

Acute drug effect, addiction potential and expression of brain proteins
involved in learning and memory after single and repeated exposure to
methadone and buprenorphine in C57BL/6J mice

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Preface

The master thesis in human toxicology is part of the master's degree in biology at the University of Oslo. Work with this master thesis was carried out at the Norwegian Institute of Public Health, Division of Forensic Sciences, Department of Drug Abuse Research and Method Development, in the period January 2014 to December 2015. Senior scientist Jannike M. Andersen (Norwegian Institute of Public Health) and Professor II Merete Grung (University of Oslo) supervised me in this work.

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Abstract

Methadone and buprenorphine are two of the most widely used substances in rehabilitation of heroin addicts. In Norway, more than 7000 people attended opioid maintenance treatment in 2013. Despite widespread use, the knowledge concerning effects of this therapy is limited. The aims of this thesis were to assess and compare the acute drug effect after single injections of methadone and buprenorphine, and also assess the ability of the drugs to induce a sensitized response (enhanced drug effect after repeated administration of a drug) following repeated exposure, by using stimulation of locomotor activity in mice as a model. Because epidemiological studies have suggested that patients in methadone maintenance treatment perform worse on various cognitive tasks than healthy controls, and abstinent heroin abusers, the second goal was to investigate how repeated exposure to methadone and buprenorphine, in a controlled setting, may affect brain proteins central for learning and memory, using western blot analysis. For comparison, heroin and morphine were also included in the experiments.

Results from the locomotor activity studies indicate that methadone has a higher acute drug effect compared to buprenorphine. Repeated administration of both stimulating and stultifying methadone doses showed a sensitized response that was not evident in the buprenorphine treated animals. The western blot analysis did not reveal any obvious changes in activation and expression of brain proteins relevant for learning and memory (CaMKII and CREB) in hippocampus, frontal cortex, dorsal striatum or nucleus accumbens following repeated exposure for methadone and buprenorphine.

Taken together, the results in the present thesis indicate, that methadone might be associated with a higher acute rewarding effect and might have a higher addiction potential following intake, compared to buprenorphine. Concerning a possible effect on brain proteins central for learning and memory, only minor effects was found.

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Abbreviations

μ	mu
μl	microliters
μmol	micromol
κ	kappa
AMPA receptor	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
BBB	Blood brain barrier
BSA	Albumin from bovine serum
CaMKII	Calcium/Calmodulin dependent protein kinase II
cAMP	Cyclic adenosine monophosphate
cm	Centimeters
CRE	cAMP-response element
CREB	cAMP-response element binding protein
DTT	dl-Dithiothreitol solution
Emax	Maximal distance travelled
g	gram
HCl	Hydrogen chloride
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
ip	Intraperitoneal
iv	Intravenous
kDa	kilo Dalton

LTP	Long-term potentiation
mM	millimolar
NARA	Norwegian Animal Research Authority
NIPH	Norwegian Institute of Public Health
NMDA receptor	N-methyl-D-aspartate receptor
OR	Opioid receptor
OMT	Opioid maintenance treatment
PBS	Phosphate buffered saline
pCaMKII	Phosphorylated calcium/calmodulin-dependent protein kinase II
pCREB	Phosphorylated cAMP-response element binding protein
PMSF	Phenylmethanesulfonylfluoride
po	Per oral
rpm	Rounds per minute
RT	Room temperature
sc	Subcutaneous
SDS	Sodium dodecyl sulphate
SEM	Standard error mean
TBS	Tris buffered saline
TBS-T	Tris buffered saline with tween 20
TTD	Three times a day
V	Voltage

1. Introduction

1.1 Background

On world basis, it has been estimated that around 32 million people use opioids. Continuous use of opioids may eventually lead to development of addiction (UNODC, 2014; Pu et al., 2002). In Norway, around 7000 people attended rehabilitation for opioid addiction in 2013, and the number of patients in treatment will probably rise during the next years (SIRUS/EMCDDA, 2015). Methadone has been used in maintenance therapy for decades (Dole and Nyswander, 1965), and has been found to be successful in stabilizing patients in treatment, reducing use of illegal opioids, and criminal activity (Warner et al., 1997; Johnson et al., 2000; Bukten et al., 2012). Buprenorphine is another available pharmacotherapy for treatment of opioid addiction (Bramness et al., 2002). Studies have indicated less negative side effects of buprenorphine compared to the use of methadone in rehabilitation of heroin addicts (Warner et al., 1997). Opioid maintenance treatment (OMT) is often a long-term treatment (Giacomuzzi et al., 2005), and despite extensive use of the substitution drugs, few studies concerning consequences of long-term use are available.

1.2 OMT for opioid addiction

OMT attempts to counteract the neural adaptations caused by extensive heroin use (Dole and Nyswander, 1967; Bramness et al., 2002) and normalize the life of previous heroin addicts (Bramness et al., 2002; Bart, 2012).

1.2.1 Methadone

During World War II, an alternative to addictive morphine was needed for the treatment of pain, and this requirement resulted in the synthesis of methadone (Sim, 1973; Tober and Strang, 2003).

Methadone exists as two enantiomeric forms, R and S (Eap et al., 2002). The most commonly used form in maintenance treatment is the racemic mixture, RS- methadone (Gorman et al., 1997), even though it is the R-isoform that gives most of the opioid effects (Eap et al., 2002; Bart, 2012). Methadone exerts its analgesic and narcotic effects through the μ -opioid receptor, and acts as an antagonist at the N-methyl-D-aspartate receptor (NMDA) (Trescot et al., 2008).

The antagonistic effect at the NMDA receptor is believed to be advantageous in preventing induction of tolerance (Callahan et al., 2004). The half-life of methadone is variable between individuals and ranges from 24 to 48 hours (Curran et al., 2001). Various enzymes are involved in the metabolism, but methadone is primarily metabolized by cytochrome P450 (CYP) 3A4 and CYP2D6 in the liver (Trescot et al., 2008).

Dole and Nyswander (1965) started using methadone in maintenance therapy in the middle of the 1960s, and it is still one of the most widely used drugs in the combat against heroin addiction (SIRUS/EMCDDA, 2015). Methadone stimulates opioid receptors (OR) and thereby reduces, or obstructs binding of other opioids to the receptors, which in turn reduces abstinence and craving for heroin (Ward et al., 1999; Enquist et al., 2012). Methadone maintenance patients receive an oral dose once daily, because metabolism varies between patients; doses are given in the range 80-120 mg/day (Dole and Nyswander, 1967; Curran et al., 2001; Eap et al., 2002).

Although considered a successful treatment because of high retention rates and for stabilizing the life of former addicts, both epidemiological and animal studies have indicated that maintenance treatment with methadone may be associated with negative consequences in respect to various cognitive functions (Tramullas et al., 2007; Mintzer and Stitzer, 2002; Verdejo et al., 2005) and this is worrying.

1.2.2 Buprenorphine

Buprenorphine has been introduced as an alternative pharmacotherapy for treatment of heroin addiction. Studies have shown that it is as effective as methadone in reducing the use of illegal opioids (Bickel et al., 1988; Johnson et al., 2000). Regarding retention in treatment it is discussed whether buprenorphine is as efficient as methadone or not (Strain et al., 1994; Fischer et al., 1999; Gerra et al., 2004; Mattick et al., 2014).

Buprenorphine differs from methadone in that it functions as a partial agonist at the μ -OR (Martin et al., 1976), and also acts as an antagonist at the κ -OR (Greenwald et al., 2007). Buprenorphine has high affinity for both the μ - and κ -OR (Huang et al., 2001). Because of rapid pre-systemic metabolism after oral administration, buprenorphine is given sublingually when used in maintenance treatment (Cone et al., 1984; Kobayashi et al., 1998; Picard et al., 2005; Bart, 2012). Buprenorphine's long half-life (up to 48 hours depending on dose

(Kuhlman et al., 1998)) allows patients to have their doses every other day (Amass et al., 1998). Doses used in treatment vary between 8-12 mg (Bramness et al., 2002). Fatal consequences like overdose and respiratory depression is thought to be less pronounced in buprenorphine patients compared to patients given methadone. It has also been reported that patients withdrawn from buprenorphine experience less discomfort than patients withdrawn from methadone, because of weaker effects on the OR. Studies in humans also suggest that buprenorphine may be associated with less negative effects (Mintzer et al., 2004; Mattick et al., 2014; Warner et al., 1997).

1.3 Opioids and opioid receptors

Opioids have been utilized for many years mainly to relieve pain, but recreational use is also widespread (Callahan et al., 2004; Trescot et al., 2008).

Opioids are a broad term that entails the natural endogenous opiate peptides (e.g. endorphins, enkephalin), the opium alkaloids (substances derived from the poppy *Papaver somniferum*, like morphine), semi-synthetic (e.g. heroin) and synthetic products like methadone and buprenorphine (McDonald and Lambert, 2008). Opioids bind to, and mediate their effects through one or more of the mu (μ), delta (δ) and kappa (κ) opioid receptors (Mao, 1999). The OR are located throughout the central nervous system and also in peripheral tissue (Feltenstein and See, 2008; Lutz and Kieffer, 2013; Martini and Whistler, 2007). OR are G-protein coupled, and upon binding of a ligand they function as signal transducers, conveying external information into the cell which stimulates a biological response (Martini and Whistler, 2007; Filizola and Devi, 2012). Biological responses may include various alternations in intracellular pathways. A ligands affinity for the receptor, its availability, and the duration of receptor stimulation are among properties that determine the degree of signal transduction (Martini and Whistler, 2007; McDonald and Lambert, 2008).

1.4 Acute drug effect and addiction

1.4.1 The initial high – the acute effect of drugs of abuse

Various drugs of abuse (e.g. heroin, amphetamine, cocaine) cause an acute rewarding effect, by producing pleasurable feelings following intake. This initial high has been thought to be mediated via the same neuronal pathway for all these types of drugs, namely the mesolimbic

pathway (Nestler, 2005). This pathway involves the ventral tegmental area (VTA) in the midbrain and dopaminergic projections to the nucleus accumbens located in the basal forebrain (Nestler, 2005). Other brain regions, including frontal cortex, amygdala and hippocampus are also thought to be involved in the rewarding effect of drugs. Agonists like methadone and buprenorphine bind to μ -OR and increases the neurotransmission from the VTA to nucleus accumbens. Doing this by inhibition of inhibitory gamma amino butyric acid (GABA) interneurons, the result is increased extracellular levels of dopamine (DA) in nucleus accumbens (Robinson and Berridge, 1993; Nestler, 2005; Di Chiara and Imperato, 1988).

Stimulation of locomotor activity in mice

In mice, increased dopaminergic neurotransmission in nucleus accumbens following administration of drugs has been shown to correlate with increased locomotor activity (Wise and Bozarth, 1987; Mørland et al., 1994). This change in locomotor activity after systemic opioid administration may be used as a measure of the acute drug effect, that might represent the rewarding and reinforcing properties of a drug. However, in mice, it is unknown whether the increase in locomotor activity following administration of a drug is due to rewarding effects, or if it is an aversive response, but it is accepted to use as a measure of acute drug effect following administration of drugs of abuse (Wise and Bozarth, 1987; Mørland et al., 1994).

1.4.2 Development of addiction

The initial high following drug intake often encourages recurrent use of drugs, which might lead to addiction. The maintenance of use and loss of ability to stop using substances, as well as recurring relapse following withdrawal are signs of drug addiction (De Vries and Shippenberg, 2002). The neurochemical and anatomical modifications underlining addiction are complex, and several theories exist (for reviews see: Taylor et al., 2013; Feltstein and See, 2008). Since brain areas involved in drug use overlap with those involved in learning, including hippocampus, frontal cortex, dorsal striatum and nucleus accumbens (Goldstein and Volkow, 2002; Hyman, 2005), it has been argued that addiction may be viewed as a negative form of learning. According to Nestler (2005), long-term use of drugs will lead to an imbalance in the DA system and eventually decreased DA levels, resulting in reduced response to natural stimuli when drug is not available. But also reduced responses upon administration of the drug, namely tolerance (Christie, 2008).

Sensitization

Sensitization is a phenomenon where an enhanced drug effect is expressed following repeated exposure to a drug (Robinson and Berridge, 2008), and the increased response may indicate persistent changes in the mesolimbic system. This increased drug effect is usually evident after intermittent exposure or after longer periods of withdrawal when addicts have less tolerance for the drug. When reintroduced to a drug of abuse the DAergic system increases the transmission of DA, the DA system is said to be sensitized (Robinson and Berridge, 2008; Pierce and Kumaresan, 2006; Nestler, 2005). It is assumed that all drugs that has the ability to increase extracellular levels of DA in nucleus accumbens after administration, may lead to addiction (Volkow et al., 2011). A sensitized locomotor activity response in mice may be used as an indication of increased activity in the dopamine circuit in the brain. This increased activity is especially important since it is thought to be one of the mechanisms central in the development of addiction (Robinson and Berridge, 2008; Wise and Bozarth, 1987).

1.5 Learning and memory

Learning can be defined as the ability to acquire new information, a new skill and understanding, while memory is defined as the ability to retain and recall learned information (Lynch, 2004).

Learning and memory formation are examples of the brains ability to change and adapt in response to repeated stimuli, both structurally and functionally. Long term potentiation (LTP) is thought to be one of the mechanisms underpinning learning and memory (Lynch, 2004; Cooke and Bliss, 2006). LTP can be explained as the outcome of increased activity or communication between two neurons, where the connection between the two neurons get strengthened (Cooke and Bliss, 2006). This process involves a signal transduction cascade that includes release of glutamate following action potentials, activation of NMDA- and AMPA-receptors leading to influx of sodium (Na^{2+}) and calcium (Ca^{2+}), that subsequently leads to activation of signaling molecules or signal cascades inside the cells (Miyamoto, 2006).

1.5.1 Hippocampus

Hippocampus is part of the limbic system (Yau et al., 2015). Various functions, including emotions and long-term memory, have been related to these structures (Isaacson, 1982). Hippocampus has been suggested to play a vital role in spatial learning, formation of memory and consolidation of memories (Morris et al., 1982; Thompson and Kim, 1996; Yau et al.,

2015). Also, neurogenesis in dentate gyrus has been shown to enhance learning and memory processes in mice (van Praag et al., 1999). Eisch et al. (2000) found in rats, that chronic opioid administration decreased the generation of neurons (neurogenesis) in hippocampus. This might affect learning and memory processes negatively.

1.5.2 Prefrontal cortex

Prefrontal cortex is part of the forebrain and is important for processing information, decision making and attention (Frith and Dolan, 1996; Nestler, 2005; Dalley et al., 2004). The connection between hippocampus and prefrontal cortex is assumed important for consolidation of memories (Siddiqui et al., 2008; Preston and Eichenbaum, 2013).

1.5.3 Dorsal striatum and nucleus accumbens

Nucleus accumbens are mainly involved in reward, selective attention and motivation, while dorsal striatum is involved in motor activity, and learning of stimulus-response associations, they are pleasure centers in the brain (Robbins and Everitt, 1996; Angulo and McEwen, 1994; O'Doherty et al., 2004). These areas are important for motivating and repeating behavior associated with drug (ab)use. It is thought that increased dopaminergic transmission from VTA to nucleus accumbens motivates the person to repeat intake because of the pleasurable effect that follows (Robbins and Everitt, 2002; O'Doherty et al., 2004).

1.5.4 Brain proteins involved in learning and memory

Calcium-calmodulin dependent protein kinase II (CaMKII)

In neurons, Ca^{2+} acts as an important intracellular second messenger. Rise in Ca^{2+} -levels in cells facilitates binding of Ca^{2+} to its main receptor, calmodulin, making the calcium-calmodulin complex (Yamauchi, 2005).

Activation of the calcium/calmodulin dependent protein kinase II (CaMKII) is accomplished by the calcium/calmodulin complex (Miyamoto, 2006), and this kinase is dependent on both calmodulin and calcium for its activity. Two, of the in total four known subunits, are greatly expressed in neural tissue, namely the α (50 kDa) and β (60 kDa) subunit (Lou et al., 1999). CaMKII phosphorylates and regulates many proteins both in the nervous system and other tissues. Synthesis and release of neurotransmitters, modulation of ion channels, transport within cells, gene expression and synaptic plasticity, important for learning and memory, are examples of functions regulated by CaMKII (Yamauchi, 2005). It is assumed that CaMKII

has an important role in LTP (Malenka and Nicoll, 1999). Studies have shown that inhibition of α CaMKII reduced LTP in mice (Silva, 2003).

Cyclic-AMP-response element binding protein (CREB)

CREB is a transcription factor located in the nucleus of cells, with a molecular weight of 43kDa. It is mainly regulated by phosphorylation at serine 133, and is activated by various protein kinases like cAMP-dependent protein kinase A (PKA) and CaMKII, in response to increased intracellular levels of cAMP and Ca^{2+} (Montminy et al., 1990; Choe and Wang, 2001). Once activated, CREB binds to cAMP-response elements (CRE) and regulates transcription of genes (De Rasmio et al., 2009).

CREB has been studied in animals, and results from these studies imply that CREB is important for learning and memory formation (Izquierdo and Medina, 1997; Yin and Tully, 1996). Studies in CREB “knockout” mice have revealed that learning and short-term memory are at a normal levels, while longer-lasting memories are disrupted, highlighting the importance of CREB in memory formation (Yin and Tully, 1996). Low levels of this transcription factor have been linked to impairment of memory formation. Opposite, overexpression of CREB has, in mice, been shown to enhance LTP, underlining its importance in synaptic changes important for memory formation (Barco et al., 2002; Silva, 2003; Bourtchuladze et al., 1994; Miyamoto, 2006).

1.6 Use of animals in experimental research – ethical considerations

In Norway, animal research is regulated by the Animal Welfare Act given by the Department of Agriculture. The Act states that animal research cannot be carried out without approval from the Norwegian Animal Research Authority (NARA), and suffering of animals needs to be weighed against the potential gain in each study. Alternative approaches for investigation must always be evaluated and suffering of animals must always be reduced to the smallest possible amount. To conduct animal research the animal facility and the project description must be approved by NARA, in addition a responsible person must be approved (Smith, 1999) All the listed criteria are satisfied at the Norwegian Institute of Public Health and all experimental protocols used during the work with this thesis were approved by NARA.

The research presented in this thesis could not have been conducted using only *in vitro* models; since studying behavior responses requires living animals. To reduce the quantity of

animals used during the work with this thesis, different brain regions and several proteins were investigated from each animal. Animals were euthanized by cervical dislocation.

1.7 Aims of the study

Epidemiological studies have reported that methadone treatment may be associated with negative side-effects, e.g. impairment of cognitive functions (Mintzer and Stitzer, 2002; Verdejo et al., 2005). Buprenorphine is an alternative pharmacotherapy assumed to be associated with less severe side-effects (Whelan and Remski, 2012). Studies on consequences of long-term treatment with these substances are rather scarce, and there is a great need for increased knowledge in this field.

Methadone and buprenorphine possess different pharmacodynamics properties, and it is reasonable to assume that the substances might be different concerning acute drug effects and the ability to induce sensitization. In other words, have unequal ability to give acute rewarding effects following administration of drug, and different potential for development of addiction.

In this thesis, there are two main aims. The first aim is to assess and compare the acute drug effect after a single injection of drug (methadone, buprenorphine, morphine and heroin), and also assess the ability of the drugs to induce a sensitized response after repeated exposure, by using stimulation of locomotor activity in mice as a model. Because it has been proposed that methadone might have negative effects on cognitive functions like learning and memory, the second aim was to investigate how repeated exposure to methadone and buprenorphine potentially may affect brain proteins central for learning and memory, using western blot analysis. In these studies morphine or heroin will be included for comparison.

Hypothesis to be tested

H₀: Methadone induces a higher acute locomotor activity response than buprenorphine

- Subcutaneous and per oral administrations will be compared

H₀: Repeated exposure to methadone causes sensitization of the locomotor activity response, this will not be evident for buprenorphine

H₀: Repeated exposure to methadone affects the expression of brain proteins important for learning and memory more than buprenorphine

- Expression and activation of CaMKII and CREB will be examined in hippocampus, frontal cortex, dorsal striatum and nucleus accumbens

2. Materials and Methods

2.1 Chemicals and animals

2.1.1 Chemicals

Chemicals used in all experiments during this master thesis listed according to manufacturer:

Bio-Rad (Oslo, Norway)

4x Laemmli Sample Buffer, Dual-color protein standard, Sodium Dodecyl Sulfate (SDS)

Braun (Melsungen, Germany)

0.9 % Sodium chloride (NaCl)

Cell Signaling Technology (Oslo, Norway)

Anti-phospho-CREB (Ser 133) (Clone name 87G3) rabbit monoclonal antibody, Anti-CREB (Clone name 48H2) rabbit monoclonal antibody, Anti-CaMKII antibody, Anti-phospho-CaMKII ((Thr286), Anti-phospho-CaMKII (Clone name D21E4), Biotinylated Protein Ladder, Anti-Biotin HRP linked antibody

Lipomed AG (Arlesheim, Switzerland)

Heroin HCl (3,6-diacetylmorphine: mol. wt. 423.9 g/mol), Buprenorphine HCl (mol. wt. 504.11 g/mol)

Merck Millipore (Billerica, USA)

Sucrose, Methanol, Folin Ciocalteu's Phenol Reagent, Re-blot Plus Mild Solution, Sodium Chloride (NaCl), Sodium hydroxid (NaOH), Glycerol

Norsk medisinaldepot AS (Oslo, Norway)

Morphine HCl (mol. wt. 345.84 g/mol)

Santa Cruz Biotechnology Inc. (Texas, USA)

Goat Anti-Mouse IgG-HRP, Goat Anti-Rabbit IgG-HRP, Anti-phospho-CREB (Ser 133) polyclonal antibody, Aporotinin, Leupeptin

Sigma-Aldrich (Oslo, Norway)

Glycine, Bromphenol blue, Hydrochloric acid (HCl) 26.5- 38%, Ponceau S, 5-Sulfosalicylic acid dihydrate, Trizma Hydrochloride (Tris-HCl), Trizma base (Tris-base), Skim milk powder, Tween 20, Monoclonal Anti- β -Actin, Phosphate buffered saline (PBS), dl-Dithiothreitol solution (DTT), 2-Mercaptoethanol, Albumin from bovine serum (BSA), Sodium Carbonate (Na_2CO_3), Copper(II)sulphate pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$), Potassium Sodium Tartrate Tetra hydrate ($\text{NaKC}_4\text{H}_4\text{O}_6 \cdot 4\text{H}_2\text{O}$), Trichloroacetic acid, Pepstatin A, Phenylmethanesulfonylfluoride (PMSF), Methadone HCl (mol. wt. 345,91 g/mol).

Thermo Scientific (Rockford, USA)

SuperSignal West Dura Extended Duration Substrate

2.1.2 Animals

All studies were carried out in male C57BL/6J-Bom mice (Bomholt, Ejby, Denmark), 7 – 8 weeks old, weighing 17.8 – 31.9g before testing. After arrival the mice were placed in cages, 5 – 8 mice per cage, with free access to water and mouse pellets (Scanbur, Nittedal). A timer regulated light – dark cycle (12:12 hours), with light period from 7:00 AM to 7:00 PM. Relative humidity ranged from 36 to 44 % and the air temperature was kept at $22 \pm 2^\circ\text{C}$. All injections and experiments were performed between 7:30 AM and 5:00 PM Monday to Friday. The mice were inspected daily, water was changed every other day and the cages were cleaned once a week. Before use in experiment, the mice were habituated in the animal facility for at least four days. The mice were sacrificed immediately after each injection regime and behavioral testing was finished. NARA approved all experimental protocols before any experiments were performed.

2.2 Methods

2.2.1 Exposure regimes

The mice were divided into groups of 5 – 8 animals. Methadone HCl, buprenorphine HCl, heroin HCl, morphine HCl or 0.9 % saline was administered either once (n=149), for four days (n=10) or repeatedly for three weeks (n=35), withdrawal Saturday to Sunday. The solutions were injected either at the lower back of the animals (sc) or directly injected into the stomach (po) with total injection volume of 0.1 ml/10 g mouse.

Mice injected once (n=149) received heroin in doses ranging from 1.25 to 10 $\mu\text{mol/kg}$, morphine from 15 to 60 $\mu\text{mol/kg}$, methadone from 12.5 to 100 $\mu\text{mol/kg}$ or buprenorphine from 0.25 to 2 $\mu\text{mol/kg}$ either subcutaneously (sc) (n=116) or per orally (po) (n=33) before behavioral testing (Section 2.2.2 Behavioral testing).

Animals repeatedly injected for four days (n=10) received either methadone 25 $\mu\text{mol/kg}$ (n=5) or saline (n=5).

Mice (n=35) were injected once a day between 07:30 AM and 09:00 AM for three weeks, Monday to Friday. This giving 14 injections in total, as the last injection day was Thursday of the third week. Methadone (25 $\mu\text{mol/kg}$) or morphine (30 $\mu\text{mol/kg}$) was given in the first round of the three weeks experiment (n=12), while methadone (50 $\mu\text{mol/kg}$), morphine (60 $\mu\text{mol/kg}$) or buprenorphine (2 $\mu\text{mol/kg}$) were given to new animals in the second round of experiments (n=16). Controls (n=7) received 0.9 % saline. Each mouse were weighed and marked before the injection regime started. The mice were also weighed every second day during the exposure regime.

The drugs were dissolved in 0.9 % saline the same day as they were used in the single injection experiments. Mice injected repeatedly, four days or three weeks, received doses from the same solution for one week. Heroin in solution was stored at 4°C while morphine, buprenorphine and methadone were stored dark at room temperature.

2.2.2 Behavioral testing - locomotor activity

Locomotor activity was tested as described in Andersen et al. (2009) using VersaMax optical animal activity monitoring system (AccuScan Instruments, Inc., Columbus, OH, USA). The activity chambers were divided into four squares (20 x 20 cm) by two perpendicular walls, and a grid of infra-red beams registered the activity. Two mice were tested simultaneously in

each chamber, using two non-adjacent quadrants. Run distance (cm/5 min), total run distance (cm) and maximal distance run (cm/5 min), was selected as expression of locomotor activity. Prior to the test each mouse, both the mice given a single injection (n=121) and the mice treated for three weeks (n=35) was habituated individually in a chamber for one hour. After habituation the mice were removed from the chambers and immediately injected in the neighboring room. The mice were administered with drugs and doses as described in Section 2.2.1. Immediately after injection, the mice were placed back into the same chamber they were habituated to and the locomotor activity was measure for 5 hours. After the locomotor activity test was finished animals receiving single injections (n=121) were sacrificed immediately and was sent to destruction, while animals injected for three weeks (n=35) were placed back into their respective home cages and sacrificed 24 hours after the last injection.

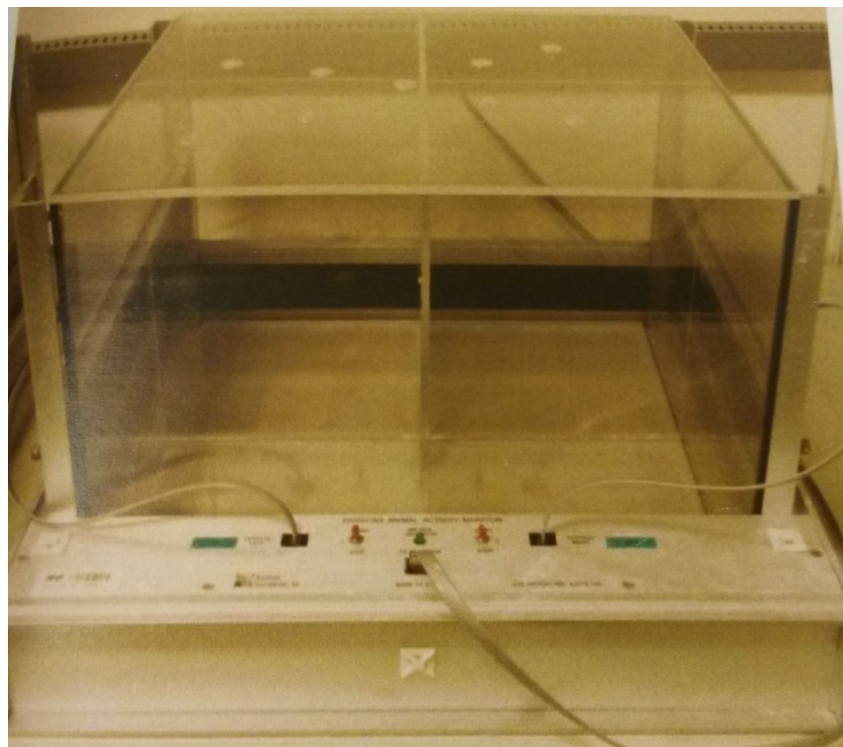


Figure 2.1 Locomotor activity chamber

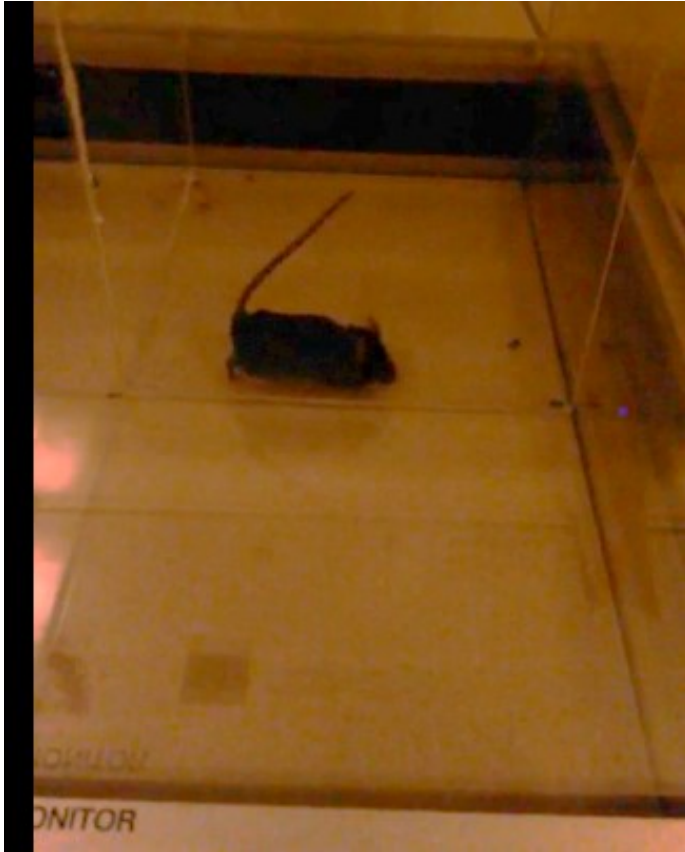


Figure 2.2 Mice, with characteristic straub tail, running in locomotor activity chamber

2.2.3 Obtaining samples and sample preparation

The animals were sacrificed by cervical dislocation either one hour after injection, for mice injected once (n=28), or 24 hours after the last injection for the mice injected repeatedly (n=28) and the brains removed. Hippocampus, frontal cortex, dorsal striatum and nucleus accumbens were rapidly dissected on ice, then immediately frozen in liquid nitrogen (-196°C) and finally stored in freezer at - 80°C.

Homogenization

After thawing on ice hippocampus, frontal cortex, dorsal striatum and nucleus accumbens were homogenized in ice cold 0.32 M sucrose containing 10 µg/µL leupeptin, 2 µg/µL pepstatin A, 10 µg/µL aprotinin and 200 mM phenylmethanesulfonylfluoride (PMSF). Hippocampus and frontal cortex were weighed and homogenized by using a glass/teflon homogenizer (500 rpm) or by sonication to make a 5 % homogenate. Dorsal striatum and nucleus accumbens weighing 5 ± 0.35 g were added 120 µL 0.32 M sucrose and homogenized

by sonication. Aliquots of 40 or 50 μL were stored at $-80\text{ }^{\circ}\text{C}$ for later determination of protein concentration and western blot analysis.

2.2.4 Lowry – determination of protein concentration

The protein concentration in different brain homogenate aliquots was determined as described by Lowry et al. (1951).

For each sample three parallels were prepared. 4 μl homogenate were mixed in 196 μl dH_2O . To start the reaction 1 ml application solution (2 % Na_2CO_4 in 0.1 M NaOH, 0.5 % $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 1 % K(Na)tartrate; 98:1:1) was added to the test-tubes. The reaction was stopped after 10 minutes by adding 100 μl stop reagent (Folin Ciocalteu's Phenol Reagent, dH_2O ; 1:1). Between each addition step the test tubes were vortexed. The samples were incubated at room temperature (RT) for at least 30 minutes before the absorbance was measured at 750 nm by a spectrophotometer (Shimadzu, Kyoto, Japan).

Bovine serum albumin (BSA) was dissolved in dH_2O and used as standard. Standards used were: Standard 1 (1 μg BSA), standard 2 (2.5 μg BSA), standard 3 (5 μg BSA), standard 4 (10 μg BSA), standard 5 (15 μg BSA), standard 6 (20 μg BSA). dH_2O was used as blank.

2.2.5 Western blotting

The Western blotting method described by Burnette (1981) and Andersen et al. (2012) was used, with some modifications, to identify proteins in the brain-tissue samples.

Sample preparation

The brain homogenates (aliquots of 40 and 50 μl) were diluted (1:2) in sample buffer (giving a final concentration of 62.5 mM Tris-HCl, pH 6.9, 4% sodium dodecyl sulfate (SDS), 5% glycerol, 0.01% bromphenolblue). The protease inhibitors leupeptin, aprotinin, pepstatin A and PMSF were also included in the sample buffer (see Table 2.1 for final concentrations in sample).

Table 2.1 Protease inhibitors with respective final concentrations in sample

Protease inhibitors	Final concentration in sample
Leupeptin	10 µg/ml
Aprotenin	10 µg/ml
Pepstatin A	5 µg/ml
PMSF	0.5 mM

Furthermore, DTT or β -mercaptoethanol was added as reducing agent (final concentration of 12% or 6%, respectively). The samples were further reduced by boiling at 100°C for five minutes and mixed well before loading onto the gels.

Gel electrophoresis – separation of proteins by molecular weight

Readymade gels were used (12% Criterion precast gels (BioRad, Oslo, Norway)). The wells were washed with 1x electrophoresis buffer (25 mM Tris-base, 0.2 M glycine, 3.5 mM SDS) before the gels were placed in a chamber filled with 1x electrophoresis buffer. Samples (7.5-30 µL), a biotinylated protein ladder (5 µL), a visible dual color standard (5 µL) and a brain homogenate used as an “internal standard” (15 µL) were loaded onto the gels using syringe pipette tips. For the first 10 minutes of the electrophoresis, the power supply was set to 100 volt (V) so that the samples could gather in the upper part of the separation gel, before the voltage was increased to 200 V. The electrophoresis continued running for around 40 minutes and was stopped before the samples ran out of the gels.

Blotting – transfer of proteins from gel to nitrocellulose membrane

After electrophoresis, the stacking gel was removed, while the rest of the gel was packed in a blotting cassette in the following order: sponge – filter paper – gel – nitrocellulose (NC)-membrane – filter paper – sponge. Sponge, filter paper and NC-membrane were presoaked in 1x transferbuffer (25 mM Tris-base, 0.2 M glycine, 20 % methanol). The blotting chamber was filled with 1x transferbuffer. A magnet and cooling element were included to prevent too high temperature and assure a continuous power current. The blotting chamber was placed on a magnetic stirrer while the power supply was set to 70 V. Blotting persisted for 75 minutes. When blotting was completed, the nitrocellulose membranes (blots) were stained with Ponceau S color for 2 minutes to verify that the proteins had been transferred successfully.

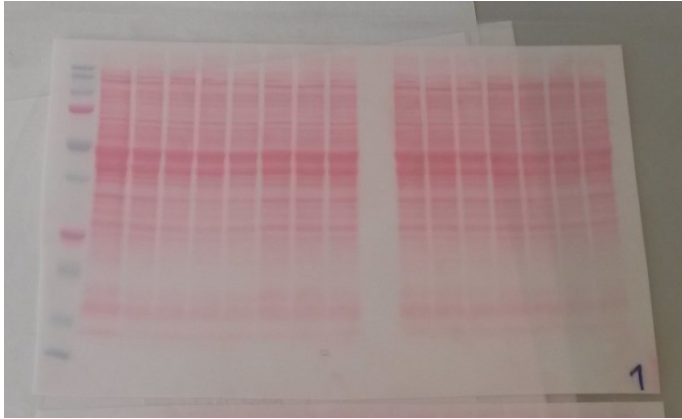


Figure 2.3 Nitrocellulose membrane stained with Ponceau S

Exposure to antibodies

The blots were incubated in 1x TBS-T (0.15 M NaCl, 0.1 M Tris-HCl, 0.5 % Tween 20, pH 7.4) for 5 minutes before incubation in blocking buffer (3% skimmed milk diluted in TBS-T) for one hour to avoid unspecific binding. Primary antibodies specific to the protein of interest were added to new blocking buffer and the blots were incubated for 2 hours at RT or overnight at 4°C with continuous shaking on a rocking table. Before adding the secondary antibodies, the blots were washed 4x 5 minutes in cold TBS-T to remove excess or unbound antibody and again incubated in blocking buffer for 30 minutes. The blots were incubated with the secondary antibodies either for 2 hours at RT or overnight at 4°C. The secondary antibodies were added to freshly prepared blocking buffer.

Primary antibodies used were: Anti-phospho-CaMKII (1:2000), Anti-CaMKII (1:2000, 1:2500), Anti-phospho-CREB (1:500, 1:1000, 1:2000), Anti-CREB (1:500, 1:1000, 1:3000) and Anti- β -Actin (1:200 000, 1:400 000). Secondary antibodies were: Anti-biotin HRP-linked antibody (1:10000), Goat-anti-rabbit IgG-HRP (1:5000) and Goat-anti-mouse IgG-HRP (1:5000).

Anti- β -Actin was included and used as a loading control.

After exposure to the secondary antibodies the blots were washed 3x 5 minutes in cold TBS-T and then in TBS (0.15 M NaCl, 0.1 M Tris HCl, pH 7.4) for 1x 5 minutes.

Detection

The blots were incubated for 5 minutes under continuous shaking in Super Signal detection solution (SuperSignal West Dura Lumniol/Enhancer, SuperSignal West Dura Stable Peroxide Buffer, 1:1) before they were positioned in a plastic folder and visualized in a ChemiDoc

XRS imager (BioRad, Oslo, Norway). Luminescence images were captured every 20 second for about two minutes.

Antibody stripping

The blots were rinsed twice in 1x TBS-T before they were stripped with 1x Re-Blot Plus Mild Solution (10 % Re-Blot Plus Mild Solution, in dH₂O) for 20 – 40 minutes. To confirm complete removal of old bands the blots were again visualized as described in the previous section. Thereafter the blots were rinsed in 1x TBS-T and washed in blocking buffer for 2x 5 minutes. The blots were now ready for exposure to new antibodies.

Analysis

The software QuantityOne Version 4.6.9 (BioRad, Oslo, Norway) was used to analyze total intensity of all the pixels in a specific band volume divided by the area of the volume in the optimized pictures captured by the ChemiDoc XRS imager. To calculate corrected band intensities, a correction factor derived from the “internal standard”, applied to every gel, was used. Only bands with equal protein amount were compared.

Determination of protein quantity

To decide how much protein should be loaded onto the gel, a test gel was loaded with increasing amounts of protein and evaluated for each antibody before a final decision was made. This is important to avoid over-saturation of signals and masking possible differences between treatment groups. The procedure was repeated for all brain areas.



Figure 2.4 Cropped section of a blot exposed to β -Actin showing increasing amounts of protein loaded (7.5 - 15 - 25 μ l sample)

2.2.6 Statistics and calculations

Statistical analyses were carried out with SPSS version 22 (SPSS Inc., Chicago, IL., USA). Data are presented as means \pm SEM unless otherwise stated.

For the locomotor activity data, total distance run was calculated by summarizing all the 5 min interval registrations of activity, E_{\max} was calculated by the excel command “MAKSA”.

Levenes test was used for evaluation of homogenous-variance in the data sets. Some of the data did not satisfy the criteria of homogenous-variance even after log-transformation, and therefore the non-parametric Kruskal-Wallis test was used to compare the groups. Pairwise comparisons were used to determine which of the groups were different. In data with homogenous-variance, the parametric one – way analysis of variance (ANOVA) was applied, using Tukey as post hoc test. For comparisons between two groups, either Mann–Whitney U or Students t-test was utilized.

p-values $(p) \leq 0.05$ were considered significant.

3. Results

3.1 Behavioral testing – locomotor activity

Locomotor activity after single, subcutaneous (sc) versus per oral (po) injections of methadone, buprenorphine, morphine and heroin.

3.1.1 Methadone

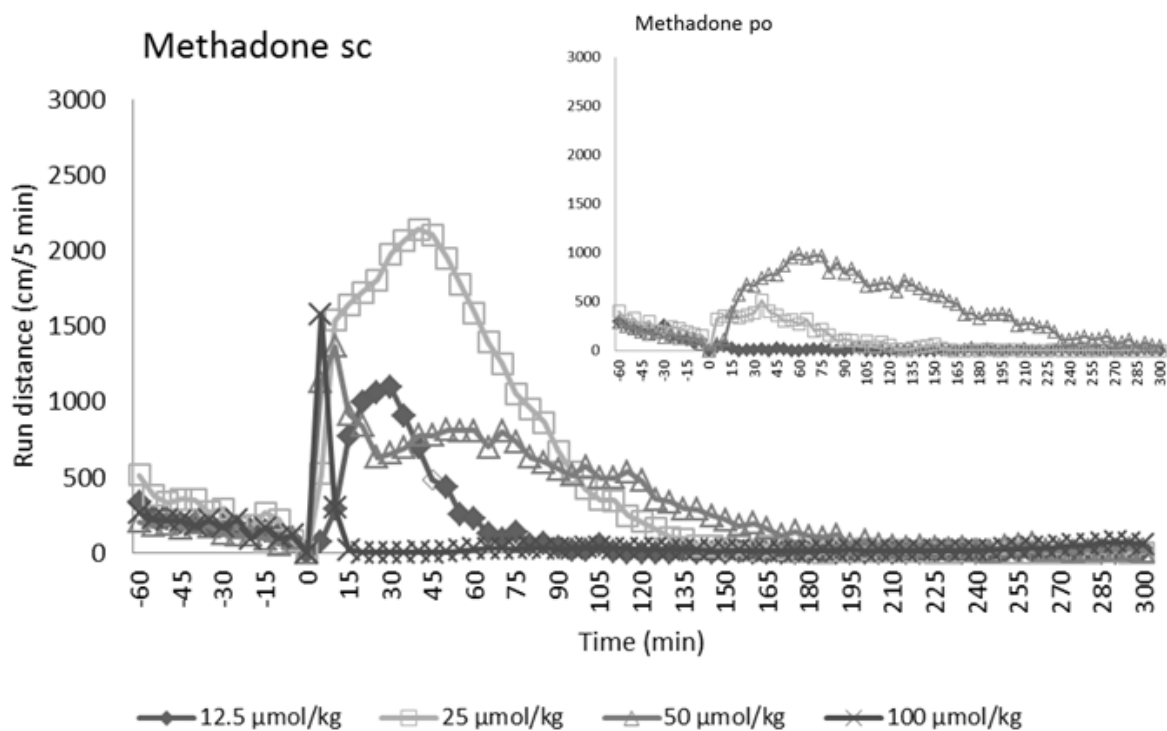


Figure 3.5 Locomotor activity in mice expressed as run distance (centimeters) per 5 minutes after subcutaneous (sc), left, and per oral (po), upper right, injections of methadone (12.5 – 100 $\mu\text{mol/kg}$). Mice were administered drugs at $t=0$, after 60 minutes of habituation. Activity was measured for five hours post administration. Data are means, $n=5 - 8$. Control animals ($n= 4$) are not shown, and SEM bars are omitted for clarity.

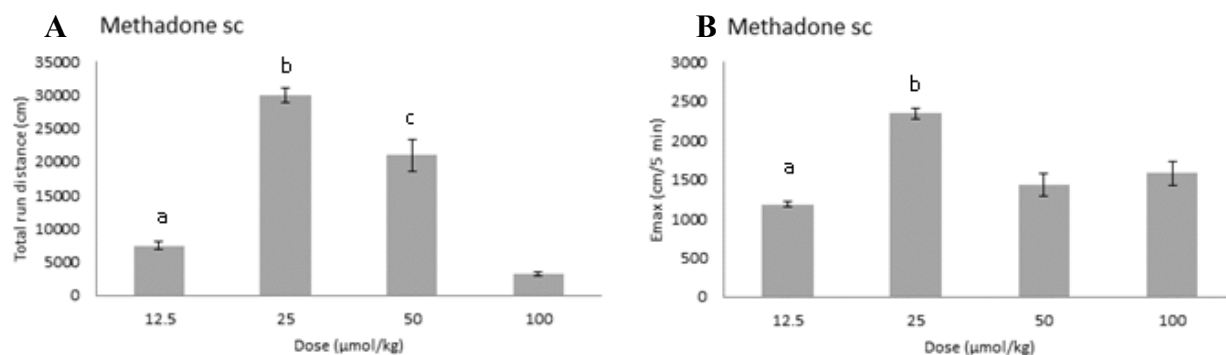


Figure 3.2 A: Calculated values for total distance run for mice injected sc with methadone (12.5 – 100 µmol/kg), a= $p \leq 0.05$ between 12.5 and 25 µmol/kg, b= $p \leq 0.0001$ between 25 and 100 µmol/kg, c= $p \leq 0.05$ between 50 and 100 µmol/kg. B: Calculated values for maximal distance run (E_{max}) for mice injected sc with methadone (12.5 – 100 µmol/kg), a= $p \leq 0.05$ between 12.5 and 25 µmol/kg, b= $p \leq 0.05$ between 25 and 50 µmol/kg. Results are presented as means \pm SEM, $n = 5 - 8$. Control animals ($n = 4$) are not shown.

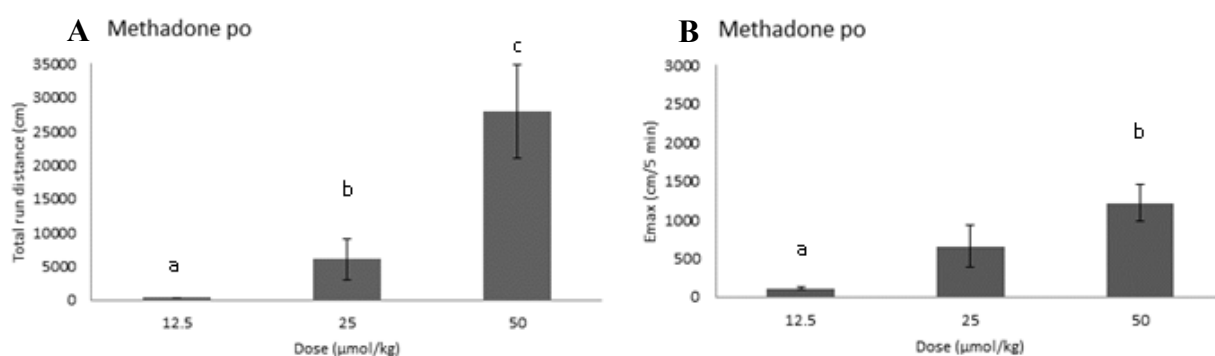


Figure 3.3 A: Calculated values for total distance run for mice injected po with methadone (12.5 – 50 µmol/kg), a= $p \leq 0.05$ between 12.5 and 25 µmol/kg, b= $p \leq 0.0001$ between 12.5 and 50 µmol/kg, c= $p \leq 0.05$ between 25 and 50 µmol/kg. B: Calculated values for maximal distance run (E_{max}) for mice injected po with methadone (12.5 – 50 µmol/kg), a= $p \leq 0.05$ between 12.5 and 25 µmol/kg, b= $p \leq 0.05$ between 12.5 and 50 µmol/kg. Results are presented as mean \pm SEM, $n = 5 - 8$. Control animals ($n = 4$) are not shown.

Sc injections of methadone stimulated locomotor activity ($H=20.71$, 3 df., $p \leq 0.0001$; Figure 3.2 A). Differences in total distance travelled after sc injections were seen between 12.5 and 25 µmol/kg ($p \leq 0.05$), 25 and 100 µmol/kg ($p \leq 0.0001$), and also between 50 and 100 µmol/kg ($p \leq 0.05$). Differences were also seen in E_{max} ($H=14.2$, 3 df., $p \leq 0.05$; Figure 3.2 B). Sc injections of 25 µmol/kg methadone stimulated a higher and more prolonged activity response compared to 12.5 and 100 µmol/kg ($p \leq 0.05$). Upon injection of 50 µmol/kg methadone a significant reduction in E_{max} ($p \leq 0.05$) was demonstrated, but total distance run was unchanged. Administration of the two highest doses resulted in stultifying effects. After

injection of 50 $\mu\text{mol/kg}$, an immediate stimulation of motor activity was seen, reaching E_{max} about 15 minutes after administration, followed by an extensive drop in the activity level. Visual observations confirmed stultifying behavior. After administration of 100 $\mu\text{mol/kg}$, the animals were too affected by the drug to move properly and showed incoherent running. Depending on the dose, the activity declined to zero after fifteen minutes to four hours.

Po injections stimulated locomotor activity ([F (2, 15) = 24.4]; $p \leq 0.0001$; Figure 3.3 A). A dose-dependent increase in total distance run was seen for all doses tested ($p \leq 0.05$). Regarding E_{max} (Figure 3.3 B), an increase was seen between 12.5 and 25 $\mu\text{mol/kg}$ ($p \leq 0.05$) and also between 12.5 and 50 $\mu\text{mol/kg}$ ($p \leq 0.05$).

Comparing sc and po injections, sc injection of 12.5 and 25 $\mu\text{mol/kg}$ resulted in higher total distance, and maximal distance travelled ($p \leq 0.05$). No differences were seen between sc and po injection of 50 $\mu\text{mol/kg}$.

3.1.2 Buprenorphine

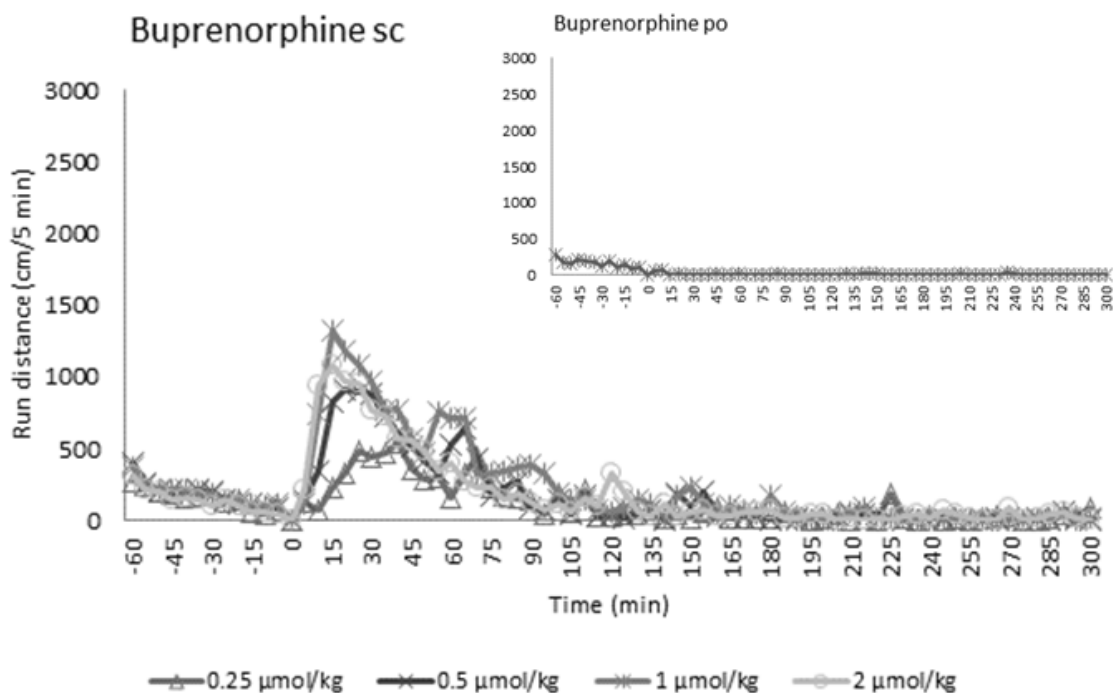


Figure 3.4 Locomotor activity in mice expressed as run distance (centimeters) per 5 minutes after subcutaneous (sc), left, and peroral (po), upper right, injections of buprenorphine (0.25 – 2 $\mu\text{mol/kg}$). Mice were administered drugs at $t=0$, after 60 minutes of habituation. Activity was measured for five hours post administration. Data are means, $n=5 - 8$. Control animals ($n=4$) are not shown, and SEM bars are omitted for clarity.

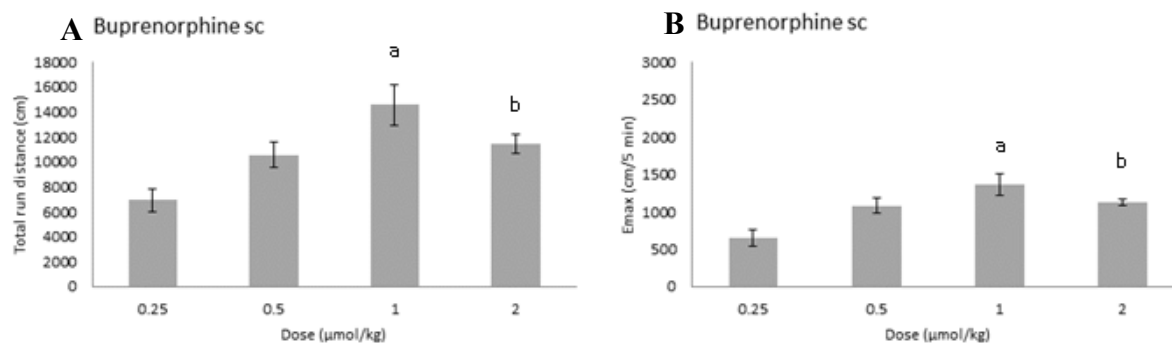


Figure 3.5 A: Calculated values for total distance run (cm) for mice injected sc with buprenorphine (0.25 – 2 µmol/kg), a= $p \leq 0.001$ between 0.25 and 1 µmol/kg, b= $p \leq 0.05$ between 0.25 and 2 µmol/kg. B: Calculated values for maximal distance run (E_{max}) for mice injected sc with buprenorphine (0.25 – 2 µmol/kg) a= $p \leq 0.01$ between 0.25 and 1 µmol/kg, b= $p \leq 0.05$ between 0.25 and 2 µmol/kg. Results are presented as means \pm SEM, $n = 5 - 8$. Control animals ($n = 4$) are not shown.

Sc injections of buprenorphine stimulated locomotor activity ([F (3, 16) = 7.86]; $p \leq 0.05$; Figure 3.5 A). Total distance run was higher after administration of 1 µmol/kg compared to 0.25 µmol/kg ($p \leq 0.001$). Total distance run was also higher after administration of 2 µmol/kg compared to 0.25 µmol/kg ($p \leq 0.05$). Maximal distance run ([F (3, 16) = 7.96]; $p \leq 0.05$; Figure 3.5 B) was higher after injection of both 1 and 2 µmol/kg (Tukey; $p \leq 0.05$) compared to 0.25 µmol/kg.

Po injections of buprenorphine did not stimulate locomotor activity (Figure 3.4 upper right).

Buprenorphine 1 and 2 µmol/kg stimulated higher total run distance when compared to the lowest dose of methadone (Tukey; $p \leq 0.0001$). Total run distance after sc injection of 0.25 and 0.5 µmol/kg buprenorphine did not differ from the lowest dose of methadone ($p \geq 0.05$). Methadone 25 µmol/kg gave higher total run distance when compared to 0.25, 0.5, 1 and 2 µmol/kg of buprenorphine. Regarding E_{max} ([F (4, 22) = 49.5]; $p \leq 0.0001$), methadone 25 µmol/kg gave higher maximal run distance compared to buprenorphine 0.25, 0.5 and also 1 and 2 µmol/kg (Tukey; $p \leq 0.0001$).

3.1.3 Morphine

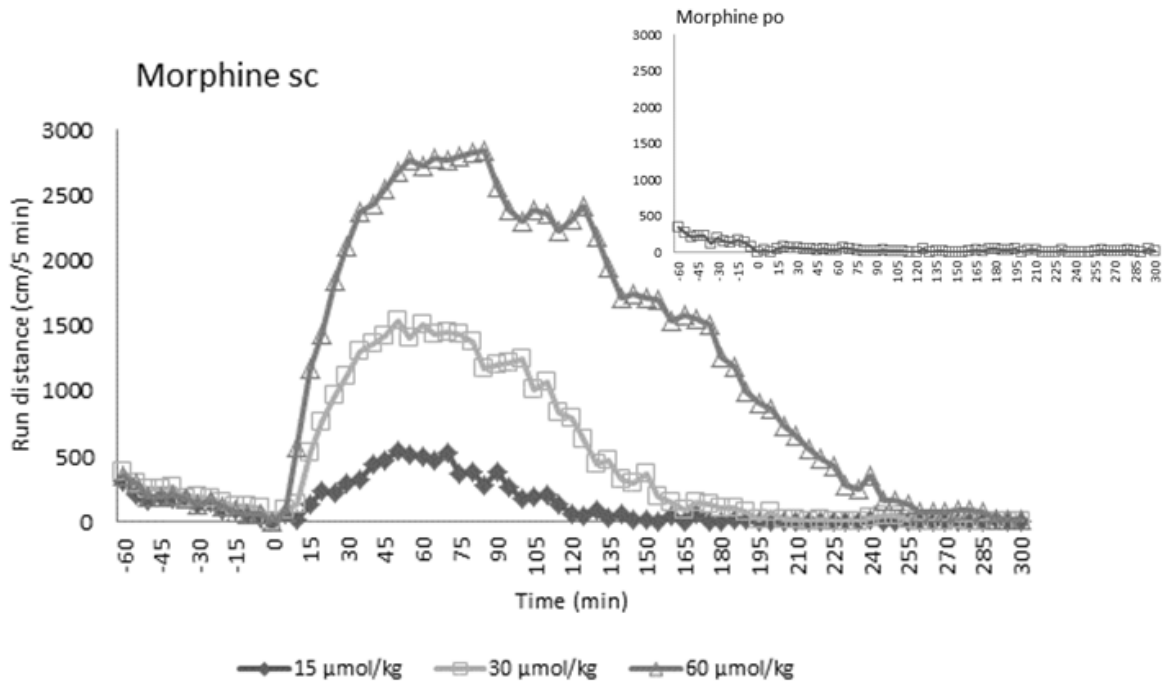


Figure 3.6 Locomotor activity in mice expressed as run distance (centimeters) per 5 minutes after subcutaneous (sc), left, and peroral (po), upper right, injections of morphine (15 – 60 $\mu\text{mol/kg}$). Mice were administered drugs at $t=0$, after 60 minutes of habituation. Activity was measured for five hours post administration. Data are means, $n=5-8$. Control animals ($n=4$) are not shown, and SEM bars are omitted for clarity.

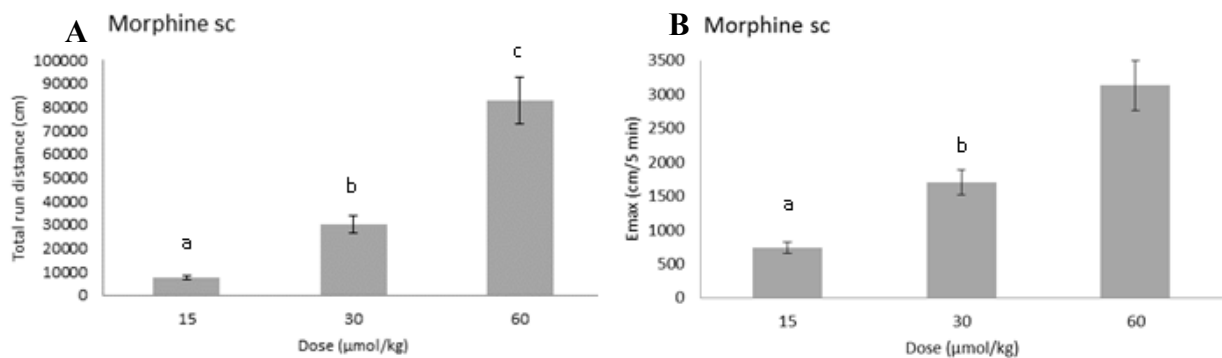


Figure 3.7 A: Calculated values for total distance run (cm) for mice injected sc with morphine (15 – 60 $\mu\text{mol/kg}$), $a= p \leq 0.0001$ between 15 and 30 $\mu\text{mol/kg}$, $b= p \leq 0.0001$ between 15 and 60 $\mu\text{mol/kg}$, $c= p \leq 0.0001$ between 30 and 60 $\mu\text{mol/kg}$. B: Calculated values for maximal distance run (E_{max}) for mice injected sc with morphine (15 – 60 $\mu\text{mol/kg}$) $a= p \leq 0.0001$ between 15 and 60 $\mu\text{mol/kg}$, $b= p \leq 0.001$ between 30 and 60 $\mu\text{mol/kg}$. Results are presented as means \pm SEM, $n=5-8$. Control animals ($n=4$) are not shown.

Locomotor activity was stimulated after sc injection of morphine ($[F(2, 17) = 62.1]$; $p \leq 0.0001$; Figure 3.7 A). A dose-dependent increase was seen in total distance run ($p \leq 0.0001$). Regarding E_{max} ($[F(2, 17) = 21.9]$; $p \leq 0.001$; Figure 3.7 B), a difference was seen between the doses 15 and 60 $\mu\text{mol/kg}$ ($p \leq 0.0001$) and also between 30 and 60 $\mu\text{mol/kg}$ ($p \leq 0.001$). Duration of activity was about 4 hours for the highest dose. Po administration of morphine (30 $\mu\text{mol/kg}$) did not stimulate locomotor activity (Figure 3.6 upper right).

3.1.4 Heroin

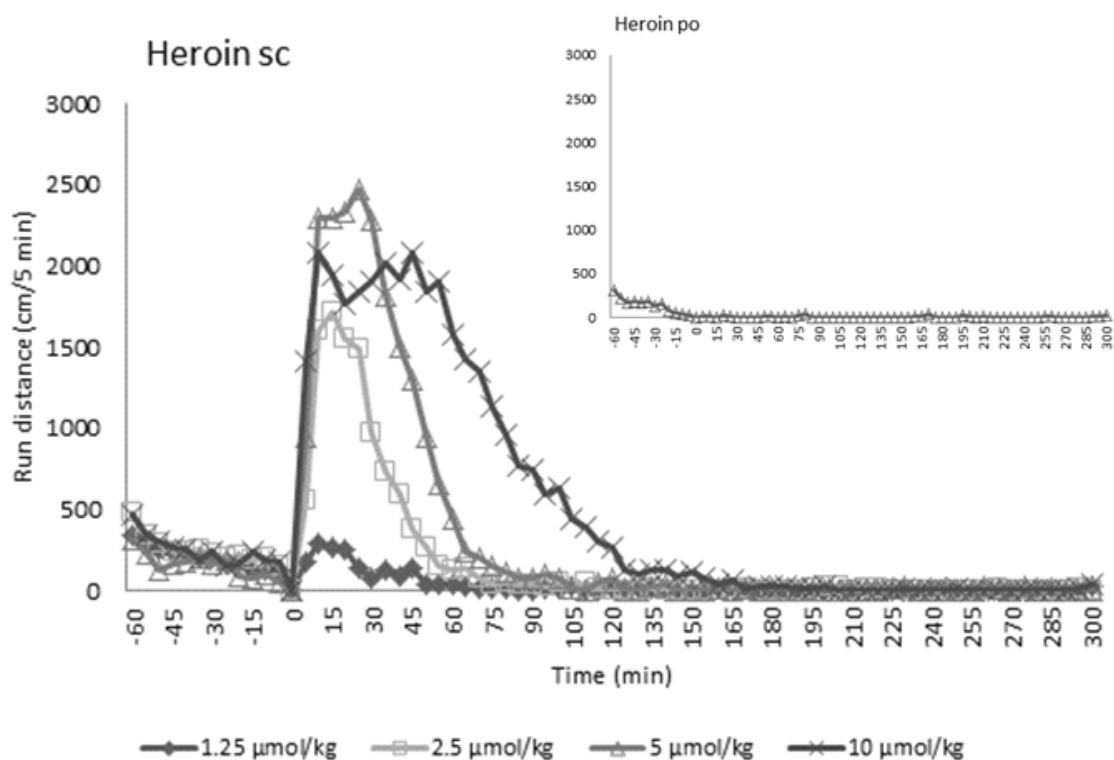


Figure 3.8 Locomotor activity in mice expressed as run distance (centimeters) per 5 minutes after subcutaneous (sc), left, and peroral (po), upper right, injections of heroin (1.25 – 10 $\mu\text{mol/kg}$). Mice were administered drugs at $t=0$, after 60 minutes of habituation. Activity was measured for five hours post administration. Data are means, $n=5-7$. Control animals ($n=4$) are not shown, and SEM bars are omitted for clarity.

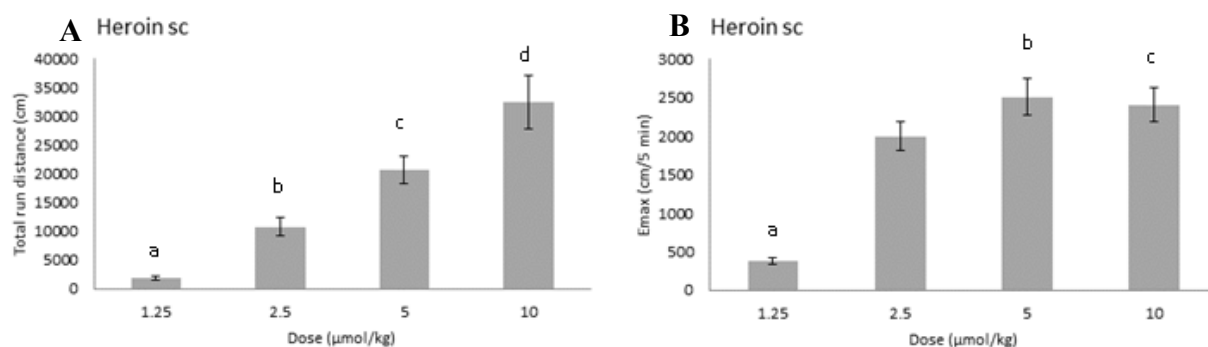


Figure 3.9 A: Calculated values for total distance run (cm) for mice injected sc with heroin (1.25 – 10 µmol/kg), a= $p \leq 0.0001$ between 1.25 and 5 µmol/kg, b= $p \leq 0.0001$ between 2.5 and 10 µmol/kg, c= $p \leq 0.05$ between 5 and 10 µmol/kg, d= $p \leq 0.0001$ between 1.25 and 10 µmol/kg. B: Calculated values for maximal distance run (E_{max}) for mice injected sc with heroin (1.25 – 10 µmol/kg) a= $p \leq 0.0001$ between 1.25 and 2.5 µmol/kg, b= $p \leq 0.001$ between 1.25 and 5 µmol/kg, c= $p \leq 0.0001$ between 1.25 and 10 µmol/kg. Results are presented as means \pm SEM, $n = 5 - 7$. Control animals ($n = 4$) are not shown.

Sc injections of heroin (1.25-10 µmol/kg) stimulated locomotor activity ([F (3, 21) = 26.4]; $p \leq 0.0001$; Figure 3.9A). A marked difference in total distance travelled was seen between the doses; 1.25 and 5 µmol/kg ($p \leq 0.0001$), 1.25 and 10 µmol/kg ($p \leq 0.0001$), 2.5 and 10 µmol/kg ($p \leq 0.0001$) and also between 5 and 10 µmol/kg ($p \leq 0.05$). Differences between doses concerning E_{max} ([F (3, 20) = 66.7]; $p \leq 0.0001$; Figure 3.9 B) were seen between the doses; 1.25 and 2.5 µmol/kg, 1.25 and 5 µmol/kg, and between 1.25 and 10 µmol/kg ($p \leq 0.0001$).

Highest E_{max} was observed for mice receiving 5 µmol/kg heroin. The activity peaked around 30 minutes after administration and the high activity gradually declined over the next hour. The highest dose of heroin (10 µmol/kg) resulted in beginning stultifying effects, and showed the longest lasting high-activity interval of all heroin doses tested. Heroin injected po did not stimulate any motor activity (Figure 3.8 upper left).

Based on these results, it was decided to use sc injections in the rest of the studies. Doses selected for repeated exposure were 25 and 50 µmol/kg methadone, 2 µmol/kg buprenorphine and 30 and 60 µmol/kg morphine.

3.1.5 Locomotor activity after single versus repeated exposure to methadone, buprenorphine and morphine

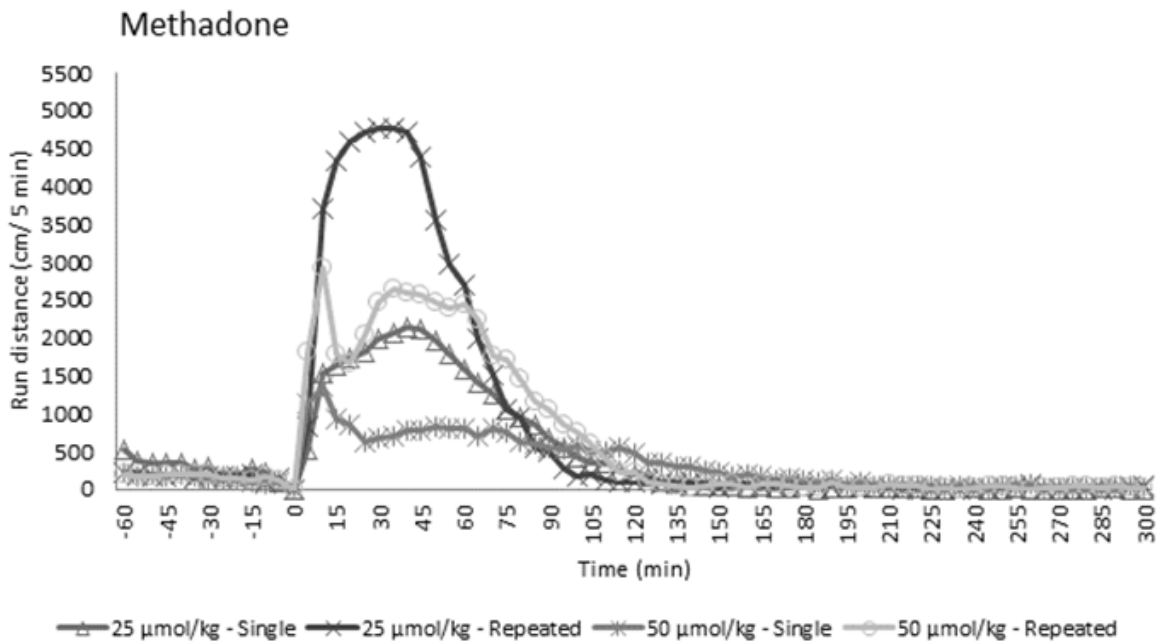


Figure 3.10 Locomotor activity in mice expressed as run distance (centimeters) per 5 minutes after subcutaneous (sc) injections of methadone. Mice were injected repeatedly (14 injections) over a three weeks period, withdrawal on Saturday to Sunday. On the last day, methadone was injected at $t=0$, after 60 minutes of habituation. Activity was measured for five hours. Data are means, $n=5-6$, Control animals ($n=2$) and SEM bars are not shown. Locomotor activity profiles after single sc injections are included for comparison.

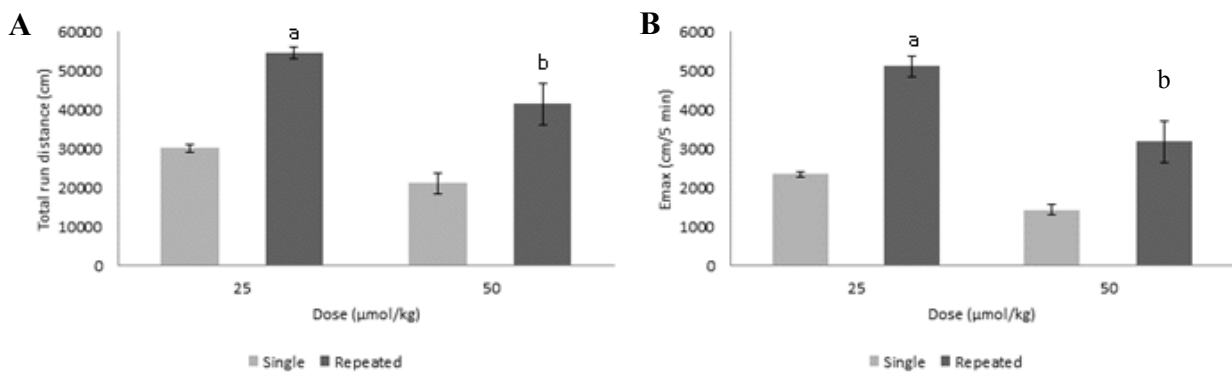


Figure 3.11 A: Calculated values for total distance run (cm) for methadone (25 – 50 $\mu\text{mol/kg}$ single and repeated injection), $a = p \leq 0.0001$ between 25 $\mu\text{mol/kg}$ single and repeated, $b = p \leq 0.05$ between 50 $\mu\text{mol/kg}$ single and repeated. B: Calculated values for maximal distance run (E_{max}) for mice injected sc with methadone (25 – 50 $\mu\text{mol/kg}$ single injection and repeated), $a = p \leq 0.0001$ between 25 $\mu\text{mol/kg}$ single and repeated, $b = p \leq 0.05$ between 50 $\mu\text{mol/kg}$ single and repeated. Calculation of values for total distance and maximal distance run after single injection (presented earlier) are included for comparison. Data are means \pm SEM, $n=5-6$. Control animals ($n=2$) are not shown.

Mice repeatedly exposed (14 injections) to 25 $\mu\text{mol/kg}$ methadone showed a significantly higher activity response compared to animals receiving a single injection of the equimolar dose (Students t-test; $p \leq 0.0001$). E_{max} was also significantly higher (Students t-test, $p \leq 0.0001$). Total distance run and maximal distance were also higher in mice repeatedly injected with 50 $\mu\text{mol/kg}$ methadone (Students t-test; $p \leq 0.05$).

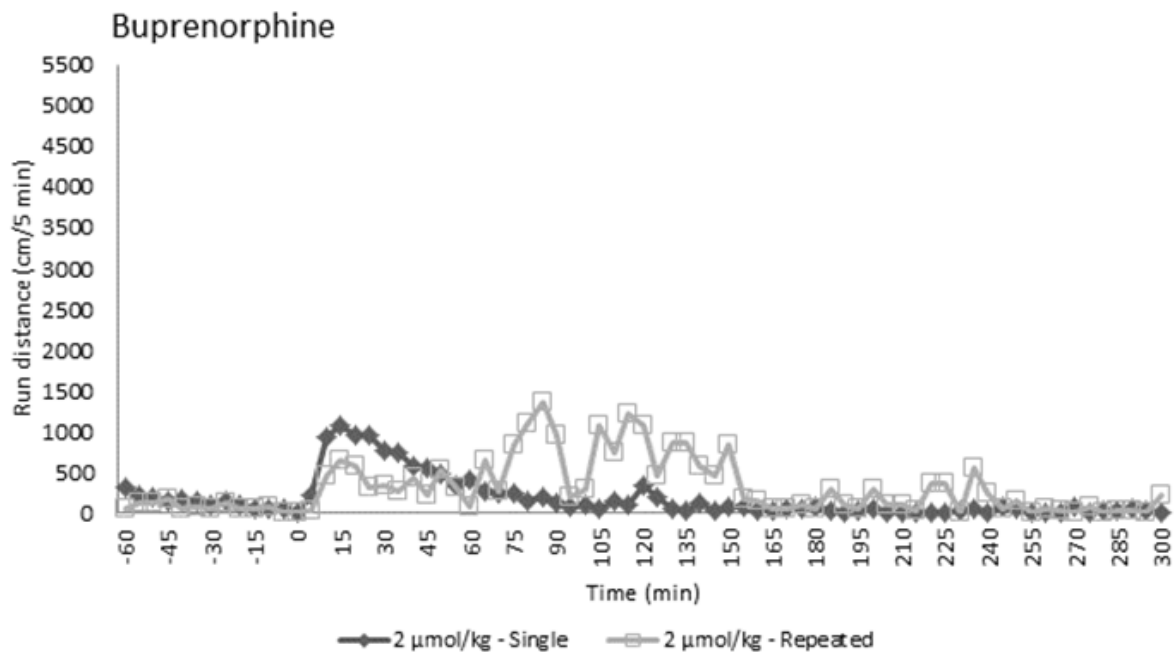


Figure 3.12 Locomotor activity in mice expressed as run distance (centimeters) per 5 minutes after subcutaneous (sc) injections of buprenorphine. Mice were injected repeatedly (14 injections) over a three weeks period, withdrawal on Saturday – Sunday. On the last day, drugs were injected at $t=0$, after 60 minutes of habituation. Activity was measured for five hours. Data are means, $n= 5 - 6$, Control animals ($n=2$) and SEM bars are not shown. Locomotor activity profiles after single sc injections are included for comparison.

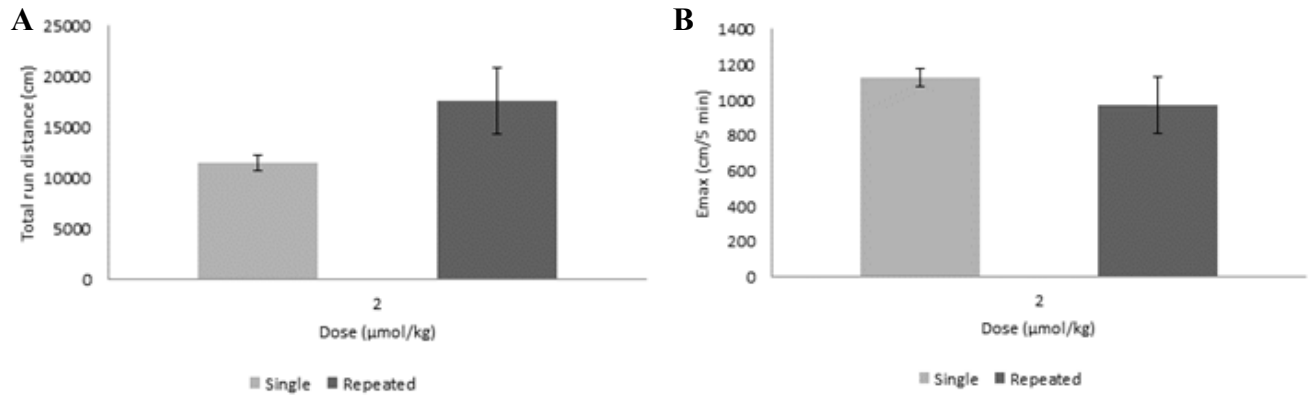


Figure 3.13 A: Calculated values for total distance run (cm) for buprenorphine (2 μmol/kg single and repeated injection). B: Calculated values for maximal distance run (E_{max}) for mice injected sc with buprenorphine (2 μmol/kg single and repeated injection). Calculation of values for total distance and maximal distance after single injection (presented earlier) are included for comparison. Data are means, $n=5 - 6$. Control animals ($n=2$) are not shown.

No difference was found in activity response between animals receiving a single versus 14 injections of buprenorphine (Students t-test; $p \geq 0.05$; Figure 3.13A and B). The response of the repeatedly treated animals tended to be delayed compared to the animals injected once, but the difference was not significant.

Compared to the methadone doses 25 and 50 μmol/kg, buprenorphine displayed a significantly lower response, both in total distance and also maximal distance run following repeated exposure (Tukey; $p \leq 0.05$).

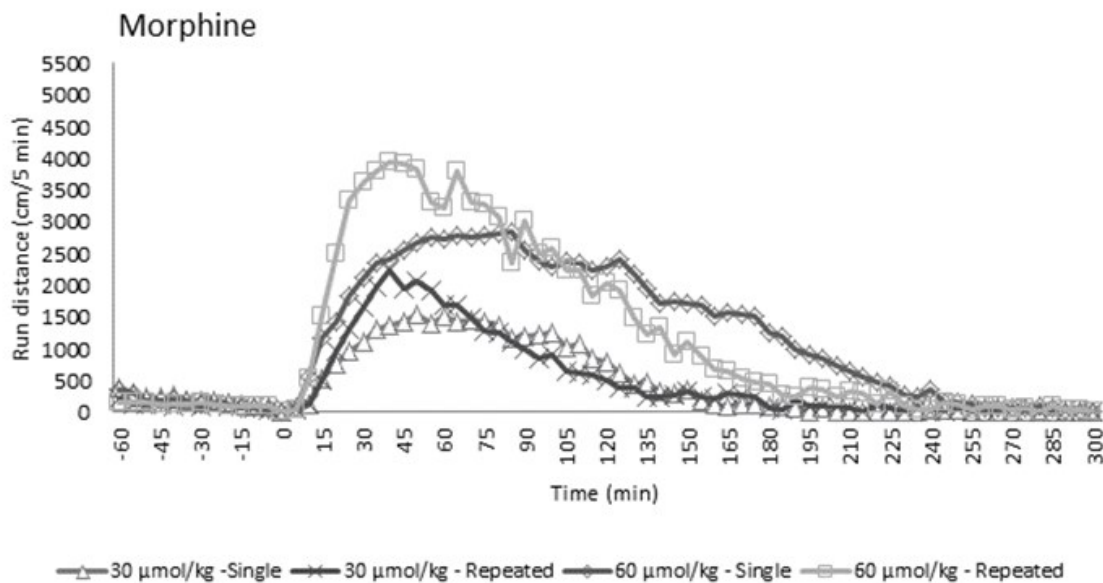


Figure 3.14 Locomotor activity in mice expressed as run distance (centimeters) per 5 minutes after subcutaneous (sc) injections of morphine. Mice were injected repeatedly (14 injections) over a three weeks period, withdrawal on Saturday – Sunday. On the last day, drugs were injected at $t=0$, after 60 minutes of habituation. Activity was measured for five hours. Data are means, $n=5-6$. Control animals ($n=2$) and SEM bars are not shown. Locomotor activity profiles after single sc injections are included for comparison.

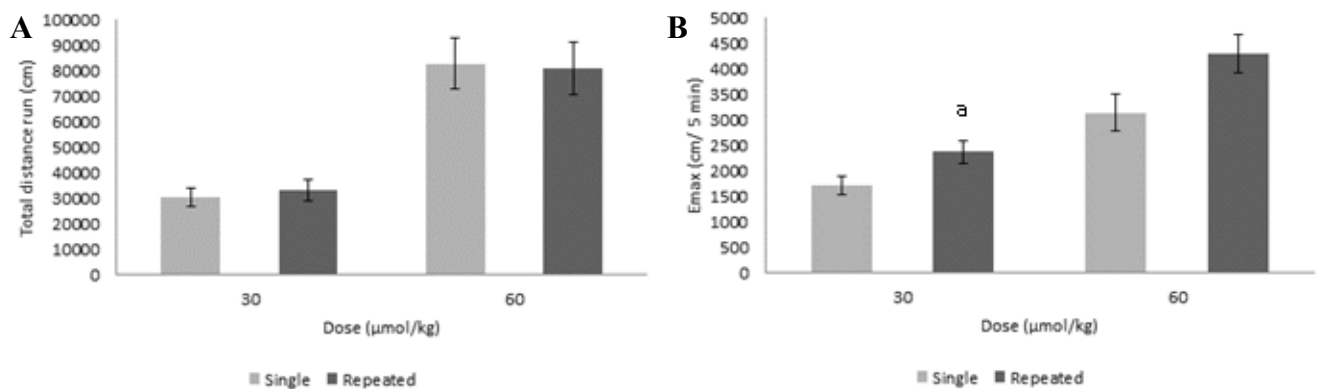


Figure 3.15 A: Calculated values for total distance run (cm) for methadone (30 – 60 $\mu\text{mol/kg}$ single and repeated injection). B: Calculated values for maximal distance run (E_{max}) for mice injected sc with morphine (30 – 60 $\mu\text{mol/kg}$ single and repeated injection), $a = p \leq 0.05$ difference between 30 $\mu\text{mol/kg}$ single and 30 $\mu\text{mol/kg}$ repeated. Calculation of values for total distance and maximal distance after single injection (presented earlier) are included for comparison. Data are means, $n=5-6$. Control animals ($n=2$) are not shown.

A slight increase in E_{max} was seen for mice repeatedly injected with 30 $\mu\text{mol/kg}$ morphine (Students t-test; $p \leq 0.05$; Figure 3.15 B). No other differences were found.

No stimulation of locomotor activity was observed for control animals injected with saline (n=2). Data are not shown.

3.2 Weight curves

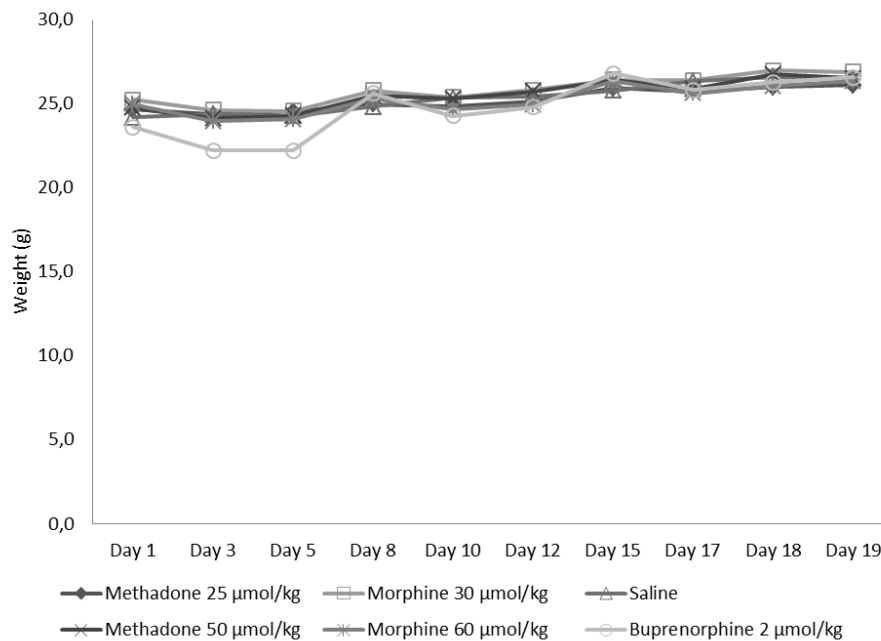


Figure 3.16 Weight curves for mice injected sc with saline, methadone, buprenorphine or morphine for four days (4 injections) and three weeks (14 injections). Mice were weighted every other day in addition to the day when locomotor activity was tested. Data are means, n = 5-12. SEM bars are omitted for clarity.

Repeated injections of methadone, buprenorphine or morphine did not affect the weight of the mice (Kruskal-Wallis test; $p \geq 0.05$; Figure 3.16).

3.3 Protein measurement

The measures of protein concentration were not significantly different between treatment groups when compared within the different brain areas (Tukey; $p \geq 0.05$).

3.4 Western blotting – protein expression

3.4.1 Expression of pCaMKII, CaMKII and β -Actin after four days of methadone exposure

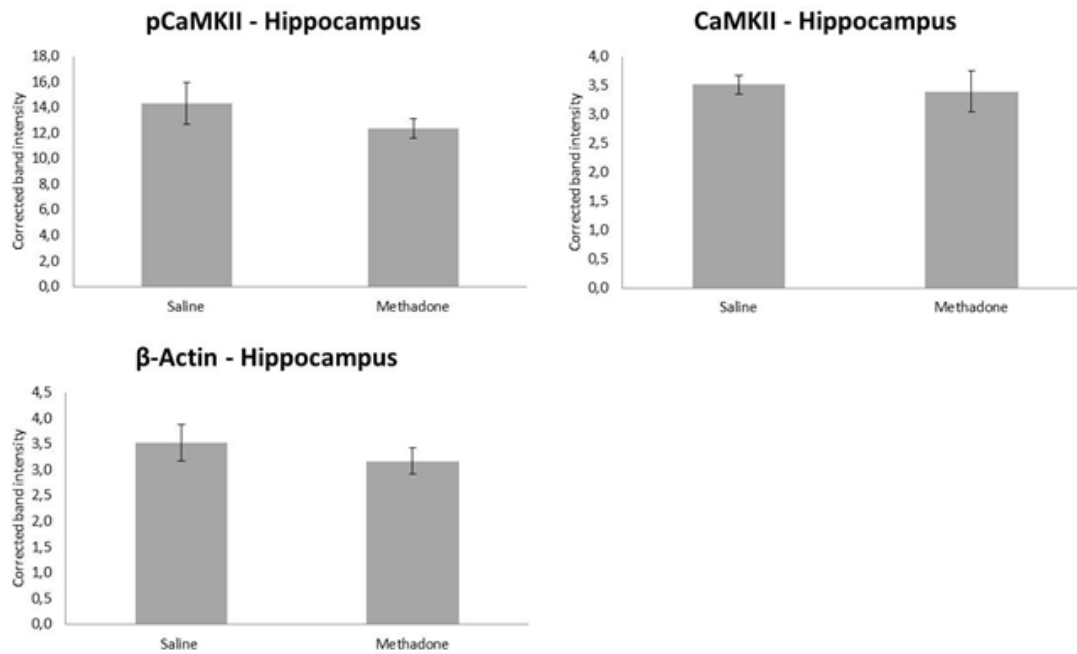


Figure 3.17 pCaMKII, CaMKII and β -Actin in hippocampus from mice one day after the last injection of a four-days exposure regime with saline or methadone (25 μ mol/kg). Data are means \pm SEM, n= 5. (Mann-Whitney U test).

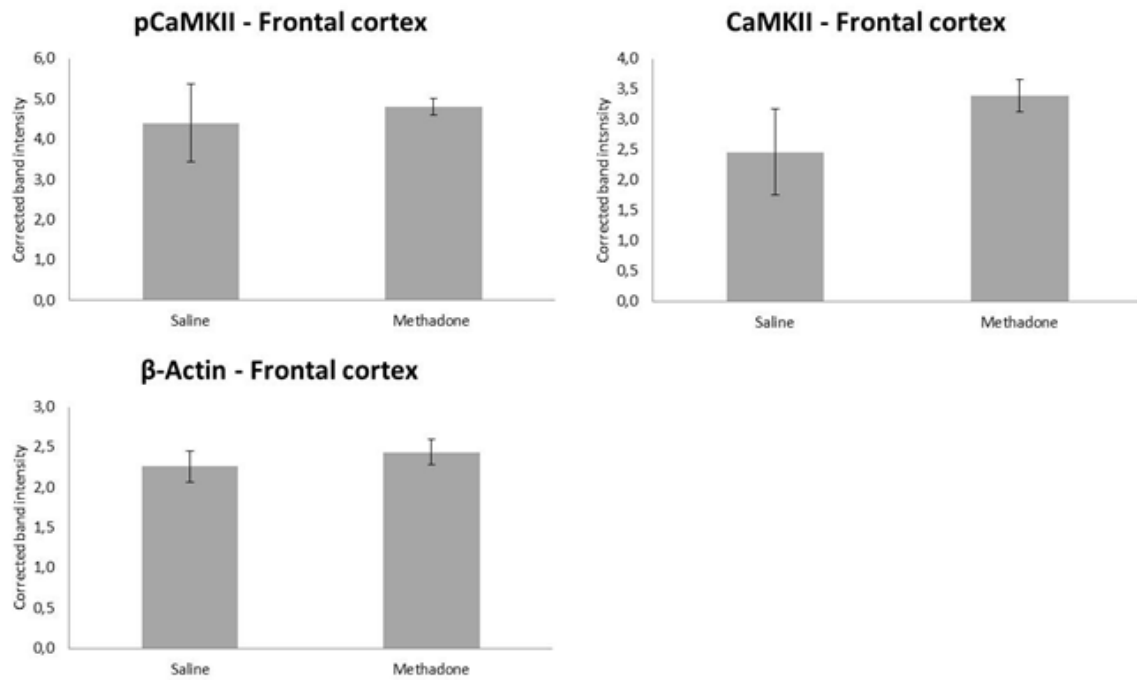


Figure 3.18 pCaMKII, CaMKII and β -Actin in frontal cortex from mice one day after the last injection of a four-days exposure regime with saline or methadone (25 μ mol/kg). Data are means \pm SEM, $n = 4 - 5$. (Mann–Whitney U test).

A four-days exposure regime, with daily injections (sc) of methadone (25 μ mol/kg) did not affect the level of pCaMKII, CaMKII or β -Actin in hippocampus and frontal cortex of mice (Mann–Whitney U test; $p \geq 0.05$).

3.4.2 Protein expression in hippocampus and frontal cortex after three weeks exposure to methadone, buprenorphine or morphine

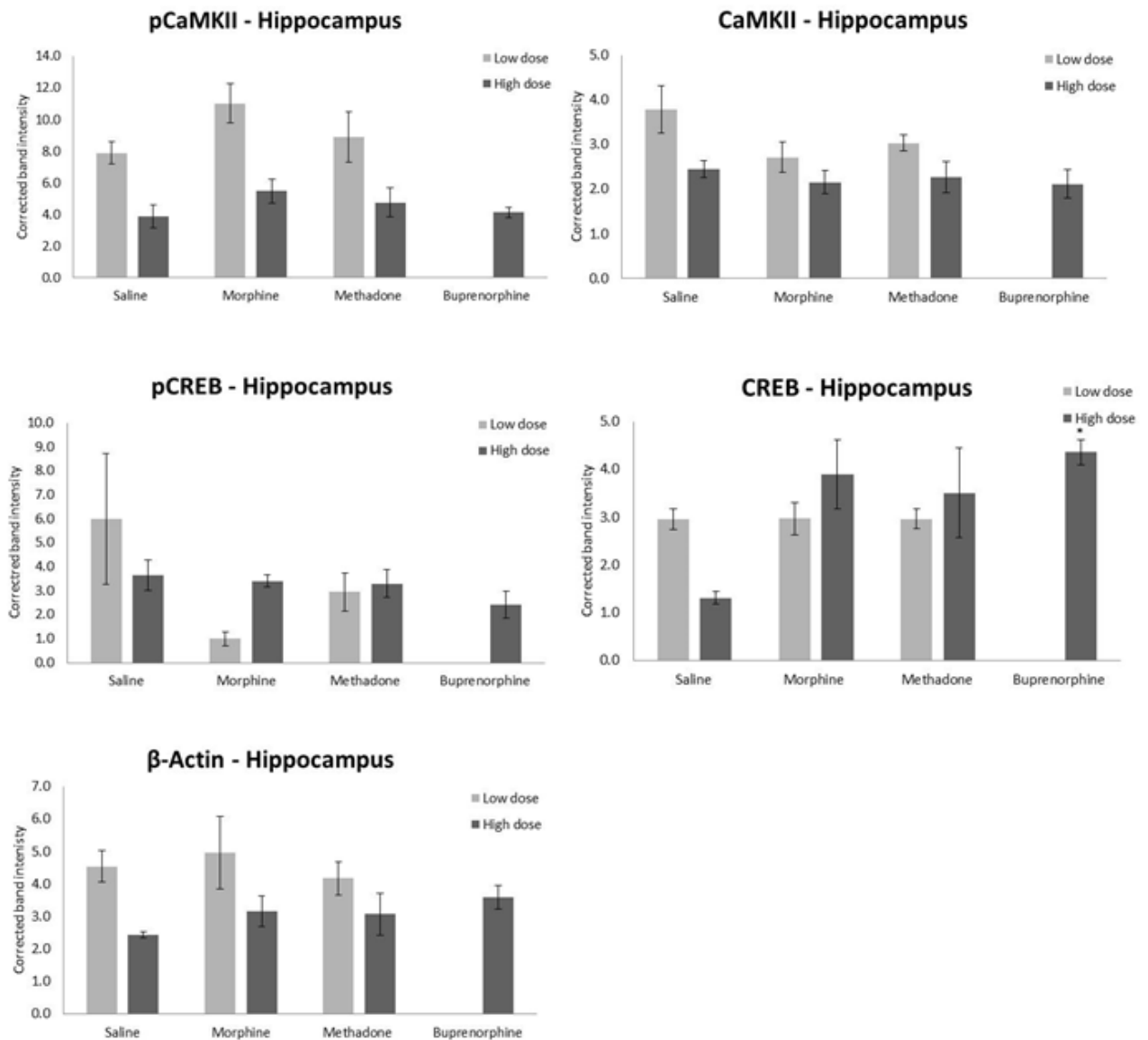


Figure 3.19 pCaMKII, CaMKII, pCREB, CREB and β -Actin in mice hippocampus one day after the last injection of a three weeks (14 injections) injection regime with saline, morphine (30 and 60 $\mu\text{mol/kg}$), methadone (25 and 50 $\mu\text{mol/kg}$) and buprenorphine (2 $\mu\text{mol/kg}$) Light grey: indicate low dose, and twice as much protein as the dark grey bars: indicating high dose. Data are means \pm SEM, $n=4 - 7$. * $p \leq 0.05$. (Kruskal-Wallis test).

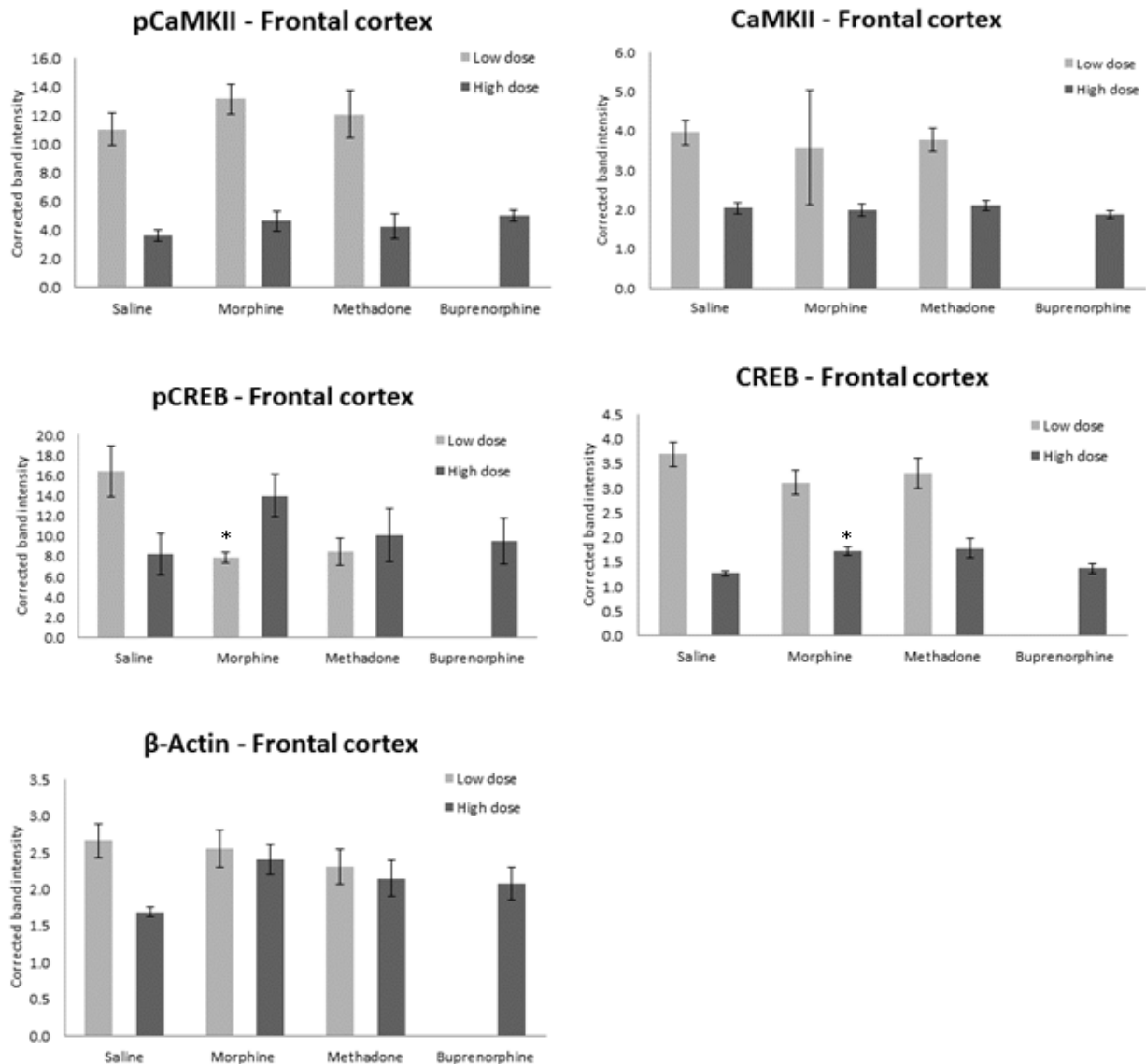


Figure 3.20 pCaMKII, CaMKII, pCREB, CREB and β -Actin in mice frontal cortex one day after the last injection of a three weeks injection regime (14 injections) with saline, morphine (30 and 60 $\mu\text{mol/kg}$), methadone (25 and 50 $\mu\text{mol/kg}$) or buprenorphine (2 $\mu\text{mol/kg}$). Light grey: indicate low dose and twice as much protein as the dark grey bars: indicating high dose. Data are means \pm SEM, $n = 4 - 7$. * $p \leq 0.05$. (Kruskal-Wallis test).

In hippocampus of mice injected with 2 $\mu\text{mol/kg}$ (high dose) buprenorphine, an upregulation in expression of CREB ($H = 12.45$, 3 df., $p \leq 0.05$) was seen when compared to mice injected with saline ($p \leq 0.05$). Expression of pCREB was significantly downregulated ($H = 7.28$, 2 df., $p \leq 0.05$) in frontal cortex of mice injected with 30 $\mu\text{mol/kg}$ morphine (low dose) repeatedly for three weeks compared to saline ($p \leq 0.05$). An upregulation in expression of CREB ($H = 10.57$, 3 df., $p \leq 0.05$) was seen in frontal cortex of mice receiving 60 $\mu\text{mol/kg}$ morphine (high dose) ($p \leq 0.05$).

3.4.3 Protein expression in hippocampus and frontal cortex one hour after a single injection of methadone, buprenorphine or morphine

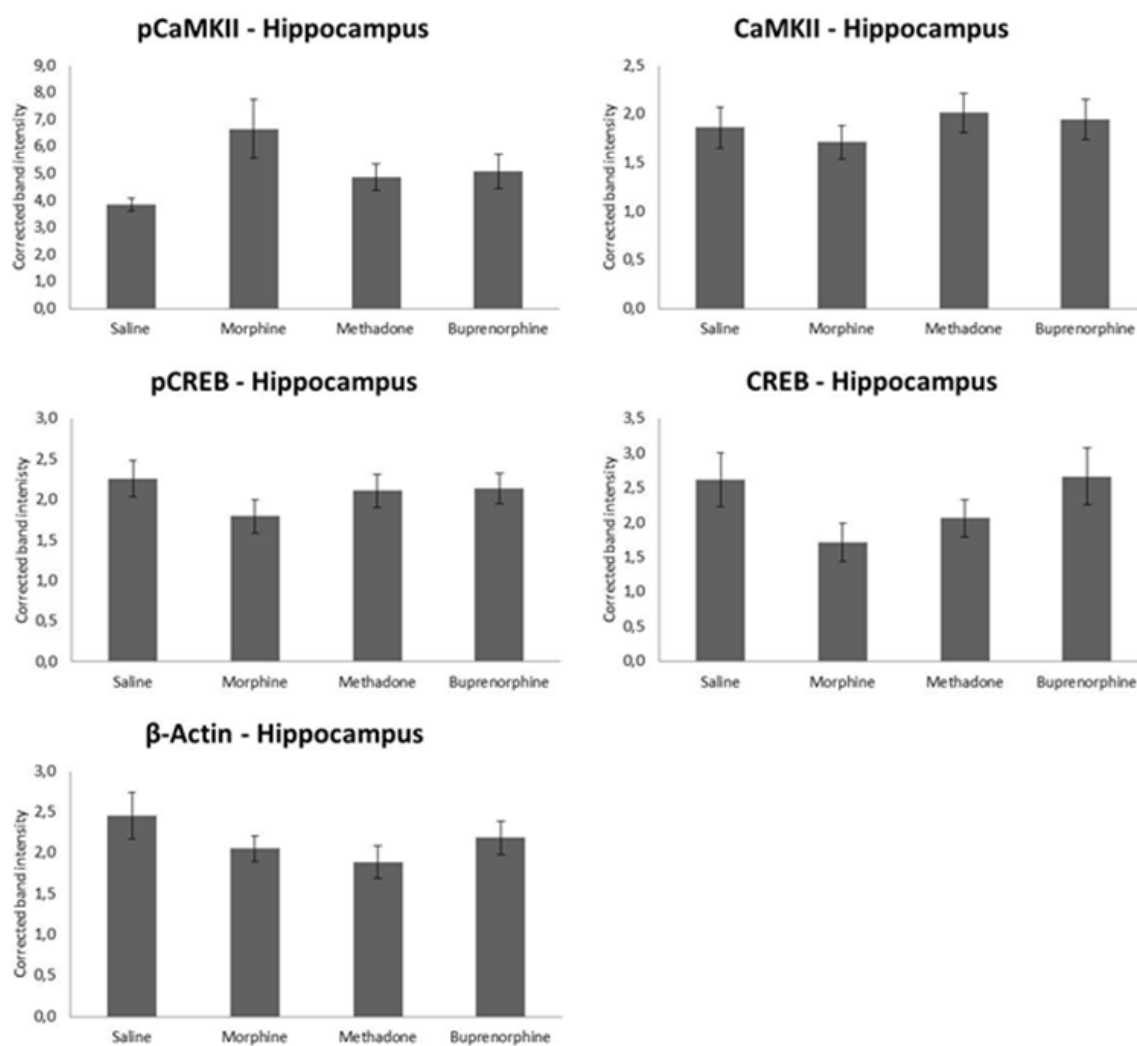


Figure 3.21 pCaMKII, CaMKII, pCREB, CREB and β -Actin in mice hippocampus one hour after a single injection of saline, morphine (60 $\mu\text{mol/kg}$), methadone (50 $\mu\text{mol/kg}$) or buprenorphine (2 $\mu\text{mol/kg}$). Data are means \pm SEM, $n=6-8$. (Kruskal-Wallis test).

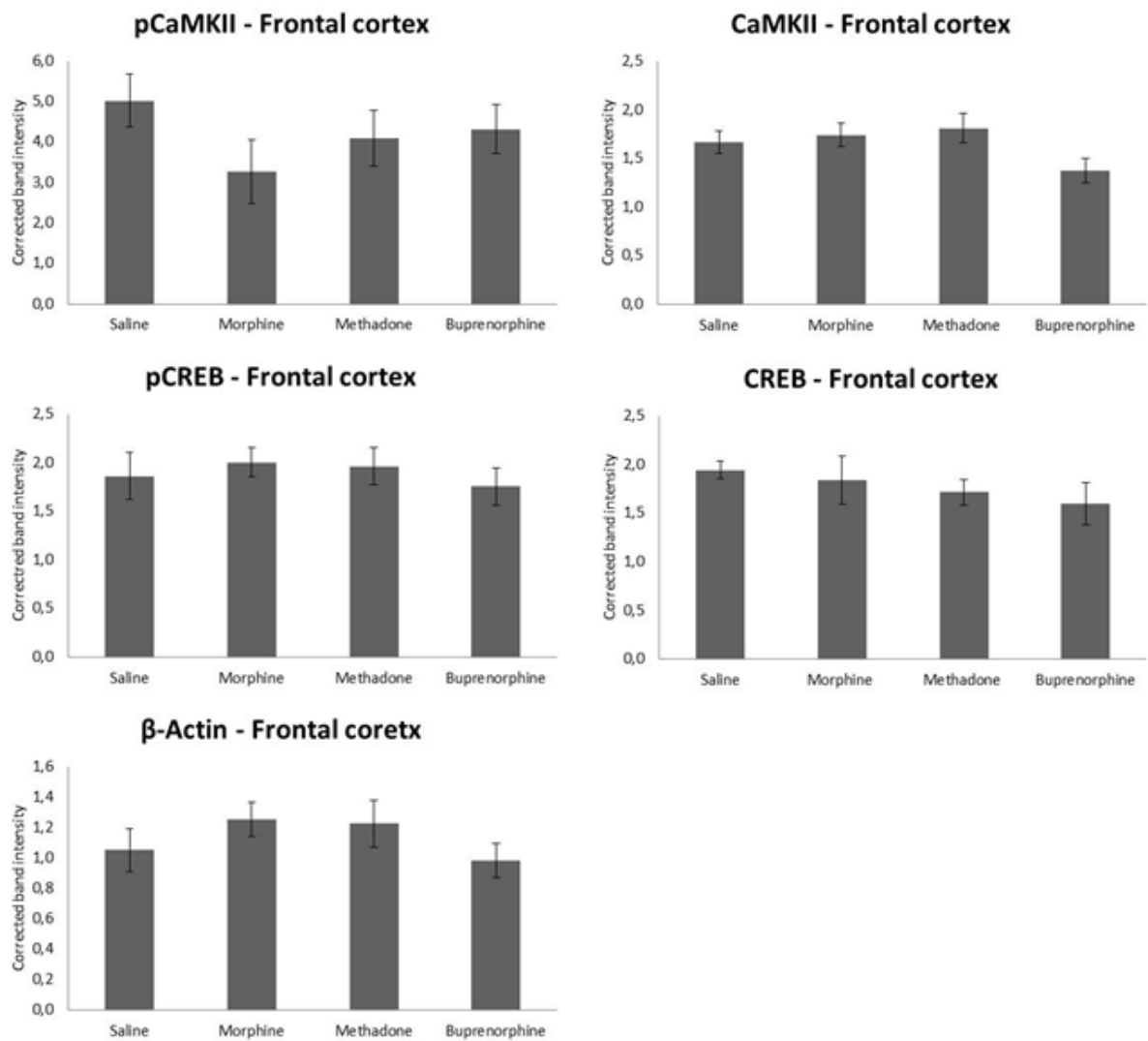


Figure 3.22 pCaMKII, CaMKII, pCREB, CREB and β -Actin in mice frontal cortex one hour after a single injection of saline, morphine (60 $\mu\text{mol/kg}$), methadone (50 $\mu\text{mol/kg}$) or buprenorphine (2 $\mu\text{mol/kg}$). Data are means \pm SEM, $n=5-8$. (Kruskal-Wallis test).

Neither pCaMKII, CaMKII, pCREB, CREB nor β -Actin was significantly affected in hippocampus or frontal cortex from mice subjected to a single injection of methadone (50 $\mu\text{mol/kg}$), buprenorphine (2 $\mu\text{mol/kg}$) or morphine (60 $\mu\text{mol/kg}$) compared to saline ($p > 0.05$) one hour after injection.

3.4.4 Expression of pCaMKII, CaMKII and β -Actin in dorsal striatum and nucleus accumbens after four days of methadone exposure

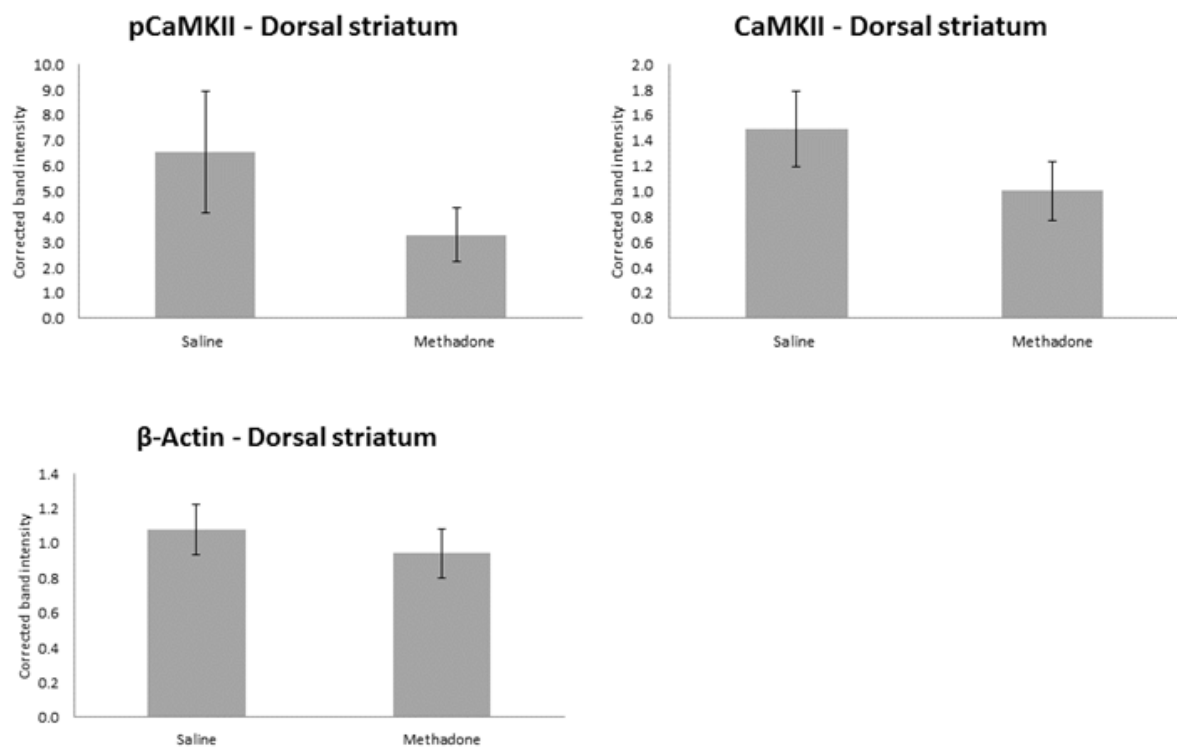


Figure 3.23 pCaMKII, CaMKII and β -Actin in dorsal striatum from mice one day after the last injection of a four-days exposure regime with saline or methadone (25 μ mol/kg). Data are means \pm SEM, n= 5. (Mann-Whitney U test).

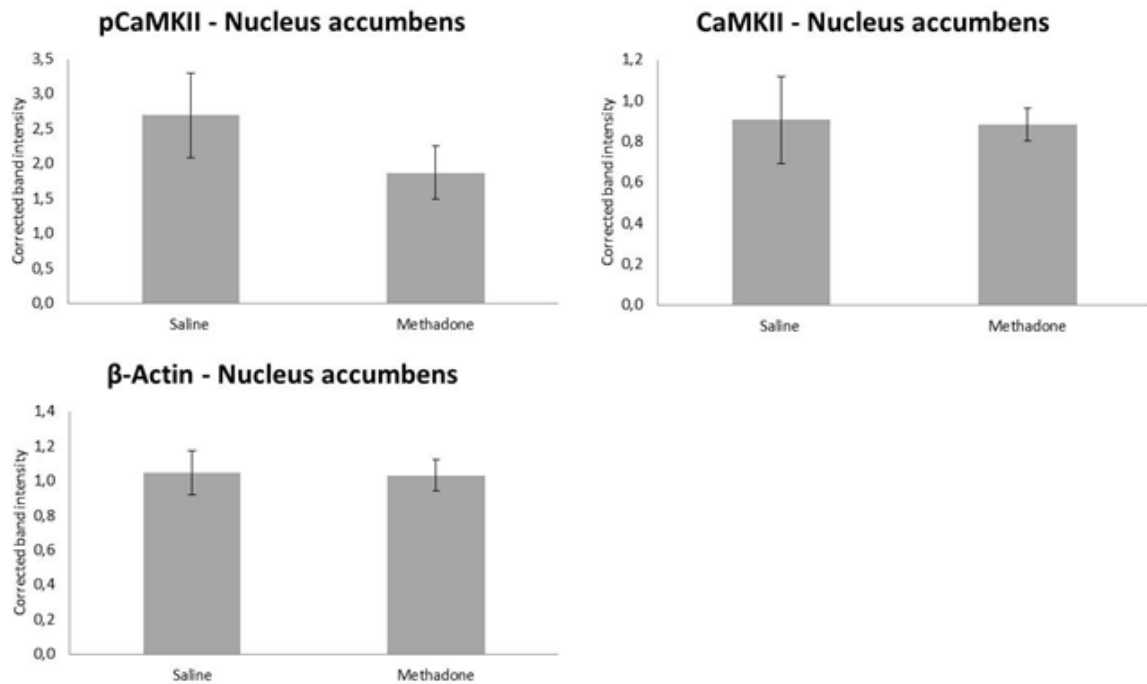


Figure 3.24 pCaMKII, CaMKII and β -Actin in nucleus accumbens from mice one day after the last injection of a four-day exposure regime with saline or methadone (25 $\mu\text{mol/kg}$). Data are means \pm SEM, $n = 4 - 5$. (Mann-Whitney U test).

No change in protein expression was found in dorsal striatum and nucleus accumbens from mice injected with methadone (25 $\mu\text{mol/kg}$) once a day for four days ($p \geq 0.05$).

3.4.5 Protein expression in dorsal striatum and nucleus accumbens after three weeks exposure to methadone or morphine

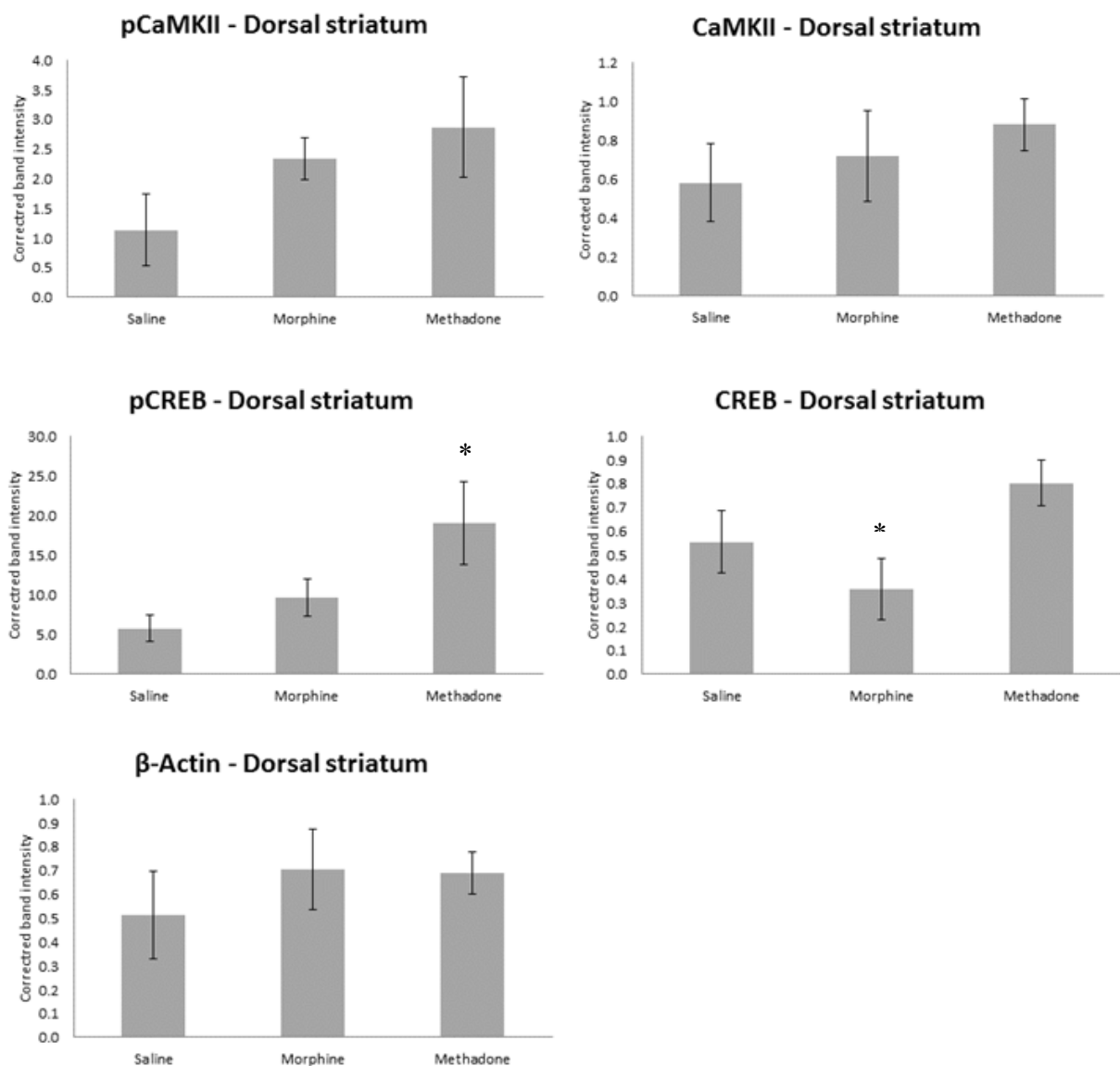


Figure 3.25 pCaMKII, CaMKII, pCREB, CREB and β -Actin in mice dorsal striatum one day after the last injection of a three weeks (14 injections) injection regime with saline, morphine (30 $\mu\text{mol/kg}$) or methadone (25 $\mu\text{mol/kg}$). Data are means \pm SEM, $n=4-7$. * $p \leq 0.05$. (Kruskal-Wallis test).

In dorsal striatum of mice injected with 25 $\mu\text{mol/kg}$ methadone, an increase in expression of pCREB was seen when compared to control mice ($H=7.76$, 2 df., $p \leq 0.05$). The expression of CREB was significantly lower in mice injected with 30 $\mu\text{mol/kg}$ morphine when compared to mice injected with 25 $\mu\text{mol/kg}$ methadone ($H=6.69$, 2 df., $p \leq 0.05$).

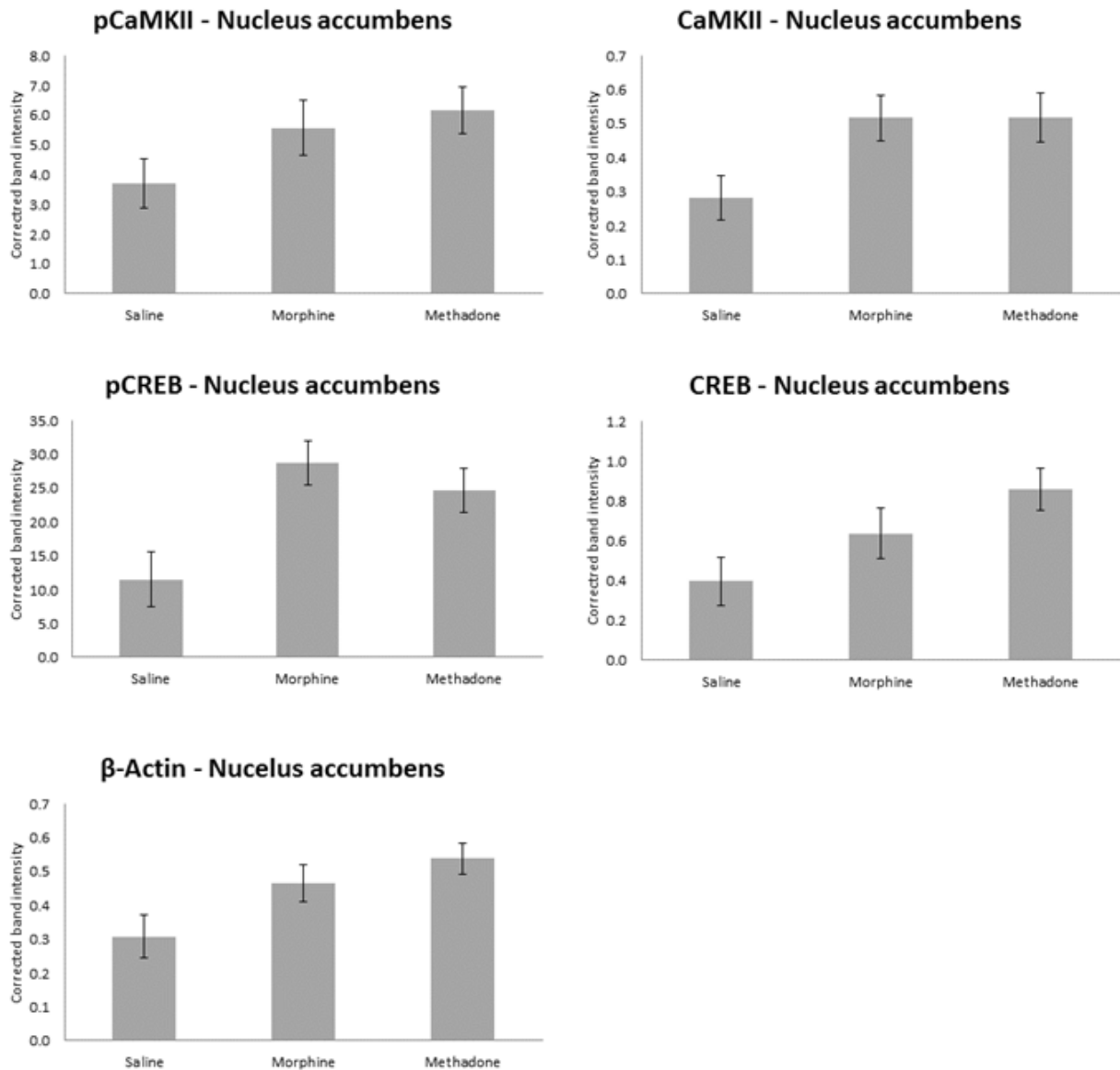


Figure 3.26 pCaMKII, CaMKII, pCREB, CREB and β -Actin in mice nucleus accumbens one day after the last injection of a three weeks (14 injections) injection regime with saline, morphine (30 μ mol/kg) and methadone (25 μ mol/kg). Data are means \pm SEM, n=4 – 6. (Kruskal-Wallis test).

Proteins in nucleus accumbens were not affected by administration of methadone or morphine ($p \geq 0.05$).

3.4.6 Protein expression in dorsal striatum and nucleus accumbens after three weeks exposure to high doses of methadone, buprenorphine or morphine

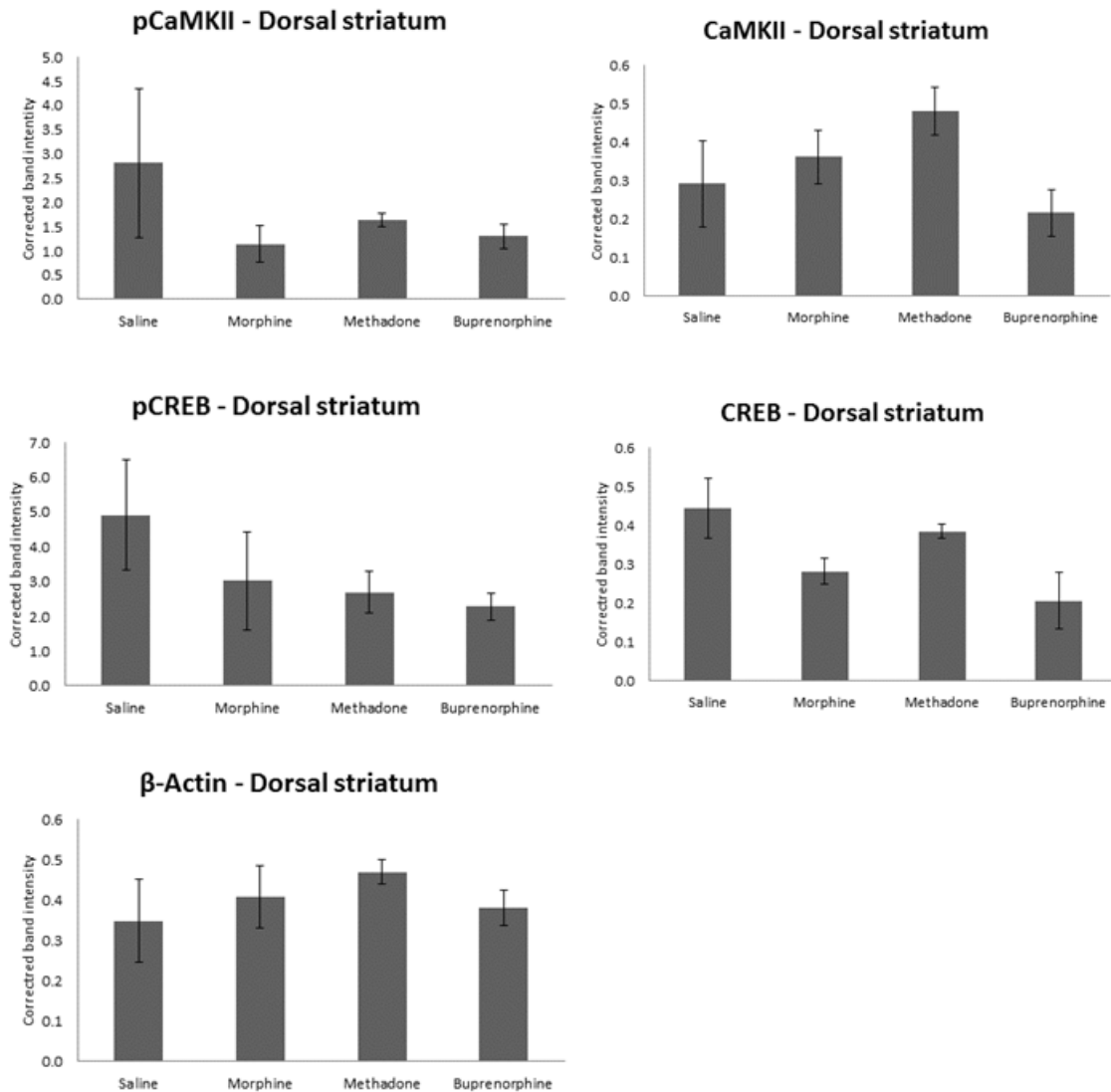


Figure 3.27 pCaMKII, CaMKII, pCREB, CREB and β -Actin in mice dorsal striatum one day after the last injection of a three weeks (14 injections) injection regime with saline, morphine (60 $\mu\text{mol/kg}$), methadone (50 $\mu\text{mol/kg}$) and buprenorphine (2 $\mu\text{mol/kg}$). Data are means \pm SEM, $n=4 - 6$. (Kruskal-Wallis test).

Expression of pCaMKII, CaMKII, pCREB, CREB and β -Actin were unaffected by injection with high doses of methadone (50 $\mu\text{mol/kg}$), buprenorphine (2 $\mu\text{mol/kg}$) and morphine (60 $\mu\text{mol/kg}$) ($p \geq 0.05$).

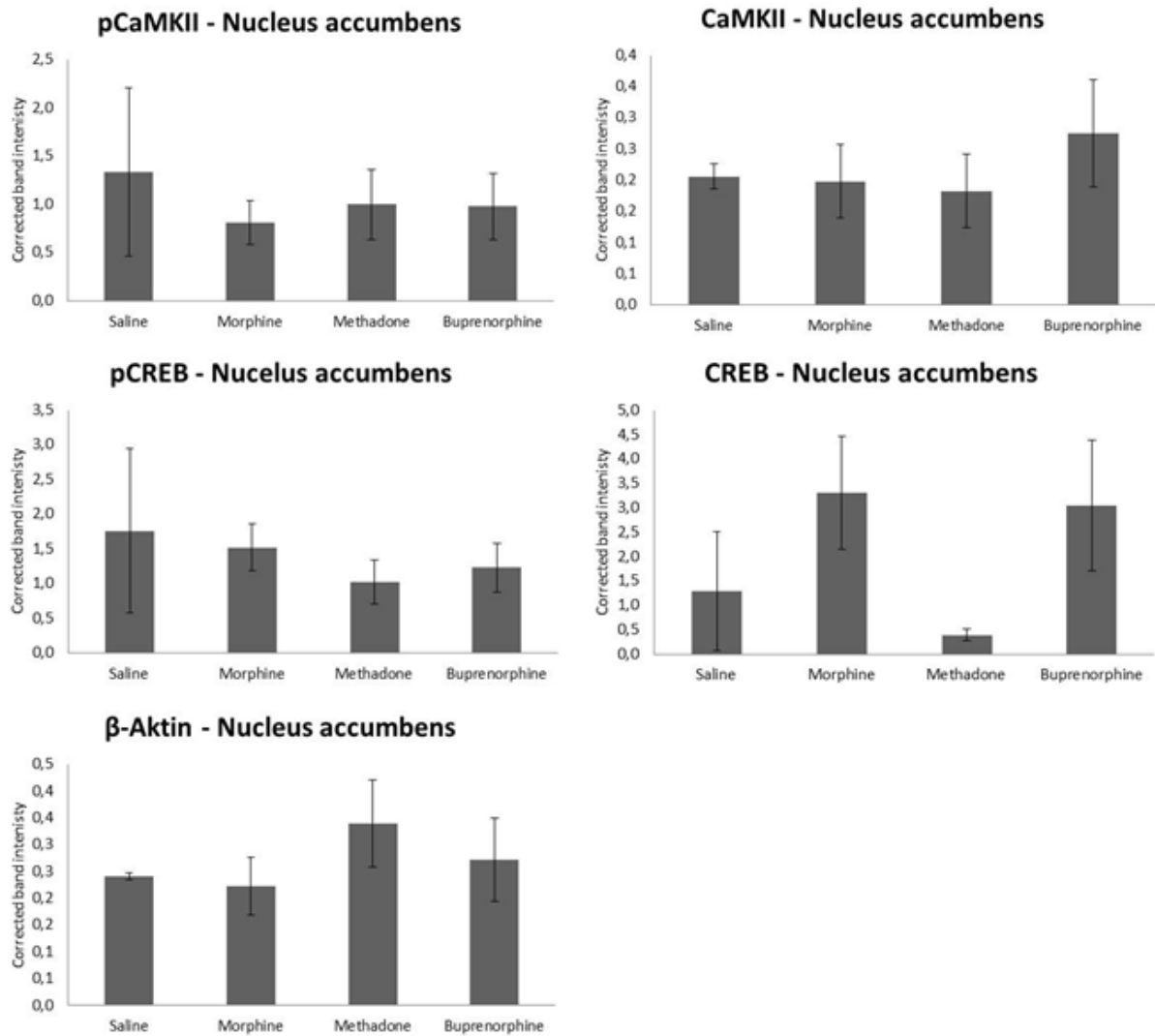


Figure 3.28 pCaMKII, CaMKII, pCREB, CREB and β -Aktin in mice nucleus accumbens one day after the last injection of a three weeks (14 injections) injection regime with saline, morphine (60 $\mu\text{mol/kg}$), methadone (50 $\mu\text{mol/kg}$) and buprenorphine (2 $\mu\text{mol/kg}$). Data are means \pm SEM, n = 2 – 6.

Because of few samples, no statistical analysis has been carried out on these results.

3.4.7 Protein expression in dorsal striatum and nucleus accumbens one hour after a single injection of methadone, buprenorphine or morphine

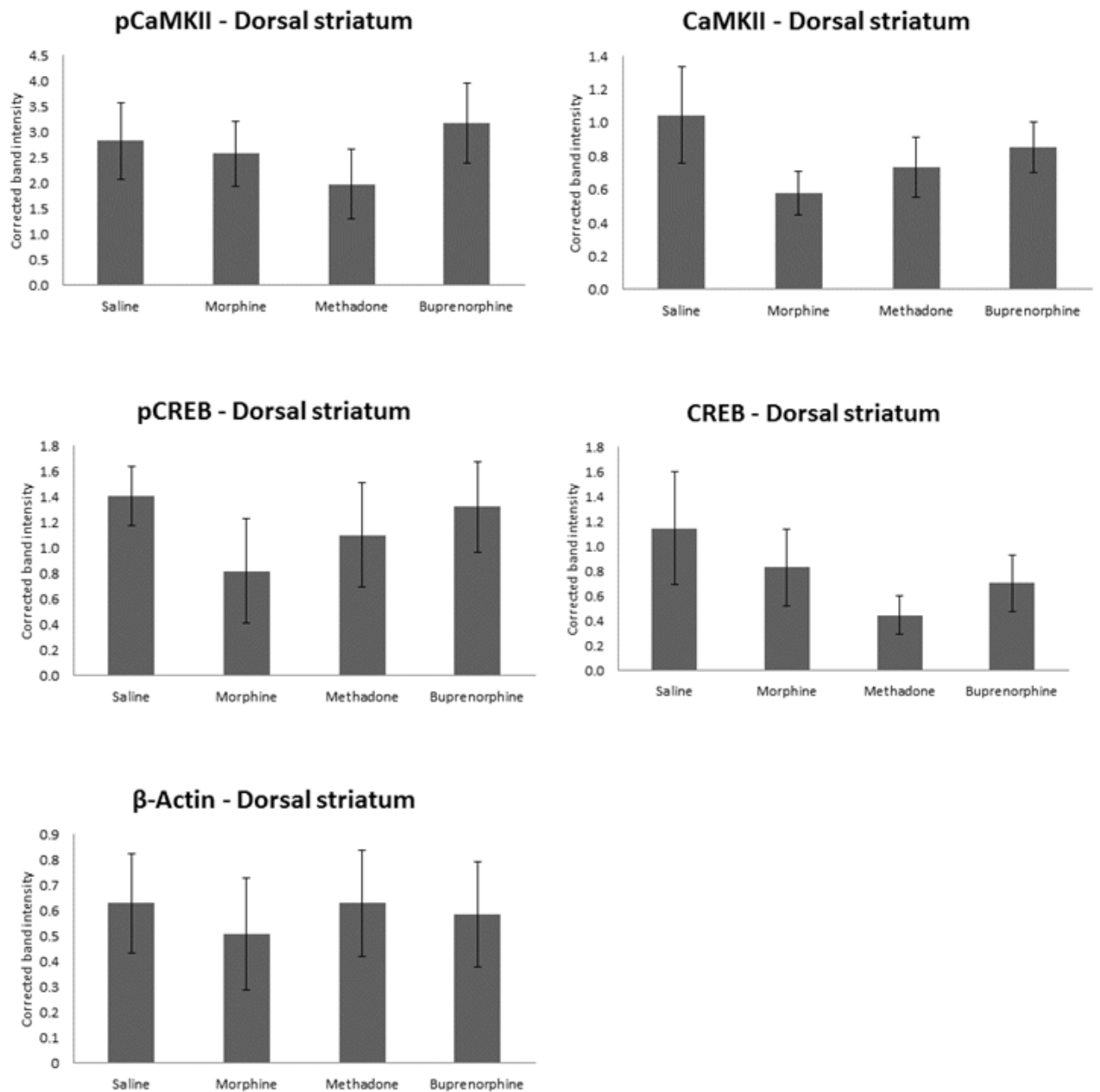


Figure 3.29 pCaMKII, CaMKII, pCREB, CREB and β -Actin in mice dorsal striatum one hour after a single injection of saline, morphine (60 $\mu\text{mol/kg}$), methadone (50 $\mu\text{mol/kg}$) or buprenorphine (2 $\mu\text{mol/kg}$). Data are means \pm SEM, $n = 4 - 8$. (Kruskal-Wallis test).

Proteins in dorsal striatum of mice given a single sc injection of methadone (50 $\mu\text{mol/kg}$), buprenorphine (2 $\mu\text{mol/kg}$) or morphine (60 $\mu\text{mol/kg}$) were not significantly affected by the treatment ($p \geq 0.05$).

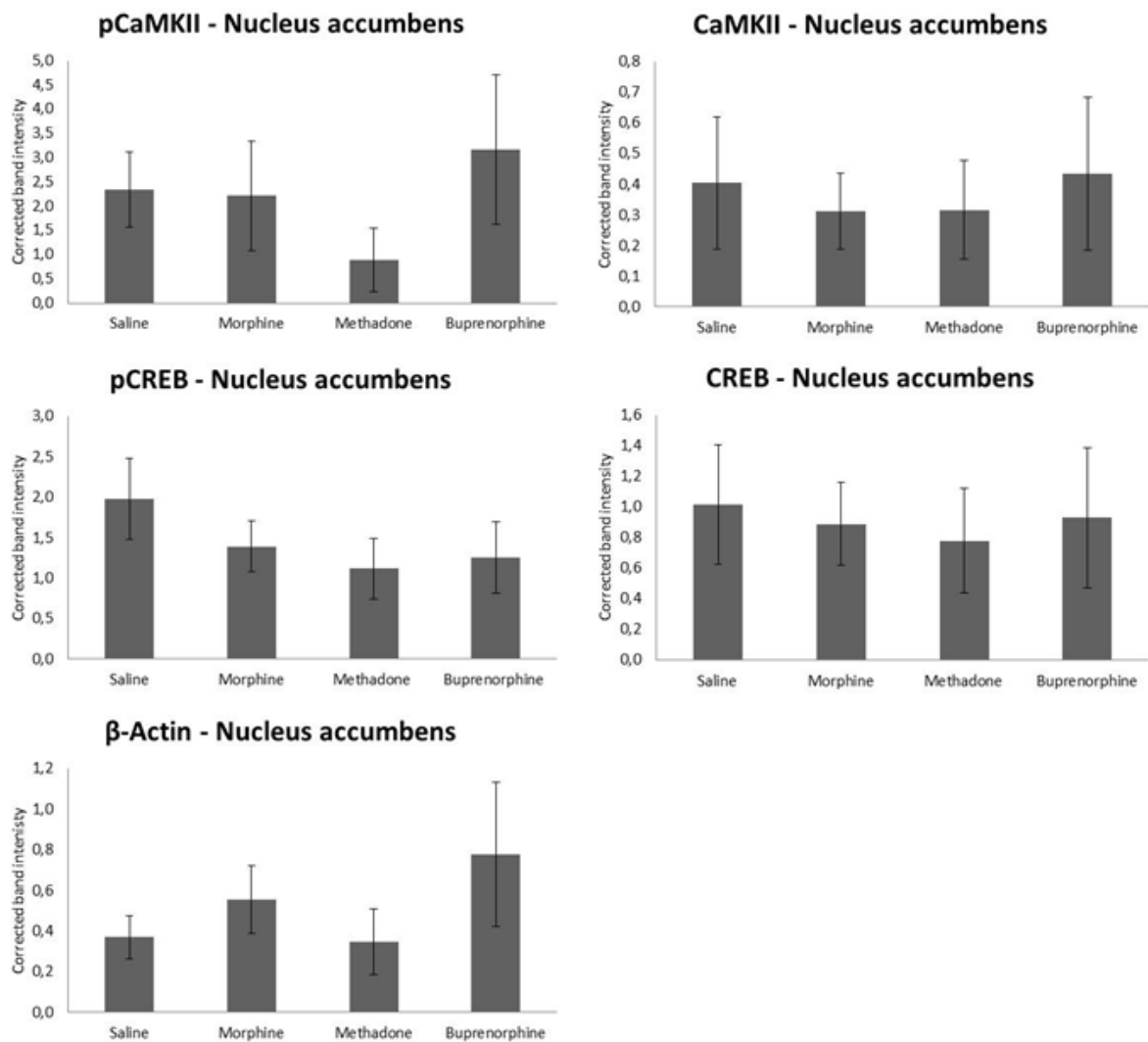


Figure 3.30 pCaMKII, CaMKII, pCREB, CREB and β -Actin in mice nucleus accumbens one hour after a single injection of saline, morphine (60 $\mu\text{mol/kg}$), methadone (50 $\mu\text{mol/kg}$) or buprenorphine (2 $\mu\text{mol/kg}$). Data are mean \pm SEM, $n = 4 - 8$. (Kruskal-Wallis test).

When compared to saline, a single injection of methadone (50 $\mu\text{mol/kg}$), buprenorphine (2 $\mu\text{mol/kg}$) or morphine (60 $\mu\text{mol/kg}$) did not change the expression of pCaMKII, CaMKII, pCREB, CREB or β -Actin in nucleus accumbens of mice.

4. Discussion

4.1 Behavioral testing – stimulation of locomotor activity

4.1.1 Sc injections are more efficient than po in stimulating locomotor activity

For all drugs and doses tested, based on earlier studies (Handal et al., 2002; Tramullas et al., 2007; Schlussman et al., 2008; Andersen et al., 2009; Smith et al., 2009; Valjent et al., 2010; Barwatt et al., 2013) and previous experiments carried out at the NIPH, we found that sc injections overall were more efficient than po administration in stimulating locomotor activity.

In maintenance treatment, methadone and buprenorphine are administered orally and sublingually, respectively (Dole and Nyswander, 1965; Cone et al., 1984; Fudala et al., 1990). Po injections are mimicking the oral administration of methadone. Since sublingual exposure in mice is challenging, sc injections were investigated as an alternative administration route. Intravenous (iv) injections of opioids give almost similar brain concentrations and acute effects as mice given sc injections (personal communication with supervisor). However, iv injections in mice are difficult. Methodologically it is more practical to inject mice sc, therefore sc and po was selected as the two routes of administration tested in the present study.

After thorough search in relevant literature, there seems to be no previous experiments conducted that have compared these two routes of exposure for methadone and buprenorphine, and their efficiency in stimulating locomotor activity in C57BL/6J mice. Handal et al. (2002), however, have previously demonstrated that sc injections of morphine promote higher activity levels when compared to intraperitoneal (ip) injections, which has been used as administration route in other similar experiments (Le Marec et al., 2011; Allouche et al., 2013; Smith et al., 2009).

The overall lower response observed after po injection of methadone might be due to rapid breakdown by enzymes in the gut of mice and thereby less available active substance, rapid first pass metabolism in the liver or poor absorption from the gastro intestinal tract because of differences in pH, enzymes or gut bacteria (Lin, 1995). The absence of response after po administration of buprenorphine was as expected, since earlier studies have found that

buprenorphine undergoes rapid first-pass metabolism in the liver when administered orally in humans (Roughan and Flecknell, 2002; Kalliokoski and Hau, 2009). It is reasonable to assume that the same is true for mice. Neither morphine nor heroin stimulated any response when injected po, and the reason for this may be the same as discussed above. Based on these studies, it was decided to use sc injections when investigating the acute drug effect of methadone, buprenorphine, morphine and heroin, as well as for finding comparable doses of the different drugs.

4.1.2 Acute drug effects of methadone, buprenorphine, morphine and heroin

Stimulation of locomotor activity was used as a measure of the acute drug effect. When opioids are administered, an increase in dopamine release in nucleus accumbens follows which induces locomotor activity. The increased activity represents an increase in stimulation of the central nervous system, and might be associated with reward (Wise and Bozarth, 1987; Joyce and Iversen, 1979; Mørland et al., 1994; Di Chiara and Imperato, 1988).

In the present study, it was found that methadone and buprenorphine gave different dose-dependent locomotor activity profiles after administration of escalating doses. Methadone had a higher stimulatory effect compared to buprenorphine both regarding maximal and total distance travelled at high dose, while buprenorphine showed higher stimulating effects at low doses.

The strong stimulating effect of methadone demonstrated with the lowest doses, was reduced upon administration of higher doses because of stultifying effects making the mice too affected to move properly. Upon administration of buprenorphine, the activity tended to increase until a certain dose before levelling off. The highest dose administered (2 $\mu\text{mol/kg}$) did not cause any change in activity, despite absence of stultifying effects. Only stimulating effects were seen for morphine and heroin, although administration of higher doses probably would have caused stultifying effects, as seen for heroin in the study by Andersen et al. (2009).

One possible explanation for the differences seen between methadone and buprenorphine may be related to their differences in the pharmacodynamic characteristics. Both methadone and morphine are full $\mu\text{-OR}$ agonists, but have different affinity for the receptor (Garrido and Trocóniz, 1999). A full agonist functions by increasing an effect with increasing dose (Rang

et al., 2014). Visual observations confirmed that the lower activity profiles found for higher doses of methadone was not due to a lower effect of the drug, rather the contrary, and the methadone mice were too drugged to run properly after administration of the highest doses. Buprenorphine on the other hand, is a partial μ -OR agonist (Selley et al., 1998; McPherson et al., 2010). A partial agonist acts as an antagonist at high doses, giving rise to an inverted U-shaped dose-response curve (Rang et al., 2014). High doses will therefore not increase the effect any further, as was seen for the highest dose of buprenorphine administered. Although the response was not significantly lower than the response of the second highest dose, it might be a distinctive partial agonist response that was observed. Testing an even higher dose might have demonstrated the characteristic partial agonist response. Visual observations of the behavior confirmed that the mice receiving the highest dose of buprenorphine did not show the stultifying behavior as observed for methadone. As shown in the present study, opioids possess two different effects, both a stimulating and a sedating or stultifying effect (Wise and Bozarth, 1987). What determines which effect to be elicited depends on the dose; the shift from stimulating to sedating effect was clearly demonstrated for methadone.

Heroin acts as a “prodrug” meaning that it mediates its acute effect mainly via the metabolites 6-monoacetylmorphine (6-MAM) and morphine, that bind to the μ -OR (Andersen et al., 2009; Boix et al., 2013). Both heroin and morphine were included in the first experiment. Compared to the other opioids, heroin showed shorter duration of action, except for the highest dose that demonstrated beginning stultifying effects. It was decided to use morphine for comparison in the rest of the experiments, since the response of morphine was more similar to methadone and also because more literature is available on morphine compared to studies on heroin.

In addition to compare the acute drug effects of methadone and buprenorphine, another goal of the first experiments was to find comparable doses of the drugs that could be used for repeated exposure. Methadone 25 μ mol/kg and morphine 30 μ mol/kg gave about equal total run distance, and was therefore chosen to use initially in the studies with repeated exposure. Based on both own experiments and literature search, a buprenorphine dose of 2 μ mol/kg was selected for repeated administration in the three weeks injection regime and also when investigating effect on protein expression after a single injection.

The results from the locomotor activity studies indicate that both methadone and buprenorphine have an acute drug effect although the dose-response curves are different.

4.1.3 Methadone and buprenorphine show different addiction potential

Increased drug effect following repeated drug administration (sensitization) expressed as increased stimulation of locomotor activity may be used as a model for predicting addiction potential of various drugs in mice (Robinson and Berridge, 2008; Feltenstein and See, 2008). Measurement of locomotor activity after three weeks with daily injections, withdrawal Saturday to Sunday, of methadone (25 or 50 $\mu\text{mol/kg}$), buprenorphine (2 $\mu\text{mol/kg}$) or morphine (30 or 60 $\mu\text{mol/kg}$) were carried out at the last day of the injection regime. A striking increase in stimulation of locomotor activity was observed after administration of methadone. This sensitized response was more pronounced for mice subjected to the stimulating dose of methadone (25 $\mu\text{mol/kg}$), but a sensitized response was also seen for the stultifying dose (50 $\mu\text{mol/kg}$), which increased both in total distance run and maximal activity level. No such response was observed for buprenorphine. Buprenorphine did not alter the activity response after long-term exposure, but the response, although not significant, tended to be delayed compared to the response in mice given a single injection, the reason for the delay is unfortunately difficult to explain. The buprenorphine response was lower compared to the response of methadone seen after repeated exposure. The highest dose of morphine showed a slight increase in maximal distance travelled, but the total distance travelled was unchanged. Control mice, injected with saline, omitted from the result chapter, showed almost no locomotor activity, which is in agreement with findings in previous studies (Schlussman et al., 2008; Andersen et al., 2009), indicating that the injections per se did not affect the behavior response.

Sensitization following methadone exposure has been reported previously by Allouche et al. (2013). However, sensitization has also been reported for buprenorphine and morphine (Marquez et al., 2007; Le Marec et al., 2011; Allouche et al., 2013) which is in contrast to the findings in the present study.

In the present study, mice were injected once a day. Because the half-life of methadone ($T_{1/2}\approx 50$ minutes), buprenorphine ($T_{1/2}\approx 25\text{-}30$ minutes) and morphine ($T_{1/2}\approx 40$ minutes) (unpublished data from studies at NIPH) in blood of C57BL/6J mice is relatively short, the mice experienced long periods each day without active substance present, giving an intermittent exposure regime. Le Marec et al. (2011) found that an intermittent administration regime with morphine produced a longer lasting and more robust sensitization response, compared to an exposure regime with three injections each day (TTD) mimicking a

continuous stimulation of receptor. They also reported that administration regime was more important in evoking the behavioral response than the specific dose injected. Allouche and colleagues (2013) found that only intermittent administration initiated sensitization in mice injected with buprenorphine, while both intermittent and TTD injections resulted in sensitization of the methadone response. Another interesting feature of the above mentioned studies (Le Marec et al., 2011; Allouche et al., 2013) is that they tested sensitization 1, 7, 14, and 35 days after treatment, and found that animals treated with methadone showed sensitization after 7 and 14 days of withdrawal when challenged with a dose of either methadone or morphine. Regarding buprenorphine, a challenge dose with buprenorphine did not induce sensitization, but when challenged with morphine sensitization was seen. These results indicate that there might be long-lasting changes in the brain after repeated opioid administration. Whether this is true for C57BL/6J mice is not yet established, in the above mentioned studies, OF1 strain of mice were used.

Discrepancy between the results in the present study and the above mentioned publications may be related to differences in mice strain used, doses administered, routes of exposure and more importantly how the sensitized response was triggered. In the studies by Allouche et al. (2013) and Le Marec et al. (2011) the sensitized response was seen when mice first were exposed to methadone, buprenorphine or morphine for a couple of days, then a drug-free period with varying length followed before the response was triggered by administration of a challenge dose of the drug. In the present study the sensitized response of methadone were seen on the last day of the injection regime, immediately following the last injection.

Results from pharmacokinetic studies carried out at NIPH demonstrate that not only the administration regime and pharmacodynamic properties of a substance, but also the pharmacokinetics of the drug may impact the behavioral response seen. Recent pharmacokinetic studies with methadone and buprenorphine from NIPH (soon to be published), show that the brain concentration of methadone and buprenorphine exceeds the blood concentration after administration. Regarding morphine, the concentration profiles are different; the blood concentration exceeds the brain concentration (Andersen et al., 2009). This means that the brain concentration of methadone is higher than the brain concentration of morphine after injection of equimolar doses. It seems reasonable to assume the brain concentration of a drug must be above a certain level to induce sensitization. This might explain why the morphine responses were not sensitized like the methadone response. The

low brain concentration of morphine may be due to morphine having lower ability to cross the BBB (Oldendorf et al., 1972). The differences in the responses seen between methadone and buprenorphine is most likely due to differences in agonist activity, being full and partial μ -OR agonists, respectively as described previously. The fact that the methadone dose (25 μ mol/kg) and buprenorphine dose (2 μ mol/kg) was 10 times different might also account for some of the difference seen between these two substances.

In summary, it seems reasonable, based on the results, to assume that the addiction potential of methadone is higher than that of buprenorphine. This difference might be explained by both pharmacodynamic and pharmacokinetic characteristics of the drugs.

4.2 Western blot analysis – protein expression

4.2.1 Effects on proteins important for learning and memory after four days, three weeks and single exposure to methadone, buprenorphine and morphine

The result of the western blot studies indicate that methadone, regardless of exposure regime, dose, and presence or absence of active drug in the brain, does not affect the expression of pCaMKII, CaMKII, pCREB or CREB in neither hippocampus nor frontal cortex of C57BL/6J mice. The same is true for mice administered buprenorphine, except for an increase in the presence of CREB in hippocampus following injections of high doses of buprenorphine. The proteins investigated in the present study have been shown to be important in the cellular mechanisms underlining learning and memory formation (Malenka and Nicoll, 1999; Silva, 2003). Seen in light of relevant literature and previous studies at NIPH, it is a bit surprising that treatment did not induce any changes.

In humans, it has been shown that methadone might impair cognitive function in patients participating in methadone maintenance treatment (MMT). Epidemiological studies have demonstrated that patients in MMT perform poorly in decision making tasks, attention, various types of memory tasks, and information processing compared to healthy controls, (Mintzer and Stitzer, 2002; Mintzer et al., 2005) and also abstinent heroin abusers (Darke et al., 2000). Whether the possible negative impact on cognition is caused by methadone use per se, or is a consequence of other lifestyle factors is still not known. Previous studies in drug-naïve rats, where confounding factors like poly-drug use, alcohol consumption and poor diet are not present, have supported the hypothesis that methadone may impair cognitive

functions. It has been found that methadone exposed rats perform worse on tasks involving spatial cognition in a Morris water maze (Hepner et al., 2002). Others have shown impaired novelty preference (attention) in rats both when methadone is present in the brain, but also one day post administration (Andersen et al., 2012), indicating that the cognitive function might be reduced even when active drug is absent. The reduced novelty preference was also found to coincide with reduced levels of pCaMKII in both hippocampus and frontal cortex (Andersen et al., 2012).

The reason why the same effect on pCaMKII previously found in rats not was found in the present study is difficult to explain since the exposure regime and the doses used were almost similar (Andersen et al., 2012). It is known that changes related to learning and memory after opioid administration may be reversible (Guerra et al., 1987; Ammon-Treiber et al., 2005). However, it seems unlikely that functional changes would have been reversed only one day post administration, and therefore it seems unlikely that the absence of effects is due to reversal of changes. One could perhaps think that alterations would have been evident if active substance were present in the brain, but western blot analysis on samples with active substance present in brain did not show any large changes in protein activation and expression (Unpublished data from NIPH). Another possible explanation may be that mice are less sensitive compared to rats. It could be that a longer exposure regime or even higher doses would have caused changes.

Regarding buprenorphine, fewer studies are available, but some researchers have found that buprenorphine may be less associated with negative effects on cognitive function compared to methadone maintenance treatment (Rapeli et al., 2007; Mintzer et al., 2004; Soyka et al., 2008). Generally, epidemiological studies are often confounded by small sample sizes, poor control groups and variations in lifestyle factors and lack information concerning long-term consequences associated with the treatment. The results from the present study might indicate that buprenorphine has minor negative consequences and are therefore in agreement with results obtained from the epidemiological studies. No changes were seen except an upregulation of CREB in hippocampus after three weeks exposure. The increase in CREB following buprenorphine exposure was unexpected and is difficult to explain. It might be that three weeks of injections in some way affected the synthesis and expression of the protein. However, no conclusions concerning this can be drawn from the present study. More studies are needed.

Morphine caused a downregulation of pCREB in frontal cortex after three weeks exposure to the low dose (30 $\mu\text{mol/kg}$). One mechanism thought to contribute to mediate phosphorylation of CREB following opioid exposure is CaMKII, however, since no changes were seen in the levels of CaMKII in the present study it seems likely that other kinases are responsible for the downregulation. PKA or cyclic-AMP-dependent protein kinase has also been proposed to cause phosphorylation of CREB (Sheng et al., 1990). The downregulation of pCREB might be explained by reduced levels of PKA, however, upon administration of the highest dose of morphine, no change was seen compared to the control animals. It is not possible to state any cause of the reduction observed before more research have been conducted. Many factors are known to affect expression and activation of CREB, like growth factors, steroid hormones, immune cell signaling etc. (for review see: Johannessen et al. (2004)) but explanations of activation or deactivation of this transcription factor is beyond the scope of this study and is therefore not discussed any further.

Overall, only minor changes were found, however, one cannot rule out that other changes in the brain may result from the various exposure regimen tested in the present study, and that other brain areas might be affected. But regarding effect on learning and memory, hippocampus and frontal cortex are most central.

4.2.2 Effects on protein expression in dorsal striatum and nucleus accumbens

Overall no changes in expression of proteins were seen, neither in dorsal striatum or nucleus accumbens, following four days or three weeks with low dose and three weeks with high dose. The proteins were unchanged also when active substance was present in the brain, after the single injection. The exception was an upregulation in both pCREB and CREB in dorsal striatum of mice injected with methadone (25 $\mu\text{mol/kg}$) for three weeks. However, no changes were seen in expression of proteins after three weeks exposure to the highest methadone dose. The upregulation of both pCREB and CREB is hard to explain, especially since no changes were seen in the level of these proteins after exposure to the high dose of the same drug. More studies are needed.

Dorsal striatum and nucleus accumbens are small brain structures which methodologically are challenging to work with. Both to obtain samples and sample preparation are laborious processes. Because of the small sample volume, large variations in the results are to be expected. Optimally, the sample size should therefore have been increased to make more

robust results, but because of a restricted time-schedule this was not possible. Results from these areas should therefore be interpreted with care.

The conditioned place preference (CPP) paradigm is another behavioral model used to study the rewarding effects of drugs of abuse. The paradigm involves association of a particular environment with a specific drug treatment followed by an association of a different environment without drug. It is said that CPP is found if the animal chooses to spend more time in the environment associated with drugs than in the environment not associated with drugs (Tzschentke, 2007; Bardo and Bevins, 2000). Researcher have found that when using a CPP paradigm, mice exposed to morphine show an upregulation of pCaMKII in dorsal striatum and nucleus accumbens, while for mice not trained for the CPP paradigm no changes in protein expression is seen after three days of exposure (unpublished data from NIPH). These results are in agreement with what was observed in the present study, where mice exposed to methadone for four days did not show any changes in the proteins, and may indicate that a conditioning or associative learning process is needed to induce cellular changes.

4.3 Thoughts concerning use of animal models for investigation of drug related issues

Use of animals in research might be an advantageous approach when studies, of ethical reasons, cannot be conducted in humans. A benefit with animal studies is that they, compared to epidemiological studies, are easier to control and may provide valuable information when a specific mechanism is investigated.

In the present study, C57BL/6J mice were used to evaluate the acute drug effects and addiction potential of various drugs, using stimulation of locomotor activity following drug administration as model (Mørland et al., 1994; Wise and Bozarth, 1987; Robinson and Berridge, 1993). However, when dealing with stultifying or sedating effects of drugs, as in the case with methadone, this model may be difficult to interpret. For future studies it could be wise to also use other models, e.g. CPP or various self-administration models (Olmstead, 2011).

Extrapolating results and findings in animal studies to humans should always be done with care. There are obvious species differences between humans and mice, like size and body

weight, but also differences in e.g. metabolism and absorption are important to account for before extrapolating from animals to humans (Lin, 1995; Yu et al., 2006).

In the present study, I found no obvious effects of methadone on the expression of the brain proteins investigated in mice. This is surprising because almost the same study has previously been carried out in rats, showing another result. In the study by Andersen et al. (2012), previously mentioned, it was found that long-term exposure to methadone reduced phosphorylation of CaMKII both in hippocampus and frontal cortex. There may be many reasons for the different outcome of these two studies, but it underlines, that one should be careful with drawing conclusions directly from animal studies to humans.

5. Conclusions

H₀: Methadone induces a higher acute locomotor activity response than buprenorphine

In this study, it was found that the highest stimulating dose of methadone induced a significantly higher acute drug effect, expressed as stimulation of locomotor activity, compared to the buprenorphine dose giving the highest stimulatory effect. The difference between the two drugs might be explained by differences in agonist activity, methadone being a full agonist at the μ -OR and buprenorphine a partial agonist. It was also found that sc injections were superior to po administration when assessing stimulation of locomotor activity in mice. The null hypothesis is retained.

H₀: Repeated exposure to methadone causes sensitization of the locomotor activity response, this will not be evident for buprenorphine

It was also found that repeated exposure for three weeks to both the stimulating and stultifying dose of methadone caused sensitization of the locomotor activity response, while the buprenorphine response remained the same as after a single injection. The null hypothesis is retained.

H₀: Repeated exposure to methadone affects the expression of brain proteins important for learning and memory more than buprenorphine

Four days or three weeks administration of methadone, buprenorphine or morphine caused only minor changes in the expression and activation brain proteins related to learning and memory (CaMKII and CREB). The null hypothesis is rejected.

Taken together, the results in the present thesis indicate, that methadone might be associated with a higher acute rewarding effect and might have a higher addiction potential following intake, compared to buprenorphine. Concerning a possible effect on brain proteins central for learning and memory, only small effects was found.

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