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„αCamKII KO Mice: Effects of Amphetamine during behavioral Testing“

verfasst von / submitted by

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Abstract

The medicinal properties of amphetamines have been known since antiquity but it was not until the mid-1960s that amphetamines have been associated with dopamine and the dopamine transporter (DAT). We now know that the psychostimulant effects of amphetamine depend on the ability to induce a dopamine efflux, thus increasing neurotransmitter concentration in the synapse. The key player in this non-exocytotic dopamine release is the protein responsible for termination of transmission by removal of dopamine from the synapse, the DAT.

DAT is a 12 transmembrane domain protein from the SLC6 family of neurotransmitter/sodium symporters. Unfortunately, up to now there is no crystal structure of DAT and the mechanism of transport reversal is not yet completely clarified as there are multiple plausible models of action. Previous research has shown that there is an interaction between the CamKII and the terminal domains of DAT which plays an important role in amphetamine induced efflux. This data is of great value as αCamKII is known to be a major player in formation of LTP and memory processes, thus linking these findings to potential mechanisms of addiction.

The goal of my master project was to follow these initial findings and provide in vivo data on amphetamine action in αCamKII KO mice. My methodology consists of in behavioural tests combined with in depth behavioural analysis. I conducted measurements of locomotor activity of KO and wt mice in an open field setting to assess the effects of amphetamine both under acute injection conditions and in sensitisation experiments over longer periods of time. Also, conditioned place preference trials were performed as a way to test to test the addictive-like proprieties of amphetamines in Wt and KO animals.

My results showed that αCamKII KO mice have naturally increased locomotion when faced with a novel environment, compared to wt mice. This observation is reminiscent of to the behaviour of DAT KO mice. I could also show a differential behaviour between Wt and KO animals regarding dosage dependent responses in acute treatment settings. My results show that KO mice have an impaired response to sensitisation but no impairment in location bound associative memory.

Zusammenfassung

Die medizinischen Eigenschaften der Amphetamine sind schon aus der Antike bekannt jedoch wurden sie erst in den 1960er mit Dopamin und den Dopamin Transporter (DAT) assoziiert. Es ist heutzutage allgemein anerkannt, dass die psychostimulierenden Effekte der Ampheramine von ihrer Fähigkeit Dopamin Efflux zu induzieren und somit die Neurotransmitter Konzentration in der Synapse zu erhöhen, abhängig sind. Der wichtigste Bestandteil in dieser non-exozytotischen Freisetzung von Dopamin ist der DAT. Der Transporter besteht aus 12 transmembran Domänen und ist Teil der SLC6 Familie der Natrium/Neurotransmitter Symporter und ist zuständig für das Entfernen des Dopamins aus der Synapse. Die Abwesenheit einer Kristallstruktur hat die Erforschung der Transportmechanismus erschwert. Bisherige Forschung hat bewiesen, dass die Interaktion der CamK II mit den Terminus Domänen des DAT, eine wichtige Rolle für den Amphetamin induzierten Effllux spielt. Da die CamK II bekannterweise ein wichtiger Bestandteil in der Entwicklung von LTP und Gedächtnisprozessen ist, könnten diese Daten einen wichtigen Beitrag zur Aufklärung von Sucht und Suchtverhalten leisten.

Ziel meiner Masterarbeit war es, die schon vorhandenen Erkenntnisse bezüglich der CamK II und deren Verbindung mit dem DAT und dem Aktionsmechanismus von Amphetamin auf ein in vivo System zu übertragen. Der Schwerpunkt meiner Arbeit besteht aus Verhaltensexperimenten mit αCamK II KO Mäusen, die zur phenotypisierung der Linie dienen. In erster Linie habe ich die psychostimulanten Effekte von Amphetamin anhand der Bewegung der Tiere im sog. Open Field Aufbau betrachtet. Dafür habe ich sowohl akute Experimente mit einmaliger Dosis, als auch Sensitisierungsversuche durchgeführt. Zusätzlich habe ich Sucht induzierenden Effekte von Amphetamin mittels Conditioned Place Preference (CPP) erforscht.

Meine Ergebnisse beweisen eine natürliche Neigung der KO Mäuse zur erhöhten Lokomotion wenn diese einer neuen Umgebung ausgesetzt sind. Dieses Verhalten erinnert an dem Phenotyp der DAT KO Mauslinie. Zusätzlich konnte ich zeigen, dass KO Tiere, im Vergleich mit Wt Tiere, keinen Dosis abhängige Verhaltensveränderungen nachweisen. Weiterhin, stellte sich fest, dass KO Mäuse Sensitisierungsfähigkeit mangeln dafür aber keine Einschränkungen in der Bildung assoziativen Gedächtnisses nachweisen.

1.Introduction

1.1 Dopamine and the dopaminergic system

1.1.1 Dopamine

Dopamine was first synthesized in 1910 by George Barger and James Ewens (von Bohlen und Halbach & Dermietzel 2006). However it took over 50 years to recognise its role as a neurotransmitter and not just simply as a precursor of norepinephrine. In 1958 the group of Arvid Carlsson in Sweden identified dopamine in the brain and by 1959 they showed that it was found in the norepinephrine poor striatum; for these discoveries, Carlsson was awarded the Nobel prize for medicine in 2000 (Marsden 2006).

DA is a neurotransmitter of the catecholamine family, together with epinephrine (or adrenaline) and norepinephrine (NE). The name, catecholamine refers to the amine group and the so called catechol structure that all the three catecholamines contain. Furthermore, they are all derived from tyrosine.

Dopamine is synthesized in special varicosities found on dopaminergic neurons (Sulzer & Rayport 2000)), in a two-step process. The synthesis pathway starts with the enzyme tyrosine hydroxylase (TH), present in all catecholaminergic neurons, which converts tyrosine into L-dopa. This reaction is rate limiting for the catecholamine synthesis, as the activity of the TH is regulated by catecholamine concentrations via a negative feedback loop. DOPA is then turned into dopamine by the DOPA decarboxylase. This enzyme is abundant in catecholaminergic neurons and the conversion rate is dependent on the availability of DOPA. Following synthesis, dopamine is transported by the vesicular monoamine transporter (VMAT) and stored into presynaptic vesicles to avoid degradation. Dopamine is the precursor of norepinephrine and further on epinephrine (Fig.1).

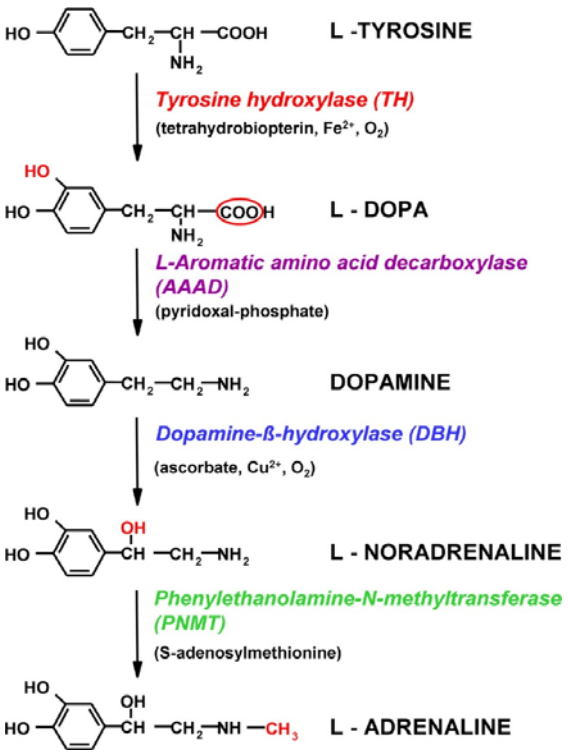


Fig. 1: Biosynthesis of dopamine from tyrosine and further on, the synthesis of norepinephrine and epinephrine. Each reaction is annotated with the matching enzyme. Adapted from (Kvetnansky et al. 2009)

Dopamine is released upon the occurrence of an action potential in a calcium-dependent way. Calcium influx thought voltage-gated calcium channels leads to a fusion of the neurotransmitter vesicles with the presynaptic membrane, leading to the exocytotic release of dopamine in the synaptic cleft. Furthermore, dopamine can also be released via a non-exocytotic mechanism facilitated by the dopamine transporter. The mechanistical details of this non-exocytotic release are not yet fully understood, despite several existing models, which will be discussed in detail further on. Once the neurotransmitter is released, it reaches the postsynaptic site via diffusion and binds to specific receptor molecules. There are a total of 5 dopamine receptors classified into two families: D1-like receptors (D1 and D5) and D2 like receptors (D2,D3 and D4).All dopamine receptors are G-protein coupled receptors, the D1 family stimulates the activity of the adenylyl cyclase and the production of cAMP while the D2 family has an inhibitory action on the same enzyme. The biochemical cascades involved in the transmission of the dopaminergic signal lead to a slow synaptic transmission. This suggests that dopamine is involved in the modulation of neural activity. In addition to the postsynaptic receptors, D2 autoreceptors are also present at the presynaptic site, with the task to measure the extracellular dopamine concentration and regulate release and synthesis (Fig.2).

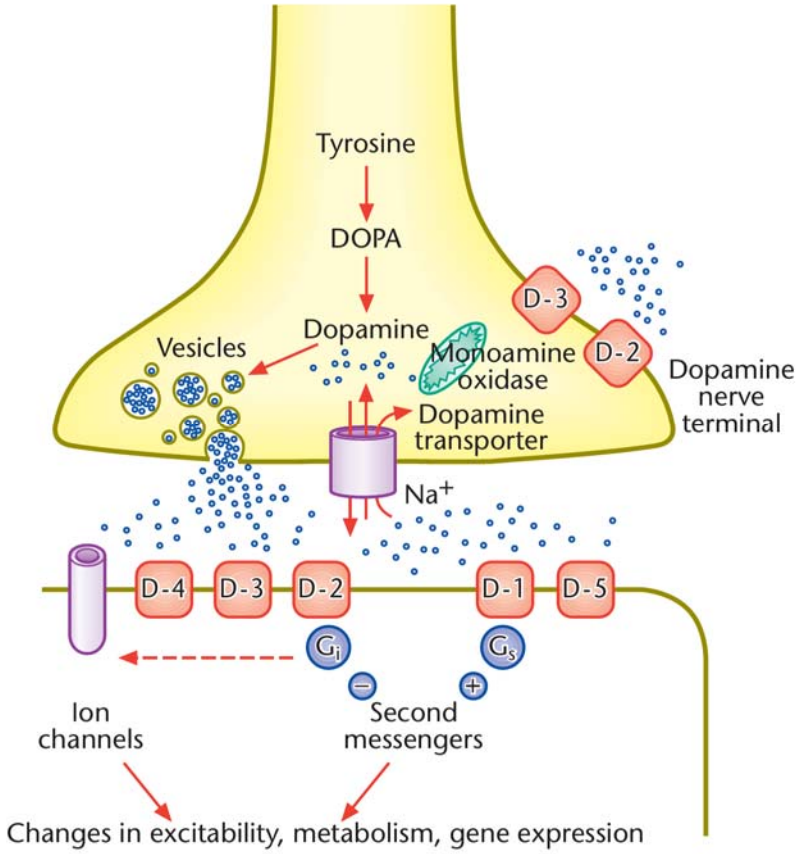


Fig 2.:Schematic model of a dopamine synapse. The synthesis, storage, release, reuptake and inactivation of dopamine are depicted. Dopamine interactions with multiple dopamine receptors are also illustrated.(Bannon, Michael J, Bannon, Erin E, Bannon 2012)

Termination of the dopaminergic signal takes place by removal of dopamine from the synaptic cleft. Unlike the cholinergic system, the catecholamine system does not employ fast extracellular degradation as the main mechanism of neurotransmitter removal from the synaptic cleft. Instead, specific transporters are employed for reuptake. Dopamine is removed from the synapse by the dopamine transporter (DAT).Once back on the intracellular side; the neurotransmitter is either recycled and packed into vesicles or degraded. Cytosolic dopamine is rapidly metabolised by either the monoamine oxidase (MAO) located on the outer mitochondrial membrane, or by the catechol-O-methyltransferase (COMT). MAO deaminates dopamine , thereby revealing DOPAC. COMT is available in two different fractions, the soluble COMT (S-COMT) and the membrane-bound COMT (MB-COMT), with S-COMT being the most common fraction in the mammalian brain. COMT catalyses the O-methlyation of dopamine .Unlike the MAO, COMT is present intracellularly only at the presynaptic site of certain brain areas such as the prefrontal cortex(Männistö & Kaakkola 1999; Myöhänen et al. 2010), but is predominantly found extracellularly or membrane bound on glia cells (Karhunen et al. 1994; Männistö & Kaakkola 1999). Dopamine which is not initially taken up by the neurons, is first metabolised to 3-methoxytyramine by COMT and further transformed into homovanilic acid (HVA) by MAO, which is present throughout all brain cells (Webster 2001) (Fig.3)

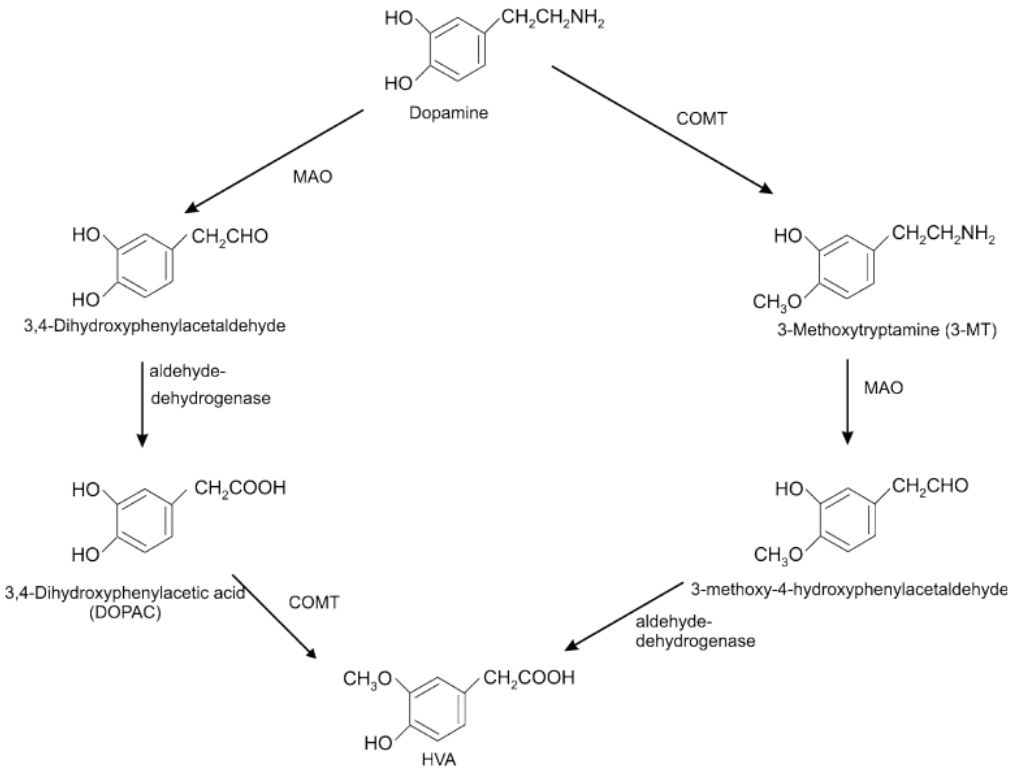


Fig.3: Degradation of dopamine to homovanilic acid (von Bohlen und Halbach & Dermietzel 2006)

1.1.2 The dopaminergic system

The dopaminergic system contains three classes of neurons, determined by the length of their projections (von Bohlen und Halbach & Dermietzel 2006)

- neurons with ultra-short projections: found in the retina and the periglomerular cells of the olfactory bulb;

- neurons with short projections with cell bodies in the areas A11 (posterior hypothalamus), A12 (arcuate nucleus of the hypothalamus), A13 (zona incerta) and A14 (periventricular nucleus of the hypothalamus)
- neurons with long projections which form the main bundle of DA neurons and have their cell bodies in areas A8 (retrorubal area) A9 (substantia nigra) and A10 (ventral tegmental area VTA)

Together these neurons form a total of 7 pathways(von Bohlen und Halbach & Dermietzel 2006), see Table 1.

System	Origin	Targets
Midbrain efferent	Retrorubral area (A8)	Caudate nucleus, nucleus accumbens
	Sustantia nigra (A9)	Caudate nucleus, putamen, globus pallidus, nucleus accumbens, olfactory bulb, cerebral cortex, locus coeruleus
	Ventral tegmental area (A10)	Caudate nucleus, putament, nucleus accumbens, olfactory bulb, cerebral cortex, hippocampus, amygdale, lateral habenular nucleus, locus coeruleus
Tubero-infundibular	Arcuate and periventricular hypothalamic nuclei (A12, A14)	Median eminence, intermediate and posterior lobes of the pituitary
Diencephalospinal	Dorsal and posterior hypothalamus (A11)	Intermedio-lateral cell columns of spinal cord
Incerto-hypothalamic	Zona incerta, posterior hypothalamus (A11, A13)	Hypothalamus, lateral septum
Periventricular	Mesencephalic and diencephalis periaqueductal	Periaqueductal and periventricular gray, thalamus, hypothalamus

	and periventricular gray (A11, A14)	
Olfactory bulb	Periglomerular cells (A16)	Olfactory glomeruli
Retinal	Inner nuclear layer (A17)	Inner and outer plexiform layers

Table 1: The seven pathways of the dopaminergic system. The table is adapted from Albanese 1986

The main dopaminergic pathways are (Fig.4): First the nigro-striatal pathway, running from the substantia nigra to the striatum (caudate nucleus and putamen). Second,the mesolimbic pathway emerging from the VTA, which runs with the negrostriatal pathway and additionally innervates the nucleus accumbens and the olfactory bulb. The third main DA pathway is the mesocortical pathway transmitting DA from the VTA to the frontal cortex. The projections of these three pathways are not completely separated from each other and intermingling is possible. Furthermore, there is a fourth completely separated pathway, arising from the arcuate nucleus (A12) which forms the tuberoinfundibular tract, which reaches the pituitary gland where it controls the release of prolactin.

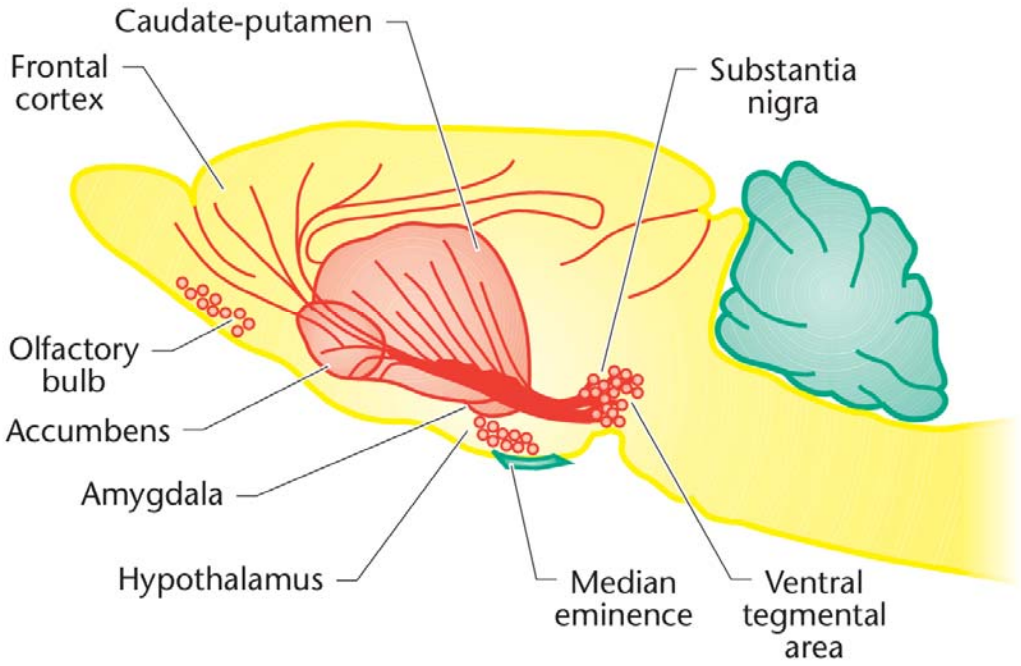


Fig. 4: Major dopamine containing cell groups in the brain and their projections (Bannon, Michael J, Bannon, Erin E, Bannon 2012)

The dopaminergic system has an important brain modulatory role and regulates a plethora of processes and behaviours. These include higher brain functions such as learning and memory as well as functions like modulation of arterial blood-flow. The nigro-striatal pathway regulates motor functions and plays a central role in Morbus Parkinson and the development of the parkinsonian

tremor (BIRKMAYER & HORNYKIEWICZ 1961)The mesolimbic and mesocortical pathways are involved in reward-mediated and motivation-dependent behaviour. Although Parkinson disease is the most prominent result of dopaminergic dysfunction, in the past few years, dopamine was linked to other psychiatric disorders such as ADHD, depression, forms of schizophrenia and bipolar syndrome. The system is also a key target for drugs of abuse, including psychostimulant drugs such as amphetamines and cocaine. This interaction is essential for the effect of these substances and plays a role in the development of addiction and addictive behaviour (Sulzer et al. 2005; von Bohlen und Halbach & Dermietzel 2006). Currently the research on drug addiction focuses on long term cellular and molecular changes that play a key role in relapse after a period of abstinence. Furthermore there is increasing interest in the connection between addiction and learning and memory processes.

1.2 Amphetamines and Addiction

1.2.1 Addiction

Addiction is associated with changes in brain function taking gradually place over a longer period of time in response to drug use. Most importantly, these changes can persist even after cessation of chronic drug use. Clinically, addiction is described as the compulsive use of a drug despite adverse consequences (Nestler et al. 1993). The "Diagnostic and Statistics Manual of Mental Disorders" (DSM) uses 4 distinctive criteria to diagnose substance abuse and 7 other criteria to diagnose addiction (substance dependence):

Criteria for Substance Abuse:

1. Failure to fulfill major role obligations at work, school, home such as repeated absences or poor work performance related to substance use; substance-related absences, suspensions, or expulsions from school; neglect of children or household

2. frequent use of substances in situation in which it is physically hazardous (e.g., driving an automobile or operating a machine when impaired by substance use)

3. Frequent legal problems (e.g. arrests, disorderly conduct) for substance abuse

4. Continued use despite having persistent or recurrent social or interpersonal problems (e.g., arguments with spouse about consequences of intoxication, physical fights)

Criteria for Substance Dependence

1. Tolerance or markedly increased amounts of the substance to achieve intoxication or desired effect or markedly diminished effect with continued use of the same amount of substance

2. Withdrawal symptoms or the use of certain substances to avoid withdrawal symptoms
3. Use of a substance in larger amounts or over a longer period than was intended
4. Persistent desire or unsuccessful efforts to cut down or control substance use
5. Involvement in chronic behavior to obtain the substance, use the substance, or recover from its effects
6. Reduction or abandonment of social, occupational or recreational activities because of substance use
7. Use of substances even though there is a persistent or recurrent physical or psychological problem that is likely to have been caused or exacerbated by the substance

Biologically, addiction can be summarised as the accumulated effects of tolerance, sensitisation and dependence (Nestler et al. 1993). Tolerance is the reduction of the effect of a drug upon repeated exposure in contrast to sensitisation, which can be considered a 'reverse-tolerance' and describes the situation in which constant exposure to the drug leads to increased effects. Last but not least, dependence is the need of continued drug exposure in order to avoid the physical and psychological symptoms of withdrawal (Nestler et al. 1993). A more recent description classifies 3 stages of drug addiction: Binge/intoxication, withdrawal/negative effect and preoccupation/anticipation (craving) (Koob & Volkow 2010).

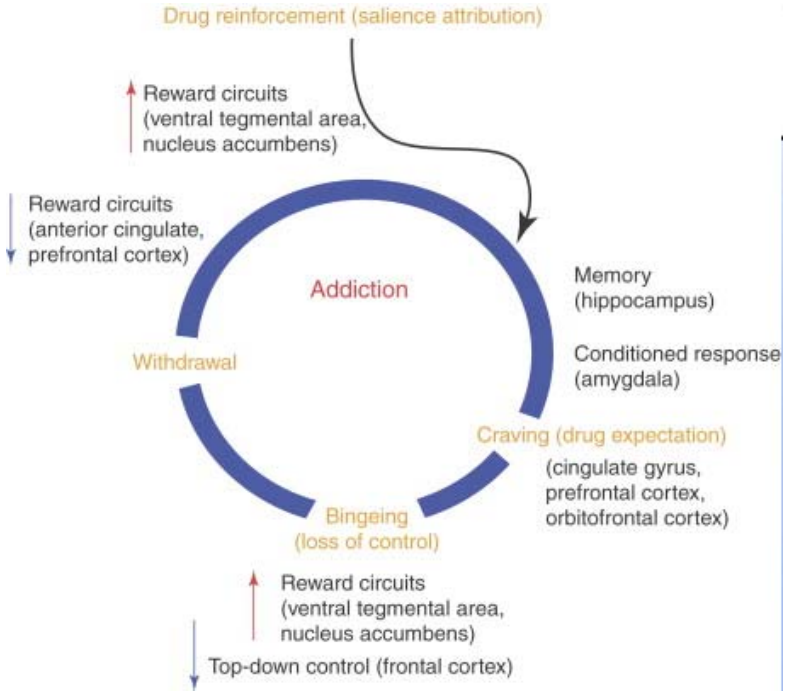


Fig.5 Integrative model of brain and behaviour (Triggle 2006).

In the past, the focus of addiction studies was to determine the physical basis of dependence, based on the assumption that compulsive drug abuse was driven by the desire to avoid withdrawal, creating a 'negative reinforcement' model of addiction(Wise 1987; Wise 1996a). Modern theories however, tend to view addiction from the psychologically rewarding point of view making addiction a form of positive reinforcement (Wise 1996b). As the view on addiction changed, the dopamine reward system became a common component for the action of different classes of drugs (Wise 1989). Addictive drugs (Fig. 6) are substances that have two main effects: they are rewarding and reinforcing (Bannon, Michael J, Bannon, Erin E, Bannon 2012). In his 1993 review, E.J. Nestler separates the anatomically defined regions of the brain responsible for the physiological (locus ceruleus) and psychological (mesolimbic dopamine system) effects of drug addiction using opiate addiction as an example. Interesting though is the fact that most drugs of abuse share, as mentioned before, a common trait: they augment the release of dopamine, especially in the nucleus accumbens. The nucleus accumbens shows great cellular heterogeneity meaning that dopamine release influences a variety of neurotransmitter systems such as the glutamatergic and the GABAergic system making stimulation of the reward system and addiction very complex matters (Robison et al. 2013)

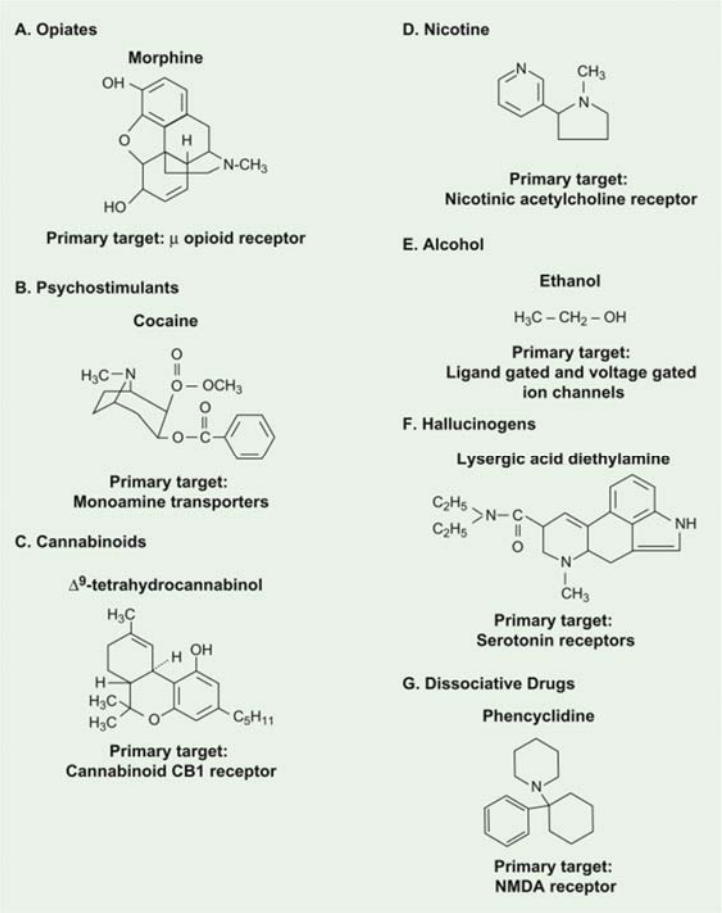


Fig.6 A list of the main classes of drugs with a representative for each class (Wolf 2012)

1.2.2 Amphetamine

The following paper focuses on the interplay between amphetamines and the dopaminergic system. The term amphetamine is the abbreviation for alpha-methyl-phenethylamine, the original compound which serves as a common motif for a large variety of other substances, generically called amphetamines (Fig.7). Another common characteristic these substances share is their ability to elevate catecholamine and serotonin levels in the synaptic cleft, independent of classical synaptic vesicle fusion and neurotransmitter release.

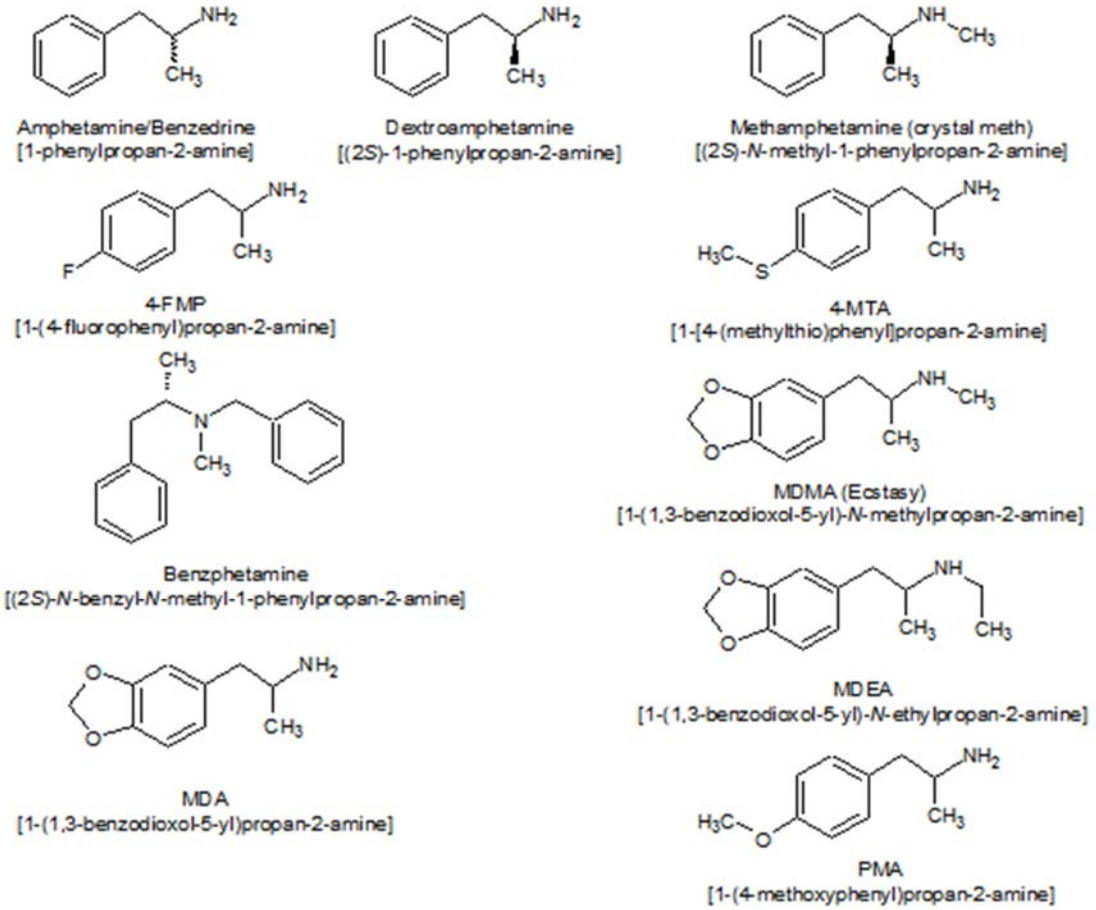


Fig 7 This figure shows a selection of substances of the amphetamine family, including well known drugs of abuse such as methamphetamine and MDMA (Sulzer et al. 2005)

Similar to nicotine, cocaine, alcohol and opium, amphetamines are found in nature and have played an important role in the social life of many cultures. The most prominent natural sources for amphetamines are the tree *Catha edulis* and the Ephedra family. *Catha edulis* is used to produce khat, containing cathinone and norpseudoephedrine. Synthetic amphetamine, was first created in 1887 by the Romanian chemist Lazar Edeleanu with the first reports on its stimulant effects coming in 1933 (Alles 1933). Amphetamine was introduced to the consumer market in 1932 under the name

Benzedrine and was recommended for the treatment of narcolepsy. Until 1946, amphetamine was promoted for over 30 different uses including the treatment of schizophrenia, seasickness, radiation sickness and persistent hiccups (Sulzer et al. 2005). Amphetamine was also given to soldiers during the Spanish Civil War and WWII to promote alertness. The first reports documenting amphetamine induced psychosis were published in 1958 and its addictive potential was recognised in the mid 1960 although it was mentioned as early as 1937 (Guttmann 1937). Nowadays, the number of adults (15-65) that have tried amphetamine or amphetamine derivatives such as MDMA (ecstasy) in a lifetime is surpassing the ones that have tried cocaine in the European Union, according to the EMCDDA European Drug Report 2016.(Sulzer et al. 2005).

Amphetamine is a so called sympathomimetic drug, meaning that it has the ability to mimic the effects of transmitter substances of the sympathetic NS, i.e. catecholamines. In humans, as well as in animal models, it increases locomotion and, with increased dosage, leads to stereotyped behaviours (Seiden et al 1993). The stimulating effects of amphetamine are a consequence of the drug's ability to increase catecholamine release by interacting with both the VMAT and NT transporters such as DAT, NET and SERT. Although these interactions have been proven (Kelly et.al 1975;Kelly & Moore 1976; Sulzer et al. 2005), it is still under debate which has the major role in behavioural effects and the development of addiction. Past research has not only linked synapthomimetic drugs to NT transporters but also the dopaminergic reward pathways to addiction (Nestler 2004; Koob & Volkow 2010; Bannon, Michael J, Bannon, Erin E, 2012). The DAT is a key target for psychoactive drugs but also a key player in the dopaminergic system. The following thesis focuses on the modulation of amphetamine induced DA efflux via the DAT.

1.2.3 The Dopamine Transporter (DAT)

The dopamine transporter (DAT) is responsible for the termination of the dopaminergic synaptic transmission by removal of dopamine from the synaptic cleft. The transporter is part of the so called monoamine transporter family together with the noradrenaline transporter (NET) and the serotonin transporter (SERT). They all belong to the SLC6 gene family of Na/Cl-dependent transporters, meaning that they rely on the co-transport of Na down its electrochemical gradient in order to accomplish the uptake of monoamine NT from the synaptic space. Analysis of DAT activity in striatal synaptosomes showed that the transport of dopamine against a concentration gradient requires the co-transport of 2 Na^+ and 1 Cl^- (Krueger 1990) Although to this day there is no crystal structure available, previous studies (Chen et al. 1998)have shown, via site-directed chemical labelling, that the monoamine transporters have 12 transmembrane domains with both N- and C-terminus located intracellularly.

Still, molecular predictions made about the structure of these proteins rely on the crystal structure of the bacterial homologue, the LeuT transporter (Fig 8).

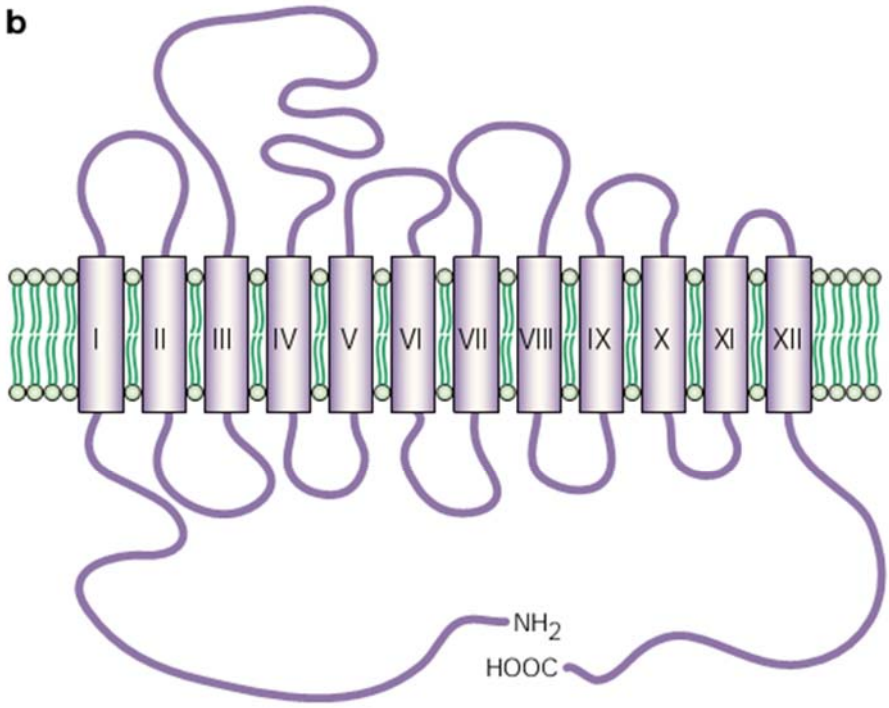


Fig. 8 A schematic depiction of the 12 transmembrane domains of DAT together with the intracellular C- and N-terminal domains (Torres et al. 2003)

DAT is a facilitated transporter, meaning that it uses an ion gradient to transport the substrate across the membrane. This also means that the transporter is capable of operating in two directions, depending on the concentration gradients and the net driving force. A model describing this reaction mechanism was described in the 1960s and was called the "alternate access model"(Jardetzky 1966) . In the original paper Jardetzky describes three structural conditions necessary for a molecule to function as a pump:

1.The molecule should contain a cavity in its structure

2.The necessity of two distinct configurations so that the cavity is sequentially open to each side of the membrane

3.Presence of a binding site for the transported substrate, which has different affinity for the substrate in the two conformations.

This basic model has been extended to also contain a transition state between the basic conformations, inward-facing and outward-facing, in which neither side is open. The “alternate access model” is mainly used to describe the inward transport of substrates from the extracellular side. This hypothesis was used to further describe the reverse transport using the analogy of a revolving door. This model was named “facilitated exchange diffusion model” (Fischer & Cho 1979) and describes outward transport as being tightly coupled to inward transport.
It is also known that the intracellular domains of the DAT contain numerous phosphorylation sites which play a crucial role in regulation and amphetamine induced reverse transport (Khoshbouei et al. 2004; Johnson et al. 2005; Fog et al. 2006).
The DAT, along with the other monoamine transporters are targets for psychostimulant substances, but the behavioural and reinforcing effects, eventually leading to addiction, have been mainly attributed to the interaction with the DAT. According to their activity we distinguish blockers and releasers. Blockers such as cocaine and benztropines only inhibit the transporter whereas releasers such as D-amphetamine (‘speed’), methamphetamine (‘ice’), 3,4-methylendioxymethamphetamie (MDMA, ‘ecstasy’) and other amphetamine derivates do not only block the transporter competitively but are also taken up.

1.2.3 DAT and Amphetamine induced Effllux

The first reports on the molecular mechanisms of amphetamine action were published in the late 1950s when it was revealed that amphetamine releases a ‘noradrenaline-like’ substance (Burn & Rand 1958). These initial findings lead to the classification of substances in either blockers such as cocaine and reserpine and releasers such as amphetamines. Further studies concluded that amphetamine interacts with both plasma membrane and vesicular monoamine transporters and induces the release of monoamines, as reviewed in Sulzer 2005 and in Robison 2013. More recent publications also imply a blocker action of amphetamine when present in high concentrations (Pifl et al. 1999; Schmitz et al. 2001; Sulzer et al. 2005) , as amphetamine is also a substrate for monoamine transporters.
The classical experiments (Axelrod et al. 1961; Glowinski & Axelrod 1965; Sulzer et al. 2005; Sitte & Freissmuth 2010) were not able to isolate and describe the specific roles of the vesicular and plasma membrane transporters in amphetamine action as they were limited by the use of complete presynaptic nerve terminals. It was not until the 1990s when cDNA availability allowed heterologous expression of the DAT and VMAT that the effects of these two components were studied separately. The human DAT the VMAT or both were transfected into cell lines lacking vesicular storage and release

mechanisms (Eshleman et al. 1994; Pifl et al. 1995). These experiments showed that expression of DAT is necessary for dopamine uptake and release (Eshleman et al. 1994) and that the presence of the VMAT greatly enhances the effect of amphetamine (Pifl et al. 1995). Amphetamine interacts with the VMAT and redistributes the vesicular dopamine storages to the cytosol (Sulzer et al. 2005). This cytosolic dopamine is the main component for DAT mediated reverse transport with newly synthesised dopamine playing a secondary role (Fig.9) Follow-up studies on transgenic mouse models showed that DAT KO mice do not exhibit amphetamine induced dopamine efflux (Giros et al. 1996; Jones et al. 1998). Similar studies on VMAT2 KO mice were not possible as VMAT2 KO mice feed poorly and die soon after birth. Interestingly though, this lethal phenotype is attenuated by amphetamine (Fon et al. 1997).

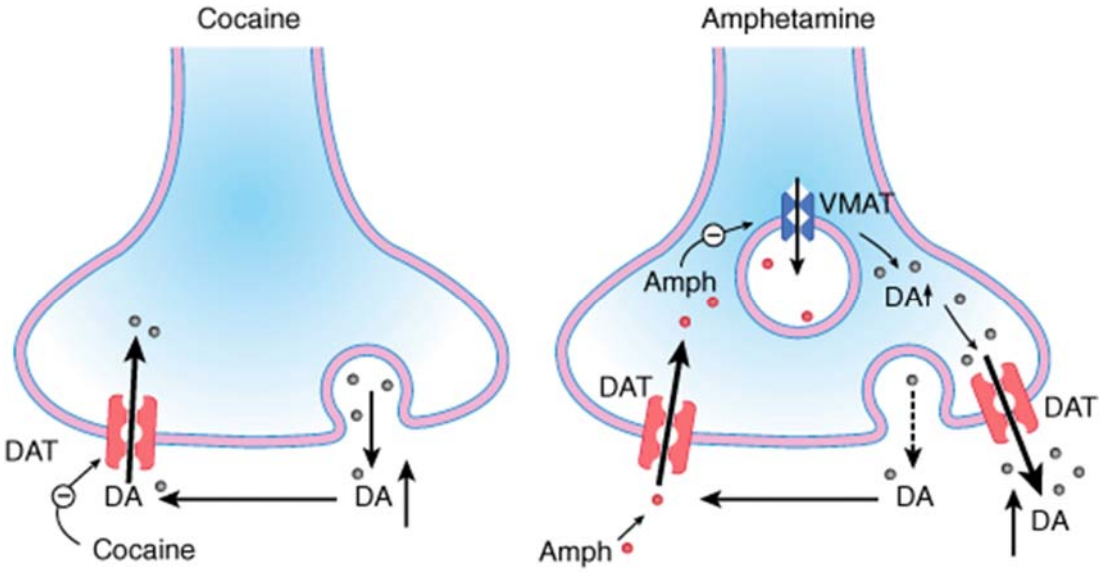


Fig. 9 A schematic comparison of the two main groups of DAT interacting drugs: blockers and releasers, exemplified by cocaine and amphetamine. (Bertram G. Katzung, Susan B. Masters 2009)

Although it is generally acknowledged that DAT is the key player for the amphetamine induced reverse dopamine transport, the mechanism underlying this effect is still not completely elucidated although several hypotheses exist. The first mechanism suggested was the above-mentioned facilitated exchange mechanism, based on the description of glucose transport. In the classical interpretation of this mechanism (Paton 1973) one molecule of dopamine is released for every molecule of amphetamine transported. This theory attributes efflux to an increase in the number of so-called inward facing transporters upon the transport of amphetamine in the pre-synaptic terminal (Fischer & Cho, 1979). In its classical form, this model predicts that one molecule of dopamine is released for each molecule of amphetamine transporter from the extracellular space. This model though, is not sufficient to explain the mechanism of dopamine efflux as there are enough instances that cannot be

explained by facilitated exchange diffusion (Sulzer et al. 1992; Khoshbouei et al. 2003; Sitte & Freissmuth 2003; Buchmayer et al. 2013). The second proposed mechanism is the more recent proposed channel-like transporter mode. Amphetamine induced channel-like dopamine released was recorded at the DAT in 2005 by Kahlig et.al. These studies suggest a dopamine efflux via a DAT-channel comparable to ion channels. As both mechanisms have been observed (Kahlig et al. 2005) a so called unified theory has been suggested by a number of publications especially in the context of DAT interaction with protein kinases(Bermingham & Blakely 2016; Khoshbouei et al. 2004; Wang et al. 2016). Neither of these attempts has managed to accurately describe the efflux mechanism. Nevertheless, a recent new 'oligomer-based counter-transport model' was suggested (Seidel et al. 2005), based on the knowledge that neurotransmitter transporters form oligomers in vivo (Hastrup et al. 2003; Hastrup et al. 2001; Scholze et al. 2002; Sorkina et al. 2003; Torres et al. 2003). According to this model, binding of amphetamine to one monomer induces the inward-facing conformation of the neighbouring transporters, thus leading to substrate efflux. It also states that once all transporters are bound by amphetamine the efflux rate ceases (Fig 10).

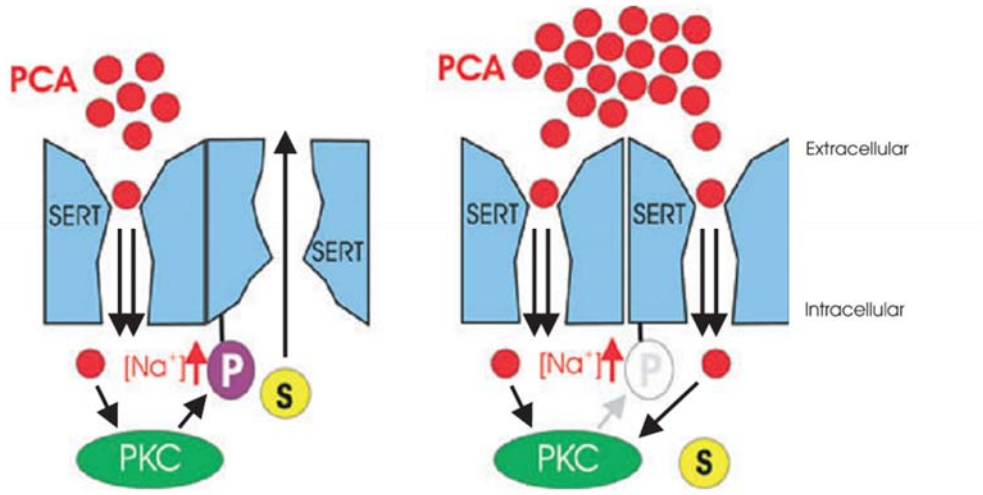


Fig.10 Schematic illustration of the oligomer-based counter-transport model (Sitte & Freissmuth 2010)*. This image exemplifies the principle using the serotonin transporter as a model.*

1.2.4 DAT regulation and interaction with kinases

Although DAT is essential for the release of dopamine under the influence of amphetamine, it is not the sole mechanism involved. Interactions between DAT and various other proteins, especially protein kinases, such as protein kinase C (PKC), protein kinase A (PKA) (Kantor & Gnegy 1998; Pristupa et al. 1998) and CamKII (Fog et.al. 2006) have emerged as important parts in this complex mechanism (Fig 11).

First studies linking PKC to amphetamine (Giambalvo 1992a; Giambalvo 1992b) showed an increase in phosphorylation and thus an increase in activation of PKC in presence of amphetamines (Gnegy 1993; Gnegy 2003; Giambalvo 1992a; Giambalvo 1992b). Further research revealed the importance of PKC in dopamine efflux: Upon specific inhibition of PKC, the amphetamine-induced dopamine release was blocked (Kantor & Gnegy 1998). It has been suggested that the upregulation of PKC activity with the PKC activator PMA (phorbol 12-myristate 13-acetate) leads to a decrease in DAT activity in transfected cells and *Xenopus* oocytes. This effect was first attributed to the cellular DAT trafficking mechanism rather than to functional alterations of the transporter (Zhu et al. 1997; Melikian & Buckley 1999). The proposed mechanism implied an increase in DAT translocation to so called endosomal recycling compartments (ERC) which suggest that activation of PKC does not lead to DAT degradation. Supporting this hypothesis, Hong and Amara published in 2013 a study comparing the surface regulation of DAT by amphetamine with PMA induced internalisation. They concluded that PKC activation leads to ubiquitination and degradation of DAT while amphetamine internalized molecule is recycled. Nevertheless, research by Eriksen et al. 2009 challenges these findings by showing that in cultured live midbrain dopamine neurons there is no observed internalisation of DAT upon PKC activation (Eriksen et al. 2009). There is no doubt that DAT trafficking plays an important role in psychostimulant action, yet the molecular mechanism is still unknown.

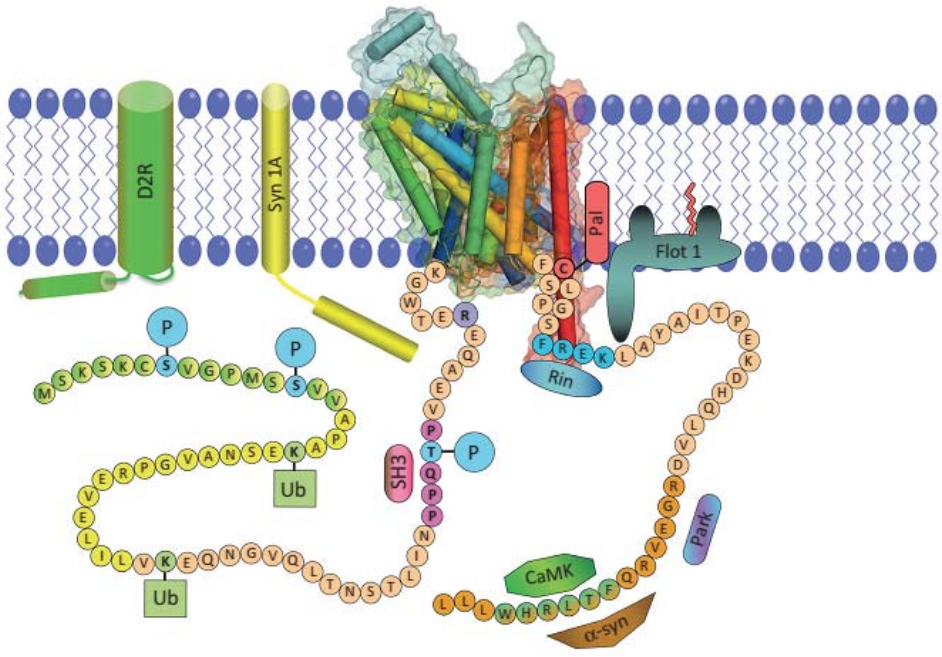


Fig.11 a diagram showing the a 3D model of the rat dopamine transporter complete with interaction sites for multiple regulatory factors, including the CaMK at the N-terminal domain (Vaughan & Foster 2013)

The main focus of this thesis though is the interaction between the CamKII and DAT. Phosphorylation of DAT has been shown to be essential for amphetamine-induced transport reversal (Khoshbouei et al. 2004; Wang et al. 2016) and was previously attributed to PKC. In 2006 Fog et.al showed that the CamKII interacts with the C-terminus of DAT and phosphorylates the N-terminus, thus playing an important role in transport reversal.

1.3 The CamK II

1.3.1 Structure and Function of CamK II

Ca^{2+} is one of the major secondary messengers as its intracellular concentrations can be maintained at very low levels. An important downstream target of Ca^{2+} is the family of calcium/calmodulin-dependent protein kinases. These enzymes translate the Ca signals into appropriate cellular responses by specifically phosphorylating serine and threonine residues of a wide variety of substrate proteins. CamKII is a ubiquitous protein in the brain where it coordinates and regulates Ca^{2+} signals. Its functions are varied and range from regulation of transcription and translation, carbohydrate, amino acid and lipid metabolisms to regulation of ion channels and synthesis and release of neurotransmitters (Hudmon & Schulman 2002a). This degree of multi-functionality is possible due to the existence of 4 isoform families (α-γ), derived from related yet separated genes. Further, these isoforms are alternatively processed giving rise to at least 30 splice isoforms currently described. The most prominently expressed isoforms in the brain are the α and the β subunits. Structurally, CamKII is a multi-subunit holoenzyme consisting of 12 subunits (Kanaseki & Ikeuchi 1991; Hudmon & Schulman 2002a) (Fig 12).

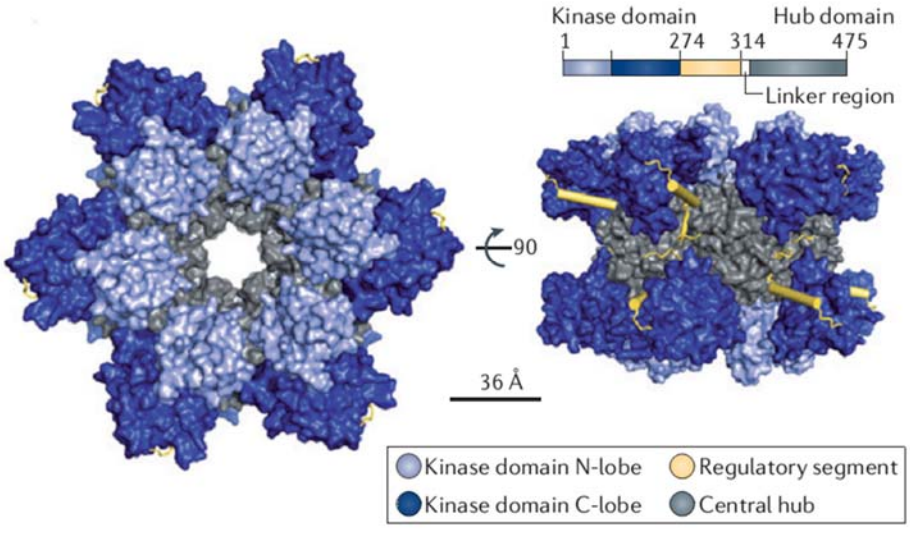


Fig. 12: Architecture of the CaMK II based on its crystal structure (Lisman et al. 2012)

Until now a high variety of holoenzyme variants were isolated, with different α-β subunit ratio. Homomers were also identified, however only formed by the α subunit and not by β (Hudmon & Schulman 2002a; Hudmon & Schulman 2002b). The composition of each holoenzyme is dictated by anatomical, developmental and local gene expression regulation rendering the CamKII a very versatile enzyme. Due to these attributes, it is not surprising that the CamKII is found throughout the body, additionally to making up to around 1% of the total amount of protein in the forebrain (Erondu & Kennedy 1985).

The prototypical CamKII subunit consists of 3 different domains: a catalytic, an autoregulatory and an association domain. The association domain is responsible for the formation of the multimeric holoenzyme. The autoregulatory domain contains autophosphorylation sites, conserved between the single isoforms. In addition, it is located N-terminally and in direct vicinity of the catalytic domain, which it regulates. The pseudosubstrate inhibition mechanism depends on the autoregulatory domain in order to bind both the substrate-binding pocket and the ATP binding pocket. The autoregulatory domain is displaced once Ca/CaM binds to one of the holoenzyme's subunits. This event leads to activation of the kinase by exposing the phosphorylation site at Thr-286 (Colbran et al. 1988) (Fig 13).

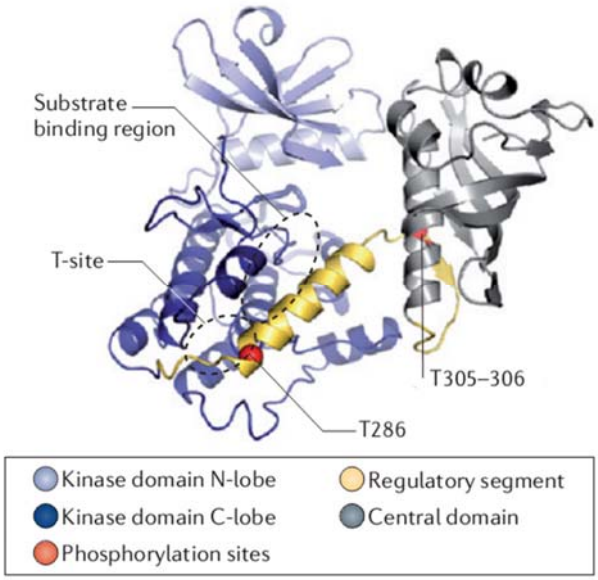


Fig 13: The crystal structure of CaMK II with highlighted phosphorylation sites, regulatory domains and C and N lobes. (Lisman et al. 2012)

By changing its conformation, the auto-inhibitory region turns from being an inhibitor to being a substrate and phosphorylation of the Thr-286 residue leads to a dramatic decrease in inhibitory potency playing an important role in the Ca/CaM independent activity of the enzyme (Hudmon & Schulman 2002b) (Fig 14).

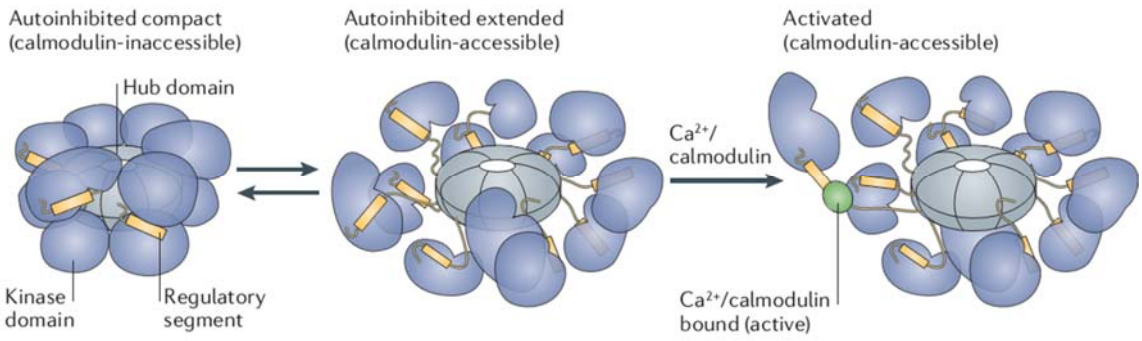


Fig. 14: Activation of the CaMK I by Ca^{2+}/calmodulin binding and a schematic depiction of the structural changes taking place (Lisman et al. 2012)

The role of CamKII in memory and learning, especially LTP (Lisman et al. 2012) and LTD, as well as behaviour itself has been described in many publications (Kennedy 1989). A review by John Lisman published in 2002 summarizes the connection between Thr-286 autophosphorylation and LTP and concludes that the CamKII is sufficient to induce LTP in the CA1 region of the hippocampus(Lisman et al. 2002). At the same time, two distinct mouse strains were described, where the amino acids at positions 305 and 305/306 were modified to create animals with either a constitutively active CamKII (TT305/6 VA, no inhibitory phosphorylation) or a constitutively inactive CamKII (T305D, mimicking phosphorylation) (Elgersma et al. 2002). Furthermore, the paper describes αCamKII KO mice which play the central part in the present thesis. Elgersma et al (2002) show that tempering with CamKII activation can lead to impairments in LTP induction as well as spatial learning. More publications report the importance of the CamKII at the post-synaptic density (PSD) and in relationship with glutamate receptors, but it was not until 2006 that a connection between CamKII and the DAT was made (Fog et al. 2006).

1.3.3 αCamKII KO mice

The first description of αCamKII KO mice was published in 1992 by Silva et al. and involved experiments on spatial learning and LTP (Silva, Wang, et al. 1992; Silva, Stevens, et al. 1992).The αCamKII KO mice used in the experiments described in this thesis were received by Ype Elgersma and have been generated in Alcino Silva's laboratory. These animals carry a deletion of the exon 2 in the αCamKII gene, thus rendering null mutants, the main difference to those created in 1992 by Silva et al. being the absence of the neomycine resistance gene. The decision to create a novel αCamKII KO mouse line was driven by the fact that the Silva αCamKII KO line retained expression of around half of the protein despite the knock-out. Research has shown that both the binding of αCamKII to the DAT as well as the

full functionality of this subunit is necessary for amphetamine induced efflux (Steinkellner et al. 2012). Possibility of functional compensation by βCamKII was dismissed by additional experiments. These showed that although βCamKII co-precipitates with DAT it is not able to substitute αCamKII function and restore DAT mediated efflux (Steinkellner et al. 2012).
An earlier report suggested a link between DAT expression and CamKII (Padmanabhan et al. 2008), nevertheless these findings could not be replicated in our studies with CamKII KO mice where the animals showed the same expression of DAT as their Wt counterparts (Steinkellner et al. 2012).

1.4 Background research and goals of this project

Fog et.al first published data demonstrating the important role of CamKII in regulating amphetamine induced dopamine efflux. They showed that CamKII interacts with the C-terminus of DAT and phosphorylates the N-terminus. As previously revealed, phosphorylation of the N-terminus is crucial for DAT efflux (Khoshbouei 2004). This knowledge is of great importance as the CamKII is a known player in the development of LTP and memory, thus providing a potential link to the development of drug addiction.
This project is a direct continuation of the data published by Steinkellner et al. in 2012. Apart from the connection between CamKII and Angelman Syndrome, Steinkellner et al presented data using preparations from the striata of αCamKII KO mice and Wt mice. Supporting Fog et al. 2006, immunoprecipitation experiments with DAT and preparations from either αCamKII KO or Wt mice showed that CamKII binds to DAT. The necessity for active CamKII in amphetamine induced efflux is tested and confirmed via superfusion of striatal synaptosomes. The natural follow up was testing this hypothesis in vivo as an important step to proving that αCamKII truly plays a crucial role in amphetamine induced effects.
Furthermore this project provides an interesting insight into the αCamKII KO mouse model phenotype. Animals were not only observed regarding their reaction to psychostimulants but also regarding their overall performance in behavioural assays routinely used in addiction studies. Our findings might help future addiction research by providing a viable model mouse strain.
In order to achieve these goals both behavioural and molecular biological techniques were employed. The animals were tested under acute and long term drug exposure conditions in an open field setting where locomotor activity, as a primary expression of amphetamine action, was monitored. Sensitisation was used as a model for reaction to long term drug exposure. Previous research showed that repeated exposure to psychostimulant drugs leads to progressive augmentation of locomotion which is the key feature of sensitisation (Licata & Pierce 2003). This form of neuronal plasticity is

thought to be the driving force leading to drug craving (Robinson & Berridge 2000). Addictive-like properties of psychostimulants were tested using the conditioned place preference set up and changes in second messengers were traced using Western Blot analysis with samples from acutely treated animals (Steinkellner et al. 2014). Data generated during this thesis was used for the publication of Steinkellner et al.2014, full version of which is to be found in the supplementary information (S3)

2. Materials and Methods

2.1 Animals

Mice were bred at the 'Himberg' Tierzucht facility. All animals used were of the C57Bl/6J background and were kept at constant temperature (24°C) and humidity with an artificial day/night light cycle of 12 hours each (lights on at 6~am). Food and water was provided ad lib. During the day radio was used to provide a white noise for the animals. The animals used in all experiments were weaned 21 days after birth. Also, no animals older than 3 months were used in any behavioural experiments. The environmental conditions in the "mouse-house", where the colonies were kept, were replicated in the room where the behavioural experiments took place.
In all behavioural experiments the animals were injected intra peritoneal. For the injection each mouse was immobilised by firm manual grip and tilted to avoid injection in the digestive tract. Sacrifice of the animals for tissue isolation was performed by cervical dislocation.
The αCamKII KO mice used were identical genetically to those described by Elgersma et al. (2002).

2.2 Genotyping

Genotyping was conducted by isolating a tissue sample from each mouse, this being either a small tail piece or the clipping from the ear marking made after weaning. All mice used in experiments were genotyped at least twice.
The tissue samples were digested either by heating for 1.5h at 95°C with 75µl Hot Shot buffer (25mM NaOH; 0,2Na^+-EDTA), followed by a neutralisation step with 75µl 40mM Tris HCl (pH=5,0) or by over-night digestion with Proteinase K (0.15mg/ml) in a one step tail digestion buffer (50mM KCl, 10mM Tris-HCl pH=9.0, 0.1% Triton X-100), at 55°C. Proteinase K was additionally inactivated by heating at 95°C for 10min. Following these procedures, the samples were used directly for PCR.

PCR

A general PCR protocol was used with slight modifications in water volume between 2 and 3 –primer reactions. Mastermix contained: 18.115/18.225µl H2O, 5µl Buffer (5x GoTaq,Promega USA), 0.5µl dNTP (Fermentas), 0.075µl Primer, 0.125µl GoTaq Polymerase (Promega, USA)

CamK II KO Genotyping

Primer Sequences

CamKa: 1 5'-CAGGCAGTGCGGTAACCTACC-3'

2 5'-GAACAGTGTGAACACCCTATCC-3'

3 5'-GGATCTGGGCCCCTGTCTGGATC-'3

PCR Program:

94°C 5min

94°C 45 sec
58°C 45 sec } x35 cycles
72°C 45 sec

72°C 10 min

Electrophoresis

After the PCR the fragments were electrophoretically examined using a 2-2,5% agarose gel. For electrophoresis 2,4g of agarose (for a 2% gel) or 3g of agarose (for a 2,5% gel) were mixed with 120ml of TAE-Buffer. Solubilisation of the agarose was achieved by heating the mixture using a standard microwave until solid agarose residues were no longer visible. This procedure was followed by a quick cooling step. Once the mixture cooled down, 6µl of ethidium bromide were added. In the following step, the mixture was poured in an electrophoresis tray and the comb was placed. The agarose was left to completely solidify for at least 15min turning into a translucent gel. Afterwards the comb was removed and the agarose gel was placed in the electrophoresis chamber and submerged in TAE buffer (TAE-Buffer containing 40mM Tris, 20mM acetic acid and 1mM EDTA).
The first lane was used for the 100kb DNA Ladder (GeneRuler^{TM} 100bp DNA Ladder provided by Thermo Scientific). No loading dye was added to the samples as it was already included in the 5X GoTaq Promega PCR buffer. A volume of 20µl of sample was added per slot. Voltage settings ranged from 134-140V for 17-25 min. Variables in time and voltage were necessary for optimal results in each gel and were established by routine checks of the gel with the transilluminator. Samples of the specific bands are shown in the figure below (Fig 15):

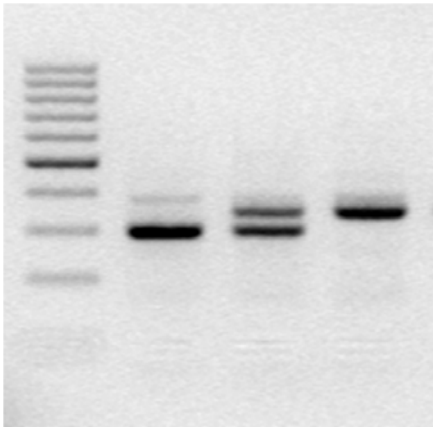


Fig 15. The figure depicts the typical result of a αCamK mouse genotyping after a gel electrophoresis. The 100bp DNA ladder is to be seen on the far left lane. Continuing to the right, in order, are the bands for homozygous wt at 290bp, heterozygous with band for both wt at 290bp and KO at 320bp and on the far left lane, the band for a homozygous KO individual with a single band at 320bp.

2.3 Open Field

In the open field set-up, the animals were placed singularly in bare chambers (L36xB36xH45) made of grey plastic with constant, indirect illumination, directly after injection. Using a camera system, the movement of the mouse was monitored over 60 min. The data were recorded and analysed with the ANY-Maze software (Version 4.7). We used these settings to execute our behavioural analysis for the acute injection and the sensitisation experiments.
The animals were left to habituate in the behavioural testing room for at least 24~h prior to testing. Also, cages were not changed for at least 24~h before the testing and the mice were not handled in any way before the open-field experiment. All experiments took place during the light-phase of the day.

2.4 Acute Injections

For the acute effect experiments the animals were tested in two consecutive days. In the first day the mice received an intra-peritoneal injection with 0.9% NaCl solution followed by 60min or activity recording in the test chambers. The settings were replicated in the second day with and i.p. injection of either Amph or MDMA solubilised in 0.9% NaCl solution.

2.5 Sensitisation

The sensitisation experiment ran over a period of 21 days. In this time the animals received an i.p. injection of a 0.9% NaCl solution on day one to establish the baseline locomotion. Over the next 6 days, the animals were injected daily with a D-Amphetamine in 0.9% saline solution. In the following 14 days the animals did not receive any treatment. On the final day of the experiment, day 21, each mouse was administrated a D-Amphetamine injection identical to the ones in days 2-7. After each injection, the locomotor activity of the animals was monitored in an open field setting for 60min.

2.6 Conditioned Place Preference (CPP)

Conditioned place preference was carried out in special designed test-boxes divided into half by a black wall containing a closable door. The two sides differed in the structure of the floor, one side containing a wire mesh while the other longitudinal metallic bars. The outer walls along with the two lids were made out of transparent plastic. These boxes were placed inside a light beam generating frame and the movement of the animals was detected via beam crossing.
The animals were tested for 30min for a total period of 8 days, including the pre-test and the challenge. In the pre-test, animals were allowed to freely pass from one chamber to the other in order to determine the natural preference of each mouse. We measured time spent on each side and assigned the least favoured chamber as the drug treatment chamber during the conditioning phase (conversely, the preferred side was assigned for vehicle treatment). The conditioning phase lasted for 6 consecutive days where animals received either drug or vehicle treatment (i.p. injection) on alternative days, making a total of 3 drug treatments and 3 vehicle treatments at the end of the phase. Notably, during the conditioning phase, mice were confined to one chamber only. In the last day of the experiment, animals were once again allowed to move freely between the chambers. As in the case of the pre-test, animals were not given any treatment during the challenge.

3. Results

3.1 Acute Injections

3.1.1 Amphetamine

To test the acute effects of amphetamine on Wt and αCamK II KO mice, a series of i.p. injections were conducted with different doses of amphetamine. An open field setting was used to measure locomotor activities in mice injected with either drugs or vehicle. The tested doses were 1 mg/kg, 2 mg/kg, 3 mg/kg and, the highest dose, 5mg/kg amphetamine. The acute injections were conducted on groups of at least 7 male mice less than 3 months of age. Each animal was injected two days in a row, on the first day with a 0.9% saline solution for the assessment of the baseline locomotion followed by an i.p. injection containing amphetamine on the second day. After injection, animals were immediately placed in the open field setting and movement was recorded for 60 min. αCamK II KO mice displayed significantly increased activity under saline treatment ($p<0.0001$ Mann-Whitney), with a mean distance moved in a novel surrounding of 173.7m (SEM 7.484, n=41) within 60 min compared to the Wt mean of 90.55m (SEM 29.89, n=40). Due to these baseline differences, normalisation was necessary, hence the data is shown as a delta value ($\Delta = ((distance_{drug} - distance_{saline})/distance_{saline}) x100$), i.e. the change in locomotion after drug treatment.
Treatment with the lowest dosage of amphetamine (1mg/kg) resulted in significant change in total distance for the KO group ($p=0.0194$ ANOVA, Tukey's multiple comparison test) but no significant change for the Wt group. Notably, significant differences were observed between groups for the saline treatment ($p=0.0233$ ANOVA, Tukey's m.c.t.). However, after normalisation of the overall performance, there is no statistically significant difference between the two test groups (Mann-Whitney) (Fig.16).
Due to the duration of the test, we additionally conducted a time dependent analysis by splitting the trial into early (0-30min) and late (30-60min) phases. Here we observe differences between early and late stages for Wt animals in both average distances travelled under saline and drug conditions as well as in delta values after normalisation. For saline trials, there are no differences between early and late stages whereas during amphetamine trials we observed a significant decrease in average locomotion between stages ($p=0.0218$, ANOVA, Tukey's m.c.t.). Furthermore, we observe significant differences between saline and drug trials despite non-significant observations in total locomotion, with increases in both stages upon drug treatment (saline early vs. amphetamine early $p=0.0019$; saline late vs. amphetamine late $p=0.0047$; ANOVA, Tukey's m.c.t) (Fig.17).

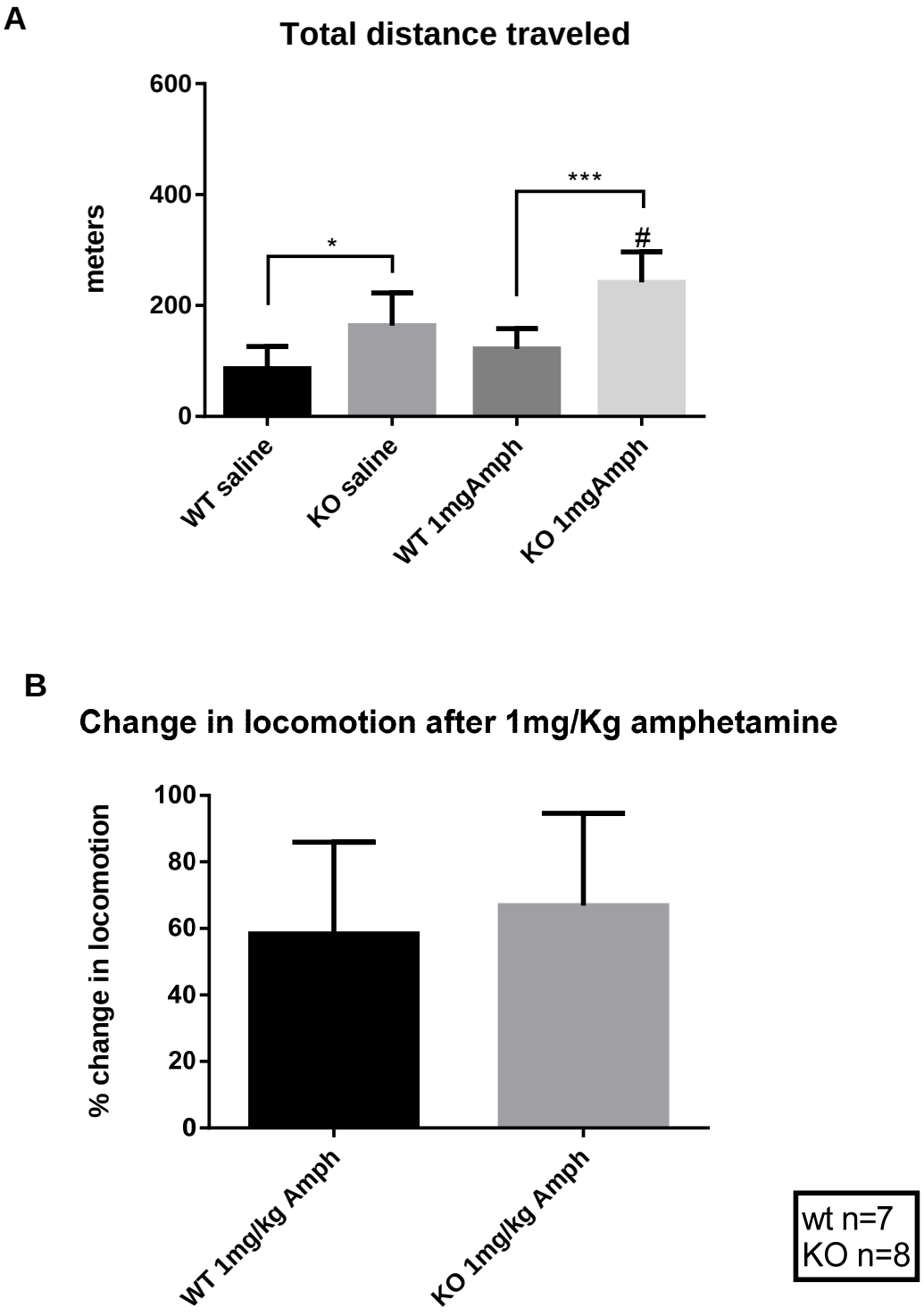


Fig. 16: Effects of acute treatment with 1mg/Kg amphetamine on CamKII KO mice and Wt controls in an open field setting. Panel A depicts total distance moved in 60 minutes for both groups under drug treatment and control saline conditions; Panel B shows the normalized data, presented as delta change. "" mark significant differences between treatment groups while "#" mark significant differences across time during one treatment session.*

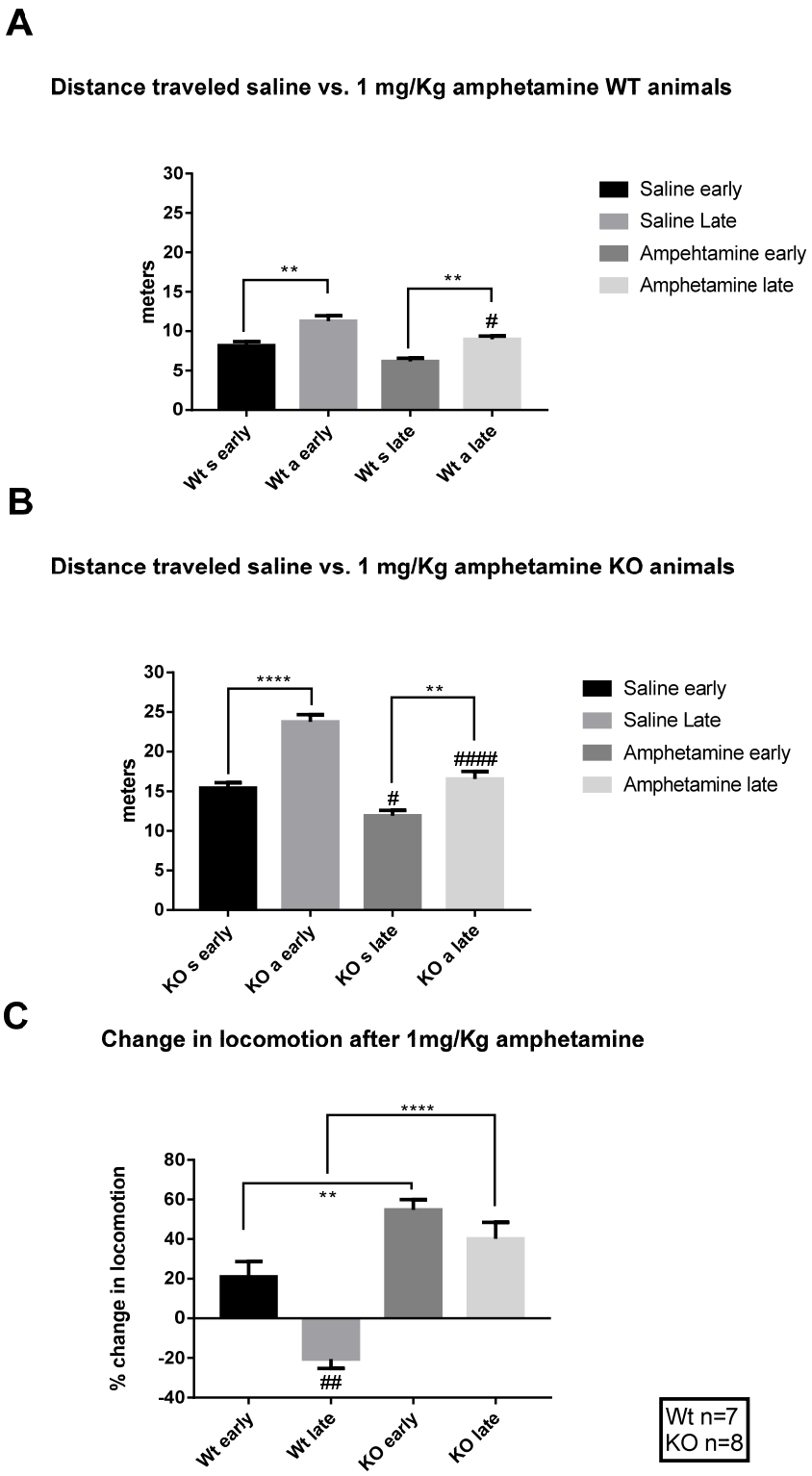


Fig.17: Time dependent analysis of distance travelled in open field under saline control and drug treatment conditions. Panel A shows results for the Wt control group. Panel B depicts results for the KO group. Panel C shows Early and late stage distance travelled across one 60min of open field testing session, shown as normalised values in form of Δ change between saline and 1mg/Kg amphetamine treatment. "" mark significant differences between treatment groups while "#" mark significant differences across time during one treatment session.*

Upon normalisation, we notice an interesting effect for the Wt group with early stage change in locomotion showing an increase and late stage change in locomotion showing a decrease relative to the saline trial (Fig 17). The difference between these two stages is statistically significant ($p=0.0014$, ANOVA, Tukey's m.c.t.). For the KO animal group average distances travelled show a significant increase upon drug treatment ($p<0.0001$ in early stage, $p=0.0032$ in late stage, ANOVA, Tukey's m.c.t.) whereas Δ-change is not significant for early and late stages of the trial.
With the 3mg/kg amphetamine dosage we observe a significant increase in total distance travelled for the KO group both compared to saline control ($p<0.0001$ ANOVA, Tukey's m.c.t.) and to the Wt treatment group ($p<0.0001$ ANOVA, Tukey's m.c.t.) with no difference in saline control between KO and Wt groups. Upon normalisation of the total distance travelled we have observed no significant differences (Fig 18). With our time dependent analysis, we observe a decrease of total distance travelled, between early and late time points, during the saline trial in Wt animals ($p= 0.0458$ ANOVA, Tukey's m.c.t.) but a significant decrease in the same measure after 3mg/kg amphetamine ($p=0.0333$ ANOVA, Tukey's m.c.t.), trial dependent (saline vs. drug) differences between time points were in both cases significant ($p<0.0001$ ANOVA, Tukey's m.c.t.). Similar results were found for the KO group with the sole difference that during the saline trial there is no difference between early and late stages of the trial. When looking at the delta change in locomotion for early and late stages we observe no difference between Wt and KO groups in either stage of the trial (Fig 19).
With the maximum dosage tested, of 5mg/kg we observed a significant ($p<0.0001$ for Wt; $p=0.0006$ for KO ANOVA, Tukey's m.c.t.) difference in locomotion compared to the saline baseline for both groups. The baseline itself was not different between the Wt and KO animals, just as there was no significant difference in locomotion after drug treatment between the groups. The normalisation of the overall locomotion revealed lower percent of change upon treatment in the KO group compared to the Wt ($p=0.0016$; Mann-Whitney test) (Fig 20).
Additionally, we analysed locomotion in 5min time intervals for the entire duration of the test. Differences in distance travelled between treatment days commenced after minute 15 for both Wt and KO animals, marking the onset of drug effect and were consistently significant to the end of the test. Interestingly, there is a significant difference ($p=0.0333$; ANOVA) between the groups in the first 5 minutes of the drug trial, with the KO animals travelling a longer distance in the open field. Upon normalisation of the time point data, we observe no group difference in the early stage of the test (0-30min) and a highly significant difference ($p<0.0001$; ANOVA, Tukey's multiple comparison test) in the percent change in locomotion (compared to saline) in the late stage (30-60min). Strikingly when comparing early and late stage locomotion within each group, Wt animals show significantly different behaviour ($p=0.0001$; ANOVA, Tukey's multiple comparison test) while KO animals do not (Fig.21).

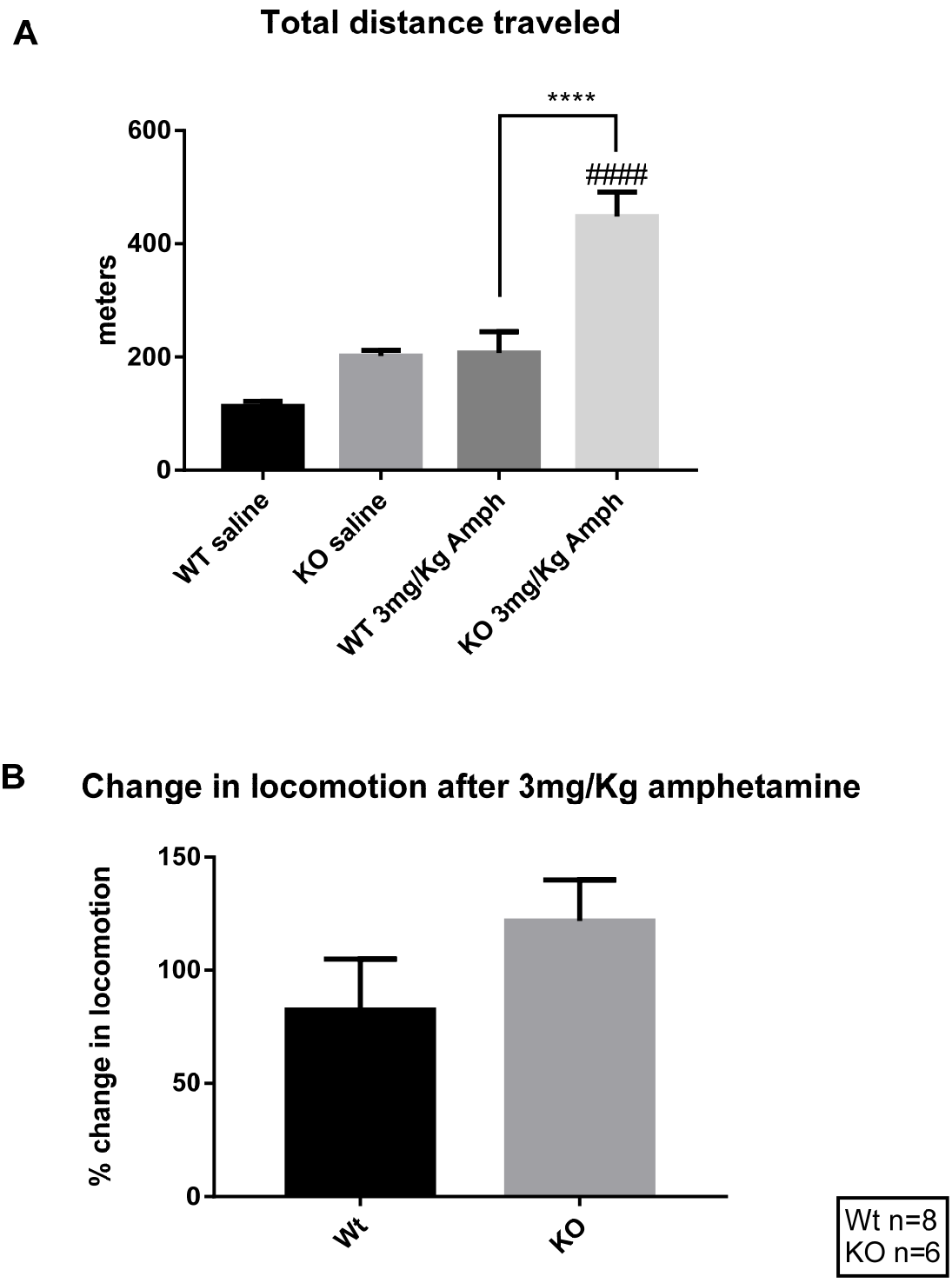


Fig. 18: Effects of acute treatment with 3mg/Kg amphetamine on CamKII KO mice and Wt controls in an open field setting. Panel A depicts total distance moved in 60 minutes for both groups under drug treatment and control saline conditions; Panel B shows the normalized data, presented as delta change. "" mark significant differences between treatment groups while "#" mark significant differences across time during one treatment session.*

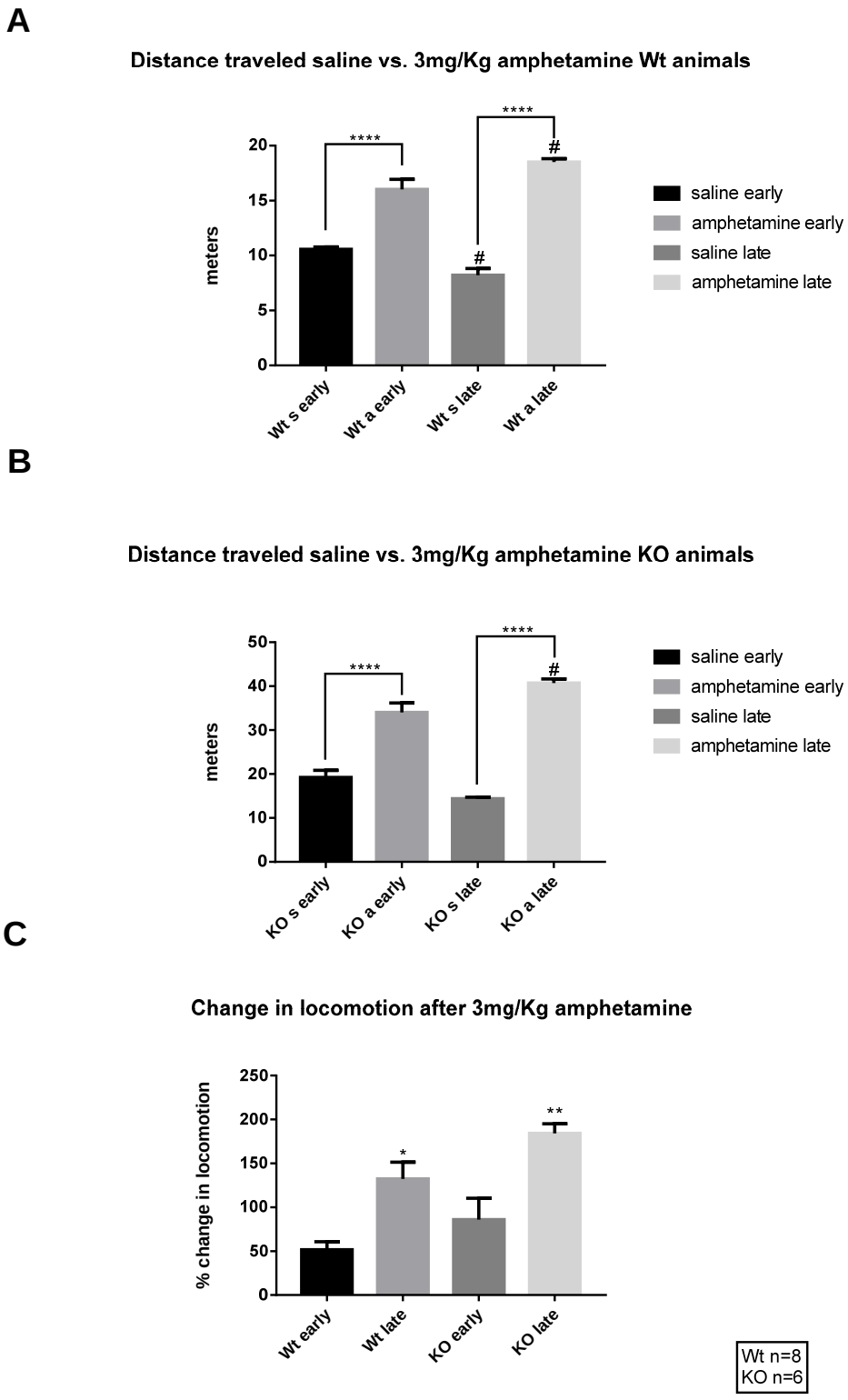


Fig. 19: Time dependent analysis of distance travelled in open field under saline control and drug treatment conditions. Panel A shows results for the Wt control group. Panel B depicts results for the KO group. Panel C shows early and late stage distance travelled across one 60min of open field testing session, shown as normalised values in form of Δ change between saline and 3mg/Kg amphetamine treatment "" mark significant differences between treatment groups while "#" mark significant differences across time during one treatment session.*

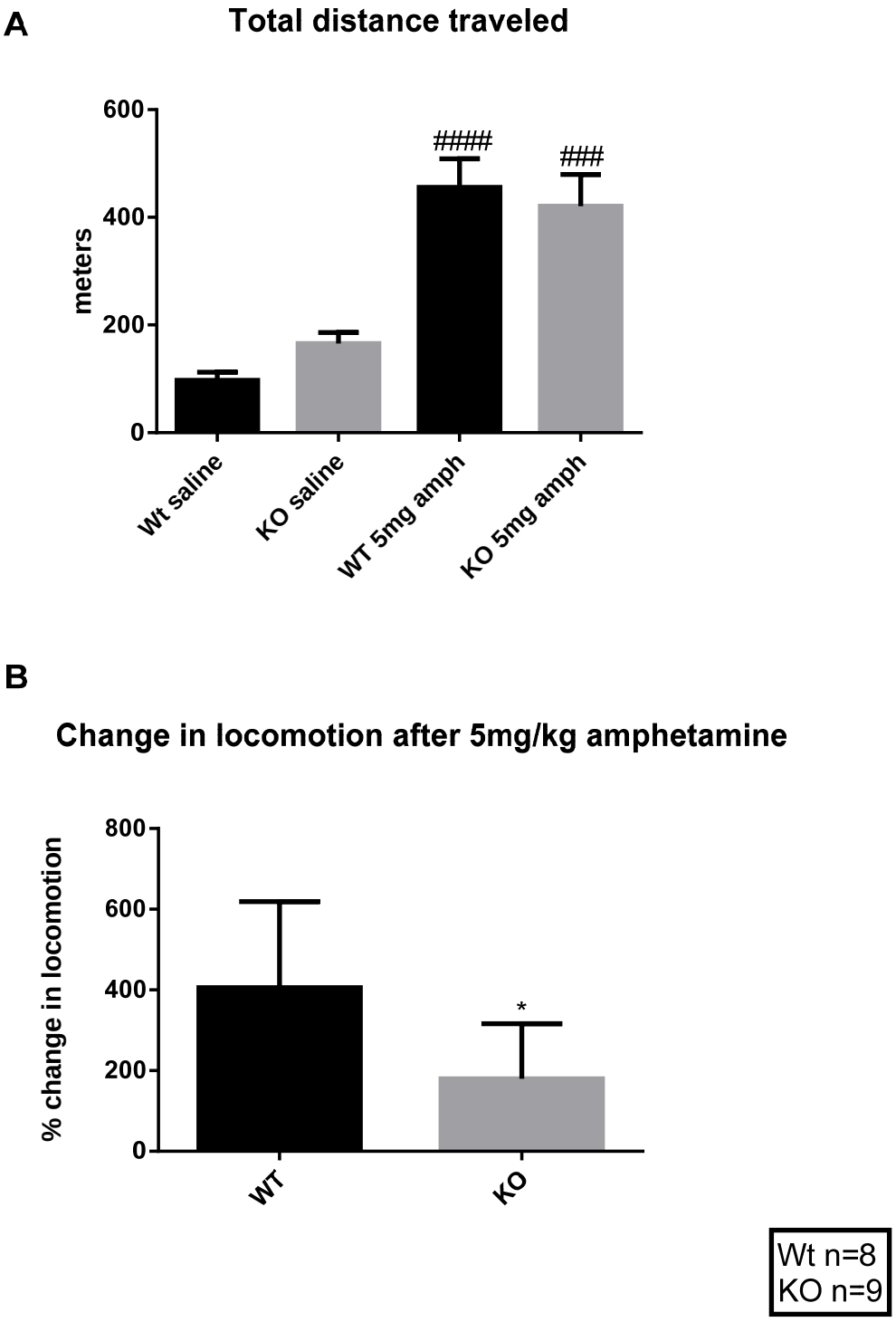


Fig.20: Effects of acute treatment with 5mg/Kg amphetamine on CamKII KO mice and Wt controls in an open field setting. Panel A depicts total distance moved in 60 minutes for both groups under drug treatment and control saline conditions; Panel B shows the normalized data, presented as delta change. "" mark significant differences between treatment groups while "#" mark significant differences across time during one treatment session*

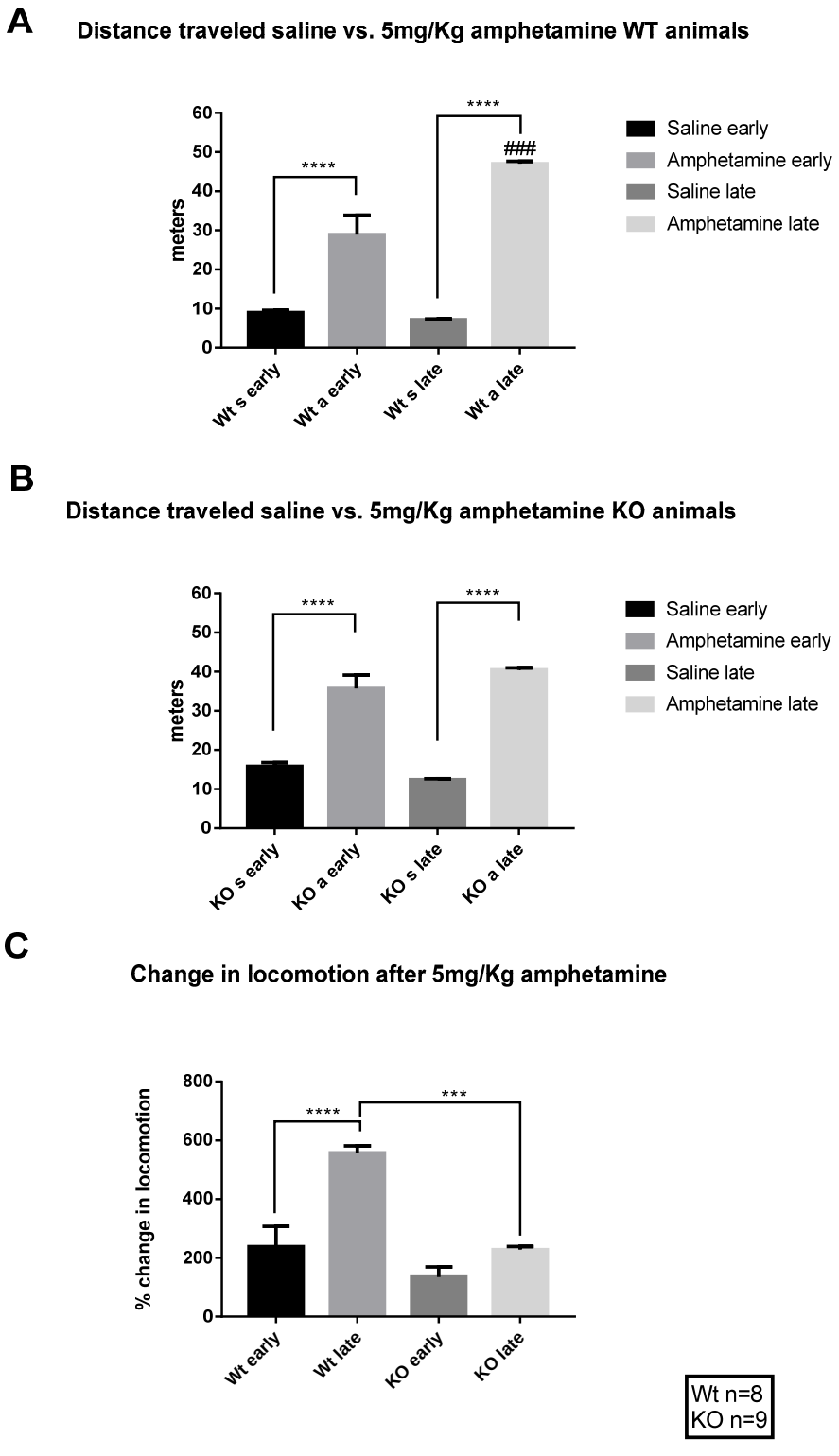


Fig. 21: Time dependent analysis of distance travelled in open field under saline control and drug treatment conditions. Panel A shows results for the Wt control group. Panel B depicts results for the KO group. Panel C shows early and late stage distance travelled across one 60min of open field testing session, shown as normalised values in form of Δ change between saline and 3mg/Kg amphetamine treatment "" mark significant differences between treatment groups while "#" mark significant differences across time during one treatment session*

3.1.2 MDMA

MDMA acute exposure experiments were conducted in identical manner to the amphetamine acute trials using the same open field settings and protocol. Two dosages were tested, 5mg/kg and 10mg/Kg. The animals were treated with 0.9% saline solution i.p, one day prior drug testing.
In the lower dosage trial, we observed significant differences in total distance travelled, between groups in both baseline saline ($p=0.0196$ ANOVA, Tukey's multiple comparison test) and drug trial ($p<0.0001$ ANOVA, Tukey's multiple comparison test). Interestingly, the Wt group showed no difference in distance travelled unlike the KO group where the difference was significant ($p=0.0047$ ANOVA, Tukey's multiple comparison test). Upon normalisation via delta value we find no difference between the two groups (Fig.22). When conducting our time dependent analysis of locomotor activity, we observe a decrease in total distance traveled between the early and late phase of the saline session ($p=0.0037$, ANOVA, Tukey's m.c.t.), as well as a significant difference between late the phases ($p<0.0001$ ANOVA, Tukey's m.c.t.), but not between early phases, for Wt animals. For the KO group we register significant differences both between trials (in early and late stages) and between stages of the same trial. Upon normalisation the only significant change is the increase in activity during the late stage for Wt animals ($p=0.0003$, ANOVA, Tukey's m.c.t.) (Fig. 23)
With 10mg/kg MDMA we observe an increase in total distance travelled, significantly different form the saline baseline, but with no difference between Wt and KO groups. No differences were observed after normalisation (Fig.24). For our time dependent analysis, we see significant differences in total distance traveled for both Wt and KO animals, both between trial stages and between treatment days. The delta normalisation values show significant increase in late stage for both groups ($p= 0.0001$ for Wt $p=0.0192$ for KO, ANOVA, Tukey's m.c.t.) but also significant late stage differences between Wt and KO ($p= 0.0105$ ANOVA, Tukey's m.c.t.) (Fig. 25).

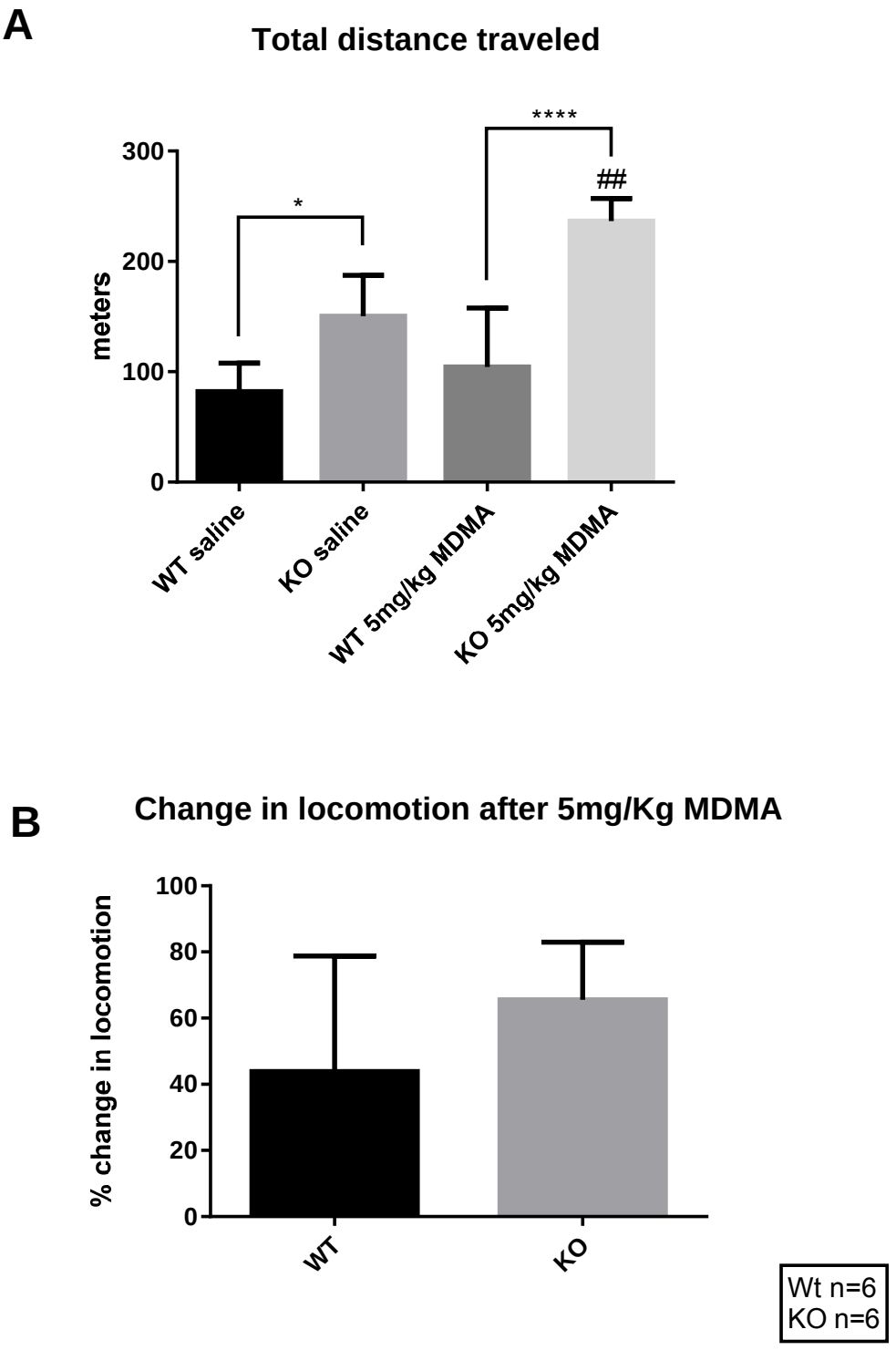


Fig.22: Effects of acute treatment with 5mg/Kg MDMA on CamKII KO mice and Wt controls in an open field setting. Panel A depicts total distance moved in 60 minutes for both groups under drug treatment and control saline conditions; Panel B shows the normalized data, presented as delta change. "" mark significant differences between treatment groups while "#" mark significant differences across time during one treatment session*

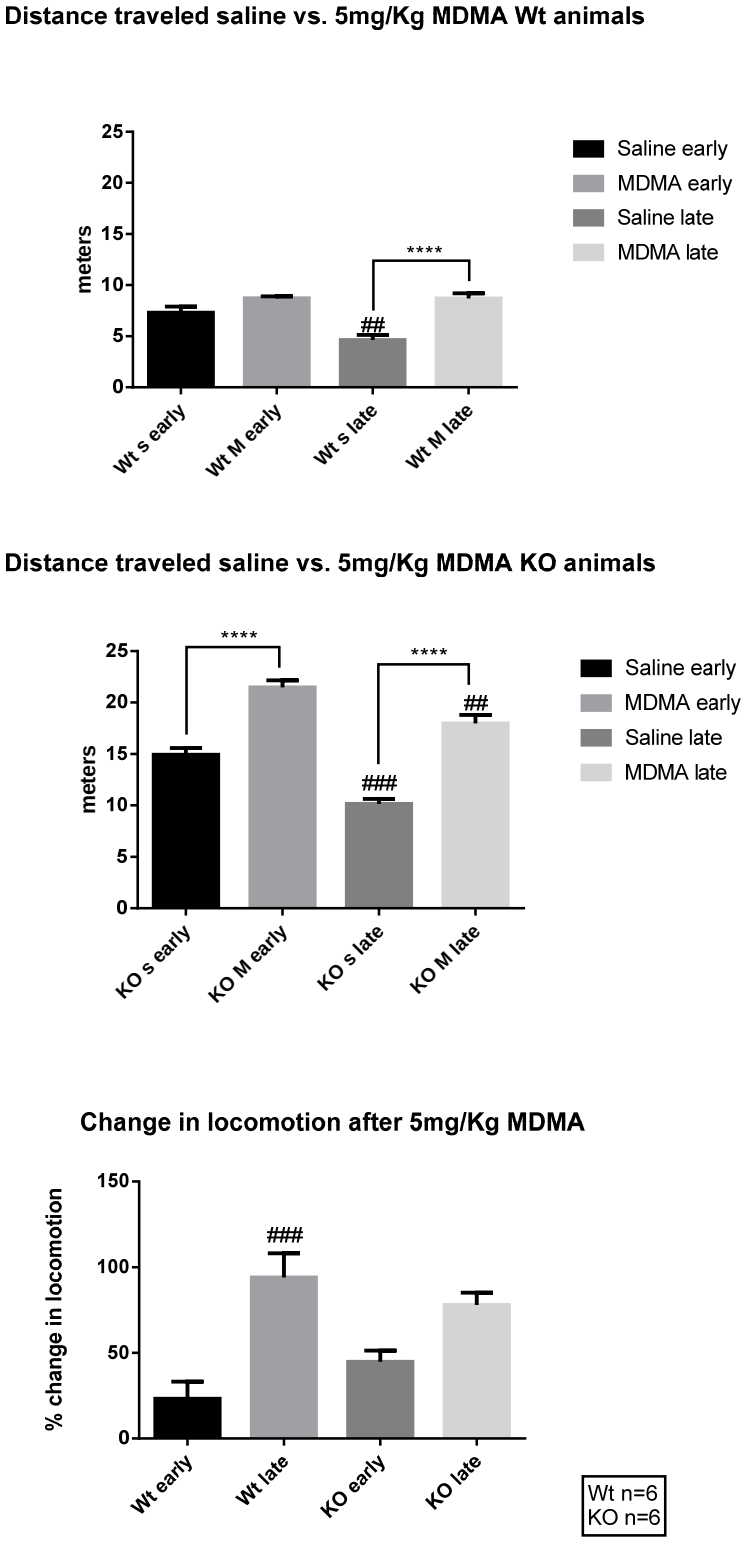


Fig. 23: Time dependent analysis of distance travelled in open field under saline control and 5mg/Kg MDMA drug tratment conditions. Panel A shows results for the Wt control group. Panel B depicts results for the KO group. Panel C shows early and late stage distance travelled across one 60min of open field testing session, shown as normalised values in form of Δ change between saline and 3mg/Kg amphetamine treatment "" mark significant differences between treatment groups while "#" mark significant differences across time during one treatment session*

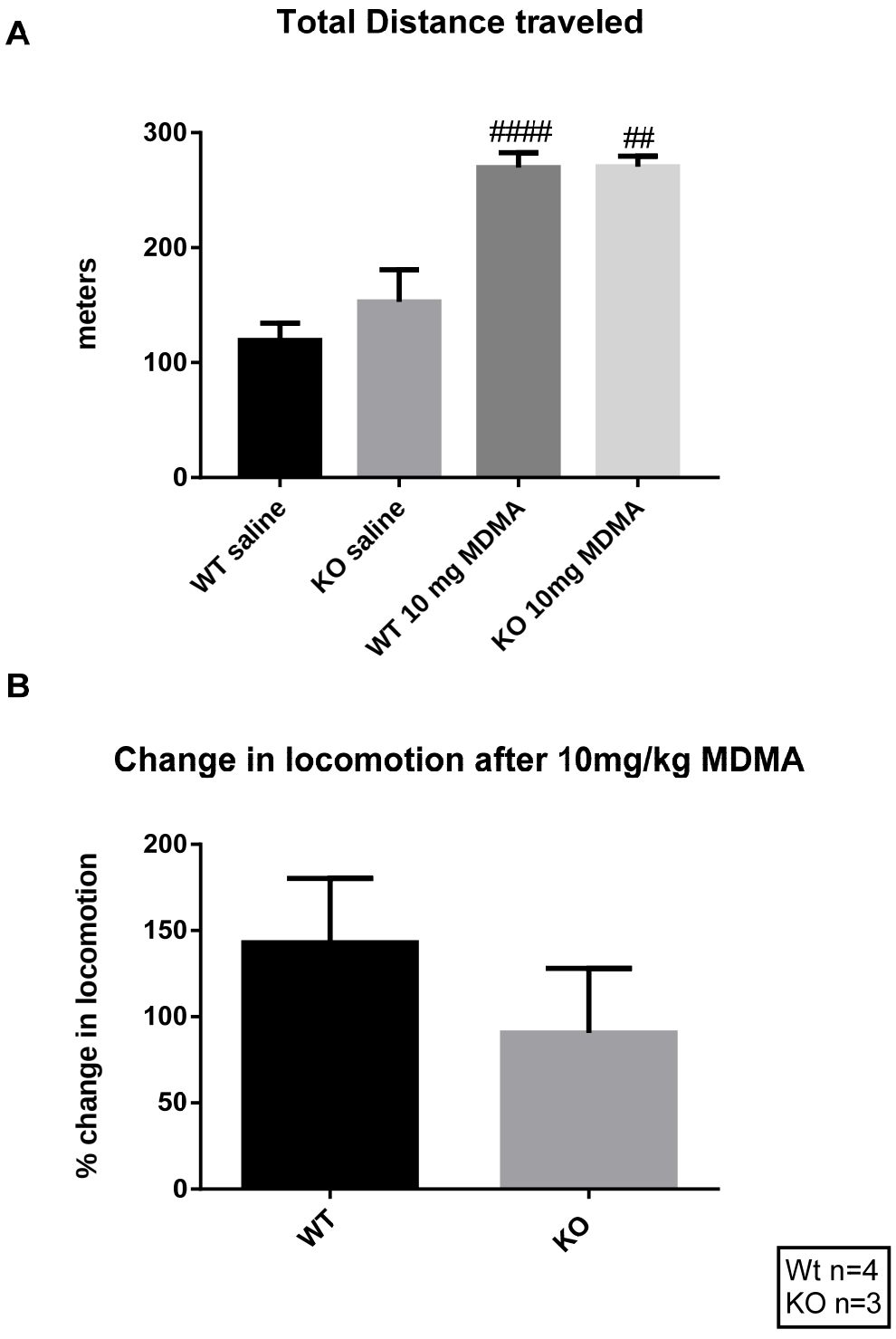


Fig.24: Effects of acute treatment with 10mg/Kg MDMA on CamKII KO mice and Wt controls in an open field setting. Panel A depicts total distance moved in 60 minutes for both groups under drug treatment and control saline conditions; Panel B shows the normalized data, presented as delta change. "" mark significant differences between treatment groups while "#" mark significant differences across time during one treatment session*

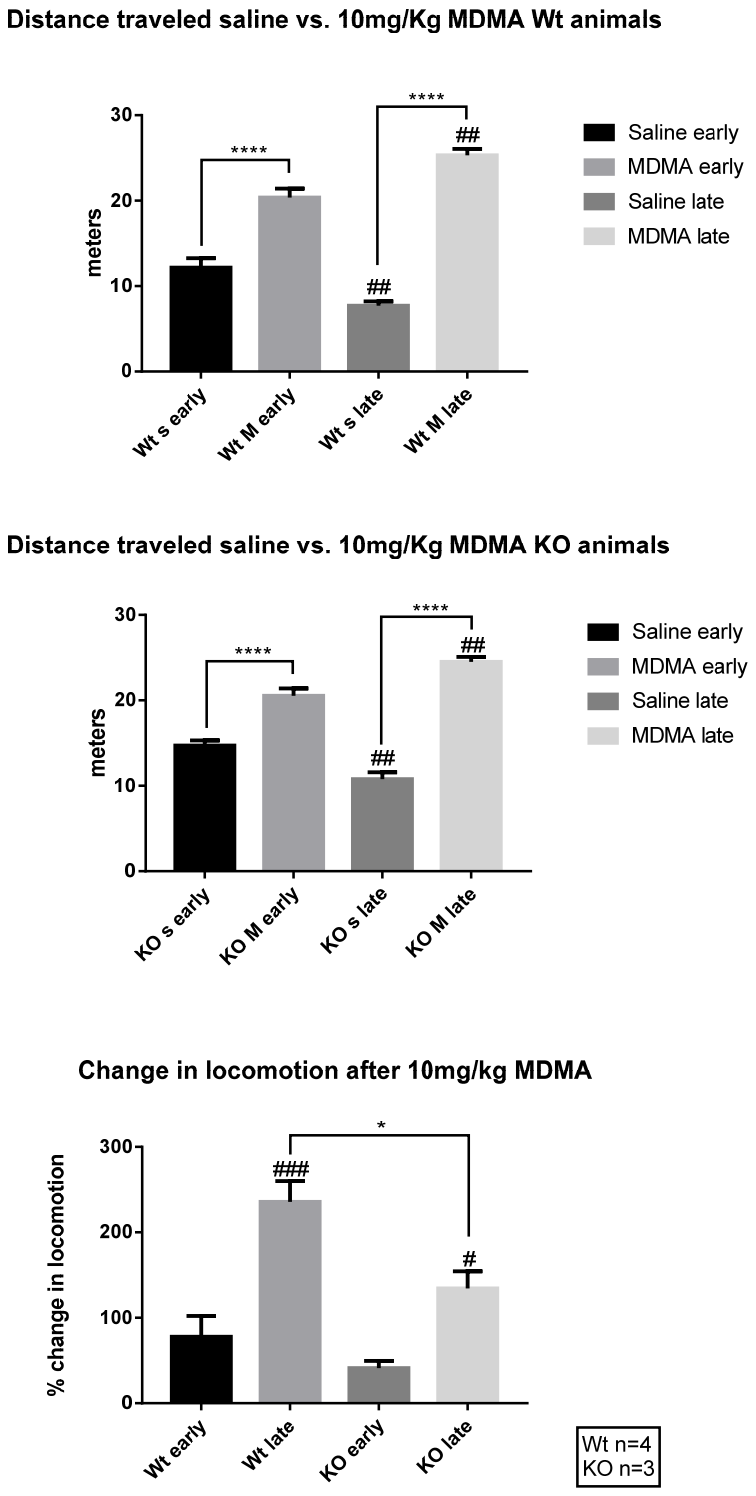


Fig. 25: Time dependent analysis of distance travelled in open field under saline control and 10mg/Kg MDMA drug tratment conditions. Panel A shows results for the Wt control group. Panel B depicts results for the KO group. Panel C shows early and late stage distance travelled across one 60min of open field testing session, shown as normalised values in form of Δ change between saline and 3mg/Kg amphetamine treatment "" mark significant differences between treatment groups while "#" mark significant differences across time during one treatment session*

3.2 Sensitisation

In order to assess long term effects of amphetamine on αCamKII KO mice sensitisation experiments with the relatively low dose of 2mg/kg were performed. After establishing the baseline activity (D0) the animals were given a dose of 2mg/kg amphetamine per day for the following 6 days (D1-D7) followed by a 2-week period of withdrawal where the mice were not injected. Finally, sensitisation to amphetamine was tested on D20/21 by administration of a last dose of 2mg/kg amphetamine. Locomotor activity was measured in an open-field setting for 60 min after each injection. The outcome of the experiment was measured by the percentage of increase in locomotor activity between D1 (first amphetamine injection) and D20/21 (last amphetamine injection). The effects of acute 2mg/kg amphetamine were also assessed by comparing performances from D0 and D1 in a similar manner to the above presented acute injections.

When looking at the total distance travelled in an acute setting, we observe a significant difference between Wt and KO groups during drug treatment ($p=0.0210$ ANOVA, Tukey's m.c.t.) but no difference in the overall normalisation (Fig.26). Analysis of the trial in a time dependent manner showed significant differences between early (minute 0-30) and late (minute 30-60) in the saline treatment for both Wt and KO groups ($p=0.0361$ for Wt, $p= 0.0113$ ANOVA, Tukey's m.c.t.) in total distance travelled. The KO group exhibits significant difference in total locomotion between saline and amphetamine sessions in both early and late stages ($p=0.0033$ early, $p<0.0001$ late, ANOVA, Tukey's m.c.t.) while the Wt animals only show similar differences only between the late stages ($p<0.0001$ ANOVA, Tukey's m.c.t.). Upon normalisation, we observe an increase in delta value for the Wt group between early and late stage ($p=0.0002$, ANOVA, Tukey's m.c.t.) as well as a between group difference in the late stage ($p=0.281$ ANOVA, Tukey's m.c.t.) (Fig. 27)

The outcome of the sensitisation experiment can be seen in in Fig. 28. We looked at both the total distance travelled and % change in locomotion between D0 and D20 (delta value).

For the raw measure of total distance travelled in the open field arena in 60 min we observe significant increases in locomotion between D0 and D20 for both Wt ($p=0.0005$, ANOVA, Tukey's m.c.t.) and KO ($p=0.040$, ANOVA, Tukey's m.c.t.) along with a significant baseline difference between Wt and KO ($p=0.0211$, ANOVA, Tukey's m.c.t.). When comparing the normalisation of D1 ("acute condition") to D0 with D20 ("sensitisation test") to D0 we observe a significant increase for the Wt group ($p=0.0455$, ANOVA, Tukey's m.c.t.), a significant difference between groups for D20 ($p=0,0303$, ANOVA, Tukey's m.c.t.) but no difference between the "acute" condition and the sensitisation test for the KO animals.

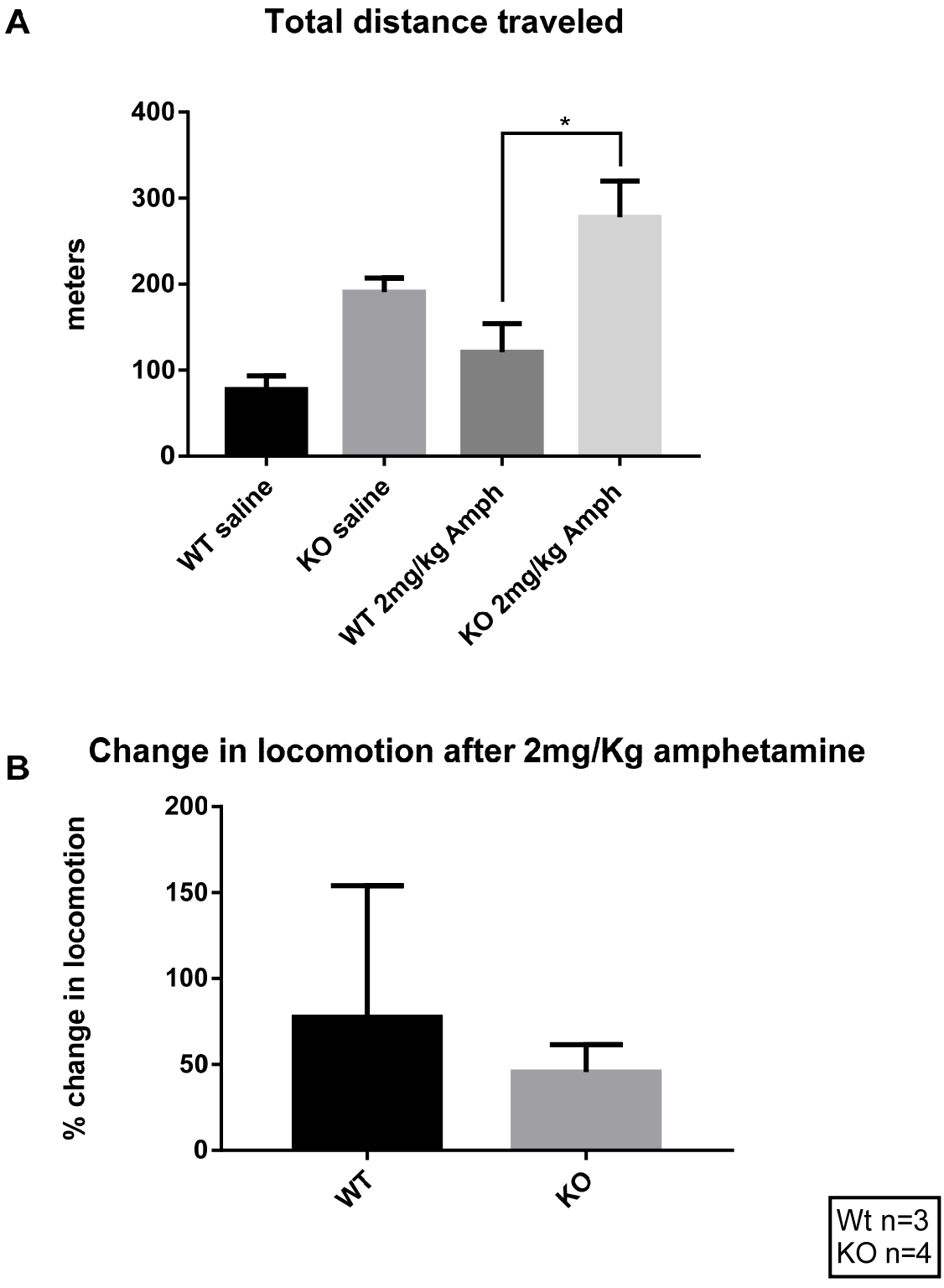


Fig.26: Effects of acute treatment with 2mg/Kg amphetamine on CamKII KO mice and Wt controls in an open field setting. Panel A depicts total distance moved in 60 minutes for both groups under drug treatment and control saline conditions; Panel B shows the normalized data, presented as delta change. "" mark significant differences between treatment groups*

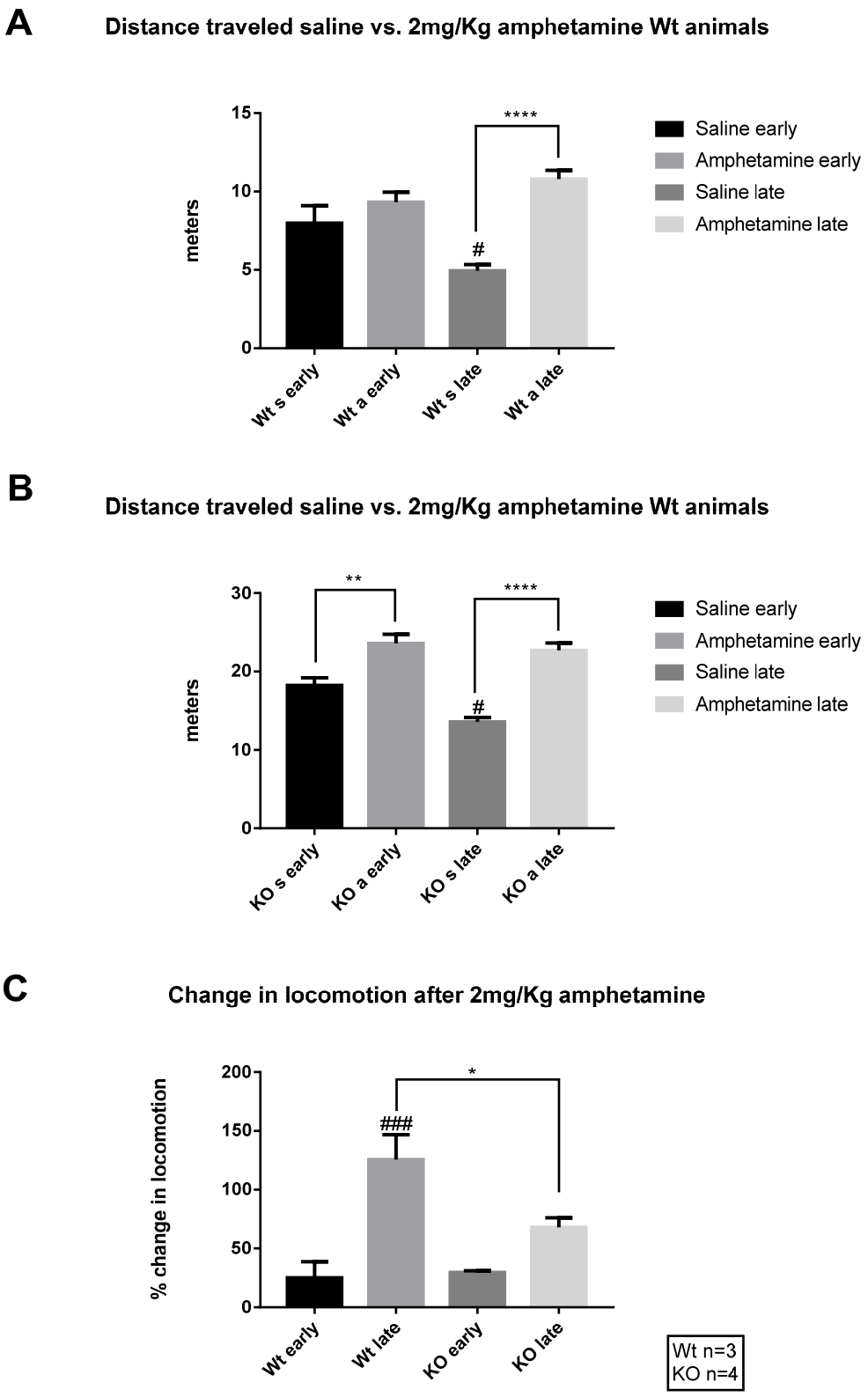


Fig. 27: Time dependent analysis of distance travelled in open field under saline control and drug treatment conditions. Panel A shows results for the Wt control group. Panel B depicts results for the KO group. Panel C shows early and late stage distance travelled across one 60min of open field testing session, shown as normalised values in form of Δ change between saline and 3mg/Kg amphetamine treatment "" mark significant differences between treatment groups while "#" mark significant differences across time during one treatment session*

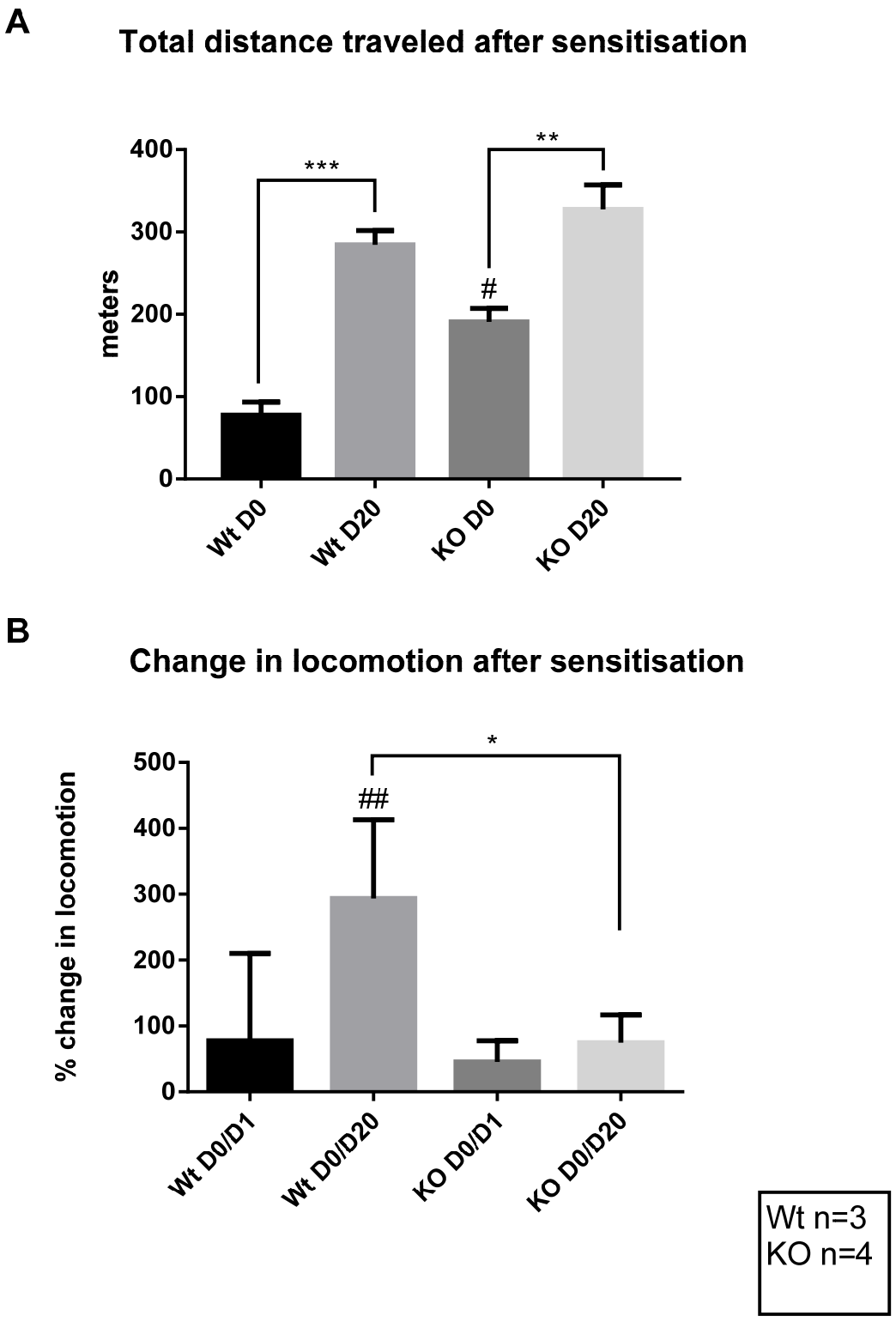


Fig.28: Effects of sensitisation represented as difference between D0 and D20 of the routine. Panel A shows the total distance travelled with "" showing significant differences across time and "#" differences between groups. Panel B shows percent change in the form of delta normalising the D1 and D20 trial to the D0 trail, with "*" showing significant differences between groups and "#" difference across time.*

3.3. Conditioned Place Preference (CPP)

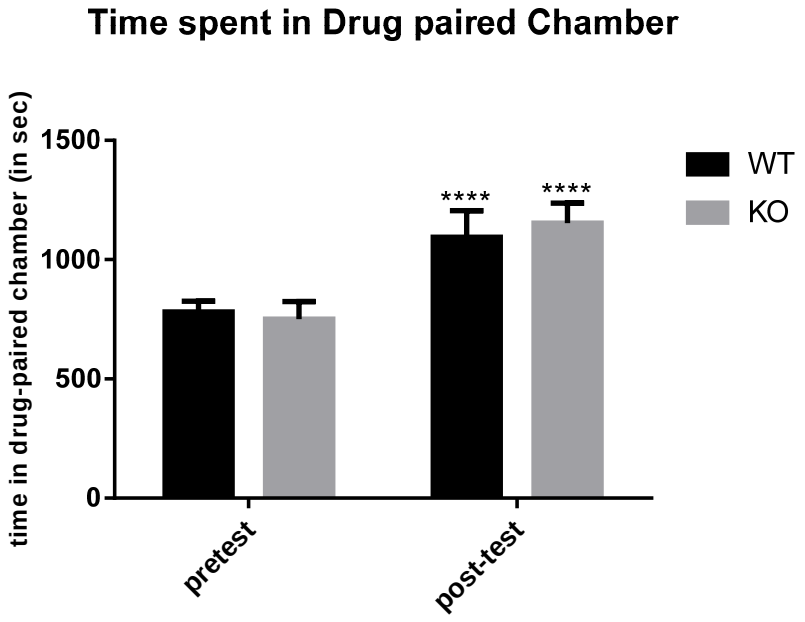
Previous studies have shown that the environment plays an important role in drug self-administration in humans and is a relevant factor influencing relapse. In order to analyse the role of αCamK II for the development of addictive behaviour the Conditioned Place Preference (CPP) paradigm was chosen. The goal of this experiment was to assess differences in drug-related place preference between Wt and KO mice.

For the CPP test a total of 10 Wt and 10 αCamK II KO male mice were used. All animals were no older than 3 months. The CPP setting consists of a chamber split in half by a wall with the possibility of free movement between the halves. The two sides of the test chamber differed in their flooring, one side contains a wire mesh and the other side longitudinal metallic bars. The pre-test was carried out with the door opened so that the animals were able to move freely between the two halves of the chamber. No injection was administrated during the pre-test. The recorded data from the pre-test was used to determine the preferred chamber for each animal. According to this information we established the drug-paired chamber (less preferred) and the saline-paired chamber (preferred). Following the pre-test, we initiated a conditioning phase lasting a total of 6 days of alternate drug or saline injections. 5mg/kg Amph in 0.9\% saline was administered i.p. on days 1, 3 and 5 while an equal volume of 0.9\% saline solution was injected on days 2, 4 and 6. During the conditioning phase the animals were confined to one halve of the testing box. After the conditioning, a so called post-test was conducted where the animals were once more set in the testing box with possibility to move freely between the two halves. During pre- and post-test trials, no injections were administrated.

All CPP sessions took 30 min and the data analysed was the individual time a mouse spent in each of the halves during pre- and post-test. Data normalisation was conducted in the same manner as for the acute injections and sensitisation experiments using the delta value.

The data shows (Fig. 29) that there is an overall increase in time spent in the drug-paired chamber after the conditioning phase, with no visible difference in the amount of time spent, between Wt and CamK II KO mice, in both pre-and post-test. Upon normalisation (percentage delta change), we observed though that the increase in time spent in the drug-paired chamber is greater, but not statistically significant (T-test $p > 0.05$) in CamK II KO mice, indicating a better response to conditioning. We also observed a slight preference of the Wt mice (7 out of 11) for the mesh ground that is contrary to the same preference of the KO mice (also 7 out of 11) for the barred ground. Although preference was present we do not believe that it affects the results of the experiment in any way.

A



B

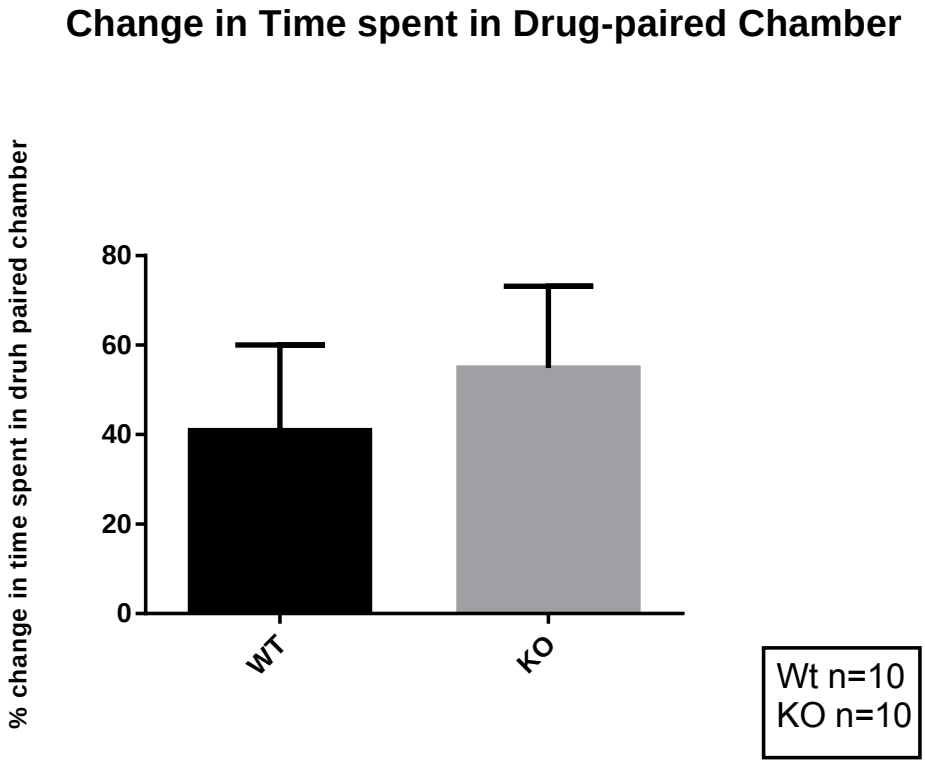


Fig.29: Conditioned place preference under treatment with 5mg/Kg amphetamine. Panel A shows total time spent in the drug-paired chamber before and after the conditioning period with "" indicating significant differences over time. Panel B shows percentage of change in form of delta normalisation regarding time spent in the drug paired chamber after conditioning.*

4. Discussion

This thesis focused on shedding light on the importance of CamKII for the mechanism of action of amphetamine in an in vivo setting. For this purpose, we used a αCamKII KO mouse strain, which in parallel lead to a phenotypic characterisation of the strain for addiction relevant behavioural tests. Our open field enquiries reveal a dosage dependent differential response for Wt and KO animals for both amphetamine and MDMA.

The lowest dosage of amphetamine (1mg/kg) induces locomotion in both Wt and KO animals, remarkably though, significant changes in raw distance travelled between baseline and saline are only visible for the KO group. Normalisation shows no difference between groups, partially due to the inherently increased baseline activity of the KO animal. Nevertheless, looking at locomotion over time during the trial, we observe an interesting effect. Wt animals move less in the second half of the test, compared to saline, denoting a wear-off of the drug along with potential fatigue. KO animals do not show this behaviour and maintain an elevated level of locomotion during the entire period of the trial, which indicates and enhancement of amphetamine action. This session time dependent effect is lost with increase of amphetamine dosage. Wt animals lose the decrease in locomotor activity during later time points of the session in trials with 2 mg/kg and 3 mg/kg. These effects are continued with the highest dosage tested, 5 mg/kg, where we observe a significant difference in the normalisation of the total locomotion data between Wt and KO. When comparing the percentage change in locomotion (delta value) we observe that the KO animals' performance remains stable throughout all acute trials while the Wt animals exhibit dramatic changes in locomotion change with the highest dosage tested (S.1). This data suggests an increased sensitivity of CamK II KO mice to even the lowest dosages of amphetamine, with the particular characteristic of sustained action throughout the entire period of the task. The most favourable model would be that due to absence of a functional CamK II dopamine efflux induced by amphetamine is truly impaired. This model would support existing data published in the past years (Khoshbouei et al. 2004, Fog et al. 2006, Steinkeller et al. 2012).

We conducted additional trials with MDMA (Ecstasy), a psychostimulant substance interacting with DAT, SERT and NET as well as with the VMAT and the 5-HT receptors (Green et al. 2003; Verrico et al. 2007; Gether et al. 2006; DeFelice & Goswami 2007). We used two dosages 5 and 10mg/Kg and observed a very similar pattern in behaviour to our amphetamine

trials. Our results suggest that αCamKII KO mice do not show the same dosage dependent change in behaviour as Wt animals do, but a rather constant behavioural pattern regardless of dosage. These findings indicate that the presence of a functional αCamKII is necessary for the function of monoamine transporters regardless of monoamine releasing agent used.

This model is further sustained by the results achieved in the sensitisation experiment. Mice lacking the CamK II strikingly lack the ability to sensitise to even a low dosage of amphetamine despite showing similar response to the same dosage of 2mg/kg with the Wt control group in the acute injection experiment. The importance of CamK II in sensitisation has been shown previously in experiments using the DAT blocker cocaine (Pierce et al. 1997; Licata and Pierce 2003; Licata et al. 2004). These studies showed that blockers of CamK II do not impair the behavioural response to cocaine but inhibit the expression of behavioural sensitisation (Pierce et al. 1997). Furthermore, blockage of CamK II activity in the VTA and enhances the acute response to cocaine (Licata et al. 2004) while over-expression of CamK II in the nucleus accumbens enhances sensitisation to cocaine (Loweth et.al 2010).

αCamK II KO mice have an elevated basal locomotion in the open field paradigm compared to Wt animals when injected with saline similar to the behaviour of DAT KO mice under similar conditions. Furthermore, it is known that DAT KO not only have increased basal locomotion, but also are less responsive to the effects of amphetamine and cocaine (Giros et al. 1996). DAT KO mice do not exhibit increased dopamine production but rather an increased dopamine concentration in the extracellular space due to lack of clearance, this could be the case for the αCamK II KO mouse model. Along with the increased dopamine levels in the synapse, studies have shown that DAT KO mice have reduced vesicular dopamine pools (Jones et al. 1998 (ex vivo); Caron et al. 2001), if this were the case for αCamK II KO mice, the lack of differential response to increasing dosages of amphetamine seems clearer.

Interestingly, we observe no difference in the ability of αCamK II KO mice to form spatial associative memories to the drug paired chamber in the conditioned place preference paradigm. This suggests that the absence of the αCamK II is not sufficient to impair drug related memory formation. These findings imply different mechanisms at work for cognitive versus motor effects of amphetamine. Further behavioural testing in more complex behavioural task would be of interest for elucidating the exact role of αCamK II in drug related as well as pathologically impaired cognitive functions.

Studies have also shown that constantly elevated dopamine concentrations in the

extracellular space leads to changes in D2 dopamine receptor sensitivity and distribution (Caron et.al 2001, Licata 2003). In order to test if changes at receptor level took place in αCamK II KO mice, a pilot test was conducted using 0.5mg/kg of the D2 agonist quinpirole in an open field setting. Eight animals were tested, 4 KO and 4 Wt under acute injection conditions. Our results for this pilot trial show a trend towards decreased sensitivity of KO animals compared to Wt controls, despite no significant decrease in locomotor activity (Supplementary figure S2). Further research will focus on the changes taking place at receptor level in αCamK II KO mice via behavioural experimentation with substances such as quinpirole and qPCR to determine the expression ratios of D1 and D2 receptors.
On a different level, a conditional KO line was created by cross-breeding Dat/cre mice with αCamK II floxed animals. The result of this crossing is ideally an animal lacking a functional CamK II in dopaminergic neurons only.

As mentioned before, CamKII plays a crucial role in modulating neural plasticity, especially in association with the development of LTP (Kennedy 1989). Being an abundant protein kinase in the nervous system the CamKII has a multitude of interaction partners, some of which have been shown to play important roles in the development of drug-related learning and addiction. At the same time, research in the past years has increasingly focused on the effects of different drugs on gene expression. The hypothesis has been postulated that modifications in gene expression are an important factor in both short and long term adaptations following drug use (Nestler 2001). Some of the best studied factors are ΔFosB a member of the *fos* gene family, cAMP response element-binding protein CREB and c-fos (Nestler 2000; Nestler 2008). Several publications already showed that CREB is a substrate for CamK and is activated via phosphorylation at Ser 133 (Sheng & Greenberg 1990; Dash et al. 1991) but inhibited by phosphorylation at Ser 142 (Carlezon et al. 2005). It is now known that CamK IV facilitates phosphorylation at Ser 133 and CamKII does so at Ser 142 (Wu & McMurray 2001; McClung & Nestler 2003). CREB is also a known mediator of drug related adaptation, together with ΔFosB. CREB activity is regulated differently depending on the brain region involved and can have a multitude of different effects. Studies have shown that CREB inhibition in the Locus coeruleus reduces behaviour associated with opiate physical dependence and withdrawal (Lane-Ladd et al. 1997; Carlezon et al. 2005). By contrast, direct activation of CREB in the Nucleus accumbens reduces the rewarding effects of stimulants such as cocaine (Carlezon Jr. et al. 1998; Self et al. 1998; Carlezon et al. 2005). ΔFosB is a product of the *fosB* gene and

comes in different isoforms that determine whether it accumulates to modulate long lasting adaptations under chronic drug consumption or not, making it a molecular switch (Nestler 2001; Nestler 2004). Stability of ΔFosB is also determined by its phosphorylation which is facilitated by different kinases (Nestler 2008). Although its importance in the development of addiction is undisputed, action mechanisms and downstream targets are not completely elucidated. Recent reports describe a connection between ΔFosB and the inhibition of c-fos where ΔFosB coordinates epigenetic machinery to silence c-fos (Nestler 2008; Nestler 2014). C-fos is one of the first so-called immediate early genes (IEG) to be identified. IEGs are genes whose expression is rapidly and transiently regulated upon stimulation. Given that these factors are known for playing a role in case of acute and chronic administration of drugs such as opiates, cocaine and amphetamine, further deeper inquiry into the specifics of the αCamKII KO strain phenotype, as well as into the molecular mechanisms of CamKII-monoamine transporter interactions would have to include analysis of IEG activation. This type of analysis would not only give insight into molecular mechanisms but would also reveal potential brain wide phenotypes as c-fos is amongst the most commonly used markers for brain activity.

Hopefully the results of this research will lead to further important acknowledgements in the research field of drug addiction and perhaps further on, to help treat complex psychiatric disorders such as depression and schizophrenia. With additional investigation, the αCamKII KO mouse model could prove to be the next important animal model in discovering the amazing complexity of the reward pathways in our brain.

5.Acknowledgements:

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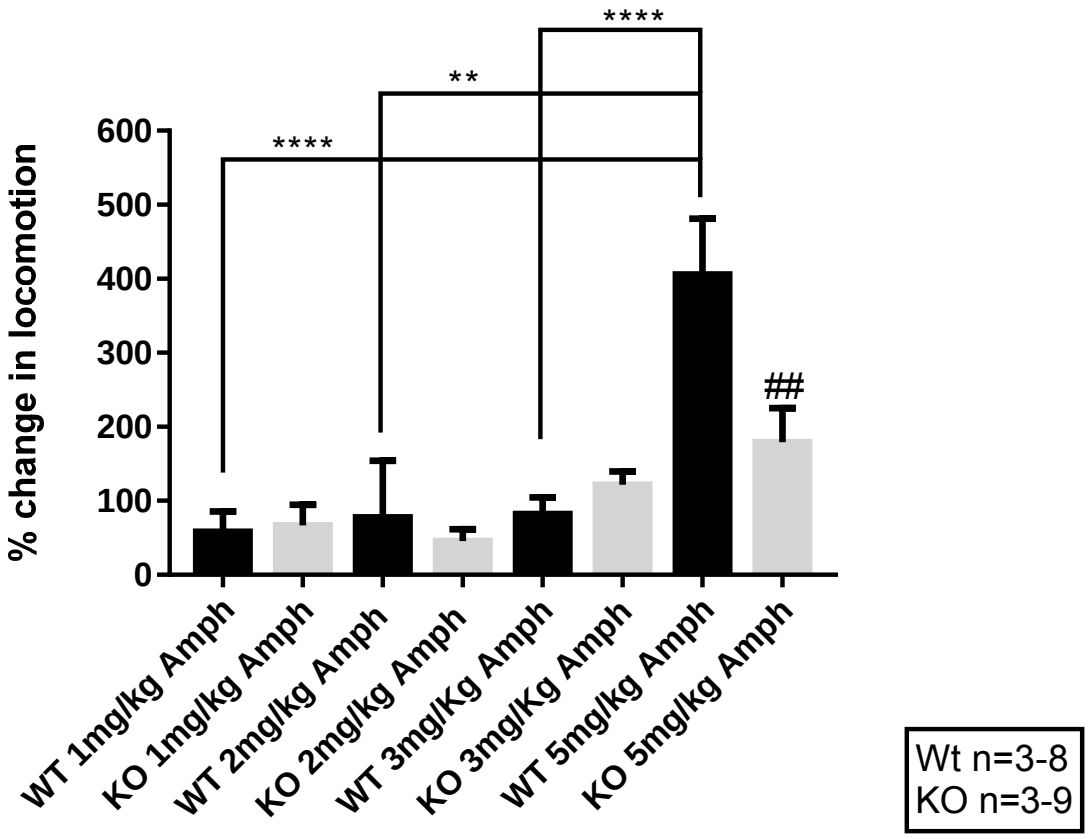
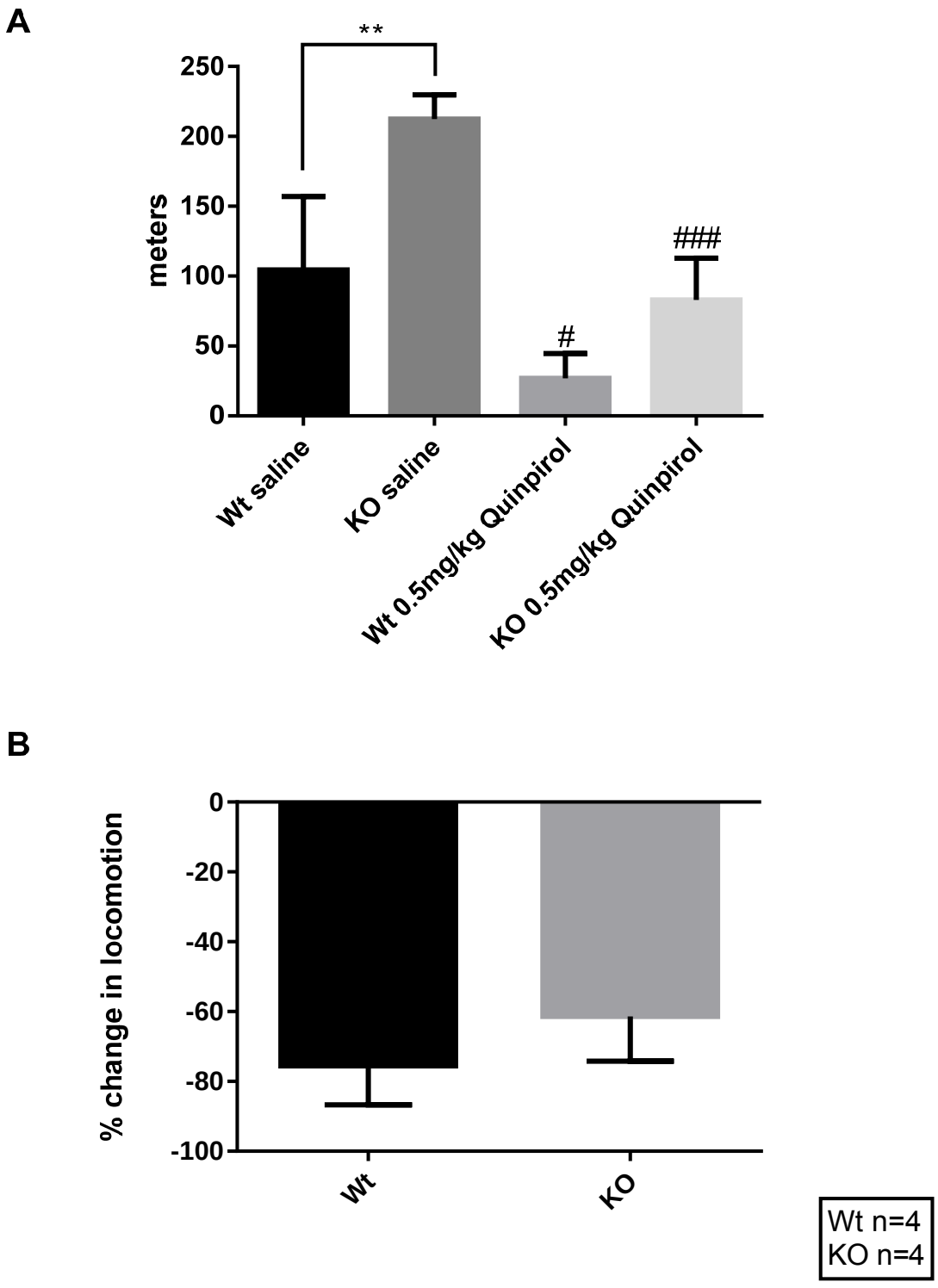
Supplementary Figures



S1: Delta values of total distance traveled for Wt and KO animals of all acute amphetamine trials. "" indicate differences between dosage in the same test group while "#" indicate significant differences between groups for a given drug dosage.*



S2 : Effects of acute treatment with 0.5mg/Kg Quinpirole on CamKII KO mice and Wt controls in an open field setting. Panel A depicts total distance moved in 60 minutes for both groups under drug treatment and control saline conditions; Panel B shows the normalized data, presented as delta change. "" mark significant differences between treatment groups while "#" mark significant differences between treatment sessions for each group.*

S3: Research article published containing the results of this study

OPEN

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In Vivo Amphetamine Action is Contingent on αCaMKII

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Addiction to psychostimulants (ie, amphetamines and cocaine) imposes a major socioeconomic burden. Prevention and treatment represent unmet medical needs, which may be addressed, if the mechanisms underlying psychostimulant action are understood. Cocaine acts as a blocker at the transporters for dopamine (DAT), serotonin (SERT), and norepinephrine (NET), but amphetamines are substrates that do not only block the uptake of monoamines but also induce substrate efflux by promoting reverse transport. Reverse transport has been a focus of research for decades but its mechanistic basis still remains enigmatic. Recently, transporter-interacting proteins were found to regulate amphetamine-triggered reverse transport: calmodulin kinase IIα (αCaMKII) is a prominent example, because it binds the carboxyl terminus of DAT, phosphorylates its amino terminus, and supports amphetamine-induced substrate efflux *in vitro*. Here, we investigated whether, *in vivo*, the action of amphetamine was contingent on the presence of αCaMKII by recording the behavioral and neurochemical effects of amphetamine. Measurement of dopamine efflux in the dorsal striatum by microdialysis revealed that amphetamine induced less dopamine efflux in mice lacking αCaMKII. Consistent with this observation, the acute locomotor responses to amphetamine were also significantly blunted in αCaMKII-deficient mice. In addition, while the rewarding properties of amphetamine were preserved in αCaMKII-deficient mice, their behavioral sensitization to amphetamine was markedly reduced. Our findings demonstrate that amphetamine requires the presence of αCaMKII to elicit a full-fledged effect on DAT *in vivo*: αCaMKII does not only support acute amphetamine-induced dopamine efflux but is also important in shaping the chronic response to amphetamine.
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INTRODUCTION

Amphetamines constitute a class of psychostimulants that share a phenylethylamine core structure. They are used illicitly for recreational purposes, but also used clinically for the treatment of attention-deficit hyperactivity disorder (ADHD) and narcolepsy (Kristensen *et al*, 2011; Steinkellner *et al*, 2011). The stimulant and addictive properties of amphetamines are thought to arise primarily from their interaction with the cocaine-sensitive dopamine transporter (DAT) (Sulzer, 2011). DAT is a member of the solute carrier 6 gene family of Na^+/Cl^--dependent neurotransmitter transporters; these transporters terminate neurotransmission by clearing the synapse of their cognate substrate(s) (Kristensen *et al*, 2011). Amphetamines are substrates of DAT and compete for reuptake with dopamine (Sitte *et al*, 1998). In addition, amphetamines can induce transport reversal leading to transporter-mediated efflux of dopamine (Sulzer, 2011; Sitte and Freissmuth, 2010). Both, competition for uptake and reverse transport lead to a pronounced increase in the extracellular concentrations of dopamine. The resulting increased dopaminergic input in the striatum has been associated with the rewarding properties of amphetamines (Schultz, 2002). Accordingly, repeated amphetamine-induced enhancement of synaptic dopamine can promote the development of drug addiction via the induction of long-term changes leading to synaptic plasticity (Nestler, 2005; Sulzer, 2011). In addition, the adaptive changes have been implicated in the emergence of stimulant-induced psychosis and schizophrenia (Snyder, 1974; Yui *et al*, 1999).

The molecular mechanism of amphetamine-induced DAT-mediated reverse transport is still a matter of debate

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(Sulzer, 2011; Sitte and Freissmuth, 2010). Reverse transport is thought to involve the uptake of amphetamines via the transporter and their passive diffusion through the membrane which is due to their lipophilic nature (Sitte *et al*, 1998; Sandtner *et al*, 2013). Besides, the weak-base hypothesis states that amphetamines are trapped within synaptic vesicles in the presynaptic specialization and deplete the vesicular stores of dopamine by dissipating the proton gradient that provides the driving force for the vesicular monoamine transporters (VMATs). Thereby, amphetamines elevate the cytosolic dopamine concentration and render dopamine available for reverse transport by DAT (Sulzer, 2011). Additionally, amphetamine is a substrate for VMATs and thereby competitively inhibits vesicular dopamine uptake. The resulting elevation of dopamine in the cytosol provides another explanation for how dopamine can efflux via DAT (Sulzer, 2011).

Undoubtedly, ion gradients are the most crucial factor in determining whether transporter reversal can occur, because the substrate-binding sites are only accessible in the presence of high Na^+ concentrations (Sitte and Freissmuth, 2010). A crucial factor for amphetamine-induced reverse transport of DAT is its contingency on the intracellular sodium concentration (Khoshbouei *et al*, 2003). However, the last years also revealed an intricate contribution of both, the membrane environment and interacting proteins (Fog *et al*, 2006; Steinkellner *et al*, 2012; Pizzo *et al*, 2013, 2014; Buchmayer *et al*, 2013) in the modulation of amphetamine-triggered reverse transport. Previous observations also indicated that dopamine efflux was regulated by cytosolic Ca^{2+} (Gnegy *et al*, 2004). Because of its abundance in neurons, Ca^{2+}/calmodulin-dependent protein kinase IIα (αCaMKII) was the candidate target of Ca^{2+}. This was subsequently verified: αCaMKII was shown to modulate reverse transport of dopamine by binding to the carboxyl terminus of DAT and to phosphorylate serines at its amino terminus (Fog *et al*, 2006). *In vitro*, inhibition of αCaMKII and its genetic ablation attenuated the amphetamine-induced substrate efflux via DAT (Fog *et al*, 2006; Steinkellner *et al*, 2012; Rickhag *et al*, 2013). These results supported the hypothesis that αCaMKII regulated the action of amphetamine on DAT.

The amphetamine-induced behavioral effects result from the complex interplay of at least three target areas, which are innervated by dopaminergic projection neurons. These include the prefrontal cortex, where dopamine impinges on executive function, the nucleus accumbens, in which dopamine encodes rewarding cues and incentive salience, and the corpus striatum, where dopamine controls locomotion. Dopaminergic projections in the brain express DAT at different levels. It is therefore not clear whether components of the amphetamine-induced behavioral response differ in their dependence on αCaMKII.

We addressed this question by exploring the action of amphetamine *in vivo* in αCaMKII-deficient mice. We found that the absence of αCaMKII blunted both the amphetamine-induced increase in locomotion and the sensitization after repeated administration of amphetamine. Surprisingly, the rewarding action of amphetamine was preserved in αCaMKII-deficient mice. These findings demonstrate that, *in vivo*, some—but not all—actions of amphetamine are contingent on functional αCaMKII.

MATERIALS AND METHODS

Reagents

D-amphetamine, cocaine, GBR12909, cis-(Z)-flupenthixol, haloperidol, ketanserin, 3-hydroxybenzylhydrazine (ND1015), reserpine, and the anti-α-Tubulin antibody were purchased from Sigma Aldrich; [^{3}H]dopamine (40 Ci/mmol), [^{3}H]SCH23390 (70 Ci/mmol), and [^{3}H]raclopride (60 Ci/mmol) were obtained from PerkinElmer Life Sciences. Anti-tyrosine hydroxylase and anti-VMAT2 antibodies were purchased from Merck Millipore. Anti-PSD-95 and anti-DARPP32 antibodies were from BD Transduction Laboratories. Anti-βCaMKII antibody was from Life Technologies. Anti-PKC antibody was obtained from Signalway Antibody LLC. Anti-phospho Akt Thr-308, anti-phospho DARPP32 Thr-34, anti-phospho ERK1/2 (p44/42) Thr-202/Tyr-204, anti-total Akt, and anti-total ERK1/2 antibodies were purchased from Cell Signaling Technology.

Animals

The generation of αCaMKII-KO mice has been described elsewhere (Elgersma *et al*, 2002). All mice were bred on a C57Bl/6J background and were housed under standard laboratory conditions (12-h light/12-h dark cycle). Food and water were provided ad libitum. Male mice were tested at 12–20 weeks of age. All experiments were conducted in accordance with protocols approved by the Animal Welfare Committee of the Medical University of Vienna and the Austrian Federal Ministry of Science and Research (license BMWF·66.009/0250-II/3b/2013).

Synaptosomal and Vesicular [^{3}H]Dopamine Uptake and Radioligand Binding

Uptake of [^{3}H]dopamine via DAT was measured in striatal synaptosomes as described (Steinkellner *et al*, 2012). Vesicular uptake was performed in striatal synaptic vesicles. Briefly, lysate pellet 2 (LP2) was isolated as described by Hell and Jahn (1994) and resuspended in uptake buffer (150 mM *N*-methyl-D-glucamine (NMDG), 10 mM HEPES, 2 mM ATP-Mg^{2+}, 2 mM KCl, and 10 mM K^+-gluconate, pH = 7.4). To measure transport, approximately 20–30 μg of vesicles were preincubated in uptake buffer for 10 min at 30 °C before the addition of 40 nM [^{3}H]dopamine and incubation for another 10 min at 30 °C. Non-specific uptake was done in the presence of 10 μM reserpine. Uptake was terminated by the addition of ice-cold uptake buffer (2 mM ATP-Mg^{2+} was substituted by 2 mM $MgSO_4$) and filtration using GF/B filters presoaked in 2% polyethylenimine.

Binding of [^{3}H]SCH23390 and [^{3}H]raclopride was performed as described (Ghisi *et al*, 2009). Briefly, striatal synaptosomes (Steinkellner *et al* 2012) were resuspended in binding buffer (50 mM Tris HCl, 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, pH = 7.4). Ketanserin (100 nM) was added to the incubation to prevent binding of [^{3}H]SCH23390 to $5HT_{2A}$ receptors. Increasing concentrations of [^{3}H]SCH23390 or [^{3}H]raclopride were added; the reaction was incubated for 1 h at 25 °C. Non-specific binding was determined in the presence of 10 μM *cis*-(Z)-flupenthixol and 50 μM haloperidol for [^{3}H]SCH23390 and [^{3}H]raclopride binding, respectively. Binding was stopped

by adding ice-cold binding buffer and filtered onto GF/B filters presoaked in 2% polyethylenimine using an automated cell harvester filtration device (Skatron Instruments AS). The radioactivity bound to the filters was measured by liquid scintillation counting.

Immunoblots

Mice were killed by cervical dislocation, decapitated, and heads were immediately immersed in liquid nitrogen for 6 s. Striata were dissected and snap-frozen in liquid nitrogen. Tissue was homogenized in RIPA buffer containing (50 mM Tris.HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, and 1% deoxycholate supplemented with protease and phosphatase inhibitors) and incubated at 4 °C for 60 min followed by centrifugation at 12 600 *g* for 30 min. For phospho-protein analysis, tissue was boiled for 10 min in 1% SDS supplemented with protease and phosphatase inhibitors (Roche). Proteins were separated on a 10% SDS-PAGE and electrotransferred onto nitrocellulose before incubation with primary antibodies overnight. IRDye 680- or 800-RD-labeled secondary antibodies were obtained from LI-COR and visualized using the LI-COR Odyssey CLx infrared imaging system. Densitometric quantification of bands was performed using NIH ImageJ software.

Behavioral Pharmacology

Horizontal locomotion (total distance traveled) was measured in 'open-field' (OF) square boxes (36 × 36 × 45 cm) using a video camera mounted above the box and analyzed using the Anymaze software from Stoelting (V.4.7). Distances traveled were recorded for 60 min. Acute drug effects were assessed by administering an intraperitoneal (i.p.) injection of saline to the mice on day zero (d0); their locomotion was measured for 60 min. On the next day (d1), mice were administered D-amphetamine (2 or 5 mg/kg) by i.p. injection. Distances traveled were again recorded for 60 min. Acute drug effects were normalized to the distances traveled upon injection of saline and expressed as fold increase in locomotion.

D-amphetamine-induced locomotor sensitization. Baseline locomotor activity of mice was assessed on day zero (d0) after injection of saline (i.p.). Mice were then sensitized to D-amphetamine (2 mg/kg) by daily (i.p.) injections for 6 consecutive days (d1–d6). After each injection, locomotor activity was recorded in the OF boxes for 60 min. After 6 days of drug sensitization, amphetamine was withheld for 14 days. Mice were challenged by injection of D-amphetamine (2 mg/kg, i.p.) on day 20 (d20) after which they were again monitored in the OF boxes.

Conditioned place preference. Conditioned place preference (CPP) was conducted in commercially available CPP chambers (MED Associates, Georgia, VT, USA) using the protocol described in Ramsey *et al* (2008). The apparatus used consisted of two chambers with distinguishable floor (grid floor *vs* rod floor). Experiments consisted of preconditioning, conditioning, and test phases. During preconditioning (d0) mice had free access to both chambers for 30 min; the time spent in both chambers was recorded. On the next day (d1), mice were injected i.p. with D-amphetamine (2 mg/kg or 5 mg/kg) or cocaine (20 mg/kg) and put into the less-preferred chamber for 30 min. On the following day (d2), saline was injected and mice were put into the other chamber for 30 min. This procedure was repeated two more times with alternating drug (d3, d5) and saline (d4, d6) injections. On the last day (d7), mice were put into the apparatus and allowed to access both chambers to test for conditioned place preference. The time spent in each chamber was recorded for 30 min.

In Vivo Microdialysis

Mice were anaesthetized using ketamine (100 mg/kg)/xylazine (10 mg/kg) and placed into a stereotactic frame (Stoelting). Concentric microdialysis probes (2-mm membrane length; cutoff 6000 Da; CMA-11, CMA/Microdialysis, Solna, Sweden) were inserted into the right dorsal striatum using the following coordinates (in mm) according to Franklin and Paxinos (2008): anterior-posterior: 0.0; lateral: −2.5; dorso–ventral: 4.4. A screw was inserted into the left hemisphere to stabilize subsequent fixation with dental cement. Twenty-four hours after surgery, freely moving animals were connected to a syringe pump and perfused with artificial cerebrospinal fluid (aCSF: 147 mM NaCl, 2.7 mM KCl, 1.2 mM $CaCl_2$, 0.85 mM $MgCl_2$; CMA/Microdialysis, Solna, Sweden). After a washout for 1 h, four 90-min fractions were collected in a low-perfusion mode (0.1 µl/min) in tubes containing 2 µl of 0.5 M perchloric acid to estimate the extracellular dopamine concentrations in the striatum (Gainetdinov *et al*, 2003).

Twenty-four hours after the low-perfusion mode, mice were again connected to the pump and perfusion was performed using 'conventional microdialysis' (1 µl/min) to measure the effect of D-amphetamine on DAT-mediated dopamine efflux in freely moving animals (Gainetdinov *et al*, 2003). After a 30-min washout, 6 × 20-min baseline fractions were collected. After that, mice were injected with saline and 6 × 20-min fractions were collected. Finally, mice were injected with 5 mg/kg D-amphetamine and 6 × 20-min fractions were collected. All these samples were collected in tubes containing 2 µl of 1 M perchloric acid

All dialysis samples were analyzed using reversed-phase high-performance liquid chromatography with electrochemical detection (HPLC–EC) to measure the levels of dopamine and its metabolites.

Neurochemical Measurement of Monoamine Tissue Levels

Striata were dissected and frozen in liquid nitrogen. For L-DOPA measurements, mice were injected with 100 mg/kg (i.p.) of 3-hydroxybenzylhydrazine (ND1015) 1 h before killing. Tissue was homogenized in 40 volumes of 0.1 M $HClO_4$, the homogenate was centrifuged at 10 000 *g* for 10 min and supernatants were filtered through 0.22 µm filters (Millipore Ultrafree-MC centrifugal filter units, 0.22 µm).

Analytical procedure. Measurements of dopamine and metabolites in collected microdialysis and tissue samples

were performed by HPLC with electrochemical detection (ALEXYS LC-EC system, Antec Leyden BV, the Netherlands) equipped with a reverse-phase column (3 µm particles, ALB-215 C18, 1 × 150 mm, Antec) at a flow rate of 200 µl/min and electrochemically detected by a 0.7-mm glass carbon electrode (Antec; VT-03). The mobile phase contained 50 mM H_3PO_4, 50 mM citric acid, 8 mM KCl, 0.1 mM EDTA, 400 mg/l octanesulfonic acid sodium salt, and 10% (vol/vol) methanol, pH 3.9. The sensitivity of the method permitted detection of ~3 fmol dopamine. All samples (11 µl) were injected into HPLC without any additional purification.

Fast-Scan Cyclic Voltammetry (FSCV)

Briefly, mice were anaesthetized with halothane and decapitated. The brain was sectioned in cold carboxygenated aCSF on a VT1000S vibrating microtome (Leica Microsystems, Nussloch, Germany) at a thickness of 300 µm. Coronal slices containing the dorsal striatum were allowed to recover for at least 1 h at room temperature in carboxygenated aCSF. For recordings, slices were superfused with 32 °C carboxygenated aCSF at a flow rate of 1 ml/min. Experimental recordings started 20 min after transfer to the slice chamber. Carbon fiber electrodes (5 µm; Goodfellow, Huntingdon, England) were made as previously described (Kuhr and Wightman, 1986). The electrodes were inserted ~100 µm into the dorsal striatal brain slice. The potential of the working electrode was held at −0.4 V *vs* Ag/AgCl between scan and was ramped to +1.3 V at 300 V/s and back at −0.4 V every 100 ms via an A-M system isolated pulse stimulator (Sequim, WA, USA). The triangular waveform was computer-controlled using HEKA EVA8 potensiostat (HEKA Elektronik Dr Schulze GmbH, Germany) and a ESA bioscience FSCV interface analog to digital converter) via TH-1 software (ESA biosciences, MA, USA). Axonal dopamine release in the dorsolateral striatum was evoked using a twisted bipolar-stimulating electrode (Plastics One, Roanoke, VA, USA). Stimulations were delivered every 2 min by a single electrical pulse (1 ms, 400 µs). Background-subtracted cyclic voltammograms were obtained by subtracting the current obtained before the stimulation from all recordings. The peak oxidation current for dopamine in each voltammogram was converted into a measure of the dopamine concentration by postcalibration of the electrode using 1 µM dopamine (Sigma Aldrich, Milan, Italy). Data were normalized to the first five recordings (10 min) of their respective control period and graphically plotted against time (means ± SEM). We reported in Figure 3b the dopamine concentration measured and we used normalized current in all the other graphs.

Statistics

The statistical significance of differences was evaluated using one-way ANOVA followed by Tukey's *post hoc* test or Student's *t*-test where appropriate. Data are shown as mean ± SEM.

RESULTS

αCaMKII-KO Mice Still Develop Conditioned Place Preference for Amphetamine

In its multimeric arrangement, individual αCaMKII moieties phosphorylate adjacent monomers and thus store information that encodes the magnitude of a preceding calcium signal. Accordingly, αCaMKII supports long-term potentiation and thus the initial steps required in the formation of some types of memory (Silva *et al*, 1992). The intrahippocampal injection of the CaMKII-inhibitor KN-93 impairs conditioned place preference (CPP) for D-amphetamine in rats (Tan, 2002). We therefore anticipated that CPP would be abrogated in αCaMKII-deficient mice. However, this was not the case. Regardless of whether D-amphetamine was administered at a dose of 2 mg/kg or 5 mg/kg, αCaMKII-deficient mice did not significantly differ from wild-type mice in their ability to recall the spatial memory associated with drug administration (Figure 1a and b). As a control, we determined CPP upon administration of the DAT-inhibitor cocaine (20 mg/kg): the rewarding properties of cocaine were similar in magnitude in wild type and αCaMKII-KO mice (Figure 1c). Taken together, these data show that αCaMKII is not required for the rewarding effect of amphetamine and cocaine as measured by CPP.

αCaMKII-KO Mice Display Increased Locomotor Activity and have Elevated Extracellular Dopamine Concentrations

A null mutation of unc-43, the *Caenorhabditis elegans* homolog of αCaMKII, causes hyperactivity in the nematode (Reiner *et al*, 1999). Hence, we recorded the baseline locomotor activities of αCaMKII-KO and wild-type mice to assess whether motor activity was affected by the loss of αCaMKII. Within the 1-h observation period in the OF, αCaMKII-KO mice covered a roughly threefold longer distance than wild-type mice (Figure 2a). Mice deficient in DAT (DAT-KO) have increased basal extracellular dopamine levels and are hyperactive (Giros *et al*, 1996; Jones *et al*, 1998). Accordingly, we also monitored locomotion of DAT-KO mice in the OF to gauge the magnitude of the effect seen in αCaMKII-KO mice. This control experiment indicated that the hyperactivity seen in αCaMKII-KO mice is approximately threefold less pronounced than that of DAT-KO mice (DAT-KO: 503 ± 71 m, $n = 5$; $p < 0.0001$). The hyperactivity of DAT-KO mice is accounted for by elevated extracellular dopamine levels (Giros *et al*, 1996). Hence, we measured striatal extracellular dopamine levels in αCaMKII-KO mice. Baseline extracellular dopamine concentrations are at low nanomolar range, but can be measured reliably by microdialysis employing a quantitative low-perfusion rate microdialysis (0.1 µl/min). Our results showed that basal dopamine levels were approximately twofold higher in the dorsal striatum of αCaMKII-KO mice compared with wild-type littermates (Figure 2b). In addition, the total tissue content of striatal dopamine was significantly increased in αCaMKII-KO mice (Figure 2c) without concomitant changes in the dopamine metabolites 3,4-dihydroxyphenylacetic acid and homovanillic acid (data not shown). At the same time, the dopamine turnover (DOPAC/dopamine ratio) was not altered (data not shown).

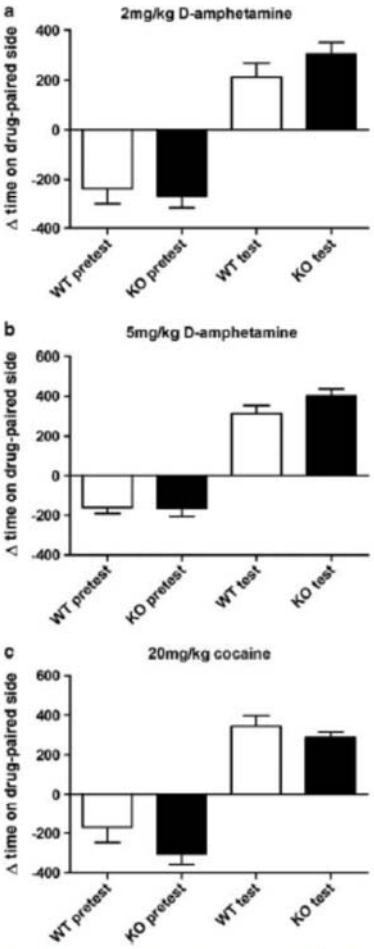


Figure 1 Conditioned place preference for 2 mg/kg (a) and 5 mg/kg (b) D-amphetamine and 20 mg/kg cocaine (c); (stimulants were administered in the less-preferred chamber): data are expressed as the time spent in the drug-associated compartment before (pretest) and after (test) D-amphetamine or cocaine treatment. D-amphetamine (2 mg/kg and 5 mg/kg) and cocaine induced significant place preference in both, wild type (WT) and knockout (KO) mice (paired two-tailed Student's *t*-test, $p<0.01$). Repeated measures two-way analysis of variance (ANOVA) revealed no significant treatment × genotype effects ($p<0.05$). (a) WT $n=7$, KO $n=13$, (b) WT $n=10$, KO $n=10$, (c) WT $n=7$, KO $n=5$.

The difference in dopamine levels may arise from a change in the dopamine synthesis rate in αCaMKII-KO mice. Accordingly, we blocked the second step in dopamine biosynthesis by pre-treating mice with 3-hydroxybenzylhydrazine (ND1015, 100 mg/kg i.p.), an inhibitor of L-aromatic amino acid decarboxylase, which readily permeates the blood-brain barrier. After 1 h, mice were killed, striata were harvested, and the levels of the dopamine precursor L-DOPA were determined as an index of the dopamine synthesis rate. L-DOPA levels were not significantly different between genotypes (αCaMKII-KO: 1.57 ± 0.14 ng/mg, WT: 1.24 ± 0.14 ng/mg; $n=7$; $p=0.1264$). In addition, we quantified the expression level of tyrosine hydroxylase by immunoblotting and found that the striata of wild type and αCaMKII-KO mice contained comparable amounts of the enzyme (Figure 2d).

In conclusion, our results indicate that the absence of αCaMKII was associated with increased extracellular and total levels of dopamine and pronounced hyperactivity.

Vesicular Dopamine Release is Increased in αCaMKII-KO Mice

An increase in extracellular dopamine can be accounted for by at least two mechanisms, which are not necessarily mutually exclusive: (i) a decrease in dopamine reuptake and/or (ii) increased vesicular release of dopamine. We previously showed that the uptake rate of the DAT substrate [^{3}H]1-methyl-4-phenylpyridinium ([^{3}H]MPP$^+$) was unchanged in striatal synaptosomes of αCaMKII-KO mice (Steinkellner *et al* 2012). [^{3}H]MPP$^+$ was used because it is more stable than dopamine and does not diffuse through the membrane. We ruled out subtle changes in handling different substrates by also carrying out uptake experiments in striatal synaptosomes of αCaMKII-KO and wild-type mice using [^{3}H]dopamine; the observations confirmed that uptake of the endogenous substrate was similar with respect to both, the maximal velocity of uptake (V_{max}) and the apparent affinity for dopamine (K_m) (Figure 3a). In line with unchanged dopamine uptake kinetics, we previously showed that DAT surface expression was not altered in the striatum of αCaMKII-KO mice (Steinkellner *et al*, 2012). Hence, reduced dopamine uptake is unlikely to account for higher basal levels of dopamine in αCaMKII-KO mice.

We also explored the alternative explanation, namely that vesicular release of dopamine was altered: striatal slices were prepared from αCaMKII-KO and wild-type mice subjected to a single electrical pulse of 400 μA and 1 ms duration. This manipulation induces dopamine release from vesicles of the readily releasable pool at the active zone of dopaminergic terminals. Dopamine released in response to the electrical pulse was measured by fast-scan cyclic voltammetry. The signals recorded in slices from αCaMKII-KO mice were consistently larger than that observed in those of wild-type mice, indicating that vesicular dopamine release was enhanced in αCaMKII-KO (Figure 3b and *c*).

The increase in vesicular dopamine release does not seem to be contingent on increased VMAT2 levels or elevated vesicular dopamine uptake: we measured both, total VMAT2 protein levels in the striatum and reserpine-sensitive dopamine uptake in purified striatal synaptic vesicles. Our results indicated that neither the VMAT2 total protein amount nor the vesicular uptake rate of [^{3}H]dopamine was significantly altered between wild type and αCaMKII-KO animals (Figure 3d and e).

Dopamine Receptors, Dopamine Signaling, and PSD-95 Protein Levels in αCaMKII-KO Mice

The findings summarized in Figures 2 and 3 *per se* suffice to account for the hyperactive phenotype of αCaMKII-KO mice, because (i) the increased dopamine tissue levels translate into (ii) enhanced vesicular dopamine release and thus drive locomotion. Elevated extracellular dopamine levels affect dopamine receptor-mediated signaling in DAT-KO mice. Therefore, we also examined the postsynaptic targets of dopamine in the striatum of αCaMKII-deficient mice, ie, dopamine D_1 and D_2 receptors as well as three prototypic dopamine-related signaling pathways (DARPP32, Akt, and ERK1/2; all of which are altered in DAT-KO mice). Furthermore, we examined the scaffolding protein PSD95 (postsynaptic density protein of 95 kD) which plays a key role in organizing signaling molecules on the postsynaptic membrane. Dopamine D_1 and D_2 receptor expression levels were quantified by binding of [^{3}H]SCH23390 and [^{3}H]raclopride to striatal membranes, respectively (Figure 3f and g). In contrast to what could have been expected with regard to the hyperdopaminergic DAT-KO mice, we did not observe any significant differences in the number of D_1 and D_2 receptors in striatal membranes of αCaMKII-KO and wild-type mice (Figure 3d and e). In line with the

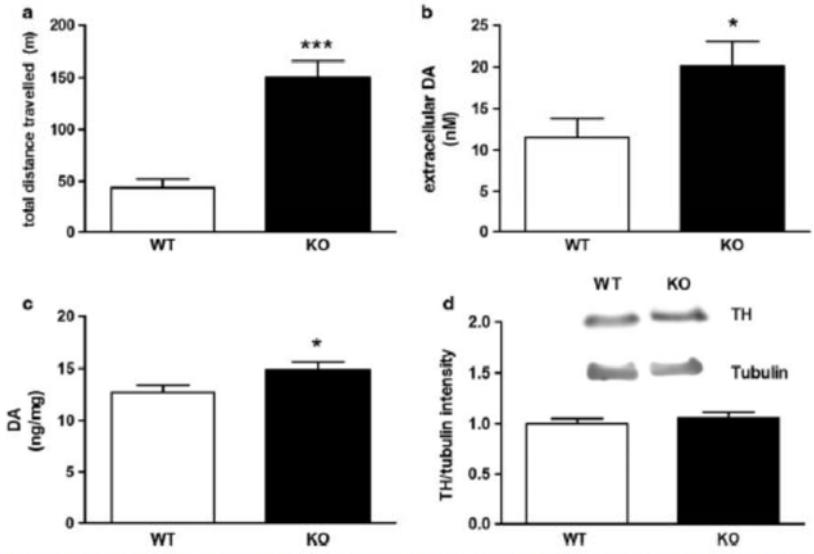
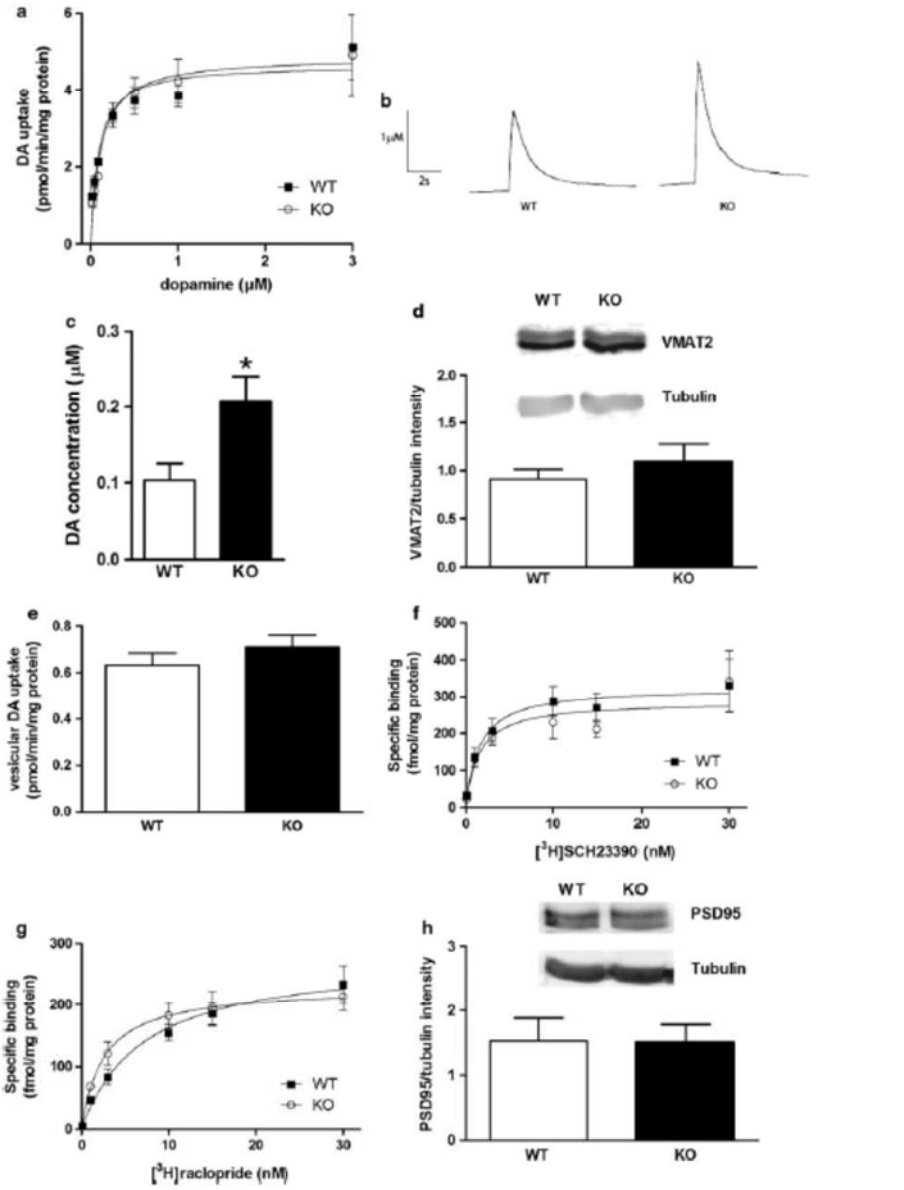


Figure 2 (a) Locomotor activity of wild type (WT) and knockout (KO) mice; horizontal distances were measured for 60 min in an open field: WT: 44.0 ± 8.3 m, $n = 13$; αCaMKII-KO: 150.6 ± 15.1 m, $n = 12$ (unpaired two-tailed Student's *t*-test; ***$p < 0.0001$). (b) Extracellular dopamine levels in WT and αCaMKII-KO mice: Dopamine concentrations in the right dorsal striatum were collected by quantitative low-perfusion microdialysis and analyzed by HPLC–EC. WT: 11.54 ± 2.31 nM, $n = 7$; αCaMKII-KO: 20.15 ± 2.92 nM, $n = 10$, unpaired Student's *t*-test; *$p < 0.05$). (c) Tissue levels of dopamine: freshly dissected dorsal striata were homogenized and extracted before measurement by HPLC ($n = 14$ per genotype; unpaired Student's *t*-test; *$p < 0.05$). (d) Densitometric analysis of western blots show no change in striatal TH expression between WT and KO animals; TH bands were normalized to tubulin; $n = 7$; Student's *t*-test, $p > 0.05$.

Figure 3 (a) [^{3}H]dopamine uptake kinetics in striatal synaptosomes of wild type (WT) and knockout (KO) animals: striatal synaptosomes were incubated with 0.1 μM [^{3}H]dopamine and increasing concentrations of unlabeled dopamine (0–3 μM) for 5 min at 37 °C. Non-specific uptake was determined in the presence of 1 μM GBR12909. K_d and B_{max} values are not significantly different (K_m: WT: 88.3 ± 30.7 nM, αCaMKII-KO: 111.2 ± 47.1 nM; V_{max}: WT: 4.66 ± 0.32 pmol/mg protein/min, αCaMKII-KO: 4.87 ± 0.45 pmol/mg protein/min; WT $n = 6$, KO $n = 5$; $p > 0.05$). (b) Fast-scan cyclic voltammetry (FSCV) was used to measure dopamine release from striatal brain slices. Representative traces in control and KO animal exemplify the higher peak height in mutants compared with control. (c) Average of stimulated dopamine release in dorsal striatum of control and KO animals show an increased evoked dopamine release in KO animals ($n = 6$; *$p < 0.05$, unpaired Student's *t*-test). (d) Densitometric analysis of VMAT2 protein levels in the striatum of WT and KO mice: bands were normalized to tubulin; $n = 9$–10; Student's *t*-test, $p > 0.05$ (e) Uptake of [^{3}H]dopamine in striatal synaptic vesicles: vesicles were incubated with 40 nM [^{3}H]dopamine for 10 min at 30 °C. Non-specific uptake was determined in the presence of 10 μM reserpine; $n = 3$; $p > 0.05$ (f) Dopamine D_1 receptor levels as assessed by [^{3}H]SCH23390 binding: WT $B_{max} = 326.6 \pm 33.2$ fmol/mg protein, KO $B_{max} = 287.1 \pm 32.9$ fmol/mg protein, K_d: WT $K_d = 1.55 \pm 0.76$ nM, KO $K_d = 1.50 \pm 0.81$ nM; WT $n = 4$, KO $n = 5$). (g) Dopamine D_2 receptor levels as assessed by [^{3}H]raclopride: WT $B_{max} = 280.2 \pm 30.0$ fmol/mg protein, $n = 6$; KO $B_{max} = 229.0 \pm 19.7$ fmol/mg protein, $n = 5$; WT $K_d = 7.18 \pm 2.15$ nM, KO $K_d = 2.55 \pm 0.84$ nM. K_d and B_{max} values are not significantly different; Student's *t*-test, $p > 0.05$. (h) Densitometric analysis of PSD-95 protein levels in the striatum of WT and KO mice: bands were normalized to tubulin; $n = 7$; Student's *t*-test, $p > 0.05$.

radioligand-binding results, we also found that there were no significant changes in pDARPP32, pAkt, pERK1, or pERK2 levels as measured by immunoblotting (pDARPP32/DARPP32 ratio: αCaMKII-KO: 1.23 ± 0.24, WT: 1.50 ± 0.26; $n = 3$; $p = 0.4856$; pAkt/Akt ratio: αCaMKII-KO: 1.43 ± 0.11, WT: 1.20 ± 0.12; $n = 6$–7; $p = 0.1875$; pERK1/ERK1 ratio:



αCaMKII-KO: 0.73 ± 0.07, WT: 0.88 ± 0.04; $n = 6$; $p = 0.1015$; pERK2/ERK2 ratio: αCaMKII-KO: 0.70 ± 0.14, WT: 0.97 ± 0.02; $n = 6$; $p = 0.0877$; Student's *t*-test).

Persistent elevations of extracellular dopamine (ie, as a result of exposing mice to cocaine or of ablating DAT) can also result in downregulation of striatal levels of PSD95 (Yao *et al*, 2004). Accordingly, we examined whether PSD95 was downregulated in αCaMKII-KO mice. However, quantitative immunoblotting for PSD95 from striatal extracts of WT and αCaMKII-KO mice did not reveal any differences between genotypes (Figure 3h).

Amphetamine-Induced Dopamine Efflux in αCaMKII-KO Mice

We previously reported that amphetamine-induced DAT-mediated substrate efflux was markedly attenuated in striatal synaptosomes and slices of αCaMKII-KO mice compared with wild-type littermate controls (Steinkellner *et al*, 2012). The decrease in substrate efflux is a functional consequence of ablated αCaMKII function rather than a result of a reduction of DAT surface expression (Steinkellner *et al*, 2012). We examined whether reverse transport by DAT was also blunted *in vivo* by implanting microdialysis probes into the dorsal striatum of these animals. This allowed to measure dopamine efflux after D-amphetamine administration (5 mg/kg, i.p.) to freely moving animals. These experiments confirmed that, in agreement with our previous *ex vivo* measurements, dopamine efflux was substantially decreased in αCaMKII-KO mice (Figure 4a). The fact that efflux was not completely abolished might be a result of other kinases involved in the modulation of reverse transport or compensatory changes during development, as the αCaMKII-KO mice used in the

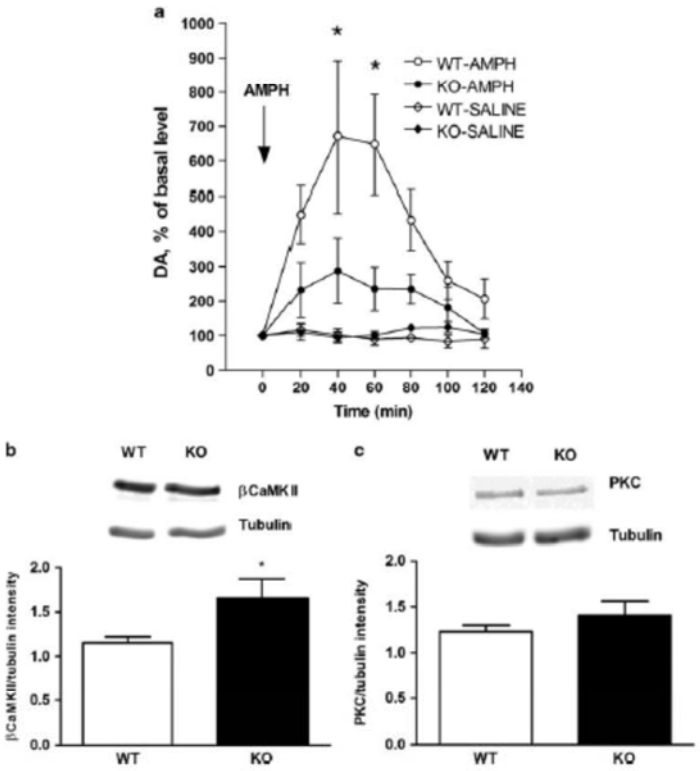


Figure 4 (a) D-amphetamine-induced dopamine release in the dorsal striatum of wild type (WT) and knockout (KO) animals *in vivo*: Microdialysis probes were inserted into the right dorsal striatum of WT and KO mice. Mice were allowed to recover for 24 h from the surgery before measurement of baseline dopamine concentrations. The day after, mice were injected with D-amphetamine (5 mg/kg i.p.) and dopamine dialysates were collected for 2 h. Amphetamine-induced dopamine release was normalized to baseline dopamine levels (see Figure 2 legend) and is presented as percentage of basal level. (b) Densitometric analysis of βCaMKII protein levels in the striatum of WT and KO mice: bands were normalized to tubulin; $n = 7$; Student's *t*-test, $^*p < 0.05$. (c) Densitometric analysis of PKC protein levels in the striatum of WT and KO mice: bands were normalized to tubulin; $n = 9$; Student's *t*-test, $p > 0.05$

experiments are global knockout mice. Hence, we also assessed whether the second most common CaMKII isoform, βCaMKII, was altered and might be able to compensate for the loss of αCaMKII. In fact, we previously reported that βCaMKII is part of the interactome of DAT (Steinkellner *et al*, 2012) and have now found that βCaMKII was upregulated in the striatum (Figure 4b). However, the presence of βCaMKII does not suffice to rescue the reduction in amphetamine-triggered efflux, as the global CaMKII-inhibitor KN-93 had no appreciable effect on amphetamine-triggered efflux in αCaMKII-KO mice as described in Steinkellner *et al* (2012). Additionally, we measured whether there were any changes in PKC, which is another Ca^{2+} sensitive protein kinase and has also been shown to modulate DAT-reverse transport. However, we did not observe any changes in PKC protein levels in the striatum of wild type and αCaMKII-KO mice (Figure 4c).

Amphetamine-Induced Locomotor Activity is Decreased in αCaMKII-KO Mice

Taken together, the findings suggested a cause-and-effect relation between the increase in steady-state extracellular dopamine levels, which were seen in the striata of αCaMKII-KO mice, and their increased locomotor activity. However, the amphetamine-induced rise in extracellular dopamine was blunted in these animals (Figure 4). It was therefore of interest to examine how αCaMKII-KO mice responded to an amphetamine challenge. We injected mice with D-amphetamine and measured the distances traveled within 60 min in the OF (Figure 5a). When administered at a dose of 2 mg/kg, D-amphetamine induced a comparable increase in locomotor activity in wild type and αCaMKII-KO mice (left hand set of bars in Figure 5a). When the dose of D-amphetamine was increased to 5 mg/kg, locomotion was substantially increased in wild-type mice. In contrast, there was no appreciable additional effect in the αCaMKII-KO mice (Figure 5a, right hand sets of bars). These observations are consistent with the conclusion that αCaMKII-dependent modulation of DAT is required to support the full-fledged acute behavioral effects of amphetamine.

Amphetamine Sensitization is Blunted in αCaMKII-KO Mice

The amphetamine-induced locomotor response is subject to sensitization, ie, repeated administration of amphetamine (or other psychostimulants including cocaine) results in an increase in the response (Steketee and Kalivas, 2011). This sensitization represents a long-lasting adaptation to the psychostimulant action and is triggered by the sequential activation of transcriptional programs (Nestler, 2005). We examined whether the blunted action of amphetamine sufficed to support the emergence of behavioral sensitization in the absence of αCaMKII: mice were injected once daily with 2 mg/kg D-amphetamine for six consecutive days, followed by a withdrawal period of 14 days and a challenge injection of 2 mg/kg D-amphetamine on day 21. This dose was chosen because wild type and αCaMKII-deficient mice did not differ in their acute response (Figure 5a). Behavioral sensitization readily developed in wild-type mice as is evident from the continuous increase in locomotor activity in the first 6 days of amphetamine treatment and the roughly fivefold increase in distance covered within 60 min upon rechallenge on day 21 (open bars in Figure 5b). In contrast, compared with the first day of treatment, αCaMKII-KO mice did not respond with any further increase in locomotor activity from day 4 on, when they had covered approximately twice the distance traveled on the first day of D-amphetamine treatment (closed bars in Figure 5b). In order to address a possible mechanistic basis for the impaired sensitization observed in the mutant mice, we investigated the transcription factor cAMP response element-binding protein (CREB), which is known to be induced after chronic exposure to addictive drugs and serves as a αCaMKII substrate (Nestler, 2005). Untreated αCaMKII-KO or wild-type mice did not differ in their basal amounts of phospho-CREB (pCREB) levels in the striatum (Figure 5c). However, we found that amphetamine sensitization did not induce an increase in pCREB levels in αCaMKII-KO, whereas it induced a significant increase in the WT (Figure 5d).

DISCUSSION

The current experiments demonstrate that the full-fledged effect of amphetamine *in vivo* is contingent on the presence of αCaMKII. This was predicted from our earlier experiments that had been conducted *in vitro* (Steinkellner *et al*, 2012). However, dopaminergic neurons project to three major brain areas (ie, the nucleus accumbens in the ventral striatum, the dorsal striatum, and the prefrontal cortex) that contribute to a different extent to the acute biological response, to the emergence of addiction and to psychotic symptoms resulting from long-term abuse. They also differ in the level of DAT expression. Accordingly, the present experiments were designed to explore which effect of amphetamine was most dependent on the presence of αCaMKII. Clearly, the absence of αCaMKII did not uniformly impair the responses elicited by amphetamine *in vivo*. It was, for instance, surprising to see that the rewarding properties of amphetamine requiring effective memory-related processes were not affected to any appreciable extent. Similarly, we found that cocaine still induced robust place preference in αCaMKII-KO animals. In contrast, the absence of αCaMKII resulted in a substantial suppression of behavioral sensitization to amphetamine. While some of these differences can be rationalized in hindsight, it is evident that this was not to be predicted *a priori*.

On a global level, αCaMKII has been implicated in synaptic plasticity (Colbran and Brown, 2004). This is, in part, accounted for by its role in shaping glutamatergic synapses in the brain (Baucum *et al*, 2013). Moreover, the establishment of addictive behavior has been hypothesized to depend in part on the activity of αCaMKII in both, animal models and people (Li *et al*, 2008). This conjecture is based on observations with several drugs of abuse such as cocaine (Pierce *et al*, 1998; Licata *et al*, 2004; Anderson *et al*, 2008), alcohol (Easton *et al*, 2013), or opioids (Lou *et al*, 1999). We used conditioned place preference (CPP) as a test to measure the addictive and rewarding potential of amphetamine and cocaine. Our findings show that the rewarding properties of amphetamine and cocaine were still preserved in αCaMKII-KO mice. This is in contrast to previous

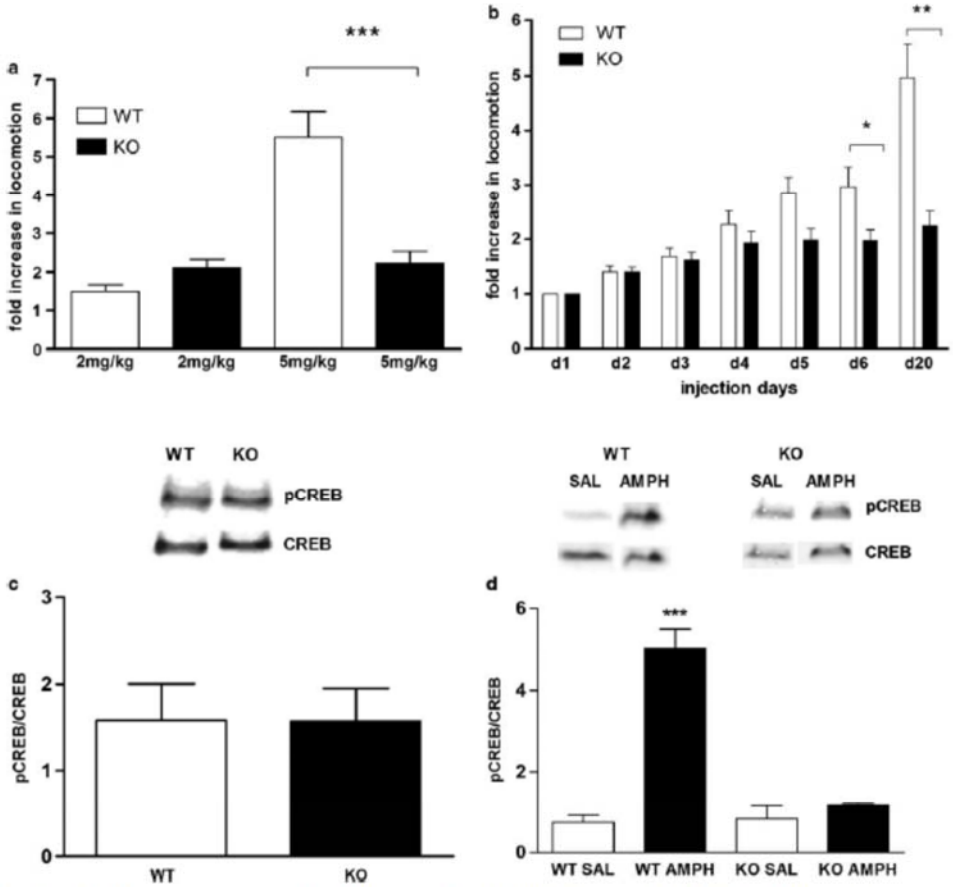


Figure 5 (a) Acute locomotor responses to D-amphetamine: mice were habituated to the open field for 60 min one day before they received an injection of D-amphetamine and subsequent recording of distances for another 60 min. Total distances traveled after D-amphetamine were normalized to baseline locomotion and are expressed as fold increase in locomotion. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test was used for statistical analysis; ***$p<0.0001$. (b) D-amphetamine sensitization: mice were habituated to open field chambers on day 0. On days 1–6, mice were injected with 2 mg/kg D-amphetamine once daily and distances traveled were recorded immediately after injection for 60 min. Mice were withdrawn from D-amphetamine for 14 days before they received an additional drug injection on day 20. The distances traveled are normalized to the first day (d1) of drug treatment; repeated measures two-way ANOVA revealed significant effects of genotype and treatment; *$p<0.05$, ***$p<0.0001$. (c) Densitometric analysis of pCREB levels in the striatum of untreated wild type (WT) and knockout (KO) mice: bands were normalized to total CREB; $n=6$–8; Student's t-test, $p>0.05$. (d) Densitometric analysis of pCREB protein levels in the striatum of amphetamine-sensitized or saline-pretreated WT and KO mice: bands were normalized to total CREB; $n=3$–4; one-way ANOVA followed by Tukey's multiple comparison test was used for statistical analysis; ***$p<0.0001$.

findings in rats, where intra-hippocampal injection of the CaMKII-blocker KN-93-attenuated amphetamine-induced CPP (Tan, 2002). However, it should be pointed out that CPP does not represent the most reliable measurement of rewarding and addictive properties of a drug. Furthermore, this discrepancy may reflect species differences or indicate that KN-93 inhibits ion channels and kinases other than αCaMKII. Irrespective of this unresolved issue, our observations support the conclusion that the interaction of αCaMKII and DAT does not play any major role in the rewarding properties of amphetamine (and cocaine). It should be noted that amphetamine (and cocaine) still demonstrate significant CPP in mice lacking the DAT (Budygin *et al*, 2004) and even cocaine self-administration

(Rocha *et al*, 1998). However, a different strain of DAT-KO mice clearly failed to acquire cocaine self-administration (Thomsen *et al*, 2009). Additionally, in mice with a cocaine-insensitive DAT, cocaine reward is lost (Chen *et al*, 2006). These observations indicate that DAT-related processes still seem to be most essential for the rewarding properties of these drugs. Potential compensatory and developmental changes in knockout mice have to be considered and certainly preclude definite conclusions. Besides, it has to be emphasized that CPP measures reward differently compared with self-administration: while CPP primarily measures the reinforcing effects of drugs, self-administration allows to discriminate between the reinforcing effects of a substance and the motivation to consume it. Hence, we cannot rule out that αCaMKII-KO mice would respond differently to amphetamine or cocaine self-administration. Regardless, small increases in psychostimulant-induced dopamine release might suffice to reach the threshold level required for the induction of reward-related behavior as measured by CPP. In fact, αCaMKII-KO mice still display dopamine efflux in response to amphetamine albeit significantly reduced compared with wild-type mice: we measured amphetamine-induced DAT-mediated dopamine efflux in the dorsal striatum by microdialysis and found that the amphetamine-induced dopamine efflux was significantly decreased in αCaMKII-KO animals. This is in accordance with our previous *in vitro* and *ex vivo* findings (Fog *et al*, 2006; Steinkellner *et al*, 2012).

Surprisingly, microdialysis also revealed that the extracellular dopamine concentrations are approximately twofold increased in the striatum of αCaMKII-KO mice and that these increased dopamine levels are the result of an elevated vesicular dopamine release without concomitant alterations in striatal VMAT2 protein levels or reserpine-sensitive VMAT2-mediated dopamine uptake into vesicles. The increase in vesicular dopamine release appears counterintuitive given that αCaMKII is the synapsin I-kinase, which defines the relative size of the reserve pool of neurotransmitters (Greengard *et al*, 1993). However, αCaMKII has also been shown to act as a bidirectional modulator in neurotransmitter release: it can both increase or decrease vesicular release (Chapman *et al*, 1995). In addition, the genetic ablation of αCaMKII in CA3 hippocampal neurons enhances stimulus-dependent vesicular glutamate release at the synaptic contact of their Schaffer collaterals with CA1 pyramidal neurons (Hinds *et al*, 2003). Thus, it was proposed that—apart from its role in mobilizing synaptic vesicles tethered to the cytoskeleton—αCaMKII can also have a nonenzymatic role and regulate the size of the readily releasable pool of vesicles at the active zone (Hojjati *et al*, 2007). In this model, αCaMKII limits this readily releasable pool; therefore its absence causes an increase in the number of vesicles at the active zone of αCaMKII-KO mice (Hojjati *et al*, 2007). The increased vesicular dopamine release that we observed in αCaMKII-KO mice is hence consistent with this expanded model of the role of αCaMKII in the presynaptic specialization. Besides, it is also in line with the finding that mesolimbic dopamine release is increased in a mouse model of Angelman syndrome, where αCaMKII is hyperphosphorylated and thereby rendered inactive (Riday *et al*, 2012).

The increased synaptic dopamine levels of αCaMKII-KO mice were accompanied by a profound hyperactivity. A similar result was obtained in *C. elegans*, where a null mutation of unc-43, a homolog of αCaMKII, caused hypermotility in the nematode (Reiner *et al*, 1999).

If αCaMKII-modulation of DAT were also important for the behavioral response to amphetamine, αCaMKII-KO animals ought to display a significantly reduced locomotor stimulation to an acute D-amphetamine injection. This prediction was verified: D-amphetamine (5 mg/kg) stimulated locomotion of wild-type mice by almost sixfold. In contrast, locomotion of αCaMKII-KO animals only doubled in response to amphetamine, consistent with the finding that amphetamine was still able to induce DAT-mediated dopamine efflux albeit to a much lesser extent than in wild-type mice. The difference in the locomotor response of αCaMKII-KO and wild-type mice provides incontrovertible evidence that αCaMKII is a modulator of DAT-reverse transport: αCaMKII favors a conformation of the transporter that is willing to efflux (Robertson *et al*, 2009). This conclusion is also supported by recently published analogous observations made in *Drosophila melanogaster* (Pizzo *et al*, 2014).

Repeated administration of amphetamine results in behavioral sensitization of locomotor responses in rodents (Steketee and Kalivas, 2011). Sensitization is also important for the emergence of addiction to psychostimulants; the underlying reprogramming of synaptic connections is orchestrated by the sequential activation of transcription factors, which creates a long-lasting memory of repeated drug exposure (Nestler, 2005). Our observations show that, in the absence of αCaMKII, sensitization to amphetamine is substantially impaired. This may arise from the reduced ability of amphetamine to cause dopamine release in mice lacking αCaMKII and to thus trigger a sustained neuronal activation, which eventually results in long-lasting synaptic facilitation. Presumably, the lack of αCaMKII limits the increase in dopamine release with repeated exposure, after a point, and therefore accounts for the reduced sensitization.

Additionally, the absence of αCaMKII may impair the emergence of a sensitized state, because αCaMKII is required for the full-fledged activation of the transcriptional program required for memory formation. The latter is supported by the finding that αCaMKII-KO mice displayed no increase in pCREB levels after amphetamine sensitization, whereas wild-type mice showed a significant increase in pCREB levels. At the present stage, it is impossible to differentiate between these two possibilities, but most likely a combination of both, a decreased dopamine efflux in response to amphetamine and an impaired αCaMKII-mediated signaling, contribute to the effects observed during amphetamine sensitization in αCaMKII-deficient mice.

Regardless of the underlying mechanism, these experiments further highlight the importance of αCaMKII in supporting the actions of amphetamine *in vivo*. Our experiments also underline that the relative contribution of αCaMKII to the behavioral actions of amphetamines might depend on the expression levels of DAT. They seem to be more pronounced in the striatum, where DAT expression levels are higher than in the nucleus accumbens, where DAT expression levels are low. Experiments looking more carefully into regional and molecular differences between the DAT/CaMKII interaction in those regions are currently being explored in our laboratories.

In spite of this inherent limitation of our approach, it is attractive to speculate that subtle variations in the relative expression levels of DAT and of αCaMKII may contribute to inter-individual differences in the susceptibility to amphetamine addiction.

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Author Contributions

MW and HHS designed the project, MW, TDS, OK, UG, MF, DDP, RRG, and HHS supervised the project, analyzed data, TS, MF, and HHS wrote the first draft of the manuscript. TS, BE, AC, LK, MR, GS, and EK designed and conducted all biochemical and behavioral pharmacology assays; LM, DL, EVE, TDS, and RRG designed and performed microdialysis and FSCV experiments and analyzed post-mortem tissue concentrations of monoamines and their metabolites. All authors contributed significantly to the writing of the final version of the article.

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