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DIPLOMARBEIT

Titel der Diplomarbeit

„In vivo sensitization by amphetamine and cocaine:

Role of Ca^{2+}/calmodulin-dependent protein kinase II α“

Verfasserin

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Summary

The dopamine transporter (DAT) exerts its physiological function in the presynaptic nerve terminal by taking up extracellular dopamine (DA) following the dopamine release after an action potential, thereby regulating the amount of free DA available for signal transmission at the post synapse. DAT belongs to the Na^+/Cl^--dependent transporters of the solute carrier 6 (SLC6) gene family, also referred to as neurotransmitter sodium symporters (NSS). It is one of the major targets for psychostimulants including amphetamines and cocaine. Amphetamines are indirect sympathomimetics and can induce euphoria and stimulation in humans and animals. Nowadays, amphetamines are used in clinics to treat narcolepsy and attention deficit hyperactivity disorder (ADHD). The Ca^{2+}/Calmodulin-dependent protein kinase II α (CamKIIα) binds to the C-terminus of DAT and phosphorylates N-terminally located serines, promoting the reverse operation of the transporter, releasing DA and therefore increasing the concentration of DA in the synaptic cleft. Elevated levels of DA are responsible for the reinforcing properties of the amphetamine action. As CamKIIα plays an important role in the process of learning and memory, Licata et al. (2004) hypothesized that CamKIIα is an important factor to acquire a drug memory.

The aim of this diploma thesis was to elucidate the role of the interaction between psychostimulants - d-amphetamine (AMPH) and cocaine - CamKIIα and the DAT, using a CamKIIα knock out (KO) mouse model. To test whether CamKIIα-KO mice display addictive-like behavior to AMPH or cocaine, behavioral sensitization to psychostimulants with an open field (OF) approach and conditioned place preference (CPP) were used. Wild type (WT) and KO mice were treated either with AMPH or cocaine for several days and measured in the OF or in the CPP boxes. Both, WT and KO mice displayed behavioral sensitization to AMPH, however the sensitizing effect of AMPH was significantly less pronounced in KO compared to WT mice. Similar results were found for cocaine, in contrast to sensitization with AMPH the sensitizing effect reached a plateau at day 3. To verify that neurochemical sensitization was present after the psychostimulant treatment, superfusion experiments were done in striatal synaptosomes of WT and KO mice. However, increased efflux was not observed in neither of the mice after treatment with d-amphetamine and cocaine. The experiments described in this thesis are part of an ongoing project.

Zuammenfassung

Die physiologische Rolle des Dopamintransporters (DAT) in der präsynaptischen Nervenzelle ist die Wiederaufnahme von extrazellulärem Dopamin (DA) nach der Ausschüttung, ausgelöst durch ein Aktionspotential. Dadurch wird die Menge des, für die Signalweiterleitung verfügbaren DA an der Postsynapse, reguliert. Der DAT gehört zu den Natrium/Chlorid-abhängigen Transportern welche der Genfamilie der „solute carrier 6“ (SLC6) angehören, die zu den Neurotransmitter:Natrium Symportern (NSS) gerechnet werden. Er ist einer der Hauptangriffspunkte von Psychostimulantien, wie Amphetamine und Kokain. Amphetamine sind indirekte Sympathomimetika, welche in Menschen und Tieren Euphorie und Anregung hervorrufen können. Heutzutage werden Amphetamine noch in der Behandlung von Narkolepsie und Aufmerksamkeitsdefizit-/ Hyperaktivitätsstörung (ADHS) verwendet. Die Ca^{2+}/Calmodulin-abhängige Protein Kinase II α (CamKIIα) bindet an den C-Terminus des DATs und phosphoryliert Serin-Reste am N-Terminus, was zu einer Umkehrung des DATs führt und dadurch die DA Konzentration im synaptischen Spalt erhöht. Die gesteigerte Konzentration von DA im synaptischen Spalt wird für die Initiierung von Drogensucht verantwortlich gemacht. Da die CamKIIα eine große Rolle beim Lernen und auch bei der Funktion des Gedächtnisses spielt, schrieben Licata et al. (2004) der CamKIIα eine wichtige Funktion beim Erlangen eines Suchtgedächtnisses zu.

Das Ziel dieser Diplomarbeit war es die Funktion der Interaktionen zwischen den Psychostimulantien - d-Amphetamin (AMPH) und Kokain - CamKIIα und dem DAT aufzuklären. Dazu wurden CamKIIα knock out (KO) Mäuse untersucht. Um aufzuzeigen, ob die CamKIIαKO Mäuse Anzeichen von suchtähnlichem Verhalten für Amphetamin und Kokain aufweisen, wurde die Sensitisierungsmethode, wie auch der „conditioned place preference“ (CPP) Ansatz gewählt. Die wild typ (WT) und KO Mäuse wurden einige Tag mit AMPH oder Kokain behandelt und anschließend in den „open field“ (OF) oder in den CPP Boxen gemessen. Beide, WT und KO Mäuse zeigten verhaltensabhängige Sensitisierung nach der Behandlung mit AMPH, jedoch war der Effekt in den KO Mäusen weniger ausgeprägt, als in den WT Mäusen. Dies gilt ebenfalls für die Behandlung mit Kokain, wobei hier am dritten Tag der Sensitisierungseffekt ein Plateau erreicht. Um diese verhaltenspharmakologischen Ergebnisse zu bekräftigen, wurden Synaptosomen aus dem Striatum von WT und

KO Mäusen superfundiert. Es konnte jedoch kein signifikanter Unterschied im Amphetamin-induzierten Efflux von den vorbehandelten Mäusen festgestellt werden. Die Experimente, die in dieser Diplomarbeit beschrieben werden, sind ein Teil eines laufenden Forschungsprojektes.

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Abbreviations

AMPH	d-amphetamine
AUC	area under the curve
ADHD	attention deficit hyperactivity disorder
Bp	base pair
Ca^{2+}	calcium
Cam	calmodulin
CamKII	Ca^{2+}/calmodulin dependent protein kinase II
cAMP	cyclic adenosine monophosphate
CNS	central nervous system
COMT	catechol-O-methyl-transferase
CPP	conditioned place preference
CREB	cAMP response element binding protein
DA	dopamine
DAT	dopamine transporter
DEPC	diethylpyrocarbonate
dNTP	deoxynucleoside triphosphate
DRD1,2,3,4,5	dopamine receptor D1,2,3,4,5 gene
EN	epinephrine
EtOH	ethanol
FDA	Food and Drug Administration
Glt	glutamate transporter

GPCR	G protein coupled receptor
i.p.	intra-peritoneal
kDa	kilo Dalton
KHB	Krebs-HEPES buffer
KO/mut	knock-out or mutant
L-DOPA	3,4–dihydroxyphenylalanin
LeuT	leucine transporter
LTD	long term depression
LTP	long term potentiation
MAO	monoamine oxidase
MDMA	3,4-methylendioxymethamphetamine, “ecstasy”
MPP^+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenylpyridinium-1,2,3,6-tetrahydropyridine
MQ H_2O	milli Q water
Na^+	sodium
NE	norepinephrine
NMDA	N-methyl-D-aspartate
NRI-KD	NMDA receptor-deficient mouse
NSS	neurotransmitter:sodium symporter
OF	open field
PBS	phosphate-buffered saline
pCA	para-chloramphetamine
PCR	polymerase chain reaction

PKC	protein kinase C
PSD	post-synaptic density
RT	room temperature
SLC6	solute carrier 6 gene family
SN	supernatant
T	threonine
TDB	tail digestion buffer
TH	tyrosine hydroxylase
TM	transmembrane domain
U.S.	United States
VMAT	vesicular monoamine transporter
VTA	ventral tegmental area
WT	wild type
5-HT	5 hydroxytryptamine, serotonin

1. INTRODUCTION

1.1 Dopamine transporter

1.1.1 Dopamine

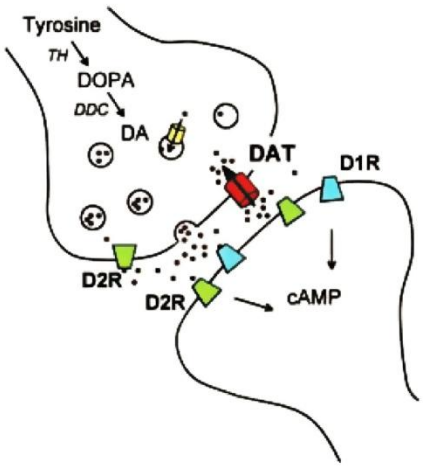
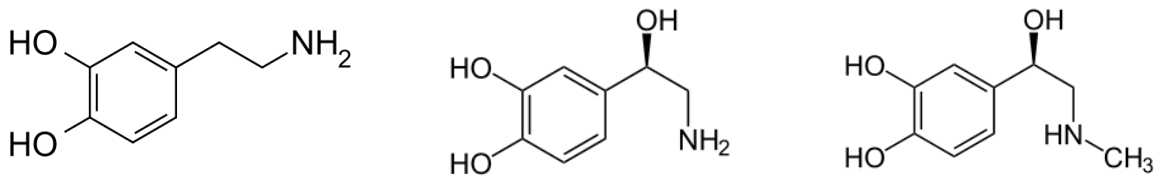


Figure 1: Scheme of a dopaminergic synapse in the striatum (Pirker and Brücke, 2004)

Dopamine (DA), epinephrine (EN) and norepinephrine (NE) are neurotransmitter belonging to the catecholamines (Fig. 2). The catecholamines together with serotonin (5-HT) represent the group of monoamines, which together with histamine are named biogenic amines (Kandel et al., 1996).

The catecholamines:



Dopamine **Norepinephrine** **Epinephrine**

Figure 2: Chemical structure of dopamine, norepinephrine and epinephrine
A common feature of catecholamines is the catechol ring displaying the distinct benzene ring with two hydroxyl groups attached.

Dopamine, norepinephrine and epinephrine share the same precursor, L-tyrosine. Tyrosine is converted to L-DOPA (3,4–Dihydroxyphenylalanin) via tyrosine hydroxylase (TH), which represents the rate limiting step in the biosynthesis of dopamine. Finally, the dopadecarboxylase converts L-DOPA to dopamine. Dopamine further is metabolized to norepinephrine and epinephrine (Kandel et al., 1996).

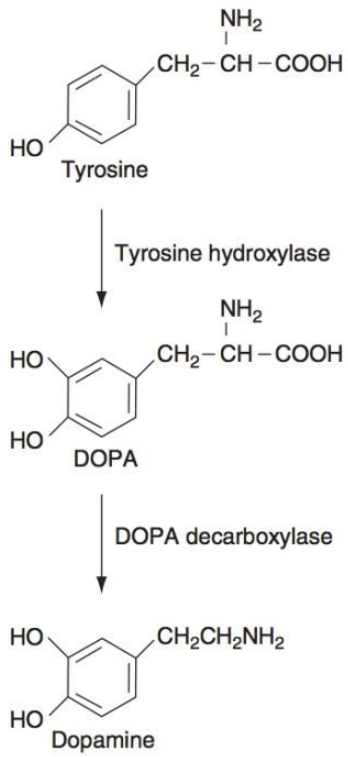


Figure3: Biosynthesis of dopamine from L-Tyrosine (Hälbig and Koller, 2007)

The dopamine transporter (DAT) takes up DA from the synaptic cleft where it is either packed and restored in synaptic vesicles or metabolized through monoamine oxidase (MAO) or catechol-O-methyl-transferase (COMT). MAO deaminates, while COMT methylates dopamine. The degradation pathway is shown in fig.4.

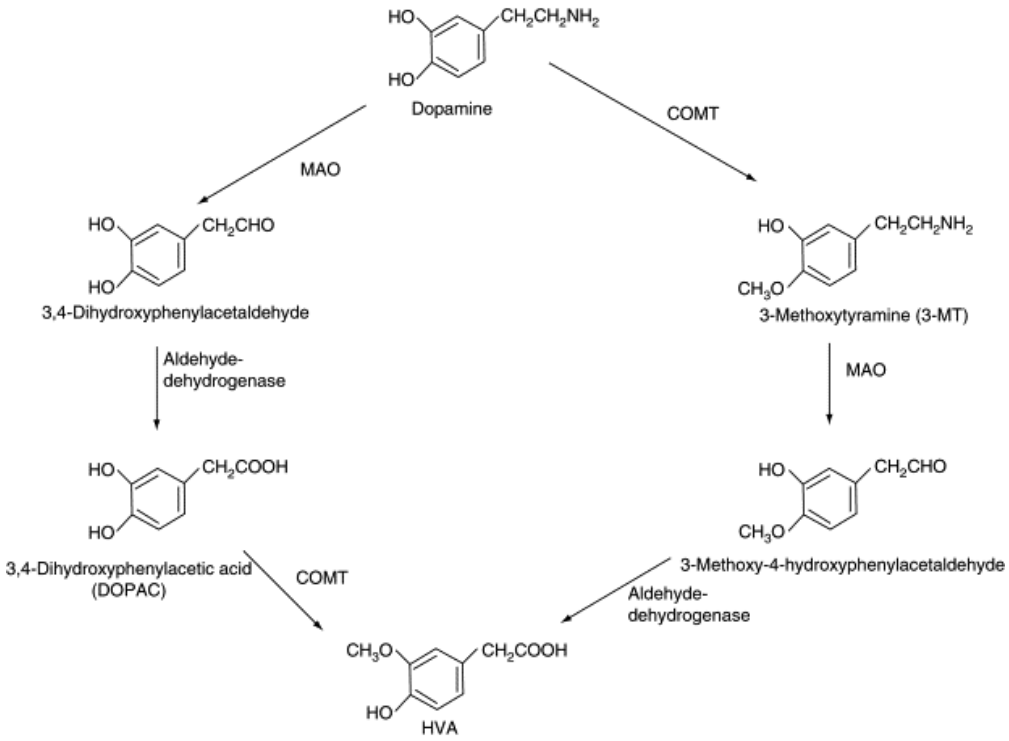


Figure 4: Degradation pathway of dopamine (Hälbig and Koller, 2007)

1.1.2 Dopaminergic system in the brain

Dopamine mainly exerts its action as a neurotransmitter in the central nervous system (CNS).

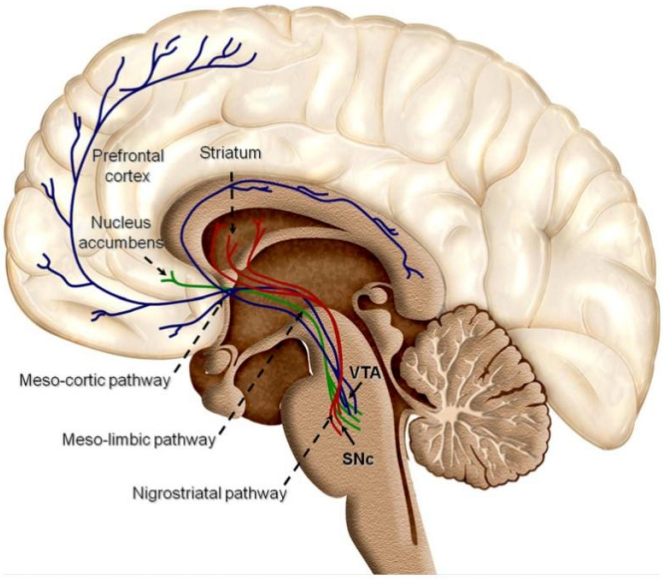


Figure 5: Localization of dopamine systems
nigrostriatal projection, mesolibical projection, mesocortical projection (Arias-Carrión et al., 2010)

The main dopaminergic systems (fig.5) in the brain are:

- **The nigrostriatal system:** It arises in the substatia nigra pars compacta and projects to the corpus striatum (dorsal striatum). The degeneration of this pathway is associated with Parkinson's disease.

- **The mesolimbical system:** The cell bodies of this system are located in the mid brain (ventral tegmental area, VTA) medial to the substatia nigra and project to the limbic system (mainly nucleus accumbens). The mesolimbic system has been implicated in regulating motivational behavior and reward.

- **The mesocortical system:** It connects the ventral tegmentum (area tegmentalis ventralis) and the prefrontal cortex. The mesocortical and mesolimbical systems are tightly connected (Arias-Carrión et al., 2010).

- **The tuberoinfudibular system (not shown):** Its cell bodies reside in the nucleus infundibularis and project to the eminentia mediana where DA inhibits the secretion of prolactin (Palmiter, 2008).

1.1.3 DAT Function and Structure

DAT belongs to the solute carrier 6 (SLC6) gene family which are Na^+/Cl^--dependent neurotransmitter transporters (Torres et al., 2003). It plays a critical role in the clearance of DA from the synaptic cleft following exocytotic release, by mediating its reuptake. When the synaptic bouton is depolarized via an action potential, DA is released into the synaptic cleft. The depolarization is generated through voltage-gated ion channels. A rapid inward flow of sodium ions changes the electrochemical gradient which leads to a depolarization of the membrane. At the peak of the action potential sodium channels are inactivated and potassium channels open exerting an outward current of potassium ions, therefore repolarizing the membrane. Following the depolarization, Ca^{2+} channels open and increase the cytosolic calcium concentration. Vesicles containing the neurotransmitter fuse with the presynaptic membrane and release the neurotransmitter into the synaptic cleft in a Ca^{2+} dependent manner. DA binds to receptors located pre- and postsynaptically and induce a signaling cascade. The neurotransmitter needs to be removed from the synapse to allow new neurotransmission. DAT rapidly takes up the extracellular

dopamine into the presynaptic terminal where DA is either restored in synaptic vesicles or enzymatically degraded.

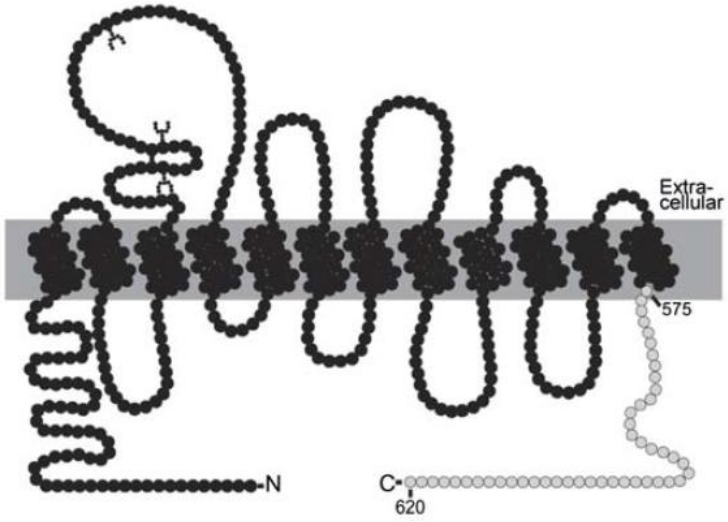


Figure 6: Diagram of human DAT topology (Fog et al., 2006)

Monoamine transporters are predicted to consist of 12 transmembrane domains (TM). The carboxy- and amino termini are located on the intracellular site, which was assessed experimentally (Chen et al., 1998). To date no crystal structure of an eukaryotic transporter is available. For the bacterial leucine transporter ($LeuT_{Aa}$) from Aqufex aeolicus a crystal structure is available, which serves as a starting point to establish homology models for DAT (Beuming et al., 2006; Stockner et al., 2013). The sequence homology between the bacterial and mammalian transporters is only 20 - 25%, however, the primary sequence of $LeuT_{Aa}$ shows clusters of high sequence conservation around the substrate binding site (Yamashita et al., 2005).

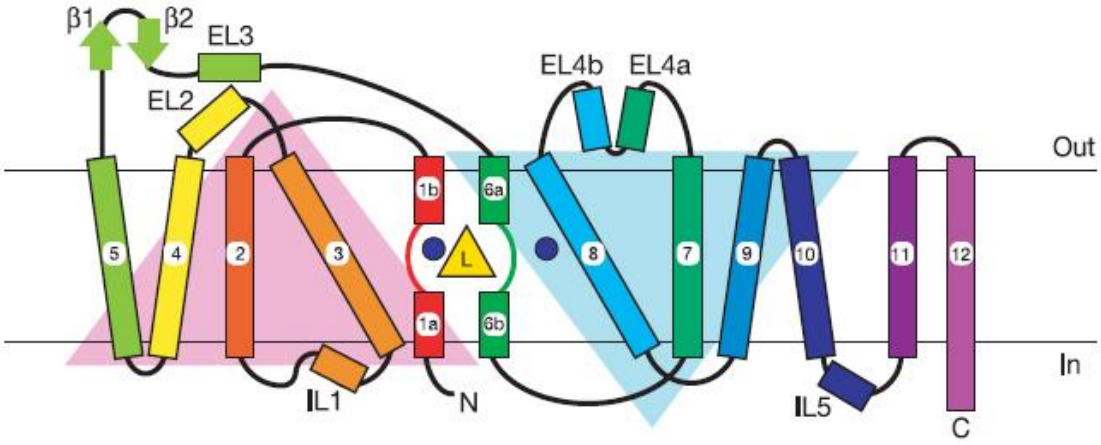


Figure 7: structure of bacterial leucine transporter from A. aeolicus ($LeuT_{Aa}$)
Shown in complex with its substrate, leucine, and two sodium ions
12 transmembrane domains, N- and C- terminus are located on the intracellular site, (Yamashita et al.,2005)

$LeuT_{Aa}$ was found to exist in at least three different states; open to outside, occluded and open to inside. It serves as a valuable template for SLC6 family members such as DAT (Yamashita et al., 2005; Singh et al., 2008; Krishnamurthy, 2012).

1.1.4 DAT Pharmacology and Reverse transport

The DAT is a site of action of several psychostimulant drugs including indirect sympathomimetic drugs like amphetamines and cocaine (Howell et al., 2007; Rothman et al., 2008). Some act as blockers of the transporter, i.e. they inhibit the monoamine transporters, such as cocaine or methylphenidate (Ritalin, see below 1.1.6.) and some are substrates and act as releasers like D-amphetamine (“Speed”), methamphetamine (“Ice”) or 3,4-methylendioxymethamphetamine (MDMA, “Ecstasy”). The releasers additionally trigger the reverse operation of the transporters leading to physiological substrate efflux (Sulzer et al., 2005).

It is important to mention that not only amphetamines are able to induce reverse transport. All compounds that are recognized by the transporter as substrates and are inwardly transported can induce reverse transport. Nevertheless, under physiological conditions, physiological substrates do not act as releasers (Sitte & Freissmuth 2010). This follows the simple fact that physiological substrates such as

5-HT or DA are released in larger quantities into the synaptic cleft. Subsequently, they are taken back up into the releasing presynaptic specialization – and immediately packaged into the vesicles again. This process has been shown to depend on a direct interaction between the plasmalemmal transport proteins and the vesicles: the associate protein synaptogyrin assists in this process and helps the vesicle dock to the transporter (Egaña et al., 2009) On the other hand, if transmembrane ion gradients are changed, like lowering the extracellular sodium concentration (Pifl et al. 1995, 1997; Pifl and Singer 1999) or the potassium concentration is raised (Scholze et al. 2000, 2002), reverse transport can be triggered.

It is hypothesized that DAT functions via an alternating access mechanism. Binding of a substrate together with sodium and chloride triggers a conformational change from an “outward conformation”, where the substrate binding site is exposed to the extracellular part, to an “inward conformation”, where the binding site faces the intracellular milieu (Sitte and Freissmuth, 2010; Tavoulari et al., 2011). The two most excepted hypotheses to explain the mechanism how amphetamines trigger the reverse action of the transporter are: the facilitated exchange diffusion model and the channel mode.

- **Facilitated exchange diffusion model**

Fischer and Cho proposed the facilitated exchange diffusion model in 1979 for amphetamine-induced DAT-mediated reverse transport (Fischer and Cho, 1979).

The model predicts that DAT transports the substrate amphetamine inside the cell, where a certain number of transporters are moved into the inward facing conformation, which in turn increases the probability to efflux DA. One molecule of DA will be released for each amphetamine molecule accumulated (Robertson et al. 2009; Sulzer 2011; Sulzer et al. 2005).

- **Channel mode**

In the so called channel mode, ion fluxes occur uncoupled from substrate translocation. Uncoupled ion conductances at the transporters are believed to be similar to those of classical ion channels. It was concluded, that neurotransmitters can pass the transporter through a pore (Galli et al., 1996; Sonders and Amara, 1996; Sitte et al., 1998; Kahlig et al., 2005; Sulzer et al., 2005).

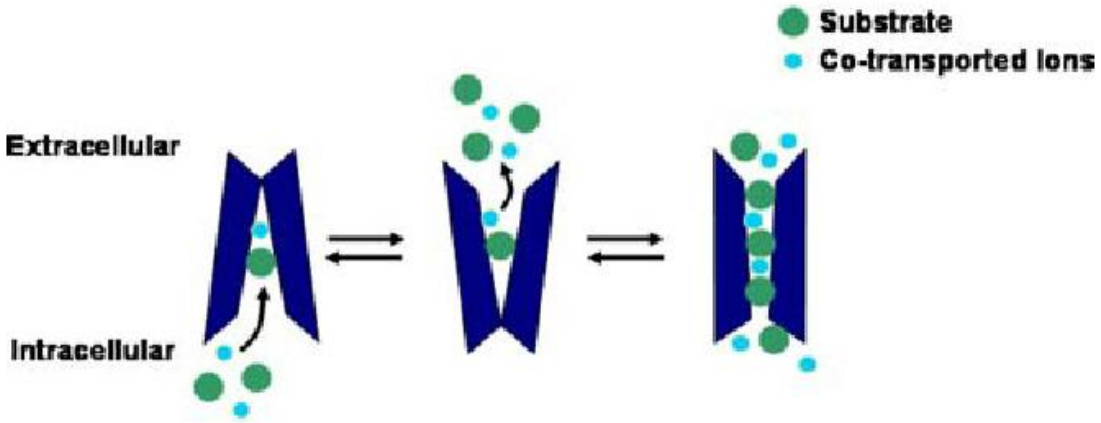


Figure 8: schematic representation of transporter mediated monoamine efflux
Inward facing transporter binding monoamines and co-transported ions in the presence of amphetamine and mediate reverse action by releasing monoamines and co-transported ions. Amphetamine can also induce release via a channel like mode (Robertson et al., 2009).

A "**unified model**" was proposed that combines features of the facilitated exchange diffusion model and the channel-like mode (Khoshbouei et al., 2004). In this model there is no limit of a one-for-one molecule exchange of substrate. It is thought that multiple substrates could be transported without the existing of a shuttling binding site, exerting channel like properties. Reverse transport probably follows an electrochemical/ionic/substrate gradient (Sulzer et al. 2005).

The **"Oligomer-based counter transport model"** relies on the finding that neurotransmitter transporters are expressed as oligomers on the surface (Sitte et al., 2004). It was stated that oligomerization is relevant for the understanding of amphetamine action on the transporters (Sitte & Freissmuth 2003; Seidel et al. 2005; Sitte & Freissmuth 2010).

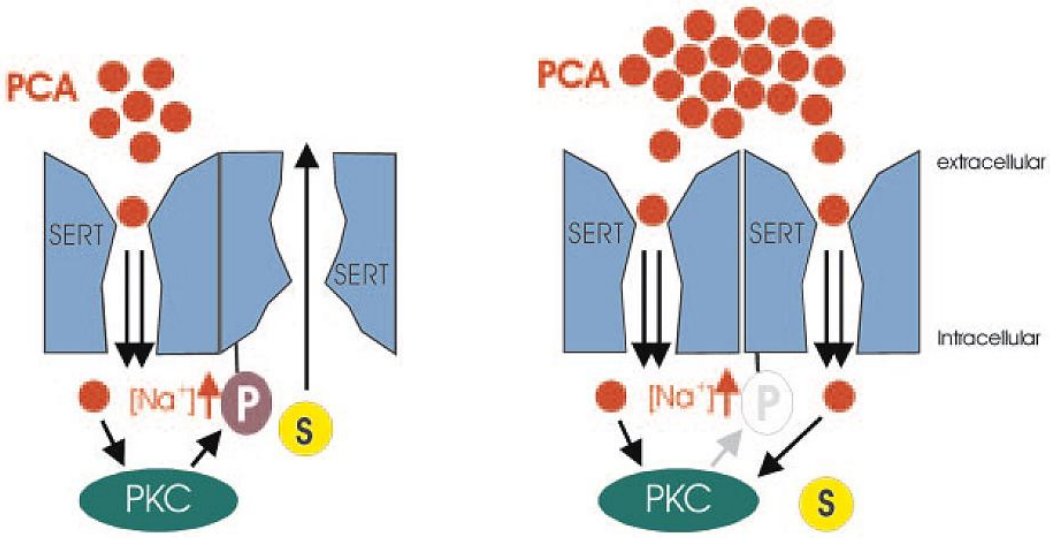


Figure 9: Diagram of the oligomer-based counter transport model
On the left side the effects of a low concentration of *p*CA are shown, on the right with high concentration of *p*CA (Seidel et al., 2005).

In this model (fig. 9), the releaser, in this case *para*-chloramphetamin (*p*CA) is taken up by one moiety of the transporter and stimulates protein kinase C (PKC). This, in turn leads to the phosphorylation of the other un-liganded moiety of the oligomeric complex which results in the outward transport of the substrate only the inactive transporter is amenable to phosphorylation (Ramamoorthy, 1999). This mechanism was proposed for lower concentrations of *p*CA (Seidel et al., 2005) while at higher concentrations of amphetamines, all transporter subunits are occupied by *p*CA. Hence, phosphorylation is prevented and substrate efflux is impaired (Ramamoorthy, 1999). According to this model, amphetamine-induced substrate efflux is highest at a concentration where half of the transporters are occupied by amphetamines (Seidel et al. 2005; Sitte & Freissmuth 2010).

Amphetamine-induced reverse transport via DAT is assumed to involve phosphorylation events. (Khoshbouei et al., 2004) Multiple protein kinases have been shown to regulate functions of DAT (Sulzer et al. 2005). Substantial experimental support for this hypothesis stems from observations that phosphorylation of N-terminal serines induce the reversal of the transporter (Khoshbouei et al., 2004). DAT shifts from a “reluctant” to a “willing” state without affecting the inward transport

(Robertson et al., 2009). It is thought to result from a shift in the voltage or sodium dependence of DA efflux (Khoshbouei et al., 2004).

As outlined above, activation of protein kinase C leads to phosphorylation of the N-terminus of DAT in rats and humans (Foster et al., 2002). Importantly, also Ca^{2+}/calmodulin-dependent protein kinase II (CamKII) is thought to play a critical role in this process, as CamKII was shown to specifically phosphorylate the DAT (Fog et al., 2006; Steinkellner et al., 2012).

1.1.5 DAT and CamKII

CamKIIα is thought to be a key component in the regulation of amphetamine-induced DAT efflux. Fog et al. (2006) observed that CamKII binds to the DAT C-terminus and phosphorylates serines at the N-terminus. By using the C-terminus of the human DAT as a bait and CamKIIα as a prey, an interaction of these two was shown in a yeast two hybrid interaction assay. This finding was confirmed by GST-pulldown. In similar experiments NET only showed a weak binding capacity for CamKIIα and the SERT C-terminus failed to interact with CamKIIα.

The binding of CamKIIα to the C-terminus of DAT and the following phosphorylation of the N-terminus was shown to promote amphetamine-induced dopamine efflux. Application of the CamKII-inhibitor KN93 significantly attenuated efflux induced by amphetamines.

1.1.6 Role of DAT in disease states

Dysfunction of Na^+/Cl^--dependent transporters can lead to many neuropsychiatric disorders including schizophrenia, depression, parkinson disease, epilepsy, attention deficit hyperactivity disorder (ADHD) or drug addiction (Yamashita et al., 2005; Fleckenstein et al., 2007; Steinkellner et al., 2011).

Chronic addiction to drugs is associated with the initiation of pharmacological tolerance, sensitization, and dependence. The impact of these processes is expressed in drug intake, craving, and relapse (Philibin et al., 2011). Neural

processes that mediate common adaptive reward-based learning are targeted by drugs of abuse, leading to repeated neural stimulation. Once, brain reward circuitry is activated, synaptic plasticity takes place, resulting in maladaptive behavior (Fig. 10) (Kalivas and O'Brien, 2008).

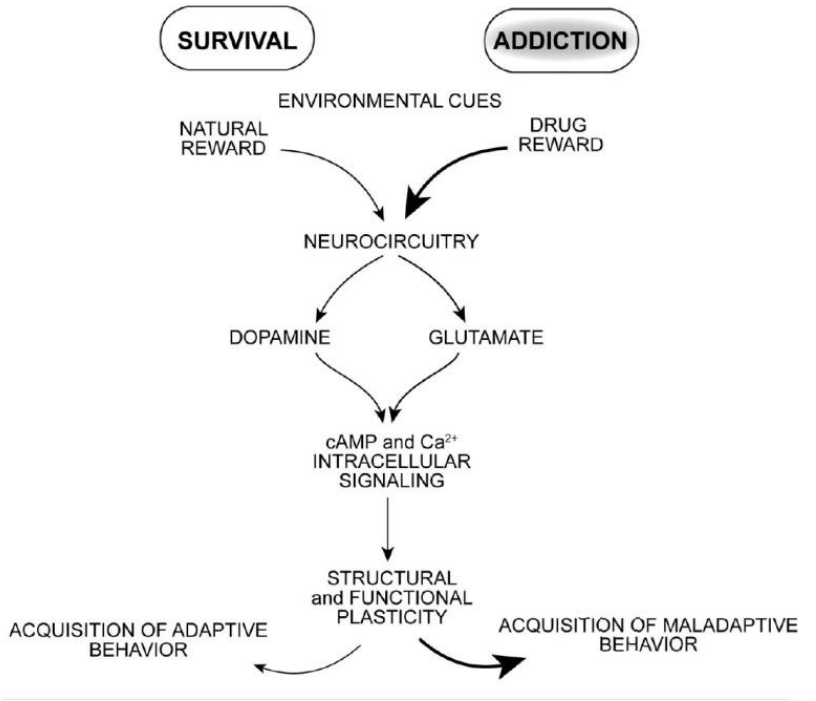


Figure 10: Neural pathways that normally mediating reward-based learning are stimulated by the pharmalogical properties of drugs of abuse, leading to alterations in the dopaminergic and glutaminergic system, resulting in the acquisition of maladaptive behavior (Philibin et al., 2011).

To reach this maladaptive behavior a great interplay between the pre- and postsynaptic site needs to take place. In addition to DAT on the presynaptic nerve terminal, the dopamine receptors on the postsynaptic site also contribute to the initiation of drug addiction (Everitt and Robbins, 2005).

1.1.7 Dopamine receptors

Dopamine receptors are implicated in a number of neuronal processes including motivation, pleasure, cognition, learning, memory and fine motor control. They are common targets of neurological drugs like antipsychotics (Beaulieu and Gainetdinov, 2011).

All five dopamine receptors (D_{1-5}) belong to the family of G-protein coupled receptors (GPCRs). They are distinguished mainly by their diverging action on the adenylyl cyclase (Philibin et al., 2011), dividing them into two families:

- **D1-like family:** The dopamine receptors D1 and D5 belong to the D1-like family. Both are Gαs coupled. Signaling via these receptors leads to a stimulation of adenylyl cyclase elevating the concentration of cyclic adenosine monophosphate (cAMP).
- **D2-like family:** The D2-like receptors (D2, D3, D4) inhibit the formation of cAMP through inactivation of adenylyl cyclase via $G_{\alpha i}$ (Philibin et al., 2011).

Dopamine receptor D1 and D2 are highly abundant in the striatum. These receptors are enriched in distinct striatal neural populations (Girault and Greengard, 2004). Psychostimulant drugs like cocaine strongly influence drug taking and seeking behavior via the regulation of D2-like class of receptors (Edwards et al., 2007). Multiple evidences show that mainly D1, D2 and D3 receptors control locomotor activity. While D1 is expressed only postsynaptically and has moderate stimulating effect on locomotion, activation of the presynaptically located D2 autoreceptor leads to a decrease in the release of DA, thereby decreasing locomotor activity. Activation of D2 at the postsynaptic bouton leads to an increase in locomotion (Missale et al., 1998), while D3 exerts moderate inhibitory action (Beaulieu and Gainetdinov, 2011).

1.2 Amphetamines

1.2.1 Chemical properties

All amphetamines share an α-methyl-phenethyl-amine motif. Biel and Bopp (1978) defined 4 structural features:

1. an unsubstituted phenyl ring
2. a two-carbon side chain between the phenyl ring and nitrogen

3. an α-methyl group and

4. a primary amino group

(for review, see Biel and Bopp, 1978)

Structure of amphetamines:

a, **b,** **c,**

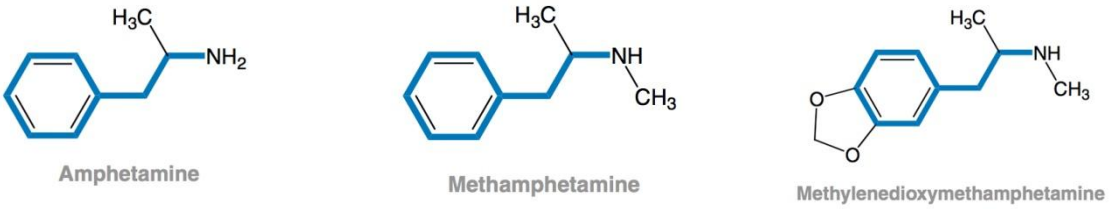


Figure 11: chemical structure of various amphetamines
a, D-amphetamine ("Speed");
b, Methamphetamine (METH,"Ice") does not obey the fourth rule, it has no primary amino group
c, 3,4-Methylenedioxymethamphetamine (MDMA,"Ecstasy") also has no primary amino group
(Fleckenstein et al., 2007)

Natural sources

Amphetamines as well as cocaine, opiates, marijuana and nicotine have been used as plant extracts for a very long time. The genus *Ephedra* and the tree *Catha edulis* contain natural amphetamines like ephedrine or cathinone. It was reported that these plants were also used to treat asthma and upper respiratory infections in former times (Sulzer et al., 2005).

1.2.2 History

Lazar Edeleano, a Romanian chemist, first synthesized synthetic amphetamine in the year 1887. (Fleckenstein et al., 2007) In 1933, the respiratory stimulant and sympathomimetic effects of amphetamines were first published. (for review, see Alles, 1933) Later it was discovered that the stimulant effects are useful for the treatment of narcolepsy (for review, see Prinzmetal and Bloomberg, 1935).

The pharmaceutical company Smith, Kline and French introduced amphetamine as Benzedrine commercially in 1932. It was available without prescription, which led to

disposal of more than 50 million tablets in the first three years. From 1939 on medical prescription was mandatory, because of the great abuse by students, artists, musicians and armed forces (Sulzer et al., 2005).

Nowadays, amphetamine and its derivates are used as a treatment in a wide variety of disorders including ADHD, obesity, narcolepsy and traumatic brain injury (Robertson et al., 2009). Importantly, amphetamines are treated as drugs of abuse and therefore have been listed as scheduled drugs worldwide. As example, Fenfluramine was approved by the Food and Drug Administration (FDA) as a prescriptive anorectic agent against obesity. Used in combination with Phentermine, it has the advantages of the need for lower doses, fewer side effects and less patients tolerance. In 1996 the total number of prescriptions of these two appetite suppressants exceeded 18 million in the United states (U.S.). Regular use of Fenfluramine and Phentermine is thought to lead to pulmonary hypertension and valvular heart disease. In consequence, Fenfluramine was withdrawn from the U.S. market in 1997 (Connolly et al., 1997).

Amphetamines cause euphoria and stimulation in humans, usage often leads to abuse (Seiden et al., 1993). With more than 35 million methamphetamine abusers worldwide, amphetamine abuse is a major social problem, as severe adverse effects like cardiac arrhythmia, psychosis and paranoia are associated with it (Rawson et al., 2002).

1.2.3 Amphetamine action

The action of amphetamine remained unclear until the late 1950s. Burn and Rand (1958) revealed that amphetamine acts by “releasing a noradrenaline-like substance” (Burn and Rand, 1958).

The monoamine transporters are the primary site of action of amphetamines and its congeners (Sitte & Freissmuth 2010; Sulzer et al. 2005), but they have additional targets including vesicular monoamine transporter 1+2 (VMAT1+2) (Fon et al., 1997) and MAO. Under physiological conditions VMAT 1 and VMAT 2 retrieve the neurotransmitter into the synaptic vesicle against its concentration gradient. If

amphetamines are available they start to compete for VMAT, interfering with the uptake of monoamines into vesicles -> **VMAT competition**.

Weak base hypothesis:

Accumulation of amphetamine (a lipophilic, weak base, pK 9.9) in the catecholamine vesicles (pH 5.0 – 5.6) changes the pH. Amphetamine gets protonated inside the acidic vesicles. Once charged it is less permeable and accumulates inside (Sulzer et al., 2005; Sitte and Freissmuth, 2010).

Monoamines that are not packed into vesicles are rapidly degraded by MAO-A and MAO-B, to a lesser extent by COMT (Seiden et al., 1993). Elimination of monoamines by MAO can be prevented by amphetamines. Amphetamine and its derivates act as competitive inhibitors on MAO (Sulzer et al., 2005; Sitte and Freissmuth, 2010).

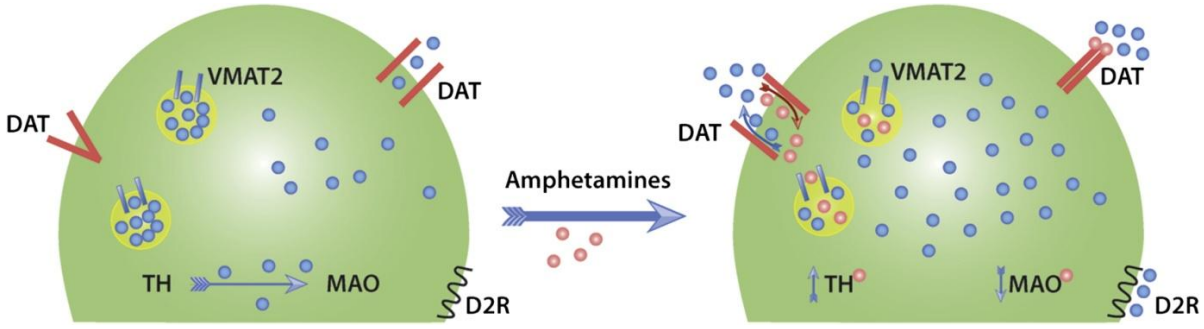


Figure 12: Amphetamine action on the dopaminergic system (Sulzer, 2011)

1.2.4 Cocaine – another indirectly-acting sympathomimetic drug

Cocaine (benzoylmethylecgonine) is obtained from the leaves of the Andean plant *Erythoxylon coca*. Bolivian natives chewed the leaves for over a thousand years. In the 1880s with the introduction of Coca Cola and the reports of Sigmund Freud cocaine became very popular in the western world. Alfred Froehlich and Otto Loewi at the Pharmacology Institute in Vienna made fundamental progress in the research on the action of cocaine, in the year 1910

In contrast to amphetamine, cocaine blocks the reuptake of monoamines into the presynaptic nerve terminal. Thereby, cocaine increases the concentration of DA in

the synaptic cleft. It mainly exerts its function in the nucleus accumbens and the nucleus caudatus (Aktories et al., 2004).

1.3 Sensitization

Consumption of amphetamines induces at first a brief “rush” followed by lasting euphoria. Tolerance develops rapidly to this effect, however there also occurs sensitization (reverse tolerance) in these individuals (Schenk and Partridge, 1997; Brust, 2010). Sensitization is the increased response to a stimulus, after a repeated stimulus exposure. (Steketee and Kalivas, 2011). It is the opposite of developing tolerance, which is characterized by the decrease of an effect of a drug due to repeated administration (Robinson and Berridge, 2003). For a sensitization effect it has furthermore been hypothesized that a withdrawal period of several days is necessary (Morgan and Roberts, 2004). Animal models for substance abuse are used to determine the sensitization effect (Steketee and Kalivas, 2011).

One key feature of sensitization is the different susceptibility of individuals to sensitization. Some sensitize rapidly, while others are more persistent. These differences are thought to be influenced by various genes and hormones. Animals exposed to predisposing factors such as stress conditions can become sensitized to drugs of abuse more easily. Further, individuals that are once sensitized, are prone to show cross-sensitization. This means that sensitization to one drug can cause sensitization effects for drugs of other classes as well. (Robinson and Berridge, 2003). Sensitization can be expressed on a behavioral and on a neurochemical level (Steketee and Kalivas, 2011).

1.3.1 Behavioral sensitization

Various addictive drugs can induce higher locomotion and willingness for exploration as a consequence of arousal, attention and motor behavior in humans and animals (Robinson and Berridge, 2003).

Behavioral sensitization is defined by the increase of locomotion that occurs after a repeated, intermittent exposure to a certain drug (Steketee and Kalivas, 2011). It is a

phenomenon that is remarkably consistent, which leads to the assumption that behavioral sensitization is an important feature of sensitization to psychostimulants. Behavioral sensitization can last for months or even years after repeated drug exposure (Robinson and Berridge, 2003). Different factors including the number of drug intake, interval between treatments, dose, sex and age can influence psychomotor sensitization (Steketee and Kalivas, 2011).

Behavioural sensitization is characterized by two major events:

- Initiation, which is the immediate neural event to the administered psychostimulant substance that triggers behavioral sensitization (Pierce and Kalivas, 1997). This event has been described to occur mainly in the ventral tegmental area (Steketee and Kalivas, 2011).
- Expression, which is the enduring neural alteration of these initiation events, i.e. increased locomotion (Pierce and Kalivas, 1997). The expression appears primarily in the nucleus accumbens (Steketee and Kalivas, 2011).

1.3.2 Neurochemical sensitization

Neurochemical sensitization is thought to be a molecular equivalent of behavioral sensitization and refers to changes in neurotransmitter release and second messenger systems following repeated administration of drugs. In our studies neurochemical sensitization is believed to be connected to an increase of dopamine efflux promoted by certain psychostimulants - d-amphetamine (AMPH) and cocaine. It involves long-lasting changes in the reward system, especially the nucleus accumbens (Robinson and Berridge, 2003).

1.4 Ca^{2+}/Calmodulin-dependent protein kinase II (CamKII)

Cellular responses to neurotransmitters are translated through dynamic second messenger systems following the activation of membrane-bound receptors. The Ca^{2+}/Calmodulin complex modulates the family of multifunctional Ca^{2+}/calmodulin

(Cam)-dependent protein kinases (CamKs), which belong to the family of serine/threonine specific protein kinases. To date CamKI, CamKII and CmKIV are known (Hudmon and Schulman, 2002). In my diploma thesis I focused on the effects that CamKII exerts on the dopamine system.

1.4.1 Structure and function of CamKII

CamKII is an ubiquitous mediator of Ca^{2+}-mediated signaling, phosphorylating a great variety of proteins including the DAT.

It is one of the most abundant proteins in the brain contributing to about 2% of the total protein content in the CNS (Solà et al., 1999). In mammals CamKII originates from a family of 4 homologous, but distinct genes (α, β, γ and δ) with more than 30 isoforms. The α (54 kDa) and β (60 kDa) subunits are the most prominent ones. Different subunits together form a hexameric ring within a dodecameric structure, the CamKII holoenzyme. The holoenzyme is composed from 8 – 12 subunits with different isoform ratios.

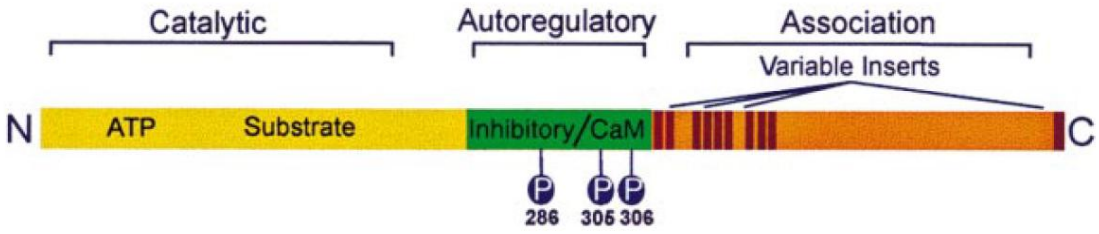


Figure 13: Schematic diagram of a CamKIIα subunit (Hudmon and Schulman, 2002)

A schematic diagram of a typical α subunit of CamKII is shown in figure 13. It consists of the N-terminal catalytic domain, the autoregulatory domain and the association domain with variable inserts at the C-terminus (Hudmon and Schulman, 2002). CamKIIα and β isoforms share a sequence similarity of about 90% in the catalytical and autoregulatory domain (Colbran and Brown, 2004).

The catalytic domain consists of an ATP-binding pocket and a substrate binding site (Colbran, 2004). It can be autoinhibited by a pseudosubstrate type of interaction until Ca^{2+}/calmodulin binding occurs. Residues in the autoinhibitory domain interact with the catalytic domain, thereby blocking the substrate binding site (Hudmon and Schulman, 2002).

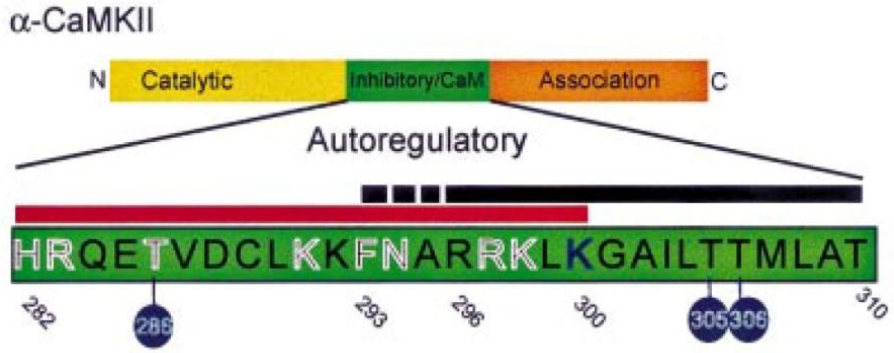


Figure 14: Detailed diagram of the autoregulatory domain of the α subunit of CamKII
Important threonine residues T286 in the core region and T305/T306 in the Cam-binding region
(Hudmon and Schulman, 2002)

Within the autoregulatory region all isoforms share autophosphorylation sites (T286 in the core region of subunit α, T287 in β, γ and δ) and in the Cam-binding domain (T305/T306 and T306/T307respectively) regulating the catalytic activity of the protein kinase (Hudmon and Schulman, 2002).

Phosphorylation of T286 residue leads to an autonomously active form of the kinase, while phosphorylation of T305/T306 can block Ca^{2+}/calmodulin from binding (Colbran, 2004).

The C-terminal part of the protein forms the association domain. Via this region the subunits bind to each other and form the holoenzyme. The variable inserts of this region contribute mainly to the many different CamKII isoforms (Hudmon and Schulman, 2002).

In contrast to CamKI and CamKIV, Ca^{2+}/calmodulin binding alone is efficient enough to produce maximal activity of CamKII. Binding of Ca^{2+}/calmodulin disturbs the interaction of the autoregulatory region with the ATP and substrate binding pocket in

the catalytic domain. This leads to an activation of CamKII, because some amino acids in the regulatory region are essential for both processes, autoinhibition and Ca^{2+}/calmodulin binding. This results in a trans-autophosphorylation of T286 leading to an enhanced affinity (over 1000-fold) to Ca^{2+}/calmodulin ("Cam trapping") (Colbran, 2004). CamKII remains autonomously active even after dissociation of the Ca^{2+}/calmodulin complex (Lou et al., 1986). Due to dephosphorylation the enzyme falls back into the Ca^{2+}/calmodulin-dependent state (Miller and Kennedy, 1986). Following these events CamKII undergoes a dramatic progression in further autophosphorylations that avoid the reactivation by Ca^{2+}/calmodulin ("Cam capping"). T305/T306 are the preferential phosphorylation sites of this inactivation process (Hudmon and Schulman, 2002).

1.4.2 The role of CamKII for learning and memory

CamKII contributes mainly to the mechanism of learning and memory, which is defined as the process to acquire knowledge, the potential of storing the acquired knowledge and to retrieve it (Kandel et al., 1996).

CamKII can act as a "molecular switch" (Miller and Kennedy, 1986) by autoactivation via autophosphorylation of T286; this mechanism induces long term potentiation (LTP) via complex-formation with the NMDA (N-methyl-D-aspartate) receptor. The main source of Ca^{2+} entry into a dendritic spine occurs through this receptor (Lisman et al., 2012). LTP is a form of neural plasticity, where the synapse becomes synchronously stimulated leading to a long lasting enhancement in signal transmission and thereby strengthening the synapse (Cooke and Bliss, 2006). The phosphorylation of T305/T306 withdraws CamKII from the postsynaptic density (PSD) causing synaptic weakening (long term depression, LTD) (Lisman et al., 2012). This process is followed by deficits in spatial learning (Elgersma et al., 2002).

1.4.3 CamKII mutant mice

Silva et al. created the first CamKIIα-KO mouse in the year 1992. The drawback of this mouse model was that half of the protein was still produced despite its gene knock out (Silva et al., 1992). Accordingly, Elgersma et al. (2002) created three different mouse models in which the CamKIIα was endogenously mutated.

- **αCamKIIKO**

In this mouse model the exon 2 in the gene of the α subunit of the CamKII was deleted which results in a translation stop due to a frameshift, leading to a premature protein. No CamKIIα subunit at all is produced. CamKIIβ expression remains at a normal level (Elgersma et al., 2002).

- **TT305/6VA and T305D**

The autophosphorylated threonine residues T305/T306 were replaced by the amino acids, valine (V) and alanine (A) respectively to prevent inhibitory autophosphorylation. Another mouse model is where the T305 residue was substituted by a negatively charged aspartate (D). Aspartate mimics the phosphorylated states of serine and threonine residues blunting in this case the binding of Ca^{2+}/calmodulin.

In our studies, only the CamKIIα knock-out (CamKIIα-KO) mouse was used.

2. WORKING HYPOTHESIS & PRELIMINARY RESULTS

2.1 Working hypothesis

Fog et al. showed that CamKIIα binds to the C-terminus of DAT and phosphorylates its N-terminus (Fog et al., 2006), which promotes the amphetamine-induced reverse operation of the transporter, leading to an increased DA concentration in the synaptic cleft. As CamKIIα plays an important role in the in-vivo sensitization and is crucial for learning and memory (Licata et al., 2004), this is thought to underlie the initiation of drug addiction.

Steinkellner et al. showed that pharmacological inhibition with KN93 as well as genetic elimination of CamKIIα significantly reduces the amphetamine-induced reverse efflux of DA an acute setting that employed in striatal synaptosomes in WT compared to KO mice (Steinkellner et al., 2012).

Based on these findings the aim of my thesis was to elucidate the role of the interaction between CamKIIα and DAT *in vivo* and *in vitro*, and its importance for the mode of action of psychostimulants.

2.2 Preliminary results

2.2.1 CamKIIαKO vs. WT

- **Superfusion experiment**

Thomas Steinkellner performed superfusion experiments with striatal synaptosomes from WT and KO mice. Synaptosomes were prepared like described in 2.4 and subsequently superfused (see 2.5) (Steinkellner et al., 2012).

Amphetamine-induced efflux in untreated CamKIIα-KO and WT mice

a, AMPH-mediated DA-efflux **b, AUC of efflux**

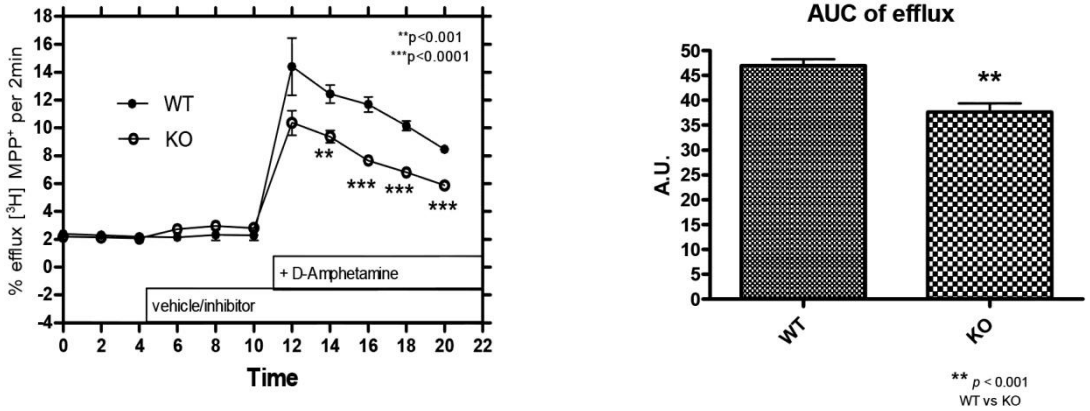


Figure 15: a, Superfusion of CamKIIα WT and KO mice with d-amphetamine
Drug-induced release was calculated normalizing total release to basal release.
b, AUC of MPP^+ efflux in striatal synaptosomes
Area under the curve of percentage of total radioactivity present in each fraction upon stimulation with d-amphetamine was calculated and normalized to basline efflux. (as published by Steinkellner et al., 2012)

Superfusion experiments showed a decreased AMPH-induced [^{3}H]-MPP^+-efflux in the KO mouse striatal synaptosomes compared to the WT.

- **Acute injections, behavioral pharmacology**

As behavioral pharmacology is an important tool to study the effects of psychoactive drugs in animals, Thomas Steinkellner used the open field approach to examine the acute locomotor response of CamKIIα-KO and WT mice to psychostimulant drugs that act on DAT including cocaine and amphetamine.

Acute injections

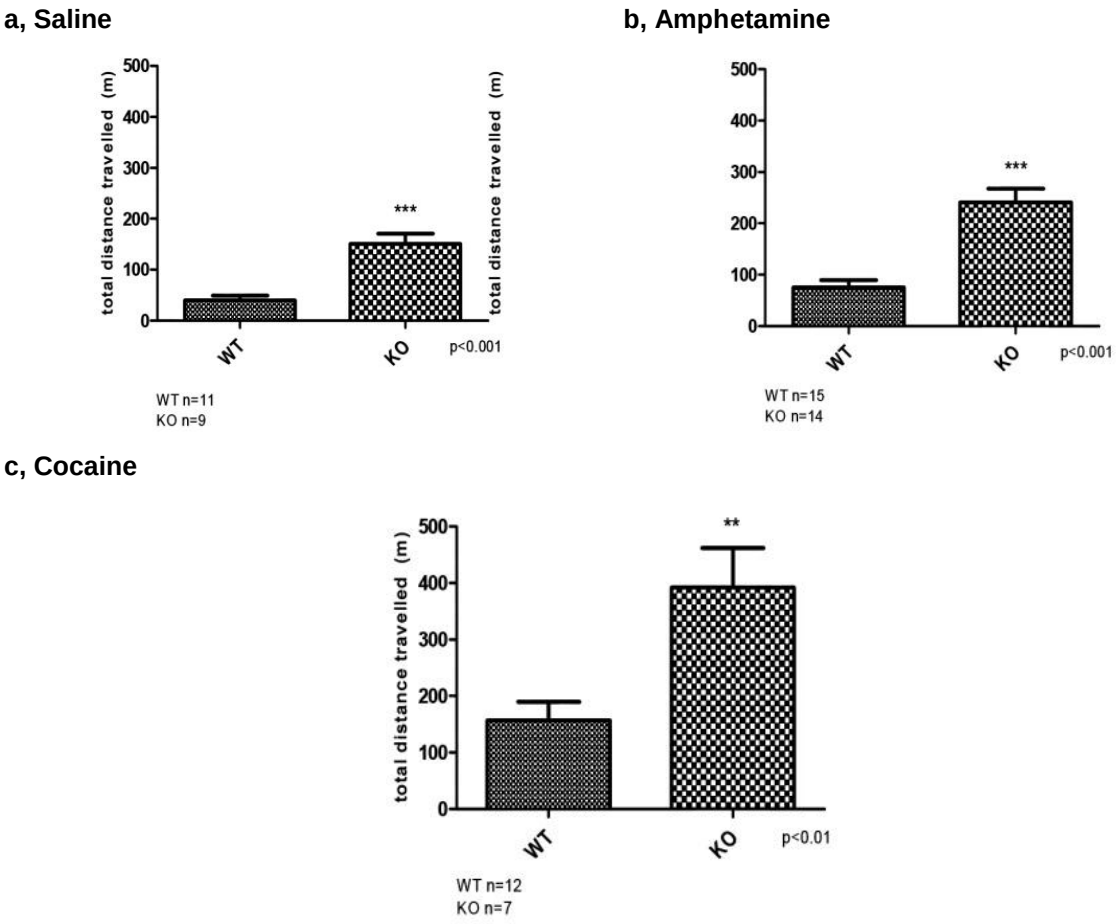


Figure 16: Acute locomotor response of WT and KO CamKIIα mice (Th. Steinkellner unpublished data)
Mice were injected with either **a,** saline; **b,** d-amphetamine (2mg/kg); **c,** cocaine (20mg/kg) intra peritoneal (i.p) and put into a box. Locomotion was monitored for 60 min. Shown is the total distance travelled in meter (m)
(Student's t-test)

Unexpectedly, acute injections of d-amphetamine and cocaine led to a significantly increased locomotor behavior in the KO animals compared to WT mice. The negative control (both genotypes treated with saline) displayed the same effect. Similar results were obtained later during the sensitization approach, see chapter open field (4.1.1.).

We started to use a psychostimulant sensitization approach to investigate, if CamKIIα-KO mice display addictive behavior to d-amphetamine and cocaine, to further shed light on the interaction between CamKIIα and DAT.

3. MATERIALS & METHODS

3.1 Animals

We used CamKIIα knock-out mice, which were kindly provided by Prof. Ype Elgersma (Department of Neuroscience, Erasmus MC, 3000 DR Rotterdam, The Netherlands). The genetic background of these mice is C57BL/6.

The CamKIIα-KO mouse has a deletion of exon 2 in the α subunit of CamKII (Ca^{2+}/calmodulin dependent protein kinase II) which results in a premature translational stop due to a frame shift (Elgersma et al., 2002).

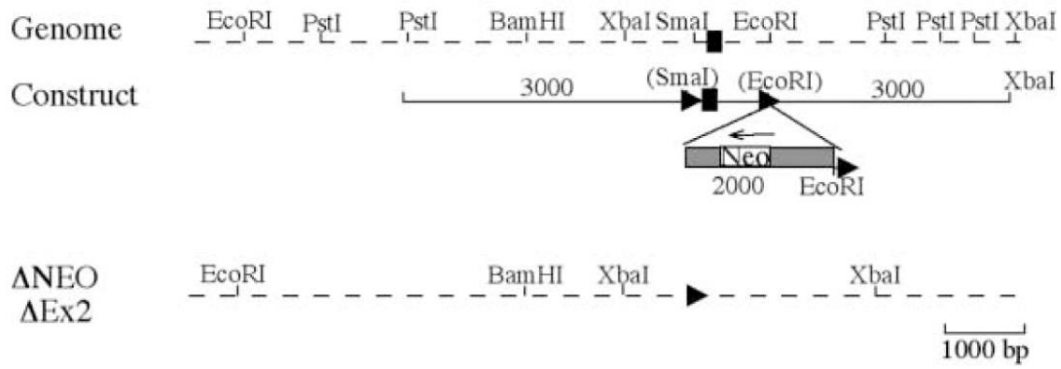


Figure 17: (Top) Diagram of CamKIIα locus displaying the targeting construct of exon 2 (including LoxP sites) used for the CamKIIα knock-out.
(Bottom) CamKIIα mutant after homologous recombination (Elgersma et al., 2002).

Heterozygous breeding pairs were used for mating, which resulted in an offspring of 25% homozygous wild type (WT/WT), 50% heterozygous (WT/KO) and 25% homozygous mutant (KO/KO) animals, which represents the expected mendelian ratio.

Only wild type (WT/WT) and homozygous mutant (KO/KO) littermates were included into the open field (OF), conditioned place preference (CPP) and neurochemical studies.

3.2 Genotyping

3.2.1 DNA Isolation

Biopsies were taken by cutting of a short piece of the mouse tail. To isolate the DNA tail digestion buffer (TDB) including proteinase K or Hot shot buffer was used.

1-step tail digestion buffer (TDB)

50mM KCL

10mM Tris-HCl (pH = 9,0)

0,1% Triton X-100

Shortly before use 0,15mg/ml proteinase K (0.15mg/ml; Sigma-Aldrich, Germany/USA) was added.

Using 1-step tail digestion buffer including proteinase K, 100µl of the buffer were added to the freshly cut tails. Afterwards they were incubated over night at 56°C. In the morning proteinase K was inactivated at 94°C for 10 minutes.

Lysate was directly used for PCR or samples were frozen at -20°C.

Hot shot buffer and neutralization buffer

Hot shot buffer (pH = 12,0)

25mM NaOH

0,2mM Na^+-EDTA

Neutralization buffer (pH = 5,0)

40mM Tris-HCL

Hot shot tail digestion was performed when the result of genotyping was needed urgently.

75µl of Hot shot buffer was used per tube and incubated at 94°C for 1 – 3 hours. 75µl of neutralization buffer was added to stop the reaction.

Most of the time the lysate was used immediately to perform PCR, otherwise the samples were frozen.

Sometimes a phenol/chloroform extraction was performed.

3.2.2 Phenol/Chloroform extraction

100µl of the lysate were diluted with 100µl of TDB. 200µl phenol/chloroform mixed 1:1 were added, inverted and centrifuged for 10 minutes at full speed. The supernatant (SN) was carefully transferred into a new tube. 200µl chloroform were put into the tube containing the SN, gently inverted and centrifuged for 10 minutes at maximum rpm. The SN was taken again and 150µl of isopropanol were added and centrifuged for 30 minutes to precipitate the DNA. The SN was discarded and the pellet was washed with 70% ethanol (EtOH) spinned down for 5 minutes and dried for several minutes at 55°C to remove the EtOH. Finally, the pellet was dissolved in 50µl 1xTris-EDTA buffer (TE buffer).

1x TE buffer

10mM Tris-HCl (pH 8.0)
1mM EDTA

3.2.3 Polymerase chain reaction (PCR)

PCR reaction:

5µl 5x Green GoTaq® buffer (Promega)

0,5µl dNTPs [10mM] (Fermentas)

0,075µl primer [100µM] (Microsynth)

0,125µl GoTaq Polymerase (Promega)

1µl DNA

18,15µl MQ H_2O (“Milli-Q PLUS 185”, Millipore)

25µl total volume

Primers (CamKIIαKO):

CamKO P1: 5’-CAG GCA GTG CGG TAA CCT ACC-3’

CamKO P2: 5’-GAA CAG TGT GAA CAC CCT ATC C-3’

CamKO P3: 5’-GGA TCT GGG CCC CTG TCT GGA TC-3’

Product WT: **290** base pairs (bp)

Product mutant: **320** bp

PCR protocol:

94°C	5 min	
94°C	45 sec	35 cycles
58°C	45 sec	
72°C	45 sec	
72°C	10 min	
4°C	∞	

After amplification the PCR products were analyzed using a 2% agarose gel (voltage: 135V). PCR products differ in their size.

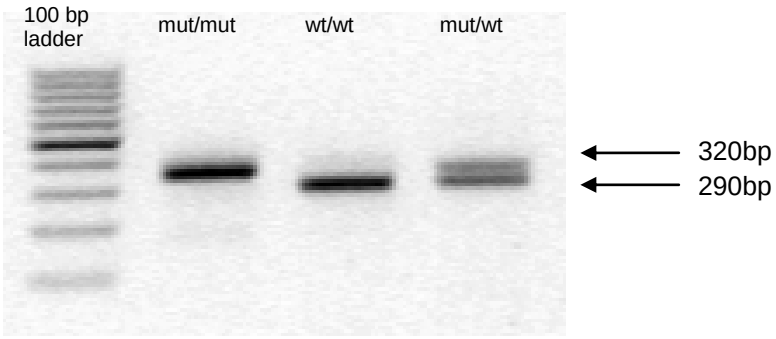


Figure 18: Genotyping
100bp ladder, KO (320bp), WT (290bp), Heterozygote (320 and 290bp)

3.3 Open field, Sensitization

The open field approach was used to study locomotor behavior of the CamKIIα-KO and WT mice under the influence of different psychostimulants. Drugs like d-amphetamine and cocaine were dissolved in 0.9% saline and injected intra-peritoneally (i.p.). Saline injection served as negative control.

A system was used that accommodated four mice at the same time in different boxes (L36xB36xH45): locomotor activity was measured by monitoring the distance travelled by the individual mice using a camera mounted above the box (Fig. 19). Anymaze (Stoelting, Version 4.7) software was used to analyze the data.

Figure 19: Scheme of an open field as described in the text
http://www.sandiegoinstruments.com/libraries/images/products/multi_unit_open_field.jpg

The mice were kept for several days in the room where the behavioral experiments were conducted to habituate the mice to the novel environment. Animals were weighted and the injection volume (V) was determined :

V(µl)= weight(g)x10

V(unit)= V(µl)/25

The syringes were labeled in units: 1 unit is 25µl

On day -1 mice received saline injections to get them used to the injection stress. The following day (day 0) they received another saline injection and were measured for one hour in the OF. On day 1 to day 6 the mice received either d-amphetamine (2mg/kg in saline solution; Sigma-Aldrich, Germany/USA), cocaine (5, 10 or 20 mg/kg in saline; Sigma-Aldrich, Germany/USA) or saline. Afterwards they were withdrawn from the respective drug for 14 days. On day 20 mice received a challenge injection and were measured again in the open field. For the superfusion experiments the pre-treated mice were killed through cervical dislocation on day 21.

Group	Weight / i.p. volume	Day -1	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 20
WT **saline** Nr.	27g = **11** Units	**saline**	**saline** + OF	saline + OF	saline + OF	saline + OF	saline + OF	saline + OF	saline + OF	challenge amph. 2mg/kg + OF
KO **saline** Nr.	32g = **13** Units	**saline**	**saline** + OF	saline + OF	saline + OF	saline + OF	saline + OF	saline + OF	saline + OF	challenge amph. 2mg/kg + OF
WT **amph** Nr.	30g = **12** Units	**saline**	**saline** + OF	amph. 2mg/kg + OF	amph. 2mg/kg + OF	amph. 2mg/kg + OF	amph. 2mg/kg + OF	amph. 2mg/kg + OF	amph. 2mg/kg + OF	challenge amph. 2mg/kg + OF
KO **amph** Nr.	25g = **10** Units	**saline**	**saline** + OF	amph. 2mg/kg + OF	amph. 2mg/kg + OF	amph. 2mg/kg + OF	amph. 2mg/kg + OF	amph. 2mg/kg + OF	amph. 2mg/kg + OF	challenge amph. 2mg/kg + OF
WT **saline** Nr.	27g = **11** Units	**saline**	**saline** + OF	saline + OF	saline + OF	saline + OF	saline + OF	saline + OF	saline + OF	challenge cocaine 20mg/kg + OF
KO **saline** Nr.	32g = **13** Units	**saline**	**saline** + OF	saline + OF	saline + OF	saline + OF	saline + OF	saline + OF	saline + OF	challenge cocaine 20mg/kg + OF
WT **coc** Nr.	23.5g = **9** Units	**saline**	**saline** + OF	cocaine 20mg/kg + OF	cocaine 20mg/kg + OF	cocaine 20mg/kg + OF	cocaine 20mg/kg + OF	cocaine 20mg/kg + OF	cocaine 20mg/kg + OF	challenge cocaine 20mg/kg + OF
KO coc Nr.	27g = **11** Units	**saline**	**saline** + OF	cocaine 20mg/kg + OF	cocaine 20mg/kg + OF	cocaine 20mg/kg + OF	cocaine 20mg/kg + OF	cocaine 20mg/kg + OF	cocaine 20mg/kg + OF	challenge cocaine 20mg/kg + OF

Figure 20: Sensitisation schedule run x

Analysis was done in Microsoft Excel and GraphPad Prism (GraphPad software, San Diego, CA, USA).

Raw data were obtained by measuring the total distance travelled of each animal per day and condition (provided by anymaze software). These data were used to calculate the average distance travelled and standard deviation for every data set. Data were normalized to day 1, the first day of drug injection. A multiple measures ANOVA followed by a Tukey's Post Hoc test was performed to show behavioral sensitization.

3.4 Preparation of synaptosomes

The sensitized mice were decapitated, their brain immediately removed and put on ice. All further steps were also performed on ice. The striatum was dissected and homogenized in 1,5mL of 0,32M sucrose solution in phosphate-buffered saline (PBS). The homogenate was centrifuged for 10 minutes at 1000xg (4°C). The supernatant was transferred into a new tube and the pellet (P1) containing cytosolic proteins was retained. Again a centrifuging step was performed (12600xg for 15 minutes at 4°C). Pellet (P2) consisting of membrane fractions was weighted and dissolved in Krebs-HEPES-buffer (KHB) (1mg/ 15µl).

10x PBS pH= 7,3

2,7mM KCl

1,5mM KH_2PO_4

137mM NaCl

4,3mM Na_2HPO_4

1x Krebs-HEPES buffer pH=7,3

10mM HEPES

120mM NaCl

3mM KCl

2mM $CaCl_2$

2mM $MgCl_2$

+ 3,96g/L D-(+)-Glucose-Monohydrate

3.5 Superfusion

Synaptosomal suspension was incubated with 0,1µM [^{3}H]-MPP^+ for 20 minutes at 37°C. MPP^+ is a substrate of DAT that is resistant to MAO degradation and can be used instead of DA under experimental conditions. Another advantage is the better signal-to-noise ratio (Steinkellner et al., 2012).

15µL of the [^{3}H]-MPP$^+$-loaded synaptosomes were transferred onto filters (Whatman, GF/B). These filters were placed into the superfusion glass chambers which were previously washed with KHB. A wash out period of 40 minutes followed to create a stable baseline.

For the first 10 min the samples were only superfused with KHB. Every 2 minutes a fraction was collected (5 fractions). They served as a baseline. To stimulate the efflux of [^{3}H]MPP$^+$, d-amphetamine (6µM) was added to the superfusion buffer.

All superfusate samples and the filters were mixed with 2mL of scintillation cocktail "Rotiszint® eco plus" (Carl Roth, Germany) to verify the total amount of the present radioactivity.

Analysis of the raw counts was performed using Microsoft Excel and GraphPad Prism.

The efflux is shown as percentage of total radioactivity present in each collected fraction (%efflux).The area under the curve (%AUC) was calculated from the total efflux of [^{3}H] MPP$^+$ every two minutes after stimulation with d-amphetamine (10-20 min) and was normalized to the basal efflux (0-6min).

3.6 Conditioned place preference (CPP)

Similar to the open field approach, we habituated the mice to the novel laboratory environment for a few days before we conducted the CPP experiments.

CPP experiments were performed in boxes with two distinguishable floors, either a grid or rods (Med Associates Inc., Georgia, VT).

Figure 21: conditioned place preference chambers

During the preconditioning (day 0) the mice were able to explore both chambers for 30 minutes. The time spent in each chamber was recorded. On day 1 d-amphetamine (2mg/kg) or cocaine (20mg/kg) was injected and the mice were placed into the less preferred chamber assessed on day 0. The following day a saline injection was paired with the other chamber. Drug and saline injections were repeated three times on alternate days. To measure whether mice developed any place preference to the injected substances, a test session was carried out on day 7 in which the mice had free access to both chambers. The time spent in either of the 2 chambers was recorded similar to the preconditioning day.

Group		Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
KO **amph** Nr.	28 g 11 Units	**30 min CPP**	**amph CPP 30min Door closed**	**saline CPP 30min Door closed**	**amph CPP 30min Door closed**	**saline CPP 30min Door closed**	**amph CPP 30min Door closed**	**saline CPP 30min Door closed**	**test CPP 30min**
KO **amph** Nr.	26 g 10 Units	**30 min CPP**	**amph CPP 30min Door closed**	**saline CPP 30min Door closed**	**amph CPP 30min Door closed**	**saline CPP 30min Door closed**	**amph CPP 30min Door closed**	**saline CPP 30min Door closed**	**test CPP 30min**
WT **amph** Nr.	26 g 10 Units	**30 min CPP**	**amph CPP 30min Door closed**	**saline CPP 30min Door closed**	**amph CPP 30min Door closed**	**saline CPP 30min Door closed**	**amph CPP 30min Door closed**	**saline CPP 30min Door closed**	**test CPP 30min**
WT **amph** Nr.	25 g 10 Units	**30 min CPP**	**amph CPP 30min Door closed**	**saline CPP 30min Door closed**	**amph CPP 30min Door closed**	**saline CPP 30min Door closed**	**amph CPP 30min Door closed**	**saline CPP 30min Door closed**	**test CPP 30min**
KO **cocaine** Nr.	23 g 9 Units	**30 min CPP**	**cocaine CPP 30min Door closed**	**saline CPP 30min Door closed**	**cocaine CPP 30min Door closed**	**saline CPP 30min Door closed**	**cocaine CPP 30min Door closed**	**saline CPP 30min Door closed**	**test CPP 30min**
KO **cocaine** Nr.	28 g 11 Units	**30 min CPP**	**cocaine CPP 30min Door closed**	**saline CPP 30min Door closed**	**cocaine CPP 30min Door closed**	**saline CPP 30min Door closed**	**cocaine CPP 30min Door closed**	**saline CPP 30min Door closed**	**test CPP 30min**
WT **cocaine** Nr.	26 g 10 Units	**30 min CPP**	**cocaine CPP 30min Door closed**	**saline CPP 30min Door closed**	**cocaine CPP 30min Door closed**	**saline CPP 30min Door closed**	**cocaine CPP 30min Door closed**	**saline CPP 30min Door closed**	**test CPP 30min**
WT **cocaine** Nr.	29 g 12 Units	**30 min CPP**	**cocaine CPP 30min Door closed**	**saline CPP 30min Door closed**	**cocaine CPP 30min Door closed**	**saline CPP 30min Door closed**	**cocaine CPP 30min Door closed**	**saline CPP 30min Door closed**	**test CPP 30min**

Figure 22: CPP schedule run x

For analysis Microsoft Excel and GraphPad Prism were used.

Differences in the time spent in either one of the two chambers was calculated by comparing preconditioning and test session.

3.7 RNA Isolation

TRIreagent (Sigma Aldrich) was used to isolate RNA from untreated wt and CamKIIαKO mouse striata.

Prepared striata were manually homogenized with 1mL TRIreagent, incubated for 5 minutes at room temperature (RT) before 200µl chloroform were added and the

tubes were gently inverted for 15 seconds. After an incubation time of 3 minutes at room temperature samples were centrifuged at 12000xg for 15 min.

To precipitate the RNA the upper aqueous phase was transferred into a new tube. 500µl isopropanol were added. The sample was shortly inverted and kept at RT for 10 min, followed by centrifugation for 10 min at 12000xg. The supernatant was discarded before the pellet was washed twice with 70% ethanol (7500xg for 5 min each). Finally, the pellet was dried and dissolved in 70µL MQ water treated with DEPC.

RNA concentration was calculated by measuring absorbance at 260nm wavelength.

RNA [mg/µl]= Abs_{260} x 40 x 100

3.8 cDNA synthesis

For cDNA synthesis the RevertAid™ First Strand cDNA Synthesis kit (Fermentas) was used.

cDNA synthesis reaction

4µl	5x Reaction Buffer
2µl	dNTP (10mM)
1µl	primer
1µl	Revert Aid™ M-MuLV Reverse Transcriptase (200u/µl)
1µg	template RNA
Xµl	MQ H_2O
20µl total volume	

Reverse Transcriptase PCR program:

for random hexameric primed synthesis

25°C	5 min	
42°C	60 min	
70°C	5 min	termination
4°C	∞	

20µl of cDNA total volume were diluted 1:4 with MQ water and stored at -20°C until usage.

3.9 Real time PCR

mRNA levels were verified by using the SensiMix™ SYBR & Fluorescein kit (Bioline) for real time PCR.

Real time PCR reaction:

12,5µl	2x SensiMix™ SYBR
1µl	forward primer
1µl	reverse primer
5µl	cDNA template
25µl total volume	

Real time PCR program:

95°C	5 min	
95°C	30 sec	} 45 cycles
61°C	30 sec	
72°C	30 sec	
72°C	10 min	

Primers:

Dopamine receptor 1

Drd 1a new left: 5’-TCT CCC AGA TCG GGC ATT TGG-3’

Drd 1a new right 3: 5’-GGC CTC TTC CTG GTC AAT C-3’

fragment size: 130bp

Dopamine receptor 2

Drd 2 left new: 5’-TCT ATC TGG AGG TGG TGG GTG-3’

Drd 2 right new: 5’-CAG CTG TGT ACC TGT CGA TGC-3’

fragment size: 140bp

GAPDH

GAPDH fw: 5‘-GGC TCA TGA CCA CAG TCC AT-3‘

GAPDH rv: 5‘-CAT ACT TGG CAG GTT TCT CCA-3‘

4. RESULTS & DISCUSSION

4.1 Open field

4.1.1 Total distance travelled

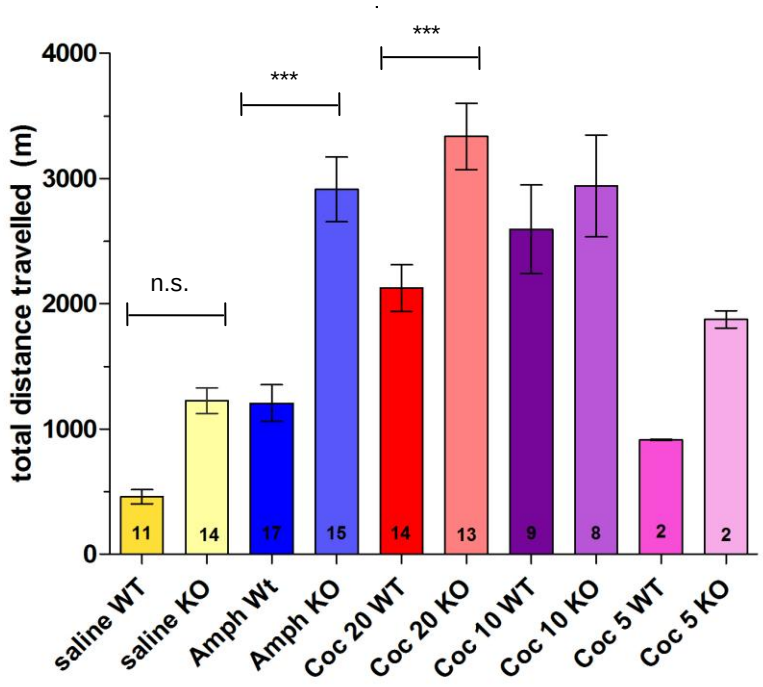


Figure 23: The total distance travelled of all mice and each day per condition is shown in meter The different colors of the bars exhibit their affiliation to the different treatment conditions. Light and dark yellow for saline, the vehicle for WT and KO; light and dark blue for d-amphetamine, red for cocaine 20mg/kg, light and dark violet for cocaine 10mg/kg and both pink for cocaine 5mg/kg. ANOVA with Tukey Post Hoc test
***p≤0,001

Figure 23 shows the total distance travelled to display the difference in their sensitization behavior. CamKIIα-KO and WT mice were sensitized with different compounds (d-amphetamine and cocaine, saline was used as a negative control) and their total distance travelled in meter (m) was measured for 60 minutes in the open field. Sample size (animals per condition) is indicated in each graph.

Against our expectations, CamKIIα-KO mice displayed a significantly higher total distance travelled compared to the WT, this holds true for d-amphetamine (2mg/kg) and cocaine (20mg/kg) treatment. However, CamKIIα-KO mice treated with saline served as negative control, also showed an increased locomotion (not significant).

At the beginning of the experiment we hypothesized that WT mice treated with d-amphetamine should cover a higher distance compared to their KO littermates. When amphetamine is taken up by DAT as a substrate, CamKII phosphorylates DAT and the transporter exerts its reverse action increasing the amount of DA in the synaptic cleft. The motor behavior becomes stimulated resulting in an increase of locomotion. As the α subunit of CamKII is knocked out in the mutant mice, DAT should not be phosphorylated anymore, attenuating its reverse transport action, followed by less extracellular DA. Unpublished observations made by Thomas Steinkellner indicate that CamKIIα-KO mice display a higher tissue content of DA in the striatum which may be the simplest explanation of the increased basal locomotion.

4.1.2 Saline

Saline treated mice serve as a negative group (control group). They are only influenced by their genetic background and several stress factors like handling of the mouse and injection stress.

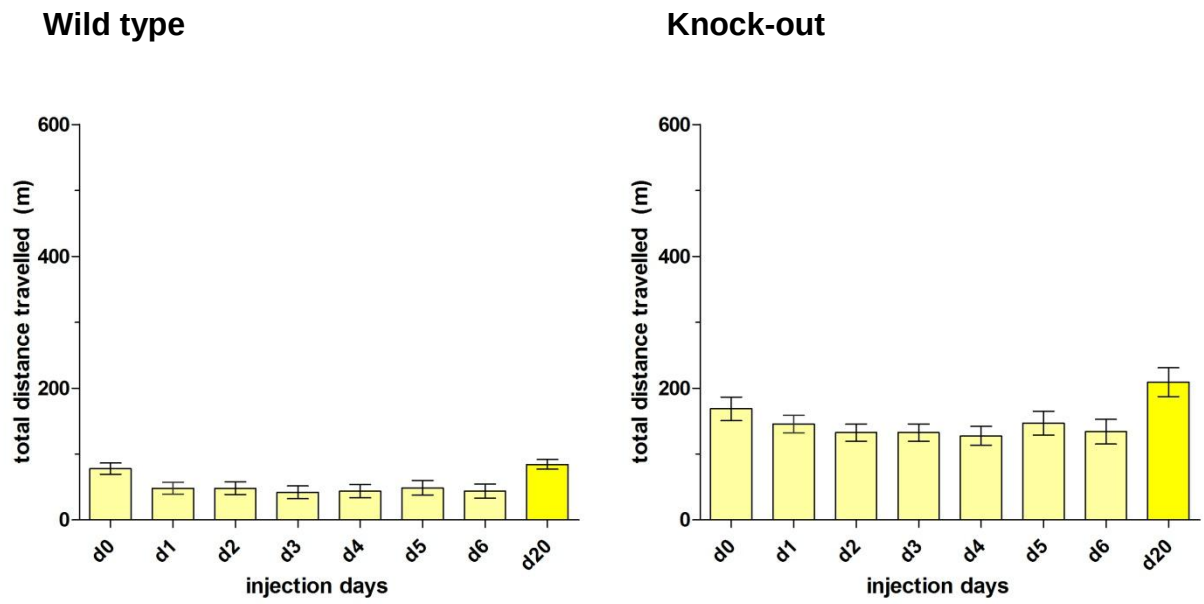


Figure 24: saline WT n = 13, KO n = 14
The total distance travelled in meter (m) was measured. Each bar represents one injection day (d0-d6). Between day 6 and day 20 there was a 14 days withdrawal period.
A multiple measures ANOVA was done, Post hoc test: Tukey test

Both, WT and KO mice were injected with saline according to their body weight (10ml/kg). After injection, they were put into the open field and monitored.

As expected, neither of the two groups showed any sensitization or habituation effect. WT mice treated with saline travelled around 50 to 100 meter during the injection period, while their KO littermates displayed a higher total distance travelled of about 190 – 220 meters.

4.1.3 Amphetamine

CamKIIα-KO mice as well as their wild type littermates were sensitized with 2mg/kg d-amphetamine. Mice received a saline injection on day 0 to accustom them to the injection stress. From day 1 to day 6, they were injected with the drug and then withdrawn for 14 days, which was shown to impact on the formation of a drug memory (Licata et al., 2004). On day 20 a challenge injection was performed.

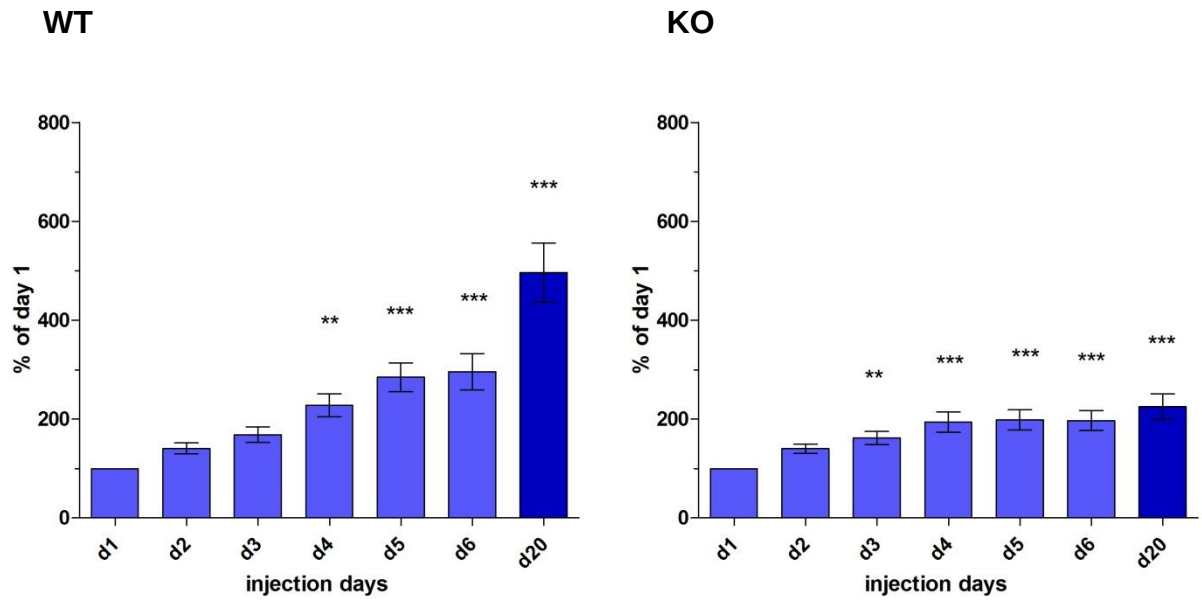


Figure 25: d-amphetamine 2mg/kg WT n = 17, KO n = 16
Shown is distance travelled normalized to day 1
A multiple measures ANOVA was performed, Post hoc test: Tukey test
**p≤0,01, ***p≤0,001

Figure 25 shows that WT mice display a gradual increase in the distance travelled (percent of day 1) from day 2 to day 6 and also on day 20. Analyzing data with a multiple measures ANOVA followed by a Tukey Post Hoc test showed a significant increase in the distance. This clearly shows that WT mice demonstrate locomotor sensitization to AMPH. Interestingly, CamKIIα-KO mice also sensitize to d-amphetamine, but to a way less extent than WT do. According to literature CamKII is not the only kinase acting on DAT and mediating amphetamine-induced DA efflux (Gnegy, 2003). In the case of the knock out of CamKIIα probably PKC plays a role as it is a major regulator of DAT (Sulzer, 2011). These findings could also be due to other factors given that the mechanism how amphetamines induce reverse transport is not fully elucidated up till now.

WT vs. KO

Next, we analyzed the data described above by two-way ANOVA followed by Bonferroni post hoc test to resolve any significant difference between the two genotypes in their locomotor behavior on the same injection day. This should highlight the sensitizing effect in the WT mice compared to the KO.

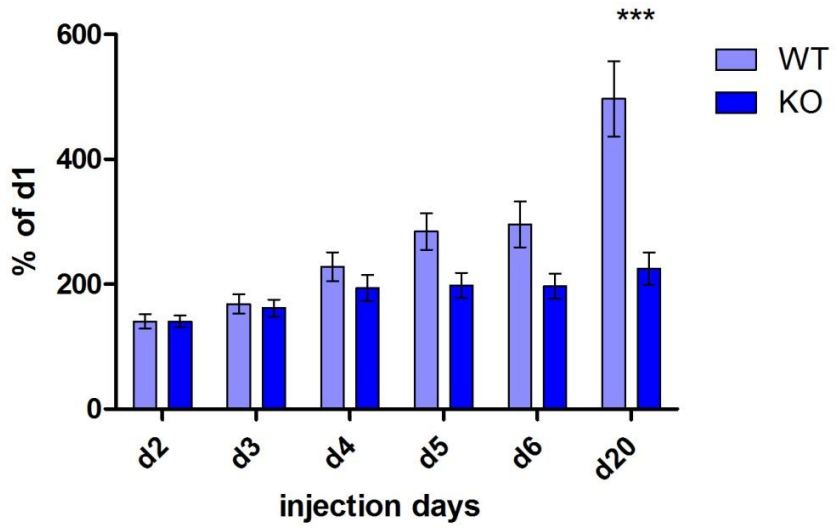


Figure 26: WT vs. KO on each sensitization day normalized to day1 (d-amphetamine)
d2 – d4 show no significance by comparing their distance travelled normalized to d1, whereas d20 does.
A two-way ANOVA with a Bonferroni post test was performed.
***p≤0,001

During the entire sensitizing regime (day 2 to 6), no statistical difference was found between WT and KO, only after the withdrawal on d20, a significantly changed locomotor behavior of the two genotypes was observed. This leads to the assumption that WT mice sensitize in this time period to d-amphetamine to a greater extent in comparison to the KO mice.

4.1.4 Cocaine

Cocaine 20 mg/kg

Licata et al. (2004) treated rats with cocaine (15mg/kg) for four days and measured them in the open field for 120 minutes. They found a significant increase in locomotion on day 4 relative to day 1.

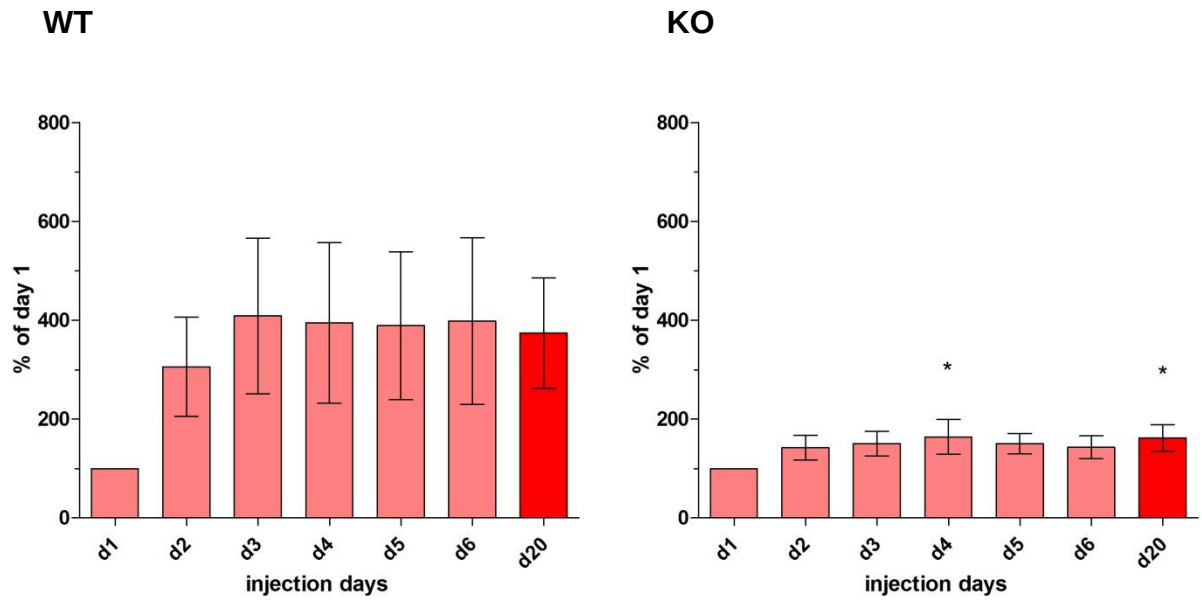


Figure 27: cocaine 20mg/kg WT n =14, KO n = 13
A multiple measures ANOVA was done, Post hoc test: Tukey test

In our studies, CamKIIα-KO mice and respective wild type littermates were used. All of them were treated with 20mg/kg cocaine and put in the open field for 60 minutes. Mice were injected with the drug for six days and afterwards withdrawn. All mice received a challenge injection on day 20. Within the first three days the WT mice show an increase in locomotion which is not significant by applying a multiple measures ANOVA and a Tukey Post Hoc test. After day 3, mice reached a plateau. Withdrawal for 14 days does not lead to any further increase in sensitization. Similar to our findings in WT mice, Ramsey et al. (2008) also sensitized mice (WT and NMDA receptor-deficient, NRI-KD) with 20mg/kg cocaine with similar effects for the WT mice on the third day of cocaine exposure, WT mice already showed full sensitization (Ramsey et al., 2008). In figure 27 the large error bars reflect the differences in both the mice themselves and in their susceptibility to sensitization with cocaine. KO mice show an approximately 1 - 2-fold increase in locomotion after sensitization compared to day 1, this remained constant all the time. On day 4 and day 20 only little significance for sensitization was displayed.

WT vs. KO

A two-way ANOVA (accompanied by a Bonferroni post test) comparing both genotypes on each day revealed: No statistical difference in the running behavior

except the amount of distance in relation to day 1 was found. As expected, no behavioral sensitization appeared in the KO mice.

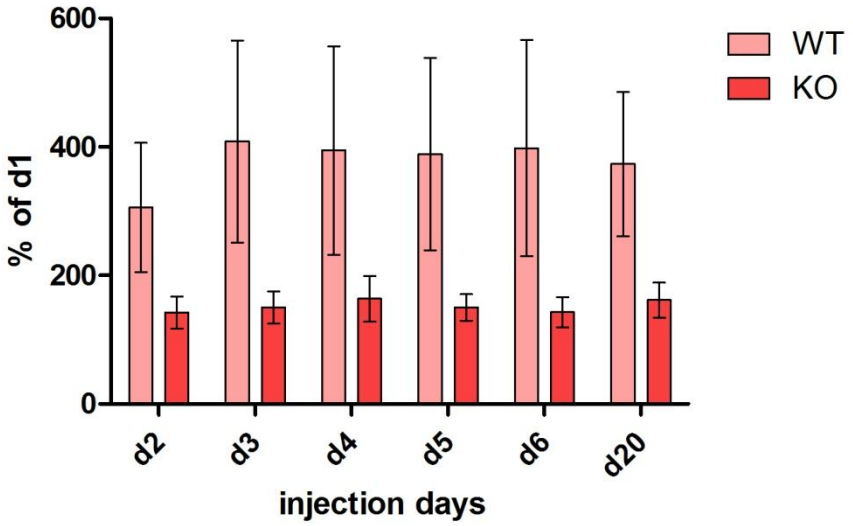


Figure 28: WT vs. KO on each sensitization day normalized to day1 (cocaine 20mg/kg)
d2 to d6 and d20 no statistical difference could be found
A two-way ANOVA with a Bonferroni post test was performed.

Cocaine 10 mg/kg, Cocaine 5 mg/kg

At the beginning of our studies, we performed a dose-finding study and tested different doses of cocaine in behavioral experiments. We used cocaine 10mg/kg and 5 mg/kg. The results obtained with 5 mg/kg and 10mg/kg were similar to that of 20mg/kg. We have finally chosen 20mg/kg, because it is also the dose which is most often used in the literature (for example Ramsey et al., 2008).

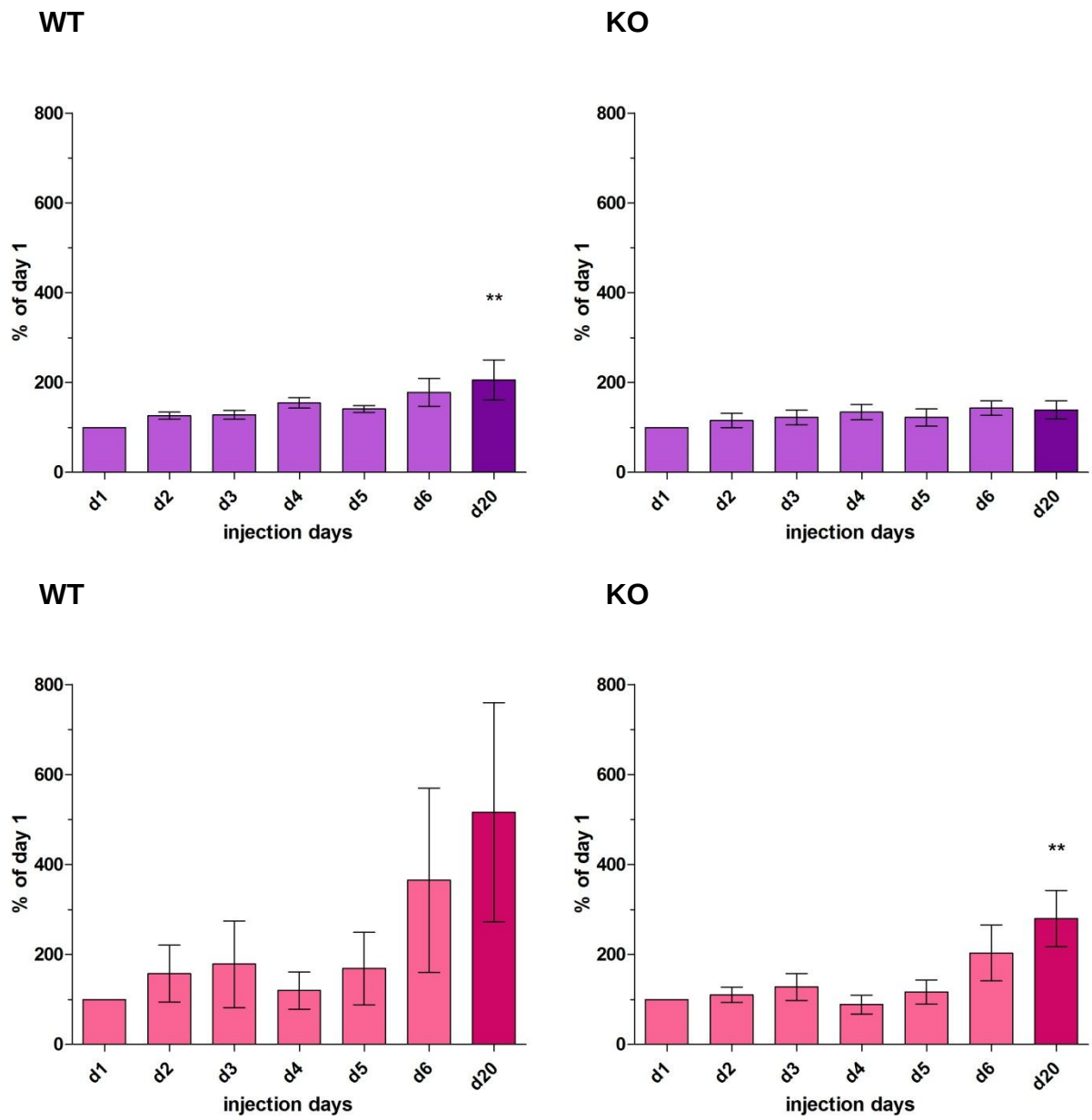


Figure 29: Top: cocaine 10mg/kg WT n = 10, KO n =7
Bottom: cocaine 5mg/kg WT n = 2, KO n = 2
A multiple measures ANOVA was done, Post hoc test: Tukey test

Animals treated with 10mg/kg cocaine showed almost similar sensitization behavior as animals injected with 20mg/kg. The WT group showed significant difference in their locomotion only at day 20.

4.2 Superfusion

Neurochemical experiments were performed in addition to the open field studies to test whether the behavioral sensitization is also reflected on a molecular level.

Sensitized mice were decapitated on day 21 and their striata dissected. The tissue was homogenized, pre-incubated with [^{3}H]-MPP$^+$, which acts as a more stable substrate for DAT compared to radioactively labeled DA. Synaptosomes were loaded onto a filter and superfused until a stable baseline was reached. AMPH was applied and fractions of two minutes were collected.

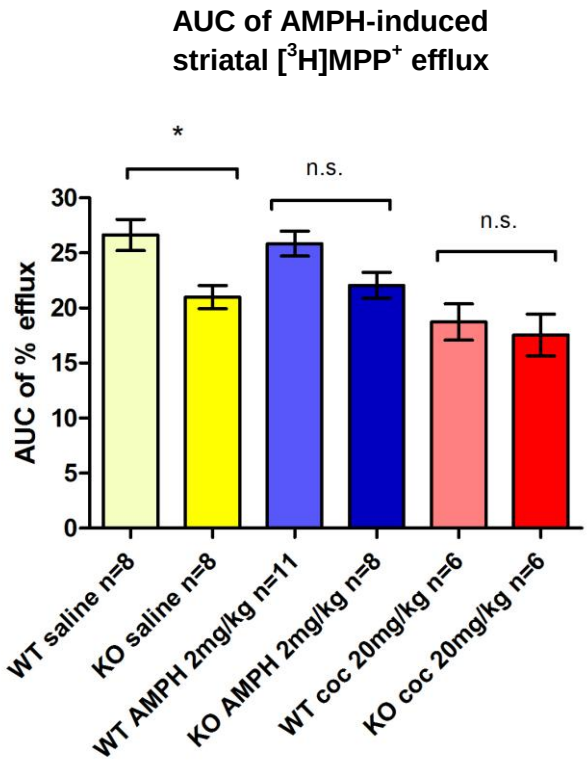


Figure 30: AUC of d-amphetamine-induced % efflux of pre-treated (sensitized) mice (saline, d-amphetamine 2mg/kg and cocaine 20mg/kg)
One-way ANOVA, Bonferroni post test

In figure 30 the AUC of % [^{3}H]-MPP$^+$ efflux is shown. Unexpectedly, no significant reduction of efflux was observed in the knock out mice treated with d-amphetamine or cocaine (Gnegy, 2003). Mice injected with saline showed a significant difference in the efflux, in line with the observations published earlier by our group (Steinkellner et al., 2012).

Possible explanations for these observations could be the sensitivity of this method: there may simply be too little difference between the two genotypes sensitized with psychostimulants in their [^{3}H]-MPP$^+$ efflux. Eventually, CamKIIα-KO mice are able to overcome the deficit on a neurochemical basis by upregulating other kinases such as protein kinase C. Furthermore, the duration of the withdrawal period could also contribute to such a rescue mechanism. Alternatively, instead of using the endogenous, physiological substrate dopamine we used [^{3}H]-MPP$^+$ as radiolabeled substrate.

4.3 Conditioned Place Preference

Conditioned place preference is the method of choice to assess the motivational and rewarding effects of drugs in genetically modified mice (Tzschentke, 2007). We used the CPP approach to gain further insights into the role of CamKIIα in the initiation of drug addiction and the acquisition of a drug memory. Three phases are involved in the conditioned place preference: habituation, conditioning and testing. The biased method was used, letting the mice first explore both chambers and choosing the preferred one.

On the first day of the experiment (pre-test) mice were allowed to explore both chambers of the CPP boxes. The less preferred chamber was chosen to be paired with the drug stimulus. Mice were injected with drug (cocaine or d-amphetamine) or saline on alternating days (3 times each) and put into the respective chambers for 30 minutes. On day 7 (post-test) mice could again explore both chambers and were measured for 30 minutes.

a, Amphetamine **b, Cocaine**

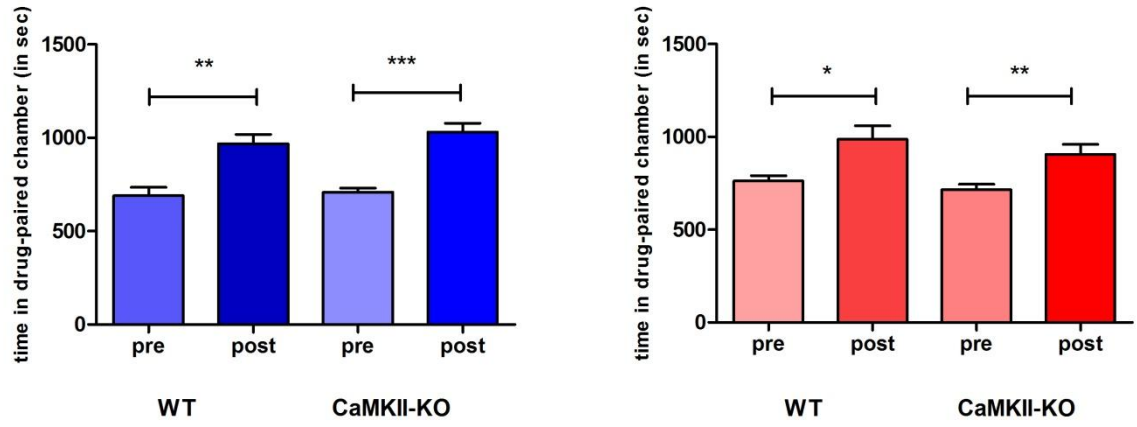


Figure 31: a, CPP time spent in drug-paired chamber in seconds
d-amphetamine 2 mg/kg
WT n =9, KO n =12
b, Cocaine 20 mg/kg
WT n =8, KO n = 8

According to the time spent (in seconds) in the drug-associated chambers during the post test, both drugs produced significant place preference in WT as well as in KO mice (fig. 31). This leads to the assumption that learning and memory was induced in both genotypes.

As previously discussed, CamKIIα is not the only protein that is involved in learning and memory. Many other factors could contribute to this result. Again, other proteins could compensate for the loss of CamKIIα, i.e. different isoforms of CamKII (eventually the β subunit), PKC or MAPK. Changes in the post-synaptic composition could also overcome the depletion of this kinase (alterations in the signaling cascade of GPCRs). Importantly, the result obtained in CamKIIα-KO mice points to the fact that the long-term effects exerted by amphetamines can not be related to CamKII.

Self-administration of the drug would be the golden standard to test whether a substance has addictive properties or not. However, this approach requires a different technical setup and is comparatively difficult in mice. The animal actively has to work to obtain the reward, it has a choice. (Steketee and Kalivas, 2011)

4.4 Real-time PCR

We started to characterize the mRNA level of the dopamine receptor 1 (D1) and 2 (D2) in WT compared to the CamKIIα-KO mice with real-time PCR. This approach followed our observation that the KO mice travelled significantly longer distances under baseline conditions in comparison to the WT mice.

Hence, we suspected a higher density of dopamine receptors in the CamKIIα-KO mice: upregulation of the dopamine receptor density could serve as a possible explanation for the hyperlocomotory phenotype of the CamKIIα-KO mice.

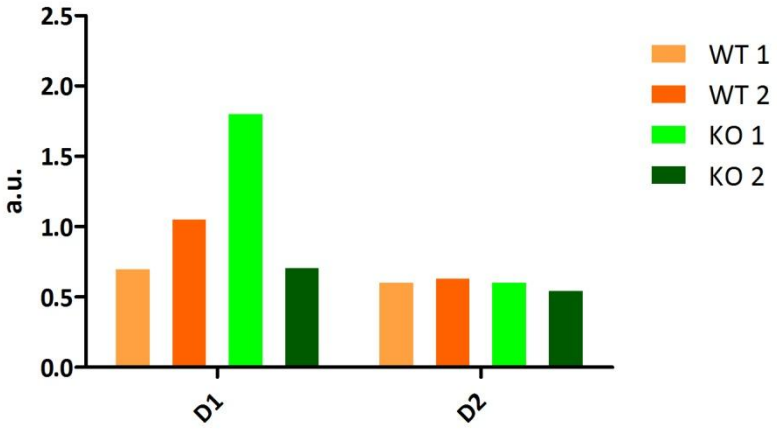


Figure 32: real time PCR of 2 WT and 2 KO mice measuring levels of dopamine receptor 1 and 2 referred to GAPDH, n = 2

Following our results, there is no change in the expression of D2 m-RNA in mouse striata between WT and KO. As both samples for D1, the WT as well as the knock out show a great variation, the sample size needs to be increased to get an established result.

The receptor density (D1,D2) of the CamKIIα-KO and their WT littermates needs to be further investigated. Thereafter, receptor regulation of each genotype sensitized with the different compounds (AMPH and cocaine) needs to be analyzed.

4.5 Conclusion

Taken together it was important for this diploma thesis to test whether the preceding in vitro findings of Fog et al. (2006) and Steinkellner et al. (2012) could also be applied in vivo. WT mice treated with d-amphetamine clearly demonstrate locomotor sensitization to AMPH, surprisingly sensitized CamKIIα-KO mice showed to a certain extent also behavioral sensitization. In mutant mice treated with 20mg/kg cocaine this effect was depleted with the knock out of the α subunit of Ca^{2+}/calmodulin kinase II. Mice, WT as well as the knock out, sensitized with cocaine (20mg/kg) reached a plateau in their locomotor behavior. Superfusion of synaptosomes originating from the sensitized mice (KO and WT treated with AMPH and cocaine) reveled no change in the d-amphetamine-induced DAT-mediated efflux, while a significant reduction could be observed in the WT compared to the KO treated with saline. To address how addictive learning and memory is influence by the absence of CamKIIα conditioned place preference experiments were performed. AMPH as well as cocaine treated KO mice showed significant place preference for the drug paired chamber, indicating that drug associated learning and memory took place. Following our observations that KO mice cover higher distances under baseline conditions the changes in the expression of dopamine 1 and 2 receptor density was measured. M-RNA levels of both genotypes were started to be analyzed by real-time PCR.

5. OUTLOOK

All of these experiments were done using mice with a global knock out of the α subunit of Ca^{2+}/calmodulin-dependent protein kinase II (Elgersma et al., 2002). CamKII is a highly abundant protein in the brain contributing to about 2% to the total protein mass of the CNS (Solà et al., 1999). Global KOs are problematic, because they could have a high influence on the outcome of our studies. The Sitte lab is currently working on mice with targeted deletion of CamKIIα solely in the dopaminergic system to find out more concisely whether CamKII-regulation of DAT is critical also in vivo.

I left the project before it was completed, so further experiments need to be considered. More data concerning the receptor density (real time PCR, dopamine receptor binding) need to be obtained and a closer look at the protein composition post-synaptically (CREB, phospho-CREB, delta Fos B) needs to be taken to gain further insight into the signaling cascade influenced by GPCRs.

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