Does long-term methadone exposure affect cognitive functions in rats?

Effects on novelty exploration

and phosphorylation of CaMKII and ERK.

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Preface

This master's thesis in toxicology is part of the master's degree in biology at the University of Oslo. The work in this thesis was carried out at the Norwegian Institute of Public Health, The Division of Forensic Toxicology and Drug Abuse – Department of Drug Abuse research, from November 2007 to June 2009. The work was supervised by senior research scientist Jannike M. Andersen (Norwegian Institute of Public Health). Steinar Øvrebø was the internal supervisor from the University of Oslo.

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ABSTRACT

Numerous studies have explored the effects of morphine on cognitive function, while knowledge concerning methadone is scarce. This study is the first in line, being part of a newly started project. The aim of this study was to examine whether long-term administration of methadone results in deficits in performance in the Novelty-test and changes in phosphorylation of calcium/calmodulin kinase II (CaMKII) and extracellular signal-regulated kinase (ERK) in the hippocampus. Rats were administrated (sc) methadone, morphine or saline once per day for three weeks (2.5-10 mg/kg), and novelty-tested one hour and one week after the last injection. Western blotting was performed on homogenate from hippocampus, taken out directly after the novelty-testing, to detect levels of phosphorylated CaMKII (p-CaMKII) and ERK (p-ERK). p-CaMKII was significantly down-regulated one week after the last injection of methadone, while no significant difference in p-ERK was observed. There were no significant differences between the groups in the novelty-test one week after the last injection, while one hour after the last injection the animals were to affected by the methadone to perform the test. Our findings indicate that long-term methadone exposure may specifically affect protein phosphorylation, but don't seem to have a persistent effect on novelty-exploration. This may also be due to the Novelty-test not being sensitive enough to pick up small differences between the exposed groups and the control group.

ABBREVIATIONS

AMPA receptor - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

Ca^{2+} - calcium ion

CaMKII - Calcium/calmodulin kinase II

cAMP - Cyclic adenosine monophosphate

ERK - Extracellular signal-regulated kinase

HRP - Horseradish peroxidase

LC-MS - Liquid chromatography-mass spectrometry

LTP - Long-term-potentiation

MAPK - Mitogen activated protein kinase

MMT - methadone maintenance therapy

MOR - μ-opioid receptor

NIPH - Norwegian Institute of Public Health

NMDA receptor - *N*-methyl-D-aspartate receptor

SDS-PAGE - sodium dodecyl sulphate polyacrylamide gel electrophoresis

1. INTRODUCTION

Methadone is a drug used in the rehabilitation of heroin addicts and has been used for the last 40 years (Dole and Nyswander, 1965). There are about 12 million heroin addicts in the world (UNODC, 2008). In Norway the corresponding number is 12-15000 (Berg, 2008), and about 4700 of these are taking part in a rehabilitation program (Bretteville-Jensen and Amundsen, 2006; Bye, 2009). During the next few years this number will probably increase. Despite the widespread use of methadone in treatment of opioid-dependent people, little is known about its acute and chronic effects on cognitive functions, such as learning and memory formation (Curran *et al.*, 2001).

1.1 Methadone

Methadone is a synthetic opioid, which was first synthesised as a morphine substitute during World War II in Germany to relieve severe pain (Tober and Strang, 2003). Methadone exerts its analgesic and narcotic effects by binding to the μ-opioid receptor (MOR) (see section 1.1.3).

1.1.1 General structure

Methadone exists in two enantiomeric forms, R and S (Sim, 1973). The racemic mixture is the form commonly used clinically. The R-form has a 10-fold higher affinity for the MOR compared to the S-form (Taracha *et al.*, 2006; Trescot *et al.*, 2008). Both the R- and the S-form have been shown to have *N*-methyl-D-aspartate receptor (NMDA receptor) antagonist activity (Callahan *et al.*, 2004; Trescot *et al.*, 2008). The S-form is also able to inhibit re-uptake of serotonin and norepinephrine from the synaptic cleft (Trescot *et al.*, 2008).

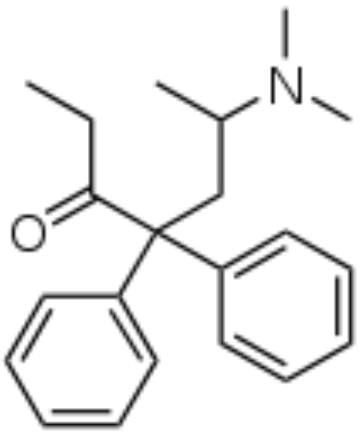


Fig. 1-1 *Chemical structure of methadone*

Methadone has a half-life of 24-48 hours in opiate-dependent people, showing large individual variation (Curran *et al.*, 2001). This half-life is considerably longer than that of morphine, 2-3.5 hours (Trescot *et al.*, 2008), and much longer than the plasma half-life of heroin, which is approximately 5 minutes (Pichini *et al.*, 1999). In rats, the half-life of methadone is about 3-4 hours (Misra *et al.*, 1973), while morphine has a half-life of approximately 120 minutes (Iwamoto and Klaassen, 1977). The metabolic rate vary greatly between individuals, and is apparently due to genetic variability in the presence of cytochrome P450 enzymes in the liver (Trescot *et al.*, 2008). Methadone is widely distributed in the tissue and highly bound to proteins, perhaps in the range 60-90% (Corckery *et al.*, 2004).

1.1.2 Use in methadone maintenance therapy

Methadone was first used in methadone maintenance therapy (MMT) in 1965 (Dole and Nyswander, 1965), and is still a cornerstone in society's efforts to reduce problems with heroin abuse. Methadone binds to opioid receptors with high affinity and prevents other opioids, like heroin, binding to the receptor (Tober and Strang, 2003). Because of the long half-life of methadone, one daily dose can maintain a steady state in the patients (Curran *et al.*, 2001). Due to the large individual variations in methadone metabolism, patients receive different doses, usually between 80-120 mg/day (Joseph *et al.*, 2000), but doses of up to 700 mg/day have been reported (Leavitt *et al.*, 2000). The right dosage of methadone will not give the subject any form of «rush», nor any abstinence or craving for heroin (Sim, 1973).

Morphine, which has a similar mechanism of action as methadone, is known to affect memory processes (Prosser *et al.*, 2006). It is therefore likely that methadone also may have negative effects on cognitive functions.

Despite its extensive use, possible negative side-effects of methadone consumption have been poorly investigated. Methadone-maintained patients perform significantly worse than controls on attention, information processing, short-term memory, long-

term memory, and problem solving (Darke and Ross, 1997). It is suggested that at least some of the reason for this is the use of methadone, rather than other factors associated with the addicts' lifestyle (e.g. poor dietary habits, repeated head injury, overdoses, concurrent abuse of alcohol and other drugs). It seems evident that an optimal cognitive function is crucial for a positive rehabilitation outcome (Spiga *et al.*, 2008). To our knowledge, there are no studies published on controlled randomised long-term methadone administration to a non-drug using human population with cognitive outcome measures. Obvious ethical considerations might have contributed to this lack of studies in healthy subjects. More surprisingly is the absence of behavioural and toxicological effects studies in experimental animals.

1.1.3 mu - opioid receptor

Opioids are a class of chemical substances that mediate their effect(s) through opioid receptors. The main use of opioids is in pain relief. The group consists of natural opiates (alkaloids of the opium plant, e.g. morphine), semi-synthetic opiates (e.g. heroin) and synthetic opioids such as methadone (Callahan *et al.*, 2004).

Opioid receptors are signal transducers located in the cell membrane, which exerts their effect through G-protein linked signalling pathways (Martini and Whistler, 2007; Henriksen and Willoch, 2008). There are 3 subgroups of opioid receptors, the μ, κ, and δ opioid receptor (Ree *et al.*, 1999; Henriksen and Willoch, 2008; Trescot *et al.*, 2008). Both methadone and morphine bind to the μ-subtype (Taracha *et al.*, 2006; Martini and Whistler, 2007; Henriksen and Willoch, 2008), although with different affinity (Kristensen *et al.*, 1995; Liu *et al.*, 1999).

Stimulation of the MOR activates intracellular second-messenger systems regulating metabolic enzymes, ion channels, transcription (Wallisch *et al.*, 2007; Henriksen and Willoch, 2008), and a number of protein kinases, including Calcium/calmodulin kinase II (CaMKII) and Mitogen activated protein kinase (MAPKs) (Ma, 2003). Acute activation of the receptor inhibits adenylyl cyclase, decreases Calcium (Ca^{2+})

influx and increases Potassium (K^+) efflux (Mestek *et al.*, 1995), while chronic stimulation has the opposite effect (Avidor-Reiss *et al.*, 1996).

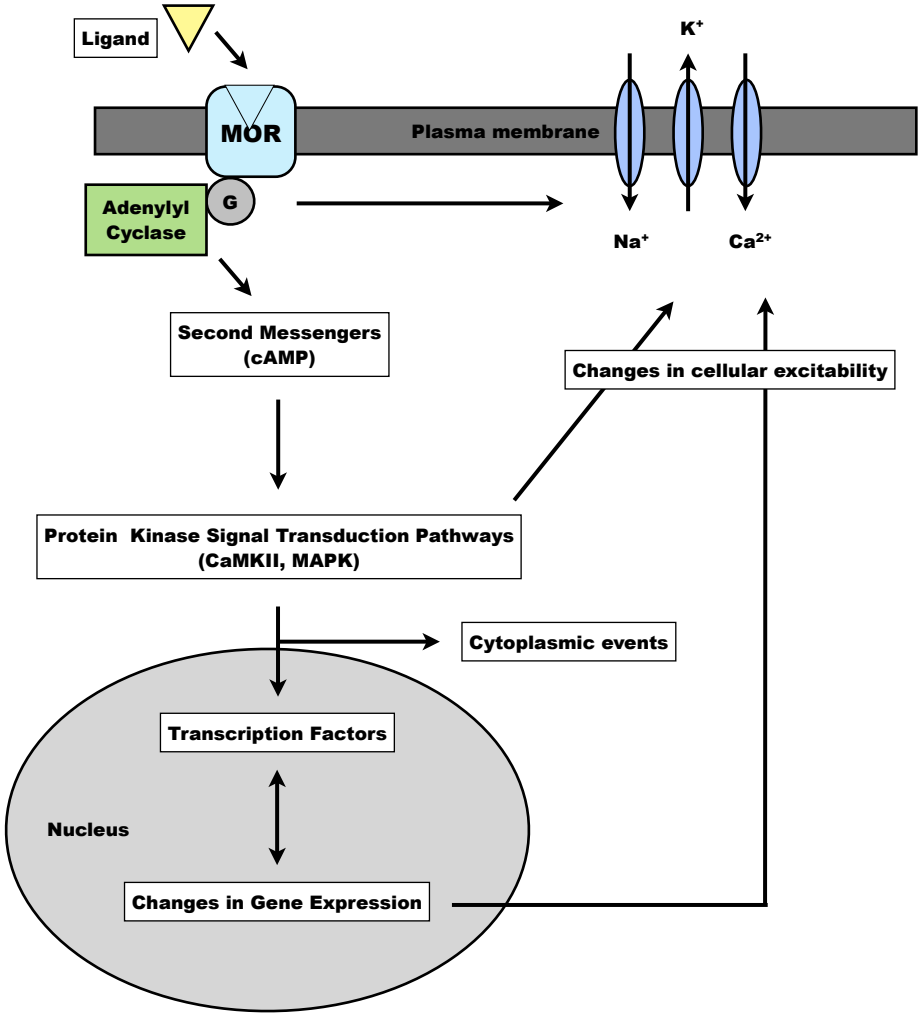


Fig. 1-2 *Simplified schematic figure of signal transduction through the MOR. MORs modulate the levels of second messengers (e.g. cAMP and Ca^{2+}), which in turn regulate the activity of different protein kinases. Such kinases affect the function of proteins located in the cytoplasm, plasma membrane, and the nucleus. Among membrane proteins affected are ligand-gated and voltage-gated ion channels. Protein kinases may also affect the activity of transcription factors. Figure modulated from Koob and Moal (2005).*

1.2 Learning and memory

Memory is the mental ability to store, retain and recall information. Learning is defined as acquiring new knowledge, behaviours, skills, values, preferences or understanding (Lynch, 2004).

Both learning and memory are examples of neuronal plasticity, the ability of the brain to change function and structure in response to internal or external stimuli (Miyamoto, 2006). In 1973, Bliss and Lømo identified long-term potentiation (LTP) (Silva, 2003; Miyamoto, 2006). LTP describes long-lasting changes in the communication between two neurons that results from simultaneous stimulation (Cooke and Bliss, 2006). LTP is widely considered to be one of the major mechanisms underlying learning and memory (Pu *et al.*, 2002; Lynch, 2004; Thomas and Huganir, 2004; Cooke and Bliss, 2006; Rudy, 2008), and involves a signal transduction cascade that includes release of glutamate, activation of NMDA receptor and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor, Ca^{2+} entry, and activation of intracellular signalling molecules/cascades (Miyamoto, 2006).

1.2.1 Hippocampus

The hippocampus is located inside the medial temporal lobe of the cerebral cortex (fig. 1-3) and is part of the limbic system. The limbic system has a variety of functions including emotions, behaviour, long-term memory and olfaction. It has been shown that the hippocampus plays a vital role in formation of memory (Pu *et al.*, 2002; Lynch, 2004).

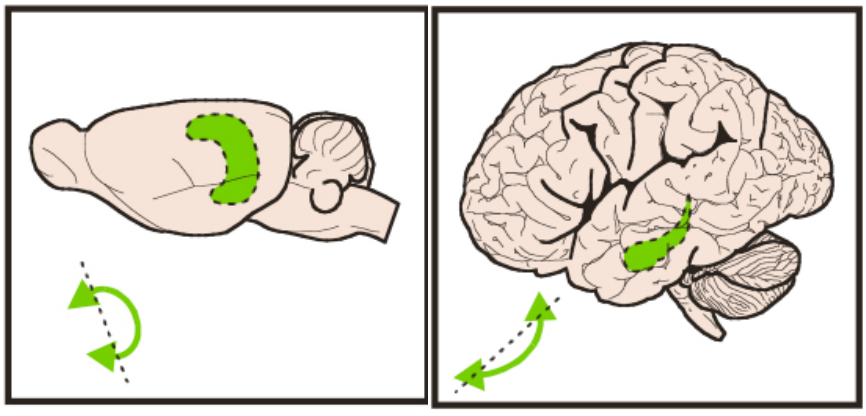


Fig. 1-3 *Location and relative size of hippocampus in the rat brain and in the human brain (Missler and Runkel, 2009)*

The hippocampus has many of the same functions in rats as in humans, i.e. memory processing and navigation (Burgess *et al.*, 2004). By inducing hippocampal damage in

animals, severe deficits in spatial learning and memory are produced (Eichenbaum, 2000).

Lifelong neurogenesis occurs for cells in the hippocampal dentate gyrus in rodents, primates and man (Altman and Das, 1965; Eriksson *et al.*, 1998; Gould *et al.*, 1999). It seems that new neurons are related to important functions in learning and memory (Gould *et al.*, 1999; Praag *et al.*, 1999), and that opioids may have a negative impact by decreasing neurogenesis (Harburg *et al.*, 2007). In rats, chronic administration of morphine reduces neurogenesis in the granular cell layer of the hippocampus by approximately 40% (Eisch *et al.*, 2000). At least *in vitro,* the inhibitory effects of morphine on the proliferation of astroglia seems to be mediated directly through the MOR (Hauser *et al.*, 1996), which the hippocampus expresses widely (Pu *et al.*, 2002).

1.2.2 NMDA receptor

The NMDA receptor is a ligand- and voltage-gated ion channel (Bear *et al.*, 2007). Glutamate is the primary ligand, but the receptor has several binding sites for different modulators (Rudy, 2008). The receptor is widely distributed in the brain, especially in the hippocampus, and it is thought to play an important role in synaptic plasticity (Cooper *et al.*, 2003; Wang *et al.*, 2006; Rudy, 2008).

The NMDA receptor is activated by the combined action of cell membrane depolarisation and glutamate binding (Rudy, 2008). Activation of the receptor results in Ca^{2+}entry and downstream activation of several proteins, e.g. CaMKII (Reul and Chandramohan, 2007), which are important for LTP induction (Miyamoto, 2006; Rudy, 2008). Methadone may affect these processes by acting as an antagonist on the NMDA receptor.

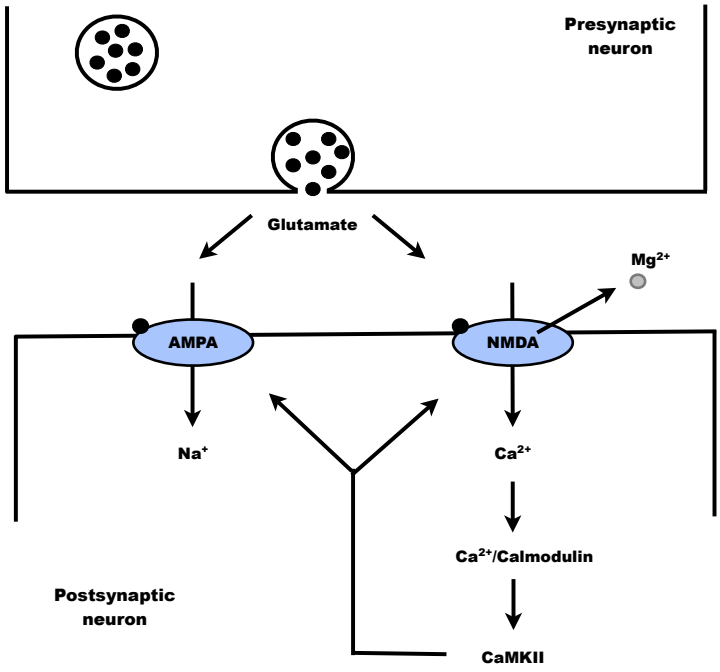


Fig. 1-4 *Simplified schematic figure of signal transduction through the NMDA receptor. Glutamate is released from the presynptic neuron, passes through the synaptic cleft and binds to the NMDA receptor and the AMPA receptor. Increased influx of sodium (Na^+)through the AMPA receptor leads to a depolarisation of the membrane, and removal of magnisium (Mg^{2+}) from the NMDA receptor channel. Opening of the NMDA receptor allows Ca^{2+} influx, which can bind to calmodulin, and further activate proteins like CaMKII. Figure modulated from Rudy (2008).*

1.3 CaMKII and ERK in learning and memory formation

A major regulatory mechanism of signal transduction is protein phosphorylation (Yamauchi, 2005).

1.3.1 CaMKII

The Ca^{2+} ion is an universal secondary messenger in eukaryotic cells. It plays an essential role in the basic operation of neurones through synaptic communication. The predominant intracellular receptor of Ca^{2+} is calmodulin (Yamauchi, 2005). The Ca^{2+}/ calmodulin complex phosphorylates the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) (Miyamoto, 2006). CaMKII exists in at least 5 isoforms throughout the body, with two of them being expressed in nervous tissue (α- and β-isoform) (Lou *et*

al., 1999). Phosphorylated CaMKII (p-CaMKII) has a wide substrate specificity and phosphorylates more than 50 different proteins (Yamauchi, 2005). Accordingly, CaMKII regulates several different neuronal functions, including neurotransmitter synthesis, neurotransmitter release, modulation of ion channel activity, cellular transport, cell morphology and neurite extension, synaptic plasticity, and gene expression (Yamauchi, 2005). There is a large body of evidence indicating that CaMKII plays an important role in LTP (Nicoll and Malenka, 1999).

CaMKII is highly expressed in the hippocampus in both man and rodents (Silva, 2003), but also in other parts of the brain (Fukunaga and Miyamoto, 2000; Yamauchi, 2005). The kinase constitutes about 2 % of the total protein amount in the hippocampus (Schulman, 1993). Genetic deletion of α-CaMKII in rats showed impaired hippocampal LTP, and impaired spatial learning in a hippocampal dependent task (Silva *et al.*, 1992; Yamauchi, 2005).

CaMKII can phosphorylate both NMDA receptors and AMPA receptors (Fukunaga and Miyamoto, 2000). This activation of the receptors may facilitate insertion of AMPA receptors in the plasma membrane and/or increase the conductance of existing channels (Cooke and Bliss, 2006), stimulating LTP.

1.3.2 ERK

The Extracellular signal-regulated kinase (ERK) MAP kinase signalling pathway plays a critical role in learning, memory and other cognitive processes (Thomas and Huganir, 2004; Davis and Laroche, 2006; Samuels *et al.*, 2009). ERKs are activated in response to a number of different extracellular stimuli e.g. Ca^{2+} (Thomas and Huganir, 2004; Cammarota *et al.*, 2008) and CaMKII (Lynch, 2004), and activate several downstream proteins. Both activation of the NMDA receptors and increased release from intracellular storage are responsible for the increase in intracellular Ca^{2+} concentration (Reul and Chandramohan, 2007), which may activate ERK.

The ERK family consists of 5 different isoforms, where ERK1 and 2 has been linked

to LTP formation (Cammarota *et al.*, 2008). Inhibition of the ERK2 signalling pathway has been shown to block both early and late LTP in the hippocampus (Lynch, 2004; Cammarota *et al.*, 2008)

ERK1 and 2 share 84% sequence identity and have the same substrate specificity. ERKs are most highly expressed in neurons compared to other cell types, and the relative expression of ERK2 is greater than that of ERK1 (Samuels *et al.*, 2009).

1.4 Animal models on hippocampal function

There are several tests available for measuring hippocampal learning and memory. The most commonly used is the Morris Water Maze, which map spatial memory (Morris, 1984). In the present work, we have used a Novelty-test. In this test, both the preference for novelty, exploratory activity and locomotor activity are measured (Myhrer, 1988). The test does not involve elements of reward, but is based on the rodents' innate tendency to explore novel objects ahead of familiar ones.

The animal species most commonly used in these tests are rats. This is because of their explorative nature and natural curiosity.

Fig. 1-5 *Picture showing housing of Wistar rats.*

1.5 Animal research and ethics

The research presented in this thesis could not have been conducted without the use of animals. The purpose of animal research is to investigate biological and medical questions related to the health of humans and animals (Hem *et al.*, 2001). Animal research in Norway is regulated by the animal rights law given by the agriculture department. The law states that animal research cannot be conducted without approval from the National Animal Research Authority (Smith, 1999). The possible suffering of the animals must be weighed against the probable gain in each study. Alternatives

must always be considered, and the level of pain must be minimised. All animals exposed to large sufferings which cannot be eased, must be put to sleep immediately. Animal research can only be conducted with the necessary approvals; approved facilities, an approved responsible person and an approved project description (Medline Plus Hem *et al.*, 2001). At the Norwegian Institute of Public Health (NIPH) these criteria are fulfilled.

1.6 Aims of the study

There is a widespread hypothesis that methadone affect cognitive functions. However, a vide variety of factors may contribute to the cognitive impairment observed in methadone users (e.g. poor dietary habits and health status, concurrent abuse of alcohol and other drugs, opiate overdoses; (Darke and Ross, 1997; Nazrul *et al.*, 2002), and controlled randomised long-term administration of methadone to a non-drug abusing human population is restricted by ethical considerations. A feasible approach to study the possible negative effects of methadone on cognitive functions is therefore experimental animal studies. Such studies can also more easily characterise the underlying neurobiological mechanisms.

In this thesis we have combined *in vivo* studies and *ex vivo* experiments to answer the following specific research questions:

1. How is novelty-preference (attention) in rats affected after long-term administration of methadone?
2. Is the possible impaired cognitive performance a result of changes in intracellular signalling molecules?

2. MATERIALS AND METHODS

2.1 Animals and Chemicals

2.1.1 Animals

Adult, male Wistar rats (n = 54), weighing 270 ± 15 g when acquisition started, were delivered by a commercial supplier (Møllegaard Breeding laboratories, Denmark). On the day of arrival the rats were approximately 60-80 days old. The rats were housed in a climatized vivarium (21°C, illuminated from 08:00 to 20:00 h), and had free access to commercial rat pellets and water. On arrival the rats were placed 2-3 rats per cage. After 5 days they were separated into their own cage (38 cm x 22 cm x15 cm) in which they lived during the injection period and the behavioural testing. All animals were handled individually by exploring a tabletop for ~3 min every day for 2-4 weeks.

All experiments were conducted during the light period of the day-night cycle between 08:00 and 14:00 h. The experiments were approved by The National Animal Research Authority.

2.1.2 Chemicals

Listed according to manufacturer:

BDH Laboratory Supplies (Poole, England)
AnalaR® ammonium formate

BioRad (Oslo, Norway)
30% Acrylamide/Bis solution 29:1, Ammonium Persulphate (APS), Precision Plus Protein Standard, Sodium dodecyl sulphate (SDS), TEMED, Tris(hydroxymethyl)-aminomethane (Tris-base)

Cell Signalling (Oslo, Norway)

Anti-biotin (HRP-linked antibody), Biotinylated Protein Ladder, anti-CaMKII, anti-Phospho-CaMKII

Lab-Scan (Gliwice, Poland)

Acetonitrile, Methanol

Merck (Drammen, Norway)

Folin Ciocalteus Reagens, Leupeptin Hemisulphate Salt, Sodium Hydroxide (NaOH), 2-propanol, Sodium chloride (NaCl), Sucrose

Millipore (California, USA)

Reblot Mild

NMD (Oslo, Norway)

Morphine hydrochloride (Morphine HCl $3H_2O$; MW 375.9)

Pierce (Rockford, USA)

SuperSignal West Dura Luminol, SuperSignal West Dura Stable Peroxide Buffer

Santa Cruz Biotechnology Inc (Heidelberg, Germany)

Anti-ERK, Anti-Phospho-ERK, Goat Anti-Mouse IgG-HRP, Goat Anti-Rabbit IgG-HRP

Sigma (Oslo, Norway)

Albumin bovine serum (BSA), Aprotinin, Bromphenol blue, Copper(II)sulphate pent hydrate ($CuSO_4$), Glycerol 2-phosphate disodium salt hydrate, Mercaptoethanol, (±)-Methadone hydrochloride (Methadone HCl; MW 345.9), Pepstatin A, Phenylmethanesulfonylfluoride (PMSF), Ponceus S, Potassium Sodium Tartrate Tetra hydrate (K(Na)tartat), Skim Milk Powder, Sodium Carbonate (Na_2CO_3), Trichloroacetic acid, Trizma hydrochloride, 5-sulfosalicylic acid dehydrate (Tris-HCl)

2.2 Methods

2.2.1 Administration of methadone

The rats were randomly assigned to one of three experimental groups. Each experimental group consisted of 7-10 animals. The animals were acclimatised to the animal house conditions for 5 days before the injection regime started. The rats were injected subcutaneous (sc) with either methadone or morphine. 0.9% NaCl was used as control. The animals were subjected to daily handling in the same room by the same experimentator. The rats were weighed before, during and after the injection regime.

The first two days, the rats received 2.5 mg/kg, the next three days the dose was increased to 5 mg/kg, and the last two weeks they were given 10 mg/kg. All injections were given in total volumes of 0.01 ml/10 g rat. The injections were given Monday to Friday in the morning (8 am - 11 am).

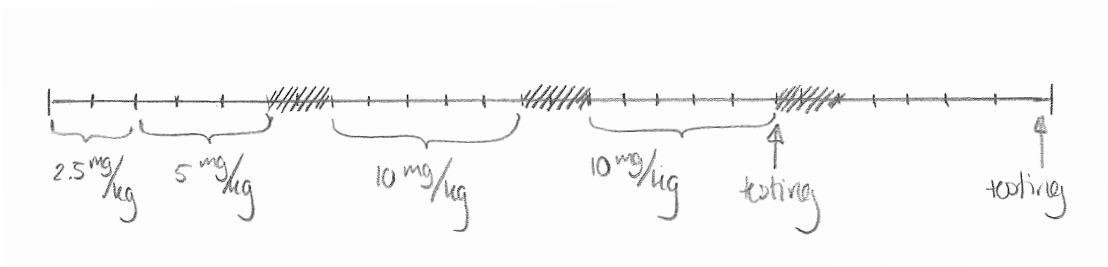


Fig. 2-1 *Injection course. One daily injection was given Monday to Friday for 3 weeks. The two first days the dose was 2.5 mg/kg, the next three days the dose was 5 mg/kg, and the last two weeks the dose was 10 mg/kg. Control animals received 0.9% NaCl.*

2.2.3 Novelty-test

Apparatus:

The behavioural testing was carried out in a Plexiglas cage (56 cm x 34 cm x 20 cm) previously described (Myhrer, 1988). The floor was divided into 18 equal squares (9 cm x 11 cm). In the middle of the cage three identical aluminium cubes (5 cm x 5 cm x 5 cm) were evenly distributed in fixed positions (neutral objects). The novel object differed in that two of its sides were uneven with tracks (visual/tactile stimuli) and its size was smaller (4.5 cm x 4.5 cm x 4.5 cm). All the four objects were painted grey.

The only light in the test room was an office lamp (15 W) placed 60 cm above the cage. The testing room was provided with a fan to make white noise.

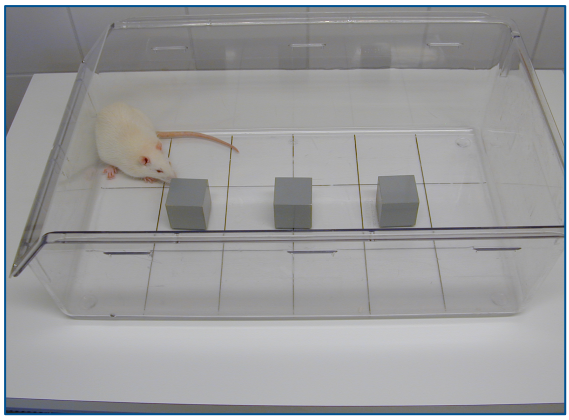


Fig. 2-2 *Picture showing the rat in the test apparatus with three neutral objects.*

Procedure:
The day before testing, the rats were allowed to individually explore the empty test cage for 15 min. Each animal was tested individually.

Prior to the testing of each rat, the apparatus and objects were carefully washed with soap (PEP) dissolved in water and allowed to dry. The test was divided into two phases. In phase 1, the rat was presented with the three neutral objects. In phase 2, the neutral object positioned in the middle was replaced by the novel object. Phase 1 and phase 2 lasted for 5 min, and was interrupted by a 10 min break, where the animal was placed back in its home cage. The following behaviours were recorded: number of sec in contact with each object, number of squares traversed (locomotor activity), and number of times animal reared (standing on their rear legs). Exploration of an object was defined as directing the snout towards the object at a distance of 1 cm or less. Bodily touch other than the snout was not considered as exploratory behaviour. Preference for novelty was calculated as the difference between exploration of the novel object versus the mean exploration of the two neutral objects in phase 2.

2.2.4 Acquiring samples and sample preparation

After the behavioural testing, the animals were decapitated under light CO_2

anaesthesia and the brains removed. The hippocampus was dissected on ice and directly frozen in liquid nitrogen (-196°C). The samples, and the rest of the brain tissue, were stored in a freezer at -80°C until use.

Homogenisation:
The hippocampus was weighed and homogenised (450 rpm) in homogenisation buffer (0.32 M sucrose, 0.01 mg/ml leupeptin, 0.01 mg/ml aprotinin, 0.005 mg/ml pepstatin A, 0.5 mM PMSF) to make a 5% homogenate. Aliquots of 50 µl homogenate were frozen (-80°C) for later analysis.

2.2.5 Protein analysis – Lowry

The amount of protein in the hippocampus homogenate was determined as described (Lowry *et al.*, 1951). In this method there will be a colour reaction between phosphomolybdic ions and proteins, if the proteins first are exposed to copper (Cu^{2+}) in an alkaline environment.

3.5 µl of the 5% homogenate was added 200 µl dH_2O. The reaction was started by adding 1 ml application solution (2% Na_2CO_3 in 0.1 M NaOH, 0.5% $CuSO_4x5H_2O$, 1% K(Na)tartrate; 98:1:1). Each sample was mixed well. After 10 minutes, the reaction was stopped by adding 100 µl stop reagent (Folin-Ciocalteus reagent, dH_2O; 1:1) and mixed well. Three parallels were made of each sample. The samples were placed on the bench for a minimum of 30 min before the absorbance was read at 750 nm by a spectrophotometer (Lambda 3; Perkin Elmer).

The standards were: blind (0 µg BSA), standard 1 (5 µg BSA), standard 2 (10 µg BSA), standard 3 (15 µg BSA), standard 4 (20 µg BSA), standard 5 (25 µg BSA) and standard 6 (35 µg BSA). BSA was dissolved in dH_2O.

2.2.6 Western blotting

The presence of proteins was semi qualitatively demonstrated by Western blotting, as

described by Burnette (1981). The method includes the following steps: separation of proteins by gel electrophoresis, transfer of proteins from the gel to a nitrocellulose membrane, identification of proteins with specific monoclonal antibodies and visualisation of protein-antibody-complexes with chemiluminescense.

Western blot samples:
50 µl of the 5% homogenate was mixed with 10 µl 10% SDS. Then 34 µl of a 3x sample buffer (0.015 mg/ml leupeptin, 0.015 mg/ml aprotinin, 0.0075 mg/ml pepstatin A, 0.7 mM PMSF) was added, and finally 3.6 µl mercaptoethanol was added. The samples were boiled for 5 min at 100 °C (under an outlet, due to the mercaptoethanol). The samples were mixed well between each step.

2.2.6.1 SDS-PAGE

SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) is used to separate proteins according to molecular weight. By binding to SDS, the proteins become negatively charged. When voltage is turned on, the SDS-polypeptide will move through the polyacrylamide gel and be separated into different bands representing distinct molecular masses. The bands can be identified by using molecular weight standards and specific antibodies.

The glass plates were washed with 70% ethanol and dried before assembling the gel mould.

The 12% resolving gel (3.3 ml dH_2O, 4.0 ml 30% Acrylamide/Bis solution 29:1, 2.5 ml 1.5 M Tris (pH 8.8), 0.1 ml 10% SDS, 0.1 ml 10% APS, 0.004 ml TEMED) was prepared and pipetted between two glass plates, slowly to avoid bubbles. Then 100 µl 2-propanol was carefully pipetted over the gel to make the surface smooth. The gel was left for 30 min to polymerise.

After the resolving gel was polymerised, the 2-propanol was washed off with dH_2O and the gel was dried carefully. The stacking gel (6.1 ml dH_2O, 1.3 ml 30%

Acrylamide/Bis solution 29:1, 2.5 ml 0.625 M Tris (pH 6.8), 0.1 ml 10% SDS, 0.05 ml 10% APS, 0.005 ml TEMED) was then prepared. This solution was pipetted on top of the separation gel. The comb was placed into the stacking gel which was left to polymerise (20-30 min).

When the stacking gel was polymerised the comb was removed and the wells washed carefully with dH_2O. The electrophoresis chamber was assembled, and filled with 1x electrophoresis buffer (25 mM Tris(hydroxymethyl)-aminomethane, 0.2 M glycine, 3.4 mM SDS). The electrophoresis was performed at 100 V the first ~10 min and then at 200 V for ~1 hour. The gel electrophoresis was concluded before the proteins went out of the gel. Two standards were used, a visible molecular standard, Precision Plus Protein Standard, and a Biotinylated Protein Ladder.

2.2.6.2 Blotting

Electrophoretic separated proteins can be transferred from a gel to a nitrocellulose membrane by electroblotting, which is a direct electrophoretic transfer of proteins.

When the SDS-PAGE finished running, the stacking gel was removed. The resolving gel was placed on top of a pre-soaked (in transfer buffer) membrane. Pre-soaked sponges, filter-papers, the gel and the nitrocellulose membrane were assembled as a «sandwich». All air bubbles were carefully removed. The chamber was then filled with 1x transfer buffer (25 mM Tris(hydroxymethyl)-aminomethane, 0.2 M Glycine, 20% methanol). The transfer was performed at 70 V for 45-60 min. After blotting the membranes (blots) were put in Ponceus colour for 2 min to verify that the proteins had been transferred and that approximately the same amount of protein was present in each well.

2.2.6.3 Antibody exposure

The following primary antibodies were used: anti-Phospho-CaMKII (1:2000), anti-Phospho-ERK (1:1000), anti-CaMKII (1:1000) and anti-ERK (1:500 000). The secondary antibodies used were goat anti-rabbit IgG-HRP (1:5000), goat anti-mouse

IgG-HRP (1:5000) and anti-biotin HRP-linked antibody (1:5000).

The blots were pre-incubated in TBS (1.54 M NaCl, 1 M Tris-HCl) for 5 min before being incubated in blocking buffer (3% skimmed milk in TBS) for one hour to avoid unspecific binding. The blots were then incubated for 2 hours at room temperature or overnight at 4°C with the primary antibody. The next day, the blots were washed for 4 x 10 min in cold TBS-T (1.54 M NaCl, 1 M Tris-HCl, 0.5% Tween 20), then incubated in blocking buffer for 30 min and exposed to the secondary antibody for 2 hours at room temperature. After exposure to the secondary antibody the blots were washed for 3 x 10 min in cold TBS-T and then 1 x 10 min in TBS.

2.2.6.4 Detection

Super Signal is a chemiluminescent substrate for Horseradish Peroxidase (HRP). The reaction between the enzyme and the substrate results in light being emitted. The emitted light is proportional with the amount of target protein, and can be detected by a molecular imager.

The blots were incubated in Super Signal detection solution (SuperSignal West Dura Luminol, SuperSignal West Dura Stable Peroxide Buffer; 1:1) for 5 min. Then the redundant fluid was removed and the blots were placed face down, on a plastic foil. The blots were wrapped in the plastic foil, and all air bubbles were removed before the blots were placed in a ChemiDoc XRS imager (BioRad; Norway). A luminescence image was captured every min for 10 min.

2.2.6.5 Antibody stripping

The blots were rinsed with 1x TBS-T (2 x 10 ml), and exposed to 20 mL 1x Re-Blot Plus Mild Solution for 15-25 min. The blots were thereafter rinsed with 1x TBS-T (2 x 10 ml), and incubated 2 x 5 min in 20 ml blocking buffer. The blots were now ready for another antibody exposure.

2.2.7 LC-MS analysis

The presence of methadone or morphine, in the brain tissue was investigated by LC-MS analysis as described (Oiestad *et al.*, 2007). LC-MS is a technique that combines the separation power of HPLC (high performance liquid chromatography) and the detection power of MS (mass spectrometry).

Brain tissue was weighed and homogenised in ice-cold dH_2O (1:2).

100 µl brain homogenate was added 100 µl dH_2O and 50 µl internal standard (5 µM methadone d_9/morphine d_9). The extractions were added to acetonitrile/methanol (85/15), mixed well and placed in the freezer for 10 min. Then the samples were centrifuged (4500 rpm, 10 min) before the organic phase was transferred to a 5 ml glass tubes. The samples were evaporated to dryness under N_2 at 50°C (water bath), added to 100 µl of 3% acetonitrile/97% 5 mM ammonium format buffer (pH 3.1) and transferred to 0.3 ml plastic autosampler tubes.

LC-MS analysis:
This is a well established analysis at The Division of Forensic Toxicology and Drug Abuse, Norwegian Institute of Public Health, and was conducted by department engineer Åse Ripel.

LC-conditions:
Injection volume: 10 µl, mobile phase: A; acetonitrile and B; 5 mM ammoniumacetat buffer pH 5, column: Xterra® MS C18 (2.1 x 150 mm), column temperature: 35°C, software: Mass Lynx, flow rate: 0.3 ml/min, and runtime: 10 min.

Table 2-1 showing the MS conditions

Positive mode (ES+), SIR

Start time:	2.5 min		
Stop time:		10.0 min	
Mass	Cone (V)	Coll (eV)	Substance
310.0	25.00	20	Methadone
319.0	20.00	20	Methadone-d9 (internal standard)
286.0	45.00	20/40	Morphine
292.0	45.00	25.00	Morphine-d9 (internal standard)

2.2.8 Statistical analysis

All figures were constructed with SPSS (Version 16.0). All results are expressed as box-and-whisker plot, showing 5, 25, 50, 75 and 95 % cumulative relative frequencies (centiles). Outliers are not shown. The data analyses were performed with SPSS (version 16.0). Some of the data were not normally distributed, therefore the Kruskal-Wallis test was used to compare the groups. The Kruskal-Wallis test is a non-parametric test that compares several populations on the basis of independent samples from each population. The test ranks the responses from all groups together and then applies one-way ANOVA to the ranks rather than to the original observations. Pairwise comparison was used to determine which of the groups were different. P-values ≤ 0.05 was stated as significant.

3. RESULTS

3.1 Novelty-test

The animals subjected to three weeks of methadone or morphine injections were tested in a novelty-test one hour and one week after the last injection.

3.1.1 Novelty-test one hour after the last injection

One hour after the last injection, there was a difference in novelty-seeking between the groups ($H = 11.75$, 2 d.f., $p \leq 0.05$, $n = 7\text{-}8$). The animals subjected to methadone had no novelty-seeking compared to the control animals ($p \leq 0.01$, fig. 3-1).

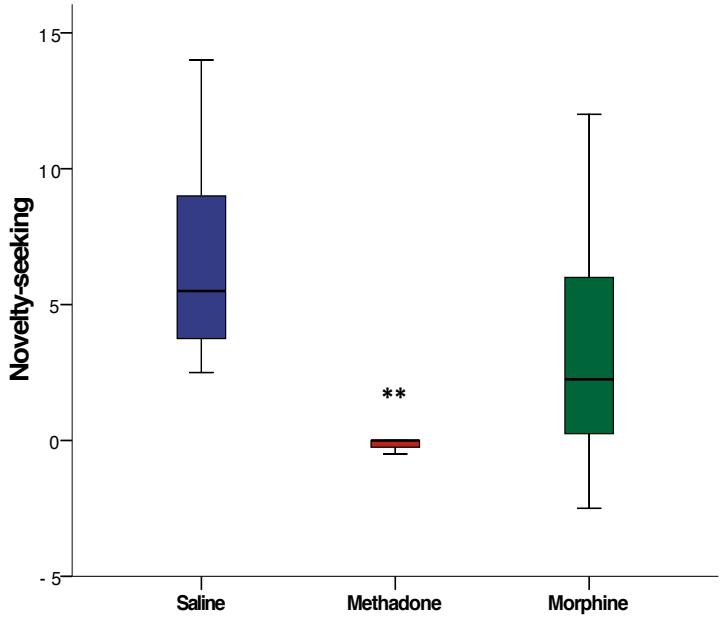


Figure 3-1 *Animals subjected to methadone (n = 8) for three weeks and tested one hour after the last injection showed no novelty-seeking. The morphine (n = 8) injected animals did not differ from the control group (n = 7). Novelty-seeking was measured as time in contact with novel object divided by the mean of time in contact with neutral objects in phase 2. ** $p \leq 0.01$, Kruskal-Wallis, compared to control.*

The locomotor activity of the groups differed from each other in both phases (phase 1: H = 20.5, 2 d.f., $p \leq 0.05$, n = 7-8, phase 2: H = 16.3, 2 d.f., $p \leq 0.05$, n = 7-8). The methadone injected animals showed little or no locomotor activity ($p \leq 0.05$, fig. 3-2a), and displayed «intoxication» behaviour such as increased salivation. The animals subjected to morphine ran around the test cage far more than the control animals and the run pattern was along the cage walls (not around the objects). The same differences were seen for rearings (standing on the rear legs, fig. 3-2b), a widely used measurement of explorative behaviour (phase 1: H = 18.0, 2 d.f., $p \leq 0.05$, n = 7-8, phase 2: H = 11.6, 2 d.f., $p \leq 0.05$, n = 7-8).

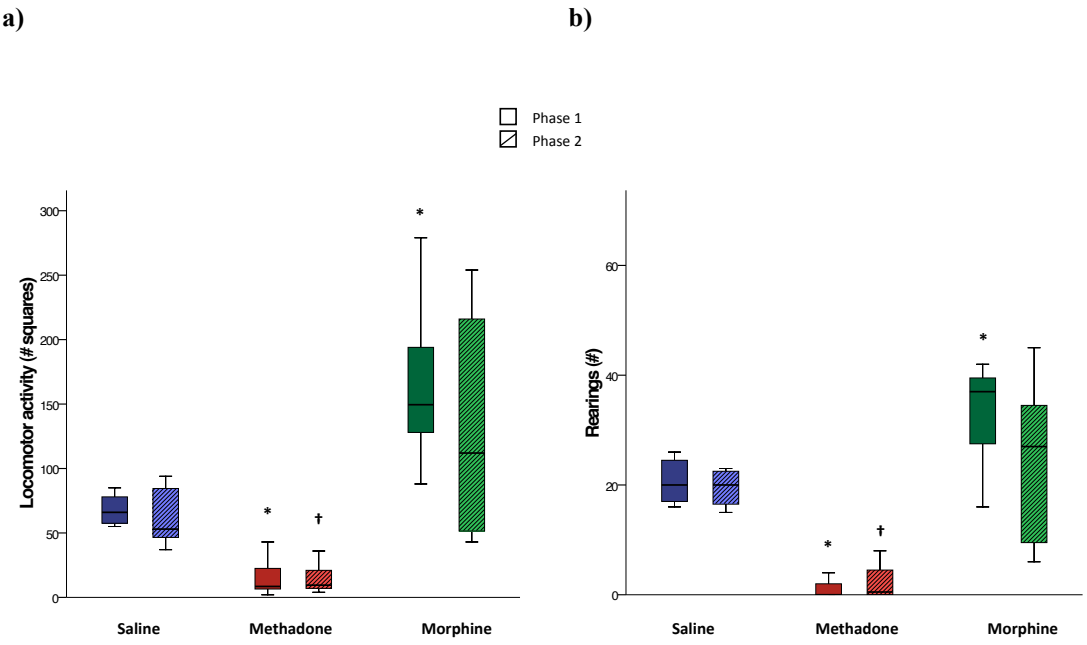


Figure 3-2 *a) Locomotor activity and b) number of rearings in animals subjected to saline, methadone or morphine for three weeks and tested one hour after the last injection. The locomotor activity was measured by counting number of squares crossed in each phase. There was a difference in locomotor activity in both phase 1 and 2 (n = 25). There was a significant difference in number of rearings in phase 1 and 2 (n = 25). * $p \leq 0.05$, Kruskal-Wallis, compared to control phase 1. † $p \leq 0.05$, Kruskal-Wallis, compared to control phase 2.*

The rats weighed 270 ± 1.5 g (n = 25) when the injections started (H = 3.88, 2 d.f., p > 0.05, n = 8-10). All the animals gained or maintained their weight during the first two weeks. The third week, the animals subjected to methadone showed a small reduction in body weight (average of 10 g, H = 7.27, 2 d.f., p ≤ 0.05, n = 3-8, fig. 3-3). At the day of testing the animals weighed between 260 and 300 g.

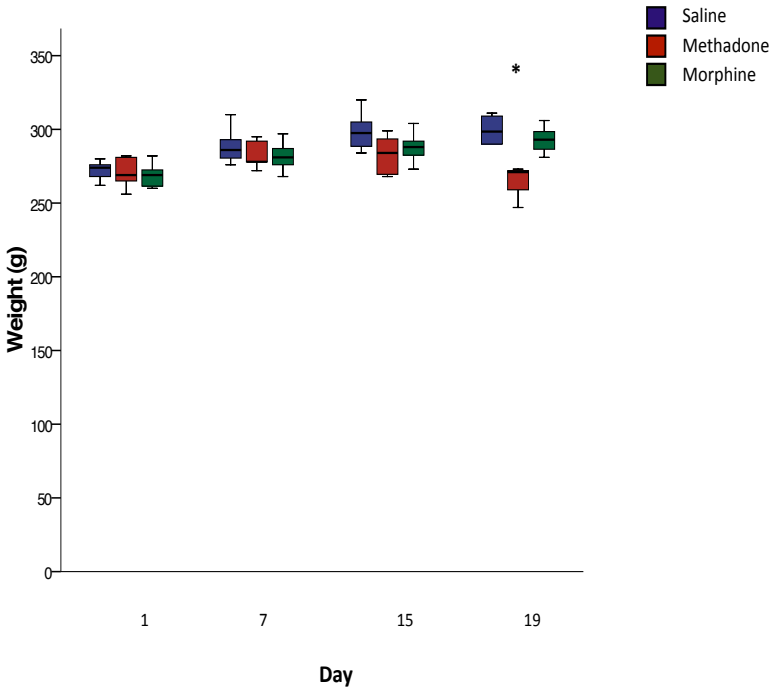


Figure 3-3 *Weight of animals subjected to saline, methadone or morphine for three weeks and novelty-tested one hour after the last injection (n =25). There were no differences between the groups on the first day of injection. At day 19, the last day of injection, there was a difference between the groups. * p ≤ 0.05, Kruskal-Wallis, compared to control.*

3.1.2 Novelty-test one week after the last injection

One week after the last injection, there were no significant differences in the novelty-seeking between the groups (H = 1.06, 2 d.f., p > 0.05, n = 7-10, fig. 3-4).

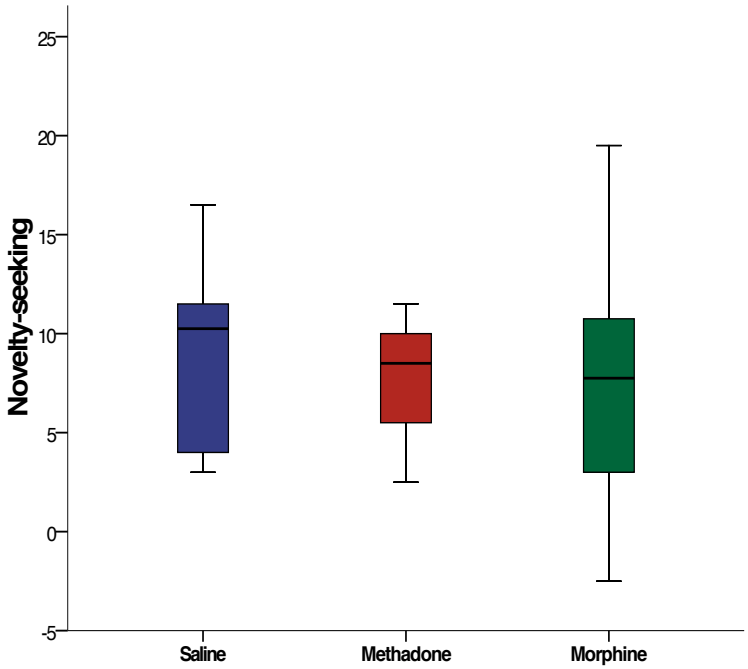


Figure 3-4 *Novelty-seeking in animals subjected to methadone (n =7) or morphine (n = 8) for three weeks and tested one week after the last injection did not differ from the control (n = 10) animals. Novelty-seeking was measured as time in contact with novel object divided by the mean of time in contact with neutral objects in phase 2.*

There were no differences in locomotor activity between the groups in each phase (fig. 3-5a) when tested one week after the last injection (phase 1: H = 2.9, 2 d.f., p > 0.05, n = 7-10, phase 2: H = 2.9, 2 d.f., p > 0.05, n = 7-10). There were no differences in the number of rearings between the three groups (fig. 3-5b, phase 1: H = 0.35, 2 d.f., p > 0.05, n = 7-10, phase 2: H = 0.72, 2 d.f., p > 0.05, n = 7-10).

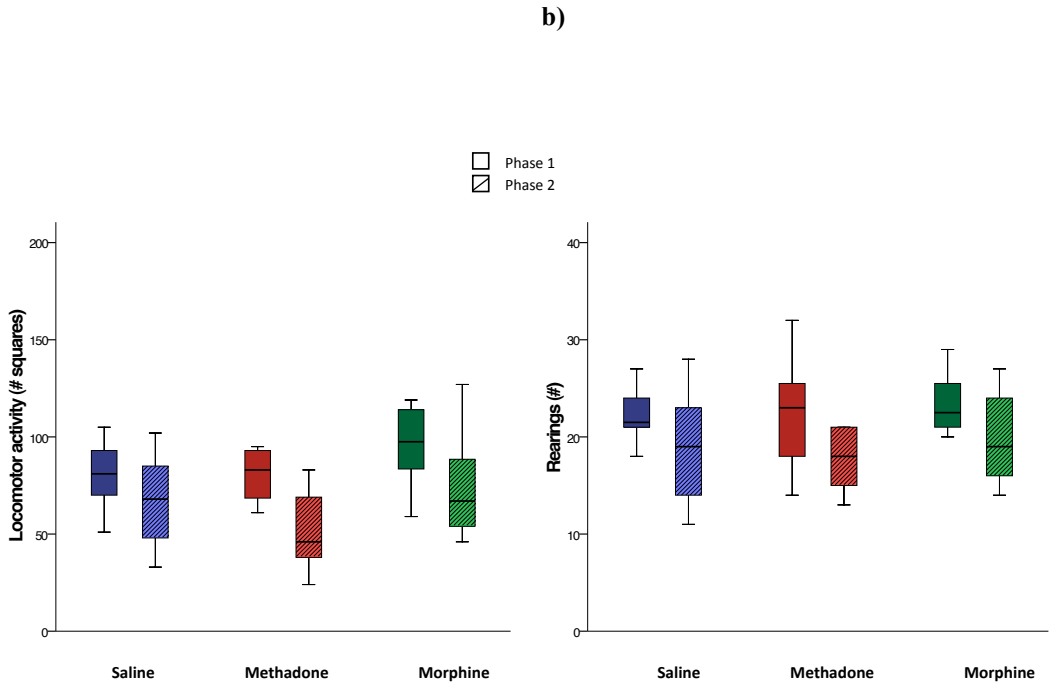


Figure 3-5 *a) Locomotor activity and b) number of rearings in animals subjected to saline, methadone or morphine for three weeks and tested one hour after the last injection. The locomotor activity was measured by counting number of squares crossed in each phase. There were no significant differences in locomotor activity or in the number of rearings between the groups in phase 1, or in phase 2 (n = 25).*

The rats weighed 274 ± 1.5 g (n = 29) when the injections started (H = 0.78, 2 d.f., p > 0.05, n = 8-12). All the animals gained or maintained their weight during the first week. The second and third week, the animals subjected to methadone maintained or showed a small reduction in body weight (average of 10 g, H = 4.5, 1 d.f., $p \leq 0.05$, n = 3-4, H = 9.9, 2 d.f., $p \leq 0.05$). After the injections were concluded (day 19), the animals exposed to methadone gained weight again. At the day of testing, the animals weighed between 300 and 340 g. Animals exposed to methadone or morphine had lower body weight compared to the control animals (H = 7.82, 2 d.f., $p \leq 0.05$, n = 8-10, fig. 3-6).

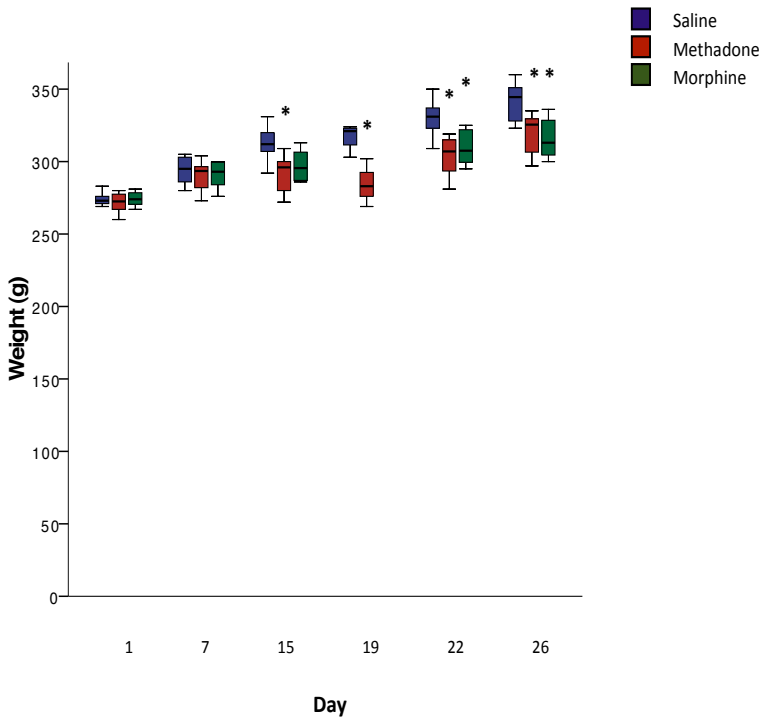


Figure 3-6 *Weight of animals subjected to saline, methadone or morphine for three weeks and novelty-tested one week after the last injection (n =29). There were no differences between the groups at the first day of injection. At day 15 and 19, the last day of injection, the methadone injected animals differed from the control. At day 22 and 26, one week after the last injection, both the methadone group and the morphine group showed a weight difference compared to the control group. * $p \leq 0.05$, Kruskal-Wallis, compared to control.*

3.2 Western blotting

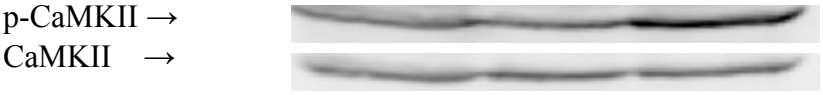
Western blotting was performed in hippocampus homogenate from the rats subjected to three weeks of methadone or morphine injection, as described in chapter 2.2.6. At least three parallels were performed for each sample.

3.2.1 p-CaMKII/CaMKII

The ability of methadone and morphine to induce phosphorylation of CaMKII was shown by use of a phospho-specific antibody that recognises phosphorylated Thr 286. The immunodetectable band of CaMKII was localised at 50 kDa. To compare the

level of p-CaMKII with the total amount of CaMKII protein, the p-CaMKII blots were reprobed with an anti-CaMKII antibody. Only small variations in the total amount of protein were observed. To be able to compare blots from different days, a control-sample were put on each gel.

One hour after the last injection of methadone or morphine, there was no significant difference in p-CaMKII/CaMKII between the control group and the exposed groups ($H = 1.73$, 2 d.f., $p > 0.05$, $n = 7$-8).



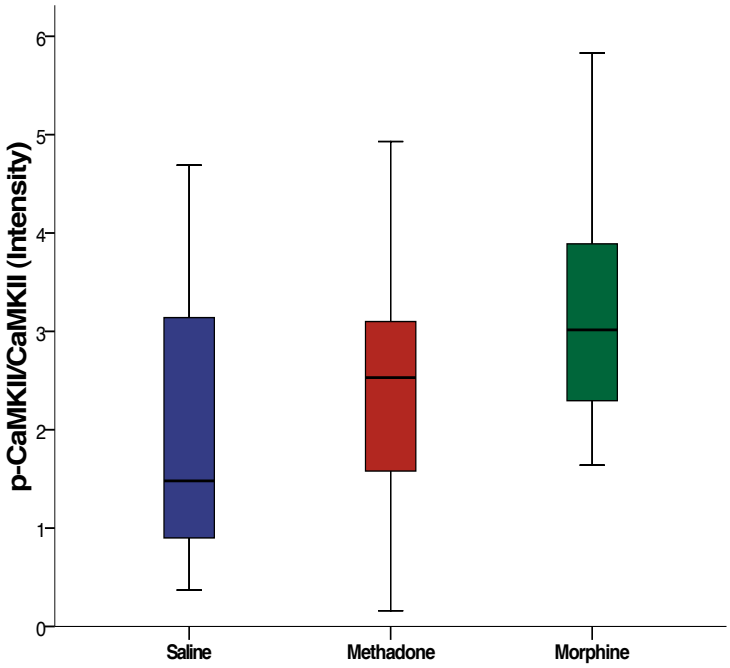


Figure 3-7 *Phosphorylation of CaMKII (p-CaMKII) in methadone (n = 8) and morphine (n = 8) exposed animals compared to control (n = 7) animals novelty-tested one hour after the last injection. Representative immunoblots for p-CaMKII and total CaMKII are shown. There were no differences between the groups. Results are shown as p-CaMKII/CaMKII. Each sample was repeated at least three times.*

One week after the last injection, p-CaMKII/CaMKII was significantly down-regulated in both methadone and morphine treated animals ($H = 11.28$, 2 d.f., $p \leq 0.01$, $n = 7\text{-}10$).

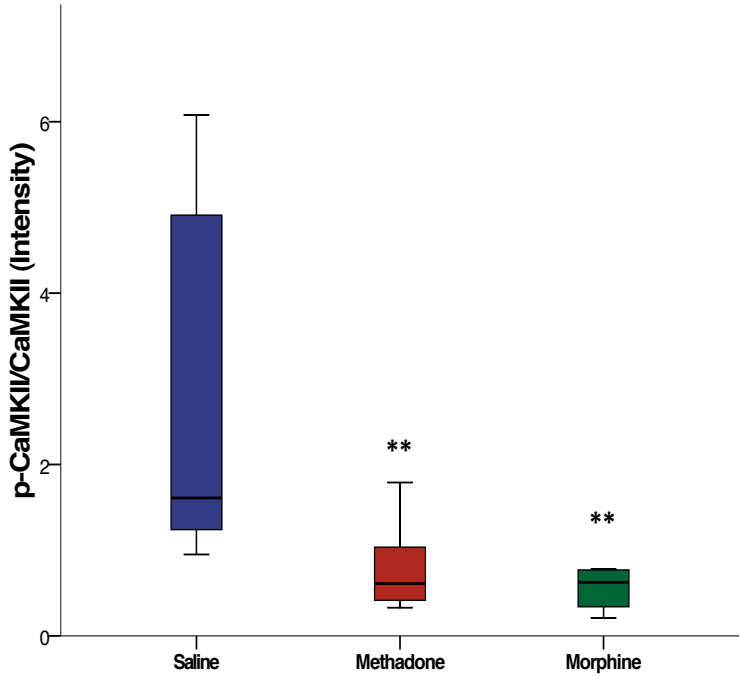


Figure 3-8 *Phosphorylation of CaMKII (p-CaMKII) in methadone (n = 7) and morphine (n = 8) exposed animals compared to control (n = 10) animals novelty-tested one week after the last injection. Representative immunoblots for p-CaMKII and total CaMKII are shown. The methadone subjected animals and the morphine subjected animals were significantly different from the control group. Results are shown as p-CaMKII/CaMKII. ** $p \leq 0.01$, Kruskal-Wallis, compared to control. Each sample was repeated at least three times.*

3.2.2. p-Erk/Erk

The ability of methadone and morphine to induce phosphorylation of ERK was shown by use of a phospho-specific antibody that recognises phosphorylated Tyr 204. The immunodetectable band of ERK2 was localised at 42 kDa. To compare the level of p-ERK with the total amount of ERK protein, the p-ERK2 blots were reprobed with an

anti-ERK2 antibody. Only small variations in the total amount of protein were observed. To be able to compare blots from different days, a control-sample were put on each gel.

One hour after the last injection of methadone or morphine there was a significant difference in p-ERK/ERK between the control group and the exposed groups ($H = 7.3$, 2 d.f., $p \leq 0.05$, $n = 7\text{-}8$). Pairwise comparisons showed that the morphine exposed animals were significantly different from the control group ($p \leq 0.05$), while the methadone exposed animals were not.

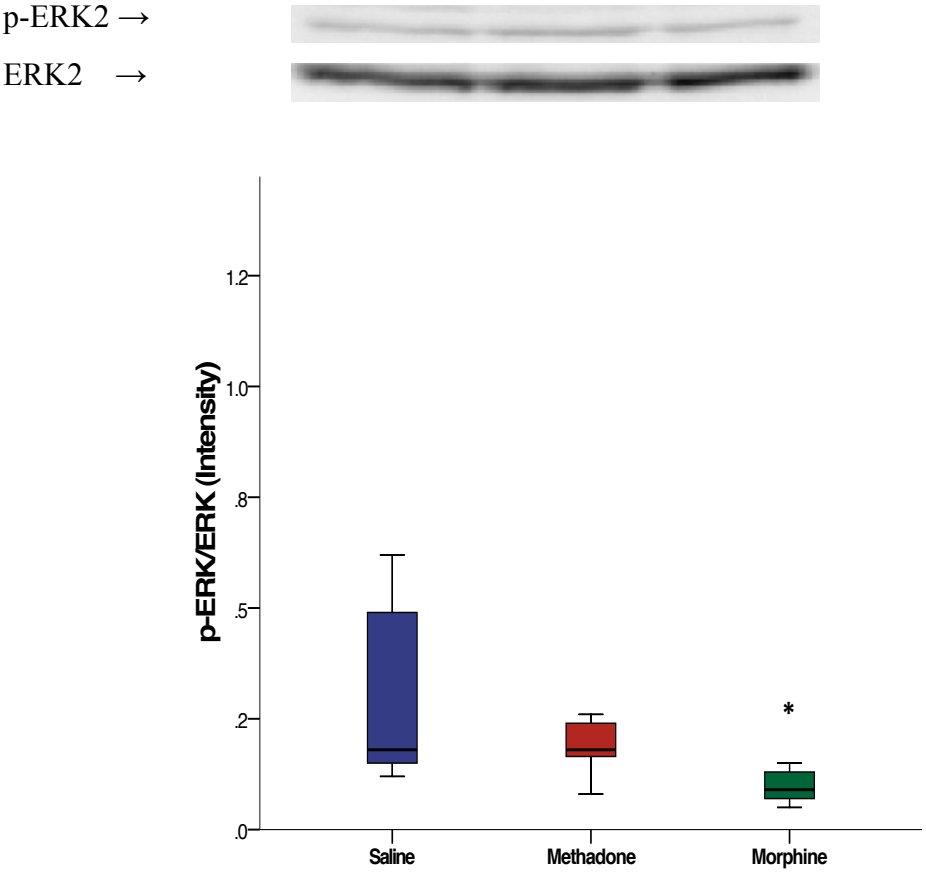


Figure 3-9 *Phosphorylation of ERK2 (p-ERK) in methadone (n = 8) and morphine (n = 8) exposed animals compared to control (n = 7) animals novelty-tested one hour after the last injection. Representative immunoblots for p-ERK2 and total ERK2 are shown. The morphine exposed animals were significantly different from the control group, while the methadone exposed animals were not. Results are shown as p-ERK/ERK. * $p \leq 0.05$, Kruskal-Wallis, compared to control. Each sample was repeated at least three times.*

One week after the last injection, there were no differences in p-ERK/ERK in a) methadone treated animals (H = 0.12, 1 d.f., p > 0.05, n = 8), or in b) morphine treated animals (H = 1.71, 1 d.f., p > 0.05, n = 2-8) compared to the control animals.

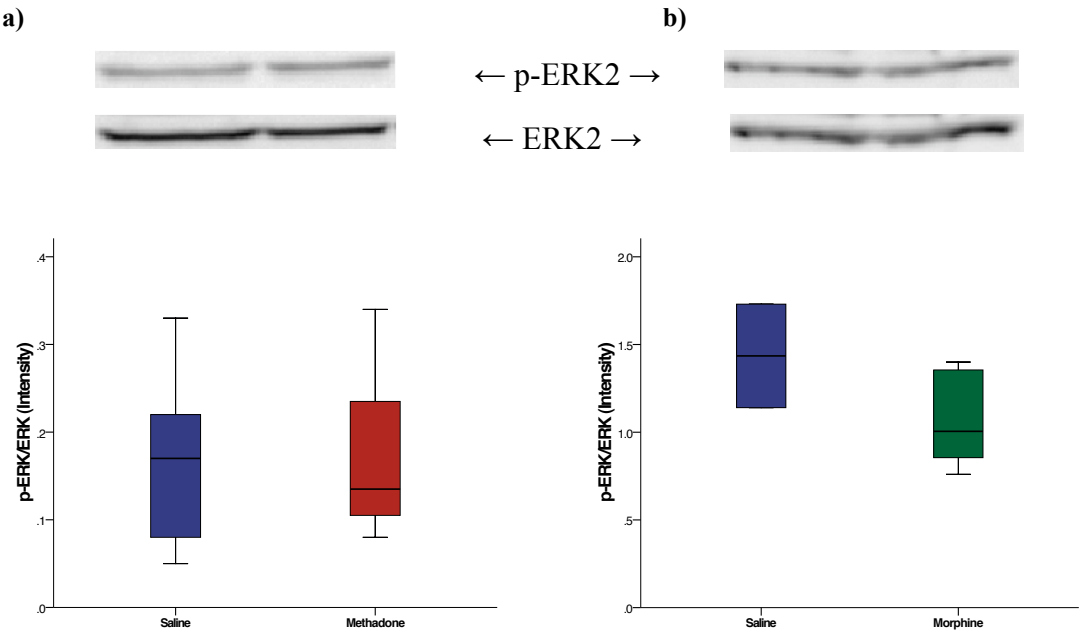


Figure 3-10 *Phosphorylation of ERK2 (p-ERK) in a) methadone (n = 8) and b) morphine (n = 8) exposed animals compared to control (n = 2-8) animals novelty-tested one week after the last injection. Representative immunoblots for p-ERK2 and total ERK2 are shown. There were no significant differences between the methadone exposed animals and the control, or between the morphine exposed animals and the control. Results are shown as p-ERK/ERK. Each sample was repeated at least three times.*

3.3 LC-MS analysis

The LC-MS analysis showed that animals subjected to three weeks of methadone injections and novelty-tested one hour after the last injection had methadone left in their brain tissue (n = 7). One week after the last injection there was no methadone (n = 7) left in the brain tissue. The morphine subjected animals had morphine left in the brain tissue when tested one hour after the last injection (n = 4), but no morphine left after one week (n = 8). Lowest detection level was set to 0.1 µM for both compounds.

Table 3-1 *LC-MS analysis of brain tissue from animals injected with methadone or morphine for three weeks. The animals tested one hour after the last injection had methadone and morphine left in the brain, while there was nothing left one week after the last injection (n = 4-8).*

Time of testing (after the last injection)	Methadone (nmol/g)	Morphine (nmol/g)
One hour	4.01 ± 1.07	0.51 ± 0.13
One week	———	———

4. DISCUSSION

4.1 Novelty-test

One hour after the last injection, the methadone animals were too affected by the methadone to perform the novelty-test. The rats were immobile and showed «intoxication» behaviour such as increased salivation. The LC-MS analysis showed that there was methadone in the brain tissue at this time of testing. This corresponds with the documented half-life of methadone in rats of 3-4 hours (Misra *et al.*, 1973). The behaviour of the morphine subjected animals were different. During the testing they were hyperactive, running around the test cage not seeming to notice the objects. Morphine has been shown to cause hyperlocomotion in rats 1-2 hours after injection (Moriyama *et al.*, 1991; Narita *et al.*, 2004). The plasma half-life of morphine in rats is about 120 min (Iwamoto and Klaassen, 1977; Lee *et al.*, 2005).

One week after the last injection, there were no differences between the exposed groups and the control group. The LC-MS analysis showed that there was no methadone or morphine left in the brain tissue at this time of testing.

In our lab at the Norwegian Institute of Public Health (NIPH), the same test has also been conducted one day after the last injection of methadone (J.M. Andersen; personal communication). At this time, the animals showed a 50% reduction in novelty-seeking. Taken together, these results indicate that long-term exposure to methadone may affect the novelty-seeking in rats, one hour and one day, but not one week after the last injection. The most likely reason for not seeing differences between the groups one week after the last injection compared to the difference one day after the last injection, may be that the effects after long-term administration of methadone and morphine are reversible. This is in accordance with previous work, showing that opioids affect different functions linked to memory and learning, but that the changes may be reversed (Guerra *et al.*, 1987; Ammon-Treiber *et al.*, 2005).

The most widely used test to map cognitive functions in rats is the Morris Water Maze (Morris, 1984). The water maze measures spatial learning and memory, which is a

hippocampal-linked function. In this test, the rat is required to swim repeatedly to an escape platform, hidden below the surface of opaque water (Morris, 1984). Novelty-seeking differs from spatial learning, but is also linked to the hippocampus. In the novelty-test, the animal can choose not to explore the objects, while in the water maze the animals are forced to perform the test, which may result in higher sensitivity. Previous studies have shown that both rats injected with a single injection of methadone (Hepner *et al.*, 2002) and repeated injections of methadone (Tramullas *et al.*, 2007) show an impaired performance in the Morris Water Maze, when tested 15 min and 2-24 hours after the last injection. The same has been seen for morphine (Gorji and Rashidy-Pour, 2008) and heroin (Tramullas *et al.*, 2008). Because of the animal house facilities at NIPH, we were not able to employ the Morris Water Maze, and therefore used the Novelty-test.

To familiarise the animals to the experimentator, the rats were subjected to daily handling. The rats were also allowed to explore the empty test apparatus the day before testing. This was done because animals not familiar with the experimentator or the test cage may not perform the behaviour-test due to fear.

The methadone subjected animals were clearly affected the first hours after injection of the 5 and 10 mg/kg doses. As in humans, there are large individual variations in how well the rats tolerate methadone. During the injection period, four animals died (18%). Methadone depresses the brain stem's respiratory control centre, reducing its sensitivity to carbon dioxide (CO_2) (Corckery *et al.*, 2004), leading to respiratory failure. This is the most likely cause of death in our animals. The deaths occurred the second week when the dose was increased from 5 mg/kg to 10 mg/kg, after two days of not receiving injections (Saturday and Sunday). The deaths may therefore, at least partly, be due to a sensitisation response to the drug (Handal *et al.*, 2008).

The animals injected with methadone for three weeks lost or did not gain weight the second and third week of the injection period. After the injections were terminated, the animals started gaining weight again. It has previously been shown that chronic methadone treatment results in a reduction of body weight in adult rats (Vajda *et al.*,

1975). A great weight loss may affect how animals perform a behaviour-test. However, the weight loss in this study was less than 10%, which is seen as an acceptable weight loss (IACUC, 2004). Moreover, the number of rearings and the locomotor activity did not differ between the groups tested one week after the last injection. It seems therefore not probable that the moderate weight loss should have any effect on the novelty-seeking.

Given that an average methadone dose to MMT patients (weighing 70 kg) is 80-120 mg/day (Joseph *et al.*, 2000) and that the average half-life is 24-48 hours (Curran *et al.*, 2001), the daily doses used in our experiments (2.5-10 mg/kg, $T_{1/2}$ = 3-4 h; Misra *et al.,* 1973) are comparable with the doses administered to MMT patients. The difference is that over time the methadone concentrations in MMT patients will probably increase because of the long elimination half-life, while this is less likely in rats.

Injection of methadone for three weeks to a rat that lives about 2-3 years, is a relative shorter exposure period compared to MMT patients that often use methadone for decades (H. Waal, SERAF; personal communication). In our laboratory, we have observed an effect after just three weeks and may question whether the effect would be even larger and more prolonged with a longer exposure period. Long-term methadone treatment has been associated with impaired cognitive functions (Darke *et al.*, 2000; Specka *et al.*, 2000). However, there has also been shown that the cognitive functions and overall health status of the MMT patients may be improved through a treatment program (Joseph *et al.*, 2000). This may be due to a reduction in alcohol and other drug use, an improved dietary and health status, an expanding social network and more intellectual challenges (Soyka *et al.*, 2008), and not directly to the methadone intake.

The rats injected with methadone showed a tendency to itch/scratch where the injections were put. This resulted in some small wounds in some of the animals. Methadone is known to cause local toxicity when given sc (Bruera *et al.*, 1991; Mathew and Storey, 1999). We therefore switched weekly between giving the

injections on the left and right side of the lower part of the back. We were also careful not to touch the stamp before the needle had penetrated the skin, and wiped the injection site carefully after each injection. In other experiments, long-term exposure to opioids has been performed by administration in the drinking water (Tiong *et al.*, 1992; Sadava *et al.*, 1997). However, we chose to use daily sc injection of methadone because this makes it easier to control the administered dose, and because methadone has an aversive taste (Chipkin and Rosecrans, 1978).

4.2 Western blotting

There were no significant differences in the phosphorylation of CaMKII between the control group, methadone group and morphine group novelty-tested one hour after the last injection. One week after the last injection, there was a down-regulation of p-CaMKII in both methadone exposed and morphine exposed animals.

There was no difference in the phosphorylation of ERK between the animals subjected to methadone, novelty-tested one hour after the last injection, and the control group, while there was a significant difference between the morphine exposed group and the control group. Chronic morphine treatment has been shown to increase (Berhow *et al.*, 1995), to decrease (Schulz and Höllt, 1998; Muller and Unterwald, 2004), or not to influence (Narita *et al.*, 2002) ERK phosphorylation. The down-regulation observed in this study shows that morphine was not able to maintain ERK stimulation one hour after the last injection of a long-term exposure paradigm.

Before the last test series, including 8 morphine injected and 2 saline injected animals, the animal house underwent changes in its procedures. Affecting this study was the ban of using CO_2 anaesthesia, which made our animals stressed during the decapitation procedure. Stress is known to induce ERK phosphorylation (Reul and Chandramohan, 2007), and high expression of ERK has been seen in the hippocampus immediately after tail-shock stress (Yang *et al.*, 2008). When comparing the intensity of p-ERK/ERK in the control animals decapitated with and without CO_2,

the intensity was 10 times higher in the rats decapitated without CO_2. Because of this high «background» we have decided to present the ERK results from this test series and the previous series separately to avoid masking a potential drug effect. There were no differences between the morphine or the methadone exposed groups compared to the control group one week after the last injection. This in accordance with another study using morphine (Valjent *et al.*, 2004). However, to conclude on the effects on ERK, the experiment should be repeated.

When comparing the western blot results from long-term methadone or morphine exposed animals tested one hour after the last injection and one week after the last injection, the phosphorylation of CaMKII was clearly different. One hour after the last injection there was still methadone and morphine left in the brain of the animal. When an opioid is present in the brain it will exert its effect, thereby maintaining a response, even though the «steady state» is changed. One week after the last injection, methadone or morphine was no longer present in the brain. The effect seen a week after the last injection may be due to modulation of cellular responses induced to compensate the long-term receptor stimulation, e.g. activation of adenylyl cyclase (Duman *et al.*, 1988; Beitner *et al.*, 1989; Nestler, 2004), decrease in G-protein coupling (Selley *et al.*, 1997), desensitisation, receptor internalisation (Jordan and Devi, 1998), and receptor endocytosis (Whistler *et al.*, 1999; Fábián *et al.*, 2002; He and Whistler, 2005). When the drug is no longer present in the system, the cell may not be able to compensate for the induced changes, resulting in differences between drug treated animals and control animals.

We cannot state if the effect seen on p-CaMKII by long-term methadone treatment (one week after the last injection) is caused by inhibition of the NMDA receptor or stimulation of the MOR, or both. A previous study supports the hypothesis that methadone may act through both the MOR and the NMDA receptor (Callahan *et al.*, 2004), due to the different receptor specificities of the R and S isomer. However, the effects of methadone and morphine observed on p-CaMKII, and also p-ERK levels, tend to go in the same direction, indicating that the MOR plays an important role since it is well established that morphine acts principally as a MOR agonist (Aderjan

and Skopp, 1998).

Exposure to methadone or morphine did not affect the amount of total CaMKII or ERK, only minor differences were observed. Therefore, it seems unlikely that the reduced level of p-CaMKII/CaMKII one week after the last injection, and p-ERK/ ERK one hour after the last injection, is a result of cell death, even though a previous study has shown that chronic methadone treatment in mice increases expression of apoptosis-related proteins (Emeterio *et al.*, 2006). A possible inhibition of the neurogenesis in hippocampus, as has been shown after chronic morphine exposure (Eisch *et al.*, 2000), cannot be ruled out.

4.3 Human relevance

MMT was intended to be a safe and effective medication to help heroin addicts starting a drug free life. However, the patients are maintained on methadone often for decades or even for life (Joseph *et al.*, 2000). Despite of this, possible negative side-effects of long-term methadone use, have been scarcely investigated.

Some of the limitations in the studies with MMT patients are the often small number of subjects, their drug use prior to and during the MMT, and the lack of suitable controls. Several studies show that MMT patients have an impaired cognitive performance (Darke and Ross, 1997; Darke *et al.*, 2000; Mintzer and Stitzer, 2002), but whether this is due to methadone or other factors associated with the life-style is not known (Soyka *et al.*, 2008). Because of the difficulties conducting studies with MMT patients and the ethical restrictions using healthy volunteers, experimental animal studies are a feasible approach in the search for more knowledge in this area. The outcome of future animal studies may affect the use of methadone in the rehabilitation of heroin addicts. If the studies reveal large damages due to long-term methadone administration, this may result in a need for additional treatment to counteract the effects. On the other hand, if future studies reveal little or no negative effect, this will be a support for continuing the treatment programs. Presently, the need for more knowledge is crucial.

5. Conclusion

This study shows that long-term exposure to methadone leads to a down-regulation of p-CaMKII in hippocampus one week after the last injection. No difference was seen in the level of ERK phosphorylation. We did not observe any effects in the novelty-seeking one week after the last injection in animals subjected to methadone. The discrepancy between reduced phosphorylation of CaMKII and no effect in the novelty-test is not known, but may be due to low sensitivity of the behavioural test.

The rats were to affected by the drug to perform the novelty-test one hour after the last injection of methadone. No differences compared to the control, were observed in phosphorylation of CaMKII or ERK at this point.

We found similar effects after morphine exposure as after methadone exposure, and may therefore speculate whether the MOR is the central receptor. More studies are needed to reveal the mechanisms involved.

Proposal for further studies

Pre-expose the animals to heroin before exposing them to methadone to better resemble the human situation.

Western blot analysis of brain tissue form animals decapitated several weeks after the last injection to see if three weeks exposure to methadone gives a persistent effect on protein phosphorylation. In the same context, phosphorylation of other proteins important for learning and memory would be interesting to investigate.

Pre-treatment with a MOR antagonist *in vivo,* and/or receptor studies *ex vivo/in vitro*, may reveal which receptor methadone exerts its effect through.

Instead of using the racemic mixture of methadone, it would be interesting to see if subjecting the animals to the different isomers would give a different result.

The Morris Water Maze could perhaps give a more accurate result, being more sensitive than the Novelty-test.

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APPENDIX

Statistics

Locomotor activity, raring and weight one hour after last injection

Ranks			
	Exposure	N	Mean Rank
Locomotion phase 1	Saline	8	12.50
	Methadone	8	4.50
	Morphine	8	20.50
	Total	24	

Test Statistics[a,b]	
	Locomotion
Chi-Square	20.507
df	2
Asymp. Sig.	.000
a. Kruskal Wallis Test	
b. Grouping Variable: Exposure	

Ranks			
	Exposure	N	Mean Rank
Locomotion phase 2	Saline	7	13.86
	Methadone	8	4.50
	Morphine	8	17.88
	Total	23	

Test Statistics[a,b]	
	Locomotion
Chi-Square	16.318
df	2
Asymp. Sig.	.000
a. Kruskal Wallis Test	
b. Grouping Variable: Exposure	

Ranks			
	Exposure	N	Mean Rank
Rearing phase 1	Saline	8	13.75
	Methadone	8	4.50
	Morphine	8	19.25
	Total	24	

Test Statistics[a,b]	
	Rearing
Chi-Square	18.015
df	2
Asymp. Sig.	.000
a. Kruskal Wallis Test	
b. Grouping Variable: Exposure	

Ranks			
	Exposure	N	Mean Rank
Rearing phase 2	Saline	7	15.14
	Methadone	8	5.44
	Morphine	8	15.81
	Total	23	

Test Statistics[a,b]	
	Rearing
Chi-Square	11.601
df	2
Asymp. Sig.	.003
a. Kruskal Wallis Test	
b. Grouping Variable: Exposure	

Ranks			
	Exposure	N	Mean Rank
Weight day 1	Saline	8	15.75
	Methadone	10	13.30
	3	8	11.50
	Total	26	

Test Statistics[a,b]	
	Weight
Chi-Square	1.254
df	2
Asymp. Sig.	.534
a. Kruskal Wallis Test	
b. Grouping Variable: Exposure	

Ranks			
	Exposure	N	Mean Rank
Weight19	Saline	4	10.75
	Methadone	3	2.00
	Morphine	8	8.88
	Total	15	

Test Statistics[a,b]	
	Weight19
Chi-Square	7.271
df	2
Asymp. Sig.	.026
a. Kruskal Wallis Test	
b. Grouping Variable: Exposure	

Locomotion, rearing and weight one week after last injection

Ranks			
	Exposure	N	Mean Rank
Locomotion phase 1	Saline	10	10.85
	Methadone	7	12.00
	Morphine	8	16.56
	Total	25	

Test Statistics[a,b]	
	Phase 1
Chi-Square	2.860
df	2
Asymp. Sig.	.239
a. Kruskal Wallis Test	
b. Grouping Variable: Exposure	

Ranks			
	Exposure	N	Mean Rank
Locomotion phase 2	Saline	10	13.90
	Methadone	7	9.07
	Morphine	8	15.31
	Total	25	

Test Statistics[a,b]	
	Locomotion
Chi-Square	2.938
df	2
Asymp. Sig.	.230
a. Kruskal Wallis Test	
b. Grouping Variable: Exposure	

Ranks			
	Exposure	N	Mean Rank
Rearing phase 1	Saline	10	12.35
	Methadone	7	12.50

	Morphine	8	14.25
	Total	25	

Test Statistics[a,b]	
	Rearing
Chi-Square	.345
df	2
Asymp. Sig.	.842
a. Kruskal Wallis Test	
b. Grouping Variable: Exposure	

Ranks			
	Exposure	N	Mean Rank
Rearing phase 2	Saline	10	13.00
	Methadone	7	11.29
	Morphine	8	14.50
	Total	25	

Test Statistics[a,b]	
	Rearing
Chi-Square	.717
df	2
Asymp. Sig.	.699
a. Kruskal Wallis Test	
b. Grouping Variable: Exposure	

Ranks			
	Exposure	N	Mean Rank
Weight1	Saline	10	16.35
	Methadone	12	13.79
	Morphine	8	17.00
	Total	30	

Test Statistics[a,b]	
	Weight1
Chi-Square	.780
df	2
Asymp. Sig.	.677
a. Kruskal Wallis Test	
b. Grouping Variable: Exposure	

Ranks			
	Exposure	N	Mean Rank
Weight day 15	Saline	10	19.55
	Methadone	9	8.11
	Morphine	8	13.69
	Total	27	

Test Statistics[a,b]	
	Weight
Chi-Square	9.862
df	2
Asymp. Sig.	.007
a. Kruskal Wallis Test	
b. Grouping Variable: Exposure	

Ranks			
	Exposure	N	Mean Rank
Weight day 15	Saline	10	13.70
	Methadone	9	5.89
	Total	19	

Test Statistics[a,b]	
	Weight
Chi-Square	9.135
df	1
Asymp. Sig.	.003
a. Kruskal Wallis Test	
b. Grouping Variable: Exposure	

Ranks			
	Exposure	N	Mean Rank
Weight19	Saline	4	5.50
	Methadone	3	2.00
	Total	7	

Test Statistics[a,b]	
	Weight19
Chi-Square	4.500
df	1
Asymp. Sig.	.034
a. Kruskal Wallis Test	
b. Grouping Variable: Exposure	

Ranks			
	Exposure	N	Mean Rank
Weight day 22	Saline	10	19.40
	Methadone	8	8.00
	Morphine	8	11.62
	Total	26	

Test Statistics[a,b]	
	Weight
Chi-Square	10.593
df	2
Asymp. Sig.	.005
a. Kruskal Wallis Test	
b. Grouping Variable: Exposure	

Ranks			

	Exposure	N	Mean Rank
Weight day 22	Saline	10	13.05
	Methadone	8	5.06
	Total	18	

Test Statistics[a,b]	
	Weight
Chi-Square	9.991
df	1
Asymp. Sig.	.002
a. Kruskal Wallis Test	
b. Grouping Variable: Exposure	

Ranks			
	Exposure	N	Mean Rank
Weight day 22	Morphine	8	6.56
	Saline	10	11.85
	Total	18	

Test Statistics[a,b]	
	Weight
Chi-Square	4.373
df	1
Asymp. Sig.	.037
a. Kruskal Wallis Test	
b. Grouping Variable: Exposure	

Ranks			
	Exposure	N	Mean Rank
Weight26	Saline	10	18.80
	Methadone	8	10.44
	Morphine	8	9.94
	Total	26	

Test Statistics[a,b]	
	Weight26
Chi-Square	7.823
df	2

Asymp. Sig.	.020
a. Kruskal Wallis Test	
b. Grouping Variable: Exposure	

Novelty-test one hour after last injection

Ranks			
	Exposure	N	Mean Rank
Novelty	Saline	7	18.21
	Methadone	8	6.31
	Morphine	8	12.25
	Total	23	

Test Statistics[a,b]	
	Novelty
Chi-Square	11.751
df	2
Asymp. Sig.	.003
a. Kruskal Wallis Test	
b. Grouping Variable: Exposure	

Ranks			
	Exposure	N	Mean Rank
Novelty	Saline	8	11.19
	Methadone	7	4.36
	Total	15	

Test Statistics[a,b]	
	Novelty
Chi-Square	9.307
df	1
Asymp. Sig.	.002
a. Kruskal Wallis Test	

b. Grouping Variable: Exposure	

Ranks			
	Exposure	N	Mean Rank
Novelty	Morphine	8	6.06
	Saline	7	10.21
	Total	15	

Test Statistics[a,b]	
	Novelty
Chi-Square	3.247
df	1
Asymp. Sig.	.072
a. Kruskal Wallis Test	
b. Grouping Variable: Exposure	

Novelty test one week after last injection

Ranks			
	Exposure	N	Mean Rank
Novelty	Saline	10	14.85
	Methadone	7	11.93
	Morphine	8	11.62
	Total	25	

Test Statistics[a,b]	
	Novelty
Chi-Square	1.064
df	2
Asymp. Sig.	.588
a. Kruskal Wallis Test	
b. Grouping Variable: Exposure	

p-CaMKII/CaMKII one hour after last injection

Ranks			
	Exposure	N	Mean Rank
pCaMKII	Saline	7	9.86
	Methadone	8	11.12
	Morphine	8	14.75
	Total	23	

Test Statistics[a,b]	
	Intensity
Chi-Square	2.147
df	2
Asymp. Sig.	.342
a. Kruskal Wallis Test	
b. Grouping Variable: Exposure	

p-CaMKII/CaMKII one week after last injection

Ranks			
	Exposure	N	Mean Rank
pCaMKII	Saline	9	19.56
	Methadone	8	9.94
	Morphine	8	8.69
	Total	25	

Test Statistics[a,b]	
	pCaMKII
Chi-Square	11.277
df	2
Asymp. Sig.	.004
a. Kruskal Wallis Test	
b. Grouping Variable: Exposure	

Ranks			

	Exposure	N	Mean Rank
pCaM	Saline	9	12.00
	Methadone	8	5.62
	Total	17	

Test Statistics[a,b]	
	pCaM
Chi-Square	6.750
df	1
Asymp. Sig.	.009
a. Kruskal Wallis Test	
b. Grouping Variable: Exposure	

Ranks			
	Exposure	N	Mean Rank
pCaM	Morphine	8	5.00
	Saline	9	12.56
	Total	17	

Test Statistics[a,b]	
	pCaM
Chi-Square	9.481
df	1
Asymp. Sig.	.002
a. Kruskal Wallis Test	
b. Grouping Variable: Exposure	

p-ERK/ERK one hour after last injection

Ranks			
	Exposure	N	Mean Rank
Intensity	1	6	14.25
	2	8	14.38
	3	8	6.56

	Total	22	

Test Statistics[a,b]	
	Intensity
Chi-Square	7.298
df	2
Asymp. Sig.	.026
a. Kruskal Wallis Test	
b. Grouping Variable: Exposure	

Ranks			
	Exposure	N	Mean Rank
pERK	Saline	6	10.50
	Morphine	8	5.25
	Total	14	

Test Statistics[a,b]	
	pCaM
Chi-Square	5.412
df	1
Asymp. Sig.	.020
a. Kruskal Wallis Test	
b. Grouping Variable: Exposure	

p-ERK/ERK one week after last injection - methadone

Ranks			
	Exposure	N	Mean Rank
pERK	Saline	7	7.57
	Methadone	8	8.38
	Total	15	

Test Statistics[a,b]	

	pERK
Chi-Square	.122
df	1
Asymp. Sig.	.727
a. Kruskal Wallis Test	
b. Grouping Variable: Exposure	

p-ERK/ERK one week after last injection - morphine

Ranks			
	Exposure	N	Mean Rank
pERK	Saline	2	8.00
	Morphine	8	4.88
	Total	10	

Test Statistics[a,b]	
	pERK
Chi-Square	1.705
df	1
Asymp. Sig.	.192
a. Kruskal Wallis Test	
b. Grouping Variable: Exposure	