mM dNTP (constituted of dCTP, dTTP, dATP and dGTP 100mM each), 1 µl primermix (containing both forward and backward primer with a concentration of 10 pmol/µl each), 0.1 µl Taq Polymerase, 5 U/µl (Biotools, Spain) and 6.75 µl MilliQ H2O). As a positive control 0.5 µl 100 ng/µl genomic DNA was used and for a negative control 0.5 µl MilliQ water. (For PCR program see appendix A:III).

The product was analyzed on 2% agarose gel in Tris-acetate-EDTA, TAE, buffer, 130 V for approximately 30 min, to detect contamination of DNA. Total RNA concentration was measured using Nanodrop® ND-1000 spectrophotometer (Nanodrop Technique Delaware, USA).

2.6 cDNA synthesis

Samples were diluted with autoclaved MilliQ water to achieve a final concentration of 5 µg RNA/µl. Volume of template and MilliQ water were 12 µl. RNA was reversed transcribed in a final volume of 20 µl containing 1xMastermix (4 µl 5x FS Buffer, 2 µl 0.1M DTT, 0.5 µl 20 mM dNTP, 0.5 µl N6 1/6.25 (random hexamers) and 1 µl MLV reverse transcriptase. Samples were incubated at 37°C for an hour, whereupon the enzyme was denatured at 95°C for 15 min.

PCR as described above was performed and the products were analyzed on 2% agarose gel in TAE buffer, 130 V for 30 min, to confirm that the cDNA synthesis had been successful. The cDNA synthesis was repeated in those samples not containing any DNA.

2.7 Real-time PCR Analysis

The cDNA was diluted with autoclaved MilliQ water to achieve a final concentration of 5 ng/µl. Real time PCR was performed in a 20 µl final volume of: 9.52 µl MilliQ water, 2 µl 10xBuffer (Mg-free), 0.20 µl 20mM dNTP, 1.6 µl 50 mM MgCl2, 0.05 µl of each primer (each with a concentration of 100 pmol/ µl), 1 µl DMSO (dimethyl sulfoxide), 0.5 µl SYBR Green (1; 50 000), 0.08 µl 5 U/µl Taq polymerase (Biotools, Spain) using an iCycler real-time detection instrument (Bio-Rad Laboratories). As a template 5 µl, cDNA was used and all PCR were carried out in duplicates on a 96 well test plate. Autoclaved MilliQ water was used as negative control. For each primer pair, 50 cycles were run with parameters of 95°C for 15 sec, 52-62°C (depending on primer) annealing for 30 sec followed by extension at 72°C for 30 sec. (For more details see appendix A:VII). After the cycling was completed, the melting curve was studied to confirm that only a single product had been amplified (figure 5).
2.8 Primer
Primers were designed using Beacon Designer v.4.0 (Premier Biosoft, USA). Each primer was diluted in autoclaved MilliQ water to achieve a final concentration of 100 pmol/µl. Primers used in this study are shown in table 2.

2.9 Statistical Analyses
qRT-PCR data were analyzed with MyiQ software, version 1.0 (Bio-Rad Laboratories, Sundbyberg, Sweden). Normalization factor from six HKG; glyceraldehyde-3-phosphate-dehydrogenase (GAPDH); histone H3, (H3b); β-actin (βAct); β-tubulin (βTUB); ribosomal protein L 19 (RPL19) and cyclophilin (Cyclo), were calculated using the geNorm method [64]. The stability of each HKG is established by measuring the average expression stability, M-value. Genes with a low M-value have more stable expression and the M-value for a control gene should be less than 1.5 (M<1.5). The geNorm program enables elimination of the most unstable gene and recalculation of new M values for the remaining genes until a stable normalization factor is achieved. The primer efficiency for each sample was calculated using linear regression analysis, LinRegPCR [65], where raw data, corrected Ct-values, for each run is used. Primer efficiency and standard deviation, SD, of the efficiency for the primer in the reaction was calculated using Grubb’s test (GraphPad Software Inc). Grubb’s test was also used to eliminate outliers within each group. Duplicates with a Ct difference of 1.5 were excluded from the statistical calculations.

Statistical analysis was performed using Prism, version 4.0 (GraphPad). Each treatment group was compared with each other using one way ANOVA. The level of significance was set to p≤0.05. The p-value is the probability that the result observed is due to a change. If the overall ANOVA was significant, Fisher’s post hoc test was performed to identify between which groups a significant value was obtained.
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*a* Annealing temperature, which is the temperature where amplification is the best.
3 Results

3.1 geNorm analysis of housekeeping genes
Six housekeeping genes (HKG) were analyzed by qRT-PCR for the hippocampus and the geNorm method was applied to determine the optimal number of HKG to achieve a valid estimation of gene expression. In this study, the best estimation was observed using five HKG; GAPDH, H3b, βAct, Cyclo and RPL19, (Appendix B).

3.2 Modulation of the mRNA level of several genes by morphine and/or cocaine treatment in rat hippocampus
Using qRT-PCR, the modulation by morphine and/or cocaine of total 26 hippocampal genes was investigated (Appendix C). Significant differences, using one-way ANOVA, were observed in six of them; Map2, Gap43, Bdnf, trkB, Flt1 and CaMK-II (figure 6).

Post hoc analysis showed significant decrease in Map2 mRNA level in the m/c group (p<0.01) and the m/s group (p<0.001) compared to the control. A significant decrease was also observed in the m/c group (p<0.01) and m/s group (p<0.001) compared to the s/c group.

The expression level of Gap43 was significantly increased in the m/c group compared to the control (p<0.05), while a significant decrease was observed in the m/s group compared to the m/c group (p<0.01).

The level of Bdnf mRNA was significantly decreased in the m/s group compared to both the s/c (p<0.001) and the m/c (p<0.05) groups. Significant alternation was also observed in the mRNA level of the Bdnf receptor, trkB. The m/c group was significant increased compared to the control (p<0.05), while a significant decrease was observed in the m/s group compared to the m/c (p<0.001) and the s/c (p<0.01) treated groups.

A significant decrease in Flt1 mRNA expression was observed in both the m/c (p<0.01) and m/s (p<0.05) groups compared to the s/c group.

The mRNA level of CaMK-II was significantly increased in the s/c treated group compared to the control (p<0.01). A significantly decrease was observed in the m/c (p<0.05) and m/s (p<0.001) compared to the s/c group.
Figure 6. Effects of morphine and/or cocaine on the mRNA level of genes associated with hippocampal plasticity, by qRT-PCR analysis. s/s, saline/saline: control group; m/s, chronic morphine (5 days, 10 mg/kg) treatment; s/c, a challenge dose of cocaine (10mg/kg); m/c, chronic morphine treatment (5 days, 10 mg/kg) followed by a challenge dose of cocaine (10mg/kg). Data represent mean ±SD (n=6-8 rats/group). Fisher’s post hoc test identifies between which groups a significant value was obtained, *p<0.05, **p<0.01, ***p<0.001.
4 Discussion

In this experiment the effect of morphine and cocaine administration of genes associated with learning and memory was studied. These results are important in expanding our knowledge of how opioid drugs and psychostimulants relate to plasticity mechanisms. The effects of each gene that showed a significant difference are discussed below.

Map2

Studies have indicated that Map2 plays an important role in the structural changes involved in LTP and that phosphorylation and dephosphorylation of Map2 is critical for protein regulation (reviewed in [48]). In this study, the mRNA level of Map2 was significantly decreased (p<0.0001) in the groups of rats treated with morphine (m/s) and morphine and cocaine (m/c) compared to the control group. This shows that repeated morphine administration decreases the mRNA level of Map2. Although it is well documented that regulation of Map2 is mainly controlled by phosphorylation and dephosphorylation changes at the protein level, this study shows that Map2 can be regulated by drugs at mRNA level. Previous studies have showed that repeated morphine administration decreases dendritic branching and cell size of neurons in VTA, NAc and neocortex of rats [9, 66]. The mechanism underlying these changes is still unclear, however alteration of neurofilament upon morphine administration has been observed [67] and it is likely that these changes could contribute to the regulation. It is therefore possible that the decreased Map2 mRNA expression observed in present study could contribute to the reduced spine size noted with morphine consumption [9, 66].

Gap43

Over-expression of Gap43 results in exuberant axonal growth in brain areas that are associated with memory processes [49, 68]. This together with findings that manipulation of Gap43 can alter LTP, supports the earlier hypothesis suggesting that Gap43 is most likely involved in the regulation of synaptic plasticity [69]. The present study of Gap43 shows that neither cocaine nor morphine severally affects the mRNA expression. However a significant increase (p=0.0276) in the m/c treated group compared to the control group could be observed. This may indicate that pre-treatment with morphine produces cross-sensitization to cocaine and that Gap43 is somehow involved in this process, showing a significant higher locomotor activity in the m/c group compared to the untreated s/s group (data not published). Vanderschuren and co-workers showed that a single dose of morphine produces behavioural as well as neurochemical sensitization in rats [70].

Bdnf and trkB

The expression of Bdnf has shown to be in coincident with its receptor trkB [71] suggesting that Bdnf may act in an autocrine or a paracrine fashion. This is consistent with present study in which the mRNA expression of Bdnf and trkB were regulated in similar ways upon drug exposure (figure 6). In this study there was a tendency to a parallel up-regulation of the mRNA level of both Bdnf (p= 0.0683) and trkB (p=0.0981) in the group giving an acute injection of cocaine compared to the control group. However, just as previous studies implicate a single injection of cocaine does not affect Bdnf nor trkB expression in rat hippocampus [72,73]. It was also seen in this study that chronic morphine treatment slightly decrease the mRNA level of Bdnf (p=0.0791). No parallel changes were however observed in trkB mRNA level and the reason for this is difficult to explain.
Bdnf and trkB have both shown to be important for LTP. Studies have proven that Bdnf is not only necessary for the induction of LTP, but also its maintenance (reviewed in [56]). Similarly LTP is impaired in trkB knockout mice [57]. Bdnf is also required for learning and memory [55, 74] and an increase in Bdnf mRNA levels have been observed in association with spatial learning [75]. This result suggests that the decrease in Bdnf mRNA level induced by morphine could contribute to impaired long-term memory.

Flt1

Previous research have shown that chronic morphine administration decreases the mRNA level of Flt1 in hippocampus [12]. To date, very little is known about this receptor and most research has focused on its substrate, VEGF. However, with the result that VEGF is regulated by negative feedback from Flt1 [59], it has been suggested that the down regulation of the receptor upon morphine administration could participate in a neuroprotective mechanism [12]. In the present study, however, morphine did not affect the expression of Flt1. This may be due to a difference length of this study as compared with previously reported studies. In this study the animals were given morphine for five days, compared with 26 days in the study by Marie-Claire et al. [12], so time might be a factor of diversity.

CaMK-II-δ

Very little is known about the δ-subunit of CaMK-II. The location of its expression however suggests that it might be involved in neurogenesis. Delta is the major subunit of astrocyte CaMK-II [76]. Astrocytes are a subtype of glia cells, known to support proliferation, survival and maturation of developing cells. Astrocytes have also been shown to be important for synaptic formation and synaptic transmission [61], and are critical for learning and memory. There are also studies that indicate that CaMK-II-δ is located in the nucleus of hippocampal granule cells [77]. Isoforms of the delta subunit have also been found in granule cells of cerebellum, where they seem to be involved in Ca$^{2+}$ signalling and in the expression of Bdnf [76]. It has been reported that Bdnf promotes the survival of neurons during development (reviewed in [52]) and it has therefore been suggested that the δ-subunit supports the survival of granule cells by increasing Bdnf expression [78]. The present study shows an increase in CaMK-II-δ mRNA levels after a single injection of cocaine (p=0.0094), while morphine did not affect the expression at all. Since it still unclear what the role of CaMK-II-δ is in neurogenesis, it is hard to draw conclusions from these results.
4.1 Conclusions

This study shows that administration of drugs affect the expression of several genes involved in structural plasticity, important for learning and memory. Few studies have been carried out in this field and the effect of repeated drug administration on genes involved in different form of plasticity is still not clear. In this study there was a tendency for down regulation in the Bdnf mRNA level after chronic morphine treatment. Together with previous finding [75] this suggests that morphine consumption could contribute to impaired long-term memory. Further enlargement and the formation of new dendritic spines has been observed during LTP [32, 33], a possible mechanism underlying learning and memory. Map2 is highly abundant in dendrites and has shown to be important for the structural properties of the dendritic spine. The decreased Map2 mRNA level observed in this study after chronic morphine consumption also suggests that morphine could have a negative effect on learning. However, the result from the present study is very limited and even though many cellular processes are regulated at the mRNA level, further analysis at the protein level is necessary for more precise evaluation.

To improve this study, the experiment could be repeated with variance in several variables such as prolonged experimental time and/or drug treatment. In addition, complementary studies to the mRNA expression, such as structural related studies, would clarify which effect drug exposure has on these genes.
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Thanks to everyone at Helgi lab, who made this time memorable.
Thanks finally to Helgi Schiöth for accepting me for this thesis project.
6 References

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7 Appendix A, Procedure protocol

I. RNA isolation from tissue

Total amount of TRIZOL:

>100 mg tissue – 1000 µl TRIZOL

It is advantageous to do the homogenisation in a smaller amount of TRIZOL, after which the remaining volume is added. This achieves a good separation.

1. Take the tissue out of the tube with a tip or a needle so that no RNAlater follows with the sample. Put the tissue in the TRIZOL in a new tube.

Amount TRIZOL for homogenisation
>100 mg tissue – homogenise in 400 µl, add 600 µl after homogenisation.

2. Homogenise
- Mechanical homogenisation with the smallest teflon stick possible.
- Ultra Sonication: 4-rotation speed, 30-pulses or continuously.
- Wash the needle with a.) EtOH, b.) H₂O.
Avoid getting the tissue warm, keep on ice.

3. Do not forget to add the TRIZOL according to above.

4. Incubate homogenates for 5 min at room temperature so that the nucleoprotein complexes dissociate. This can also be done while the remaining TRIZOL is added.

5. Add 200 µl chloroform per 1000 µl TRIZOL added earlier.

6. Upend the tubes gently for 15 seconds, do not vortex.

7. Incubate for 2-3 min at 15-30 °C.

8. Centrifuge at 13 000 rpm for 20 min at 2-8 °C. After the centrifugation, the RNA is in the upper aqueous phase. Do not put the samples on ice after centrifugation.

9. Transfer the RNA in the water phase to new clean tubes.

10. Precipitate RNA with isopropanol
    a) Add 500 µl isopropanol per 1000 µl TRIZOL
    b) Incubate for 2 h in -20 °C or leave overnight.

11. Centrifuge the samples at 13 000 rpm for 20 min at 2-8 °C.

12. Remove the supernatant. Wash the RNA twice.
    a) Wash 1: 75% room temperature ethanol, 1000 µl ethanol per 1000 µl TRIZOL
    b) Centrifuge at 13 000 rpm for 10 min at 4 °C.
    c) Wash 2: 80% ice cold ethanol.
    d) Centrifuge at 13 000 rpm for 10 min at 4 °C.
13. Remove all of the ethanol.

14. Let the RNA pellet air dry in the fume cupboard at room temperature, not to long, for approximately 15-30 min.

15. Dissolve the RNA pellet in 14-40 µl 1xDNAase buffer, the magnitude depends on the size of the pellet.

The final amount of RNA is often 40-100 µg. Store the RNA in -80°C freezer.

II. DNAase treatment for rat, brain tissue

ALL RNA is used for the DNAase treatment.

1. The volume of 10xDNAase buffer depends on the amount of water the RNA is dissolved in. DNAase buffer shall have the concentration 1x the final solution for the DNAase enzyme to be active. If the RNA already is dissolved in 1xDNAase buffer then only the enzyme DNAaseI is added.

2. Add DNAaseI, 2 µl

3. Incubation of the samples
   - 37°C for 1.5 h.
   - 75°C for 15 min, for inactivation
   - cool the samples on ice then centrifuge down the condensation drops

4. Store in an -80°C freezer.

III. PCR

A) PCR on isolated RNA
B) PCR after CDNA synthesis with Reverse Transcriptase

PCR ingredients per 10µl PCR sample (for both A and B)
- 0.5 µl Template, which shall be one of the following options:
  - RNA, in which no DNA shall be present (alternative A above)
  - DNA, which shall contain DNA (alternative B above)
  - Positive control for example genomic DNA 100ng/µl 0.5 µl
  - Negative control (water), 0.5 µl
- 9.5 µl Mastermix

Mastermix reagents per 9.5 µl
- 1 µl 10xBuffer
- 0.3 µl 50mM MgCl₂
- 0.25 µl Tween (W)
- 0.1 µl 20mM dNTP
- 1 µl primermix, each primer with a concentration of 10 pmol/µl (both forward and backward primer; for example 100bp or 300bp)
- 0.1 µl Taq Polymerase Enzyme
- 6.75 µl H₂O

Most efficient is to do one mastermix for all samples, which contains all reagents above except the template.

1. Prepare the mastermix, vortex and put on ice
2. Dispense the mastermix into PCR-tubes; 9.5 µl per tube
3. Add 0.5 µl template to each tube.

**PCR program:**
1. 1. 95°C 3min-repeat 1
2. 1. 95°C 30 sec} – repeat 35
   2. 58°C 30 sec}
   3. 72°C 45 sec}
3. 1. 72°C 5 min- repeat 1

Hold time 4°C

**IV. Gel electrophoresis for Rat tissue.**

1. A 2% agarose solution, containing 4 g Agarose + 200ml 1xTAE buffert and 4 µl EtBr, is used.
2. Add 0.5 µl 10xDNA dye per 5 µl sample
3. Run the gel in 1xTAE solution, 130 V for 30 min.
4. Take a photo of the gel.

**V. RNA concentration measurement with nanodrop**

Unfreeze the samples on ice, vortex and centrifuge down the condensation drops. 0.7 µl RNA is added to the lens and the concentration is measured.

**VI. cDNA synthesis**

A solution of 20 µl total is used:
- 12 µl template + MilliQ water (5 µl total RNA)
- 8 µl Mastermix

8 µl Mastermix
- 4 µl 5xFS buffer
- 2 µl 0.1 M DTT
- 0.5 µl 20mM dNTP
- 0.5 µl Random hexamers, diluted to 1/6.25 (N6)
- 1 µl Reverse Transcriptase (MLV)

1. Incubate the samples at 37°C for 1 hour, after which you denature the enzyme at 95°C for 15min. Put immediately on ice.
2. Centrifuge down the condensation drops
3. Run PCR and electrophoreses according to protocol III and IV!
4. If DNA is present, dilute the samples to a final concentration of 100 ng/µl. For example; if 5 µl RNA have been used for RT-PCR in 20 µl, additional 30 µl autoclaved MilliQ water shall be added to the newly synthesized DNA samples.

VII. qRT-PCR

In every well on the test plate:
- 15 µl master mix
- 5 µl 5 ng/µl template or MilliQ water (for negative controls)
A solution of total 20 µl.

Master mix per 15 µl:
- 9.52 µl MQ water
- 2 µl 10x buffer
- 0.2 µl dNTP
- 1.6 µl MgCl₂
- 0.05 µl forward primer
- 0.05 µl reverse primer
- 0.5 µl SYBR green
- 0.08 µl 5U/µl Taq Polymerase

1. Add 5 µl template to the test plate and 5 µl MilliQ water to four wells for negative controls.
2. Prepare the master mix, vortex and spin down.
3. Dispense the master mix on to the test plate; 15 µl/well.
4. Place a plastic film over the test plate and centrifuge.
5. Choose plate setup and temperature, and start the machine.

qRT-PCR Program:

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(1x) Step 1</td>
<td>95.0°C</td>
</tr>
<tr>
<td>2</td>
<td>(50x)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Step 1: 95.0°C</td>
<td>00:15</td>
</tr>
<tr>
<td></td>
<td>Step 2: Ta</td>
<td>00:30</td>
</tr>
<tr>
<td></td>
<td>Data collection and real-time analysis enabled</td>
<td></td>
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<tr>
<td></td>
<td>Step 3: 72.0°C</td>
<td>00:30</td>
</tr>
<tr>
<td>3</td>
<td>(1x) step 1:</td>
<td>Ta</td>
</tr>
<tr>
<td>4</td>
<td>(84x) step 1:</td>
<td>55.0°C</td>
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<tr>
<td></td>
<td>Increase setpoint temperature after cycle 2 by 0.5°C</td>
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</tr>
<tr>
<td></td>
<td>Melt curve data collection and analysis enabled</td>
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</tr>
<tr>
<td>5</td>
<td>(1x) Step 1:</td>
<td>20.0°C</td>
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</tbody>
</table>

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geNorm method was used to determine the most stable housekeeping genes, HKG, in the hippocampus. The most stable HKG were RPL19, Actb, H3b, GAPDH and Cyclo.
### 9 Appendix C, Alternation in mRNA expression

Changes in mRNA expression between the different treatments groups by morphine and/or cocaine administration

<table>
<thead>
<tr>
<th>Gene</th>
<th>1-way ANOVA</th>
<th>Fisher's Post hoc test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p-value</td>
<td>s/s vs s/c</td>
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<tr>
<td>Arc</td>
<td>0.5519</td>
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<tr>
<td>Flna</td>
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<tr>
<td>Gap43</td>
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<tr>
<td>Gfap</td>
<td>0.0951</td>
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<tr>
<td>Snca</td>
<td>0.0597</td>
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<tr>
<td>Stmn1</td>
<td>0.0677</td>
<td>-</td>
</tr>
<tr>
<td>Dnm1</td>
<td>0.0811</td>
<td>-</td>
</tr>
<tr>
<td>Sfxn4</td>
<td>0.1877</td>
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<tr>
<td>Fli1</td>
<td>0.0402</td>
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<tr>
<td>Fe65</td>
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<tr>
<td>Pcdh9</td>
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<tr>
<td>Cltc HC</td>
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<tr>
<td>Map2</td>
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<tr>
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<tr>
<td>Mss4</td>
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<tr>
<td>Zwint-1</td>
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<tr>
<td>Hsp90B</td>
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<td>Cdk5</td>
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<tr>
<td>Bdnf</td>
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<tr>
<td>M18k</td>
<td>0.0025</td>
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<tr>
<td>NR1</td>
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<tr>
<td>NR2A</td>
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<tr>
<td>NR2B</td>
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</tr>
<tr>
<td>CaMK-II</td>
<td>0.0043</td>
<td>↑ (p&lt;0.01)</td>
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<tr>
<td>CREB</td>
<td>0.9512</td>
<td>-</td>
</tr>
</tbody>
</table>

s/s, saline /saline; s/c, saline/cocaine; m/c, morphine/cocaine; m/s, morphine/saline.
10 Appendix D, ANOVA results

![Graphs showing mRNA expression for various genes.](image-url)