CaMKII is activated in opioid induced conditioned place preference, but αCaMKII Thr286 autophosphorylation is not necessary for its establishment

Jannike M. Andersen, Siri H. Opdal, Christian P. Müller, Fernando Boix

Section for Drug Abuse Research, Department of Forensic Sciences, Oslo University Hospital, Norway
Section for Pediatric Forensic Medicine, Department of Forensic Sciences, Oslo University Hospital, Oslo, Norway
Department of Psychiatry and Psychotherapy, University Hospital, Friedrich-Alexander-University Erlangen-Nuremberg, Erlangen, Germany

GRAPHICAL ABSTRACT

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ABSTRACT

Activation of calcium/calmodulin-dependent protein kinase II (CaMKII), particularly its α isoform, is known to be important for neuronal processes central for learning and memory and has also been implicated in the maladaptive learning involved in drug addiction. Thr286 autophosphorylation of αCaMKII has been shown to be indispensable for establishment of cocaine-induced CPP (Easton et al., 2014). To study the contribution of CaMKII in opioid induced conditioned learning, we examined how establishment of conditioned place preference

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Activation of calcium/calmodulin-dependent protein kinase II (CaMKII), particularly its α isoform, is known to be important for neuronal processes central for learning and memory and has also been implicated in the maladaptive learning involved in drug addiction. Thr286 autophosphorylation of αCaMKII has been shown to be indispensable for establishment of cocaine-induced CPP (Easton et al., 2014). To study the contribution of CaMKII in opioid induced conditioned learning, we examined how establishment of conditioned place preference

Abbreviations: CaMKII, calcium/calmodulin-dependent protein kinase II; CPP, Conditioned Place Preference; M6G, morphine-6-glucuronide; PCR, polymerase chain reaction; TBS, tris-buffered saline; WT, wild type

* Corresponding author.
E-mail address: fernando.boix@ous-hf.no (F. Boix).

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CaMKII

(CPP) induced by 10 or 30 \( \mu \text{mol/kg} \) morphine or its active metabolite morphine-6-glucuronide (M6G) affects the levels and Thr286 autophosphorylation of the \( \alpha \)- and \( \beta \)-isoforms of CaMKII, as well as \( \beta \)-actin levels, in dorsal and ventral striatum and hippocampus of mice. An acute and a sub-chronic treatment were used as controls. Whereas an acute single administration of morphine or M6G caused increases in CaMKII levels and phosphorylation at Thr286 and \( \beta \)-actin in striatal areas, CPP induced by these opioids was accompanied primarily by an increase in the protein levels of both CaMKII isoforms and \( \beta \)-actin in dorsal striatum and hippocampus. Decreases in CaMKII Thr286 phosphorylation were observed in dorsal striatum after the sub-chronic pharmacological treatment. Despite the changes observed in CaMKII activity in wild type mice, morphine-induced CPP was not affected in \( \alpha \text{CaMKII}^{T286A} \)-autophosphorylation-deficient mice. These results indicate that opioid-induced CPP is accompanied by activation of \( \alpha \)- and \( \beta \text{CaMKII} \) in striatum and hippocampus, but, in opposition to what has been observed with cocaine, \( \alpha \text{CaMKII} \) autophosphorylation is not essential for establishment of opioid-induced CPP.

1. Introduction

The \( \alpha \)- and \( \beta \)-isoforms of calcium/calmodulin-dependent protein kinase II (CaMKII) are richly present at the postsynaptic density [1], where \( \alpha \text{CaMKII} \) is activated by intraneuronal Ca\(^{2+} \) through binding to calmodulin [2]. CaMKII is also able to interact with actin and alter its bundling and assembly [3], and thereby to affect the structure of synaptic spines [4]. Maintained activation of \( \alpha \text{CaMKII} \), due to autophosphorylation at Thr286, has been shown to play a key role in hippocampal long-term potentiation, synaptic plasticity and, hence, in learning and memory processes in animals [5] and humans [6].

Drug abuse and addiction is considered to involve a maladaptive form of learning and memory [7,8], where a psychoactive drug is strongly paired with environmental stimuli by recruiting brain circuits involved in reinforcement learning [9]. Conditioned place preference (CPP) and drug self-administration, the most applied animal models for the study of drug abuse, are essentially based on this concept [10,11]. This form of learning is provided by long term changes in the activity of striatal neurons resulting from the interaction between mesolimbic dopamine and cortical glutamate inputs, combining reward-related signalling with cortical information [12]. Accumulated evidence suggests that CaMKII is an important component of the synaptic plasticity processes leading to the long term changes in neuronal activity after exposure to psychoactive drugs of abuse [13]. CaMKII becomes overexpressed and/or phosphorylated after exposure to alcohol [14] and psychostimulant drugs [15,16]. Actually, the activation and \( \alpha \text{CaMKII} \) autophosphorylation at Thr286 have been shown to be critical for the regular establishment of addiction-related behaviours, at least for psychostimulants and alcohol [17,18]. Less is known about the role CaMKII activity plays in the formation of opioid related memories, and thus opioid addiction [13], even though recent evidence suggests its involvement in opioid abuse in humans [19].

From this evidence, it can be postulated that CPP induced by opioids should be accompanied by CaMKII activation, particularly \( \alpha \text{CaMKII} \) which should be indispensable for the acquisition of CPP. In order to test this hypothesis, we have examined if CaMKII levels and its phosphorylation at Thr286 (Thr287 for \( \beta \text{CaMKII} \)) were increased in the dorsal and ventral striatum after CPP induced by the opioids morphine or morphine-6-glucuronide (M6G), or after an acute or sub-chronic administration of these opioids. Changes were also analysed in the hippocampus, a brain area linked to learning and memory [20]. M6G is an active morphine metabolite which, similar to morphine, has analgesic properties [21] and abuse potential in animal models [22,23]. In order to explore if Thr286 phosphorylation of \( \alpha \text{CaMKII} \) is necessary for the establishment of CPP produced by opioids, morphine-induced CPP was also studied in \( \alpha \text{CaMKII} \) autophosphorylation deficient mice (\( \alpha \text{CaMKII}^{T286A} \), [24]).

2. Material and methods

2.1. General

2.1.1. Animals

Male C57BL/6 JOlalHsd (Harlan Sprague Dawley Inc – currently Envigo, Horst, Netherlands) or C57BL/6 JBomTac mice (Taconic Biosciences, Bomholt, Denmark), about 9 weeks old upon arrival, were used in experiment 1 and experiments 2 and 3, respectively. The animals were housed in transparent polycarbonate 1290D Eurostandard Type III cages (5 animals per cage), including a red mouse house (both Techniplast, 21020 Buguggiate, Italy), under standard conditions (lights on 08:00 to 20:00; food and water ad libitum) in the animal facility for at least one week before each experimental block.

Male \( \alpha \text{CaMKII}^{T286A} \) gene modified mice were bred in our facilities from three heterozygous breeding pairs from Paco Herson lab at the Department of Pharmacology (University of Colorado Denver, USA, [25]). After genotyping, the animals were housed individually in PET Innocage cages with a plastic enrichment dome and wheel (all from Innovive, San Diego CA, USA). In the homozygous mutants (MT) the autophosphorylation of \( \alpha \text{CaMKII} \) is prevented due to the insertion of a missense mutation (threonine-286 changed to alanine; T286A) within the autoinhibitory domain. Mutants were generated using a gene-targeting strategy which utilizes a replacement vector containing the point mutation and a neo gene flanked by loxp sites; after homologous recombination the neo gene was removed by Cre recombination. R1 ES cells (F1 between 129/Sv and 129/Sv-Cp) were used. The mice were subsequently backcrossed for at least eight generations into C57BL/6 J and then crossed once with 129S2/SvHsd mice and kept in this mixed background by interbreeding [24]. The mice used in the experiment were obtained from interbreeding of heterozygotes and genotypes were determined by PCR as described in the section “Genotyping” in Material and methods.

The cages with the animals were kept in a ventilated Scantainer Classic cabinet coupled to a ScanClime Basic (both from Scanbur, Karlslunde, Denmark) for air handling and humidity control. The animals were arbitrarily selected and sequentially allocated to each treatment group. Due to the limited capacity of the testing equipment, up to 10 animals (with equal representation for each treatment group) were tested in a week period. The place and context of the injection procedure was identical for the animals in all the experiments. All the experiments were conducted in accordance with the Norwegian Animal Welfare Act and the protocols approved by the Norwegian National Animal Research Authority (FOTS id 2451, 2846, 4716, and 7512).

2.1.2. Drugs

Morphine-HCl (Norsk Medisinaldepot AS, Oslo, Norway) and morphine-6-ß-D-glucuronide hydrate (R3M-57-HY, Lipomed, Arlesheim, Switzerland) were dissolved in 0.9% saline. The doses used were 10 or 30 \( \mu \text{mol/kg} \) for both drugs, corresponding to 3.8 or 11.3 mg/kg for morphine and for 4.6 or 13.8 mg/kg for M6G, respectively. All injections were administered subcutaneously (sc) in a volume of 0.01 mL/gram.
mouse.

2.2. Experimental protocols

A graphical timeline of each experiment is represented in Fig. 1.

2.2.1. Changes in proteins triggered by CPP

In order to study how CaMKII levels and autophosphorylation were affected by the establishment of opioid-induced CPP, the effect of morphine or M6G administration was tested in three different experimental conditions:

2.2.1.1. Experiment 1. Acute administration

In order to test if a single administration of the opioids was able to induce changes in the proteins of interest, fifty C57BL/6 J mice (ten animals in each experimental group) were acutely injected subcutaneously (sc) either with 0.9% saline, or 10 or 30 μmol/kg morphine or M6G. The animals were returned to their home cage immediately after administration and subsequently to the cabinet. Thirty minutes later, the animals were sacrificed by cervical dislocation, the brain extracted, and samples from ventral and dorsal striatum and from hippocampus taken for later protein analysis with western blotting. The purpose was to explore if an acute administration was capable to engage the neuronal mechanisms assumed to be associated with the acquisition of CPP induced by the opioids.

2.2.1.2. Experiment 2. CPP

To analyse the effects of opioid-induced CPP on CamKII, forty mice (eight animals in each experimental group) were submitted to the CPP procedure as described below. At the end of the test session on day four, the animals were sacrificed by cervical dislocation, the brain extracted, and samples from ventral and dorsal striatum and from hippocampus taken for later protein analysis with western blotting. Two animals (one for the 10 and one for the 30 μg/kg morphine) were excluded due to questionable health status.

2.2.1.3. Experiment 3. Sub-chronic administration

The purpose of this experiment was to examine if the neurochemical changes observed in experiment 2 were due to the repeated administration of the drugs independently of the CPP procedure. With this aim, forty mice (eight animals in each experimental group) were subjected to the same

![Graphical time-line of the experimental designs.](image-url)
pharmacological treatment as in experiment 2 but without being exposed to the CPP apparatus. During three consecutive days, animals were injected daily with a sc administration of the experimental drug (0.9% saline, or 10 or 30 μmol/kg morphine or 10 or 30 μmol/kg M6G) in the morning (8:00 am) and with 0.9% saline in the afternoon (2:00 pm). Following each administration, the animals were returned to their home cages and to the cabinet thereafter. On the fourth day, animals were injected sc with 0.9% saline and returned to their home cages. After 20 min, the animals were sacrificed by cervical dislocation, the brain extracted, and samples from ventral and dorsal striatum and from hippocampus taken for later protein analysis with western blotting.

2.2. CPP in autophosphorylation-deficient αCaMKII<sup>T286A</sup> mice

To test if αCaMKII phosphorylation at T286 blocks its autophosphorylation without affecting the Ca<sup>2+</sup>-dependent activity [24]. For conditioning, mice received 0.9% saline, or 10 or 30 μmol/kg morphine sc.

2.3. Experimental methods

2.3.1. Conditioned place preference

The 40 × 40 cm cages used for the CPP procedure were divided into two distinct compartments connected by an opening in a wall at the centre of the box. One of the compartments had white walls with vertical, 1.2 cm wide black stripes and a wrinkled plastic plate as its floor; the other compartment had black walls with horizontal, 1.2 cm white stripes and a metal plate with holes (4 mm Ø) as the floor. The position of the animals in the cage was registered by infrared beams spaced 2.5 cm and situated at floor level. The sensors were connected to a Versamix animal activity monitoring system (AccuScan Instruments Inc., Columbus, USA).

The CPP protocol involved three consecutive days of conditioning sessions and a test session one day later. During three days, mice received a sc administration of the experimental drug (0.9% saline, or 10 or 30 μmol/kg morphine, or 10 or 30 μmol/kg M6G) in the morning (8:00 am) and then immediately carried to a room next door and placed into the conditioned compartment. In the afternoon (2:00 pm), the procedure was repeated but the mice received a sc injection of 0.9% saline and were placed into the other compartment (unconditioned compartment) of the same cage. The connection between the compartments was closed during the conditioning sessions, and the animal confined to the corresponding compartment. Each session lasted for 20 min and afterwards the animals were returned to their home cages. Drug-naïve mice did not show any significant preference for any of the compartments in previous tests. Half of the animals in each experimental group were conditioned in one of the two compartments, which were randomly allocated. In the morning (8:00 am) of the fourth day, and after a sc administration of 0.9% saline, the animals were placed in the open connection between the compartments with free access to both compartments. During the 20 min test session, the time spent in each compartment was automatically registered. In order to reduce the possible interference from the injection and enhanced exploratory behaviour during the first minutes, only the last 15 min was used as a measure of place preference.

2.3.2. Western blotting

Immediately after extraction of the brain, a 2 mm slice was cut frontal from the chiasma opticus with the help of an Alto acrylic brain matrix (#68-1175-1, CellPoint Scientific, Gaithersburg, MD, USA), and two punches (0.5 mm Ø each) were taken from the ventral (comprising mainly the Nucleus accumbens) and from the dorsal striatum, one from each hemisphere. For each striatal area, the punches from both hemispheres were collected in the same vial and processed together. Afterwards, the right hippocampus was rapidly dissected. The left hippocampus was also dissected and later used as internal standard for the western blotting (see below). The sampling procedure, which took about three minutes from sacrifice to removal of the hippocampus, was carried out on ice. The samples obtained were immediately frozen in liquid nitrogen and stored at −80 °C.

After thawing on ice, each sample was homogenised using a Virstonic 300 sonicator (#175893, Virtis, Gardiner, NY, USA) in 300 μl (for right hippocampus samples) or 120 μl (for samples from the dorsal striatum and nucleus accumbens) ice-cold 0.32 M sucrose containing 10 μg/ml leupeptin, 10 μg/ml aprotinin, 5 μg/ml pepstatin A, and 0.5 mM phenylmethylsulfonyl fluoride. The final protein concentrations were 4.18 ± 0.18 μg/μl for hippocampus, 1.38 ± 0.07 μg/μl for dorsal, and 1.38 ± 0.06 μg/μl for ventral striatum samples. After homogenization, each sample was divided in 50 μl aliquots and stored at −80 °C. The samples from the left hippocampus were also homogenised in 300 μl of the sucrose solution, and all the samples from the different treatment groups in each of the experiments combined as one, stirred, and divided in 50 μl aliquots which were later used as internal standard controls for each blot.

Loading buffer was added to each homogenate (final concentrations: 3% sodium dodecyl sulphate, 5% glycerol, 62.5 mM Tris/HCl, pH 6.9, 0.1% bromophenol blue and 6% 2-mercaptoethanol), and incubated at 100 °C (5 min). Samples were separated in sodium dodecyl sulphate-polyacrylamide SDS gels (self-prepared 3% stacking/12% separating gel, or Criterion TGX, 12%, 18 wells (#5671044, Bio-Rad Laboratories, Inc, Hercules, California, USA)) by electrophoresis at 200 V (#1645052 PowerPac HC 300 W, Bio-Rad Laboratories, Inc, Hercules, California, USA). Lines were filled with 20 μl of sample from dorsal or ventral striatum or 25 μl of sample from hippocampus, independent of protein content in the samples. Subsequently, samples were electrophoretically transferred to a nitrocellulose membrane. Transfer was confirmed by Ponceau S staining. The proteins of interest were consecutively analysed using antibodies against c-Fos (1:1000, #4384), CaMKII (1:2000, #3362), CaMKII (1:2000, #3361; all three from Cell Signaling Technology Inc., Danvers, MA, USA), and β-actin (1:200000, #A 5316, Sigma-Aldrich Inc., St. Louis, MO, USA). For each protein assay, the membranes were incubated (1 h, room temperature) in blocking buffer (Tris-buffered saline containing 0.05% Tween 20 (TBS-T) and 3% low-fat dry milk for c-Fos or 5% BSA for the other antibodies) and probed with the corresponding primary antibody (2 h at room temperature or overnight at 4 °C) diluted in blocking buffer. Thereafter, the blots were washed in TBS-T (4 × 5 min), and incubated for 2 h at room temperature with polyclonal HRP-conjugated secondary antibody. After washing (3 × 5 min TBS-T and 1 × 5 min TBS), the blots were exposed to SuperSignal West Dura Stable Peroxide Buffer; 1:1, Thermo Scientific, Waltham, MA, USA), and visualised by a ChemiDoc XRS imager (BioRad, Hercules, CA, USA). The strips were stripped with Reblot Plus Mild Antibody Stripping Solution (#2502, Millipore, Billerica, MA, USA) and proceeded again with the next primary antibody. Protein concentrations were assayed by the method of Lowry [26] in aliquots not used for western blotting.

Each blot contained five samples corresponding to one of the dissected areas and each of the five experimental groups matching animals tested and/or sampled at the same time. In addition, three lanes were occupied by samples from the combined left hippocampus (diluted 50% with sucrose solution in the blots containing dorsal or ventral striatal samples to compensate for the different tissue content), which were used as internal standard controls. The luminescence from each of the five experimental samples on each blot was standardised by relating its intensity to the average of the intensity of the three quality control hippocampus bands in the blot, which were treated as internal standards. A later analysis of the data showed differences in β-actin levels between the treatment and control groups that were not related to protein content in the samples, as measured by the Lowry method. It
has already been shown that β-actin, or other “housekeeping” proteins, can be affected by the experimental conditions and therefore are not suitable as loading control [27,28]. Besides, correlations between β-actin and the other proteins measured did not show any consistent statistical relationship, neither at the level of all the samples nor at group level, excluding a possible direct association between the differences observed in β-actin and the differences obtained in the other proteins. Consequently, β-actin was considered not suitable as a loading control, as was the initial purpose. Instead, the results were normalised by dividing the standardised luminescence of each experimental sample by its protein content (µg/µl) measured by the Lowry method, which was used as proxy for loading control since the loading volume was the same for each line in the blots and not corrected for protein content in the sample. The western blotting procedure was carried out by a person unaware of the treatments.

The antibody against c-Fos detected prominently two bands around 47 and 58 kDa, in the molecular weight range known for c-Fos [29], and total c-Fos, as the sum of both bands, is presented. The antibodies for CaMKII and its phosphorylated form detected two bands around 50 and 60 kDa, corresponding to the α- and β-isoforms, respectively. The antibody for β-actin exposed a band at 40–42 kDa (Supplementary Fig. 1).

2.3.3. Genotyping

The T286A mutation in the αCaMKII gene was analysed using the Thermo Scientific Phire Animal Tissue Direct PCR Kit (#F14005, Thermo Fisher Scientific, Waltham MA, USA), with DNA extraction followed by polymerase chain reaction (PCR), according to the manufacturer’s instructions.

For DNA extraction, a small fragment of the outer ear (1 × 1 mm) was covered in 20 µL Dilution buffer and 0.5 µL DNARelease Additive, followed by 5 min incubation at room temperature and 2 min incubation at 95 °C. The remaining tissue was spun down, and the supernatant used as template in the following PCR reaction.

The PCR reaction mix consisted of 7.6 µL H₂O, 10 µL 2x Phire Animal Tissue PCR buffer, 0.5 µL of each primer, 0.4 µL Phire Hot Start II DNA Polymerase, and 1 µL template. The Primer sequences were 5′-CTGTACCAGCAGATCAAAGC and 5′-ATCAGGACCATGTTGTGTC. The temperature profile for the PCR reaction was initial denaturation at 95 °C for 5 min, followed by 21 cycles with 95 °C for 5 s, 65 °C -0.5 °C per cycle for 5 s, 72 °C for 20 s, followed by 21 cycles of 95 °C for 5 s, 55 °C for 5 s, and 72 °C for 20 s.

The amplified products were detected by gel electrophoresis on 2% agarose gels in the presence of GelRed (#4100, Biotium, Freemont CA, USA). The DNA bands were visualised under ultraviolet light. In this system, the fragment corresponding to 286T was 194 bp long, while the fragment corresponding to 286A was 230 bp long [24].

2.4. Data analysis

In order to assess the establishment of statistically significant CPP, the time spent in the conditioned compartment during the last 15 min of the test session was compared against the time in the unconditioned compartment for each experimental group using a paired Wilcoxon Signed Ranks Test. To simplify the presentation, the differences in sojourn times between the conditioned and unconditioned compartments are presented in the figures.

To facilitate the comparison between the different proteins, brain areas, and experimental conditions, the results from western blotting are presented as the normalised luminescence of each data value divided by the normalised luminescence of the saline control value of the corresponding blot. All extreme values (more than 3xIQR) in each group were eliminated as suspects for technical inaccuracies. These results were analysed using a generalized linear mixed model with drug (saline vs opioid), drug type (saline vs morphine or M6G) or treatment (saline vs morphine 10 µmol/kg, morphine 30 µmol/kg, M6G 10 µmol/kg, or M6G 30 µmol/kg) as fixed effects and blot as subject in a cluster design modelling the random effects between blots [30]. This statistical procedure takes into account the correlation due to subject bundling (clustering) both during testing, sampling and blotting. Further, in comparison to traditional analysis of variance, linear mixed models do not require normality of data (Kolmogorov-Smirnov tests showed that a large number of variables did not fit normality) and is not compromised by the presence of missing data [31].

The IBM SPSS Statistics v.23 package (IBM Corp, Armonk, NY, USA) was used for statistical analysis. A p ≤ 0.05 is considered as statistically significant, but 0.1 < p < 0.05 are also shown in the Figs. 3–8.

3. Results

3.1. Changes in proteins triggered by CPP

3.1.1. Conditioned place preference

Unlike the saline control group, the animals treated with 10 or 30 µmol/kg morphine or M6G subjected to the CPP procedure showed a statistically significant preference for the conditioned compartment during the CPP test session (Fig. 2). There was no statistically significant locomotor sensitization, defined as increased locomotor activity through the conditioning sessions (data not shown).

3.1.2. Changes in cFos, CaMKII, and β-Actin

The levels of total c-Fos protein (Fig. 3) were elevated in dorsal (p = 0.039) and ventral (p = 0.043) striatum after the acute administration of morphine or M6G, but not in hippocampus. In the animals exposed to the CPP procedure, total c-Fos was generally elevated in dorsal striatum (p = 0.014) and hippocampus (p = 0.032), whereas in the ventral striatum only the animals administered with M6G showed statistically significant higher levels of total cFos. No statistically significant differences were observed in both striatal areas or in hippocampus after the sub-chronic administration.

The levels of αCaMKII (Fig. 4) were generally increased in the two striatal areas (p = 0.045 and p = 0.001), but not in hippocampus after the acute administration of the opioids. The animals treated with M6G
showed a statistically significant increase in the dorsal and ventral striatum, whereas the morphine treated animals showed statistically significantly higher levels also in the ventral striatum. The CPP procedure triggered a statistically significant increase in the dorsal striatum and the hippocampus, but not in ventral striatum, of the animals conditioned with morphine or M6G. No noteworthy changes were observed after the sub-chronic treatment.

Phosphorylation of αCaMKII at Thr286 was statistically significantly increased in dorsal (p = 0.053) and ventral (p = 0.038) striatum (Fig. 5) after the acute injection of the opioids, being statistically significant in dorsal striatum for the animals receiving M6G and in ventral striatum for the animals receiving morphine. No statistically significant differences were observed in the hippocampus. After the CPP procedure, no differences were observed in striatum or hippocampus, but the groups treated with morphine showed a tendency (p < 0.1) to a higher phosphorylation of αCaMKII in ventral striatum and hippocampus. A general decrease in αCaMKII phosphorylation was observed after the sub-chronic treatment which was statistically significant (p = 0.028) in dorsal striatum. Indeed, the three regions studied presented lower levels of phosphorylated αCaMKII after treatment, particularly in the animals treated with 30 μmol M6G.

βCaMKII levels (Fig. 6) were significantly (p = 0.004) increased in the ventral striatum after the acute administration of both opioids, but no differences were observed in the other areas, except a trend (p = 0.092) to higher levels in dorsal striatum, particularly after M6G which was statistically significant. After the CPP procedure, the levels of this

Fig. 3. Levels of total c-Fos, standardised to the saline control of the corresponding blot (mean + s.e.m., arbitrary units), in samples from dorsal and ventral striatum, and hippocampus from mice taken 30 min after a single administration (Acute), after the test session of the CPP procedure, or after the last drug administration following the CPP pharmacological treatment protocol (Sub-chronic). Mice were treated with saline (black bars), or 10 (hatched bars) or 30 (no hatched bars) μmol/kg morphine (grey bars) or M6G (white bars). * 0.05 < p ≤ 0.1 drug type (saline vs morphine or M6G), linear mixed models; # p ≤ 0.05 drug type (saline vs morphine or M6G), linear mixed models; + p ≤ 0.05 against saline, linear mixed models. p = 0.XXX at the up left corner shows significance of fixed effects drug factor (saline vs opioid), linear mixed models.
protein were statistically significant higher ($p = 0.022$) in the dorsal striatum, particularly after M6G conditioning, and in the hippocampus ($p = 0.019$), specifically in the morphine conditioned animals. In the ventral striatum, only the animals conditioned with M6G showed statistically significant increases in $\beta$CaMKII levels. After the sub-chronic administration, the levels of this protein were statistically significantly reduced in dorsal striatum ($p = 0.015$), particularly in the groups treated with M6G.

After the single acute administration of both opioids, a statistically significantly greater phosphorylation of $\beta$CaMKII (Fig. 7) was observed in both dorsal ($p = 0.032$) and ventral ($p = 0.035$) striatum. After the CPP procedure, a trend ($p = 0.072$) to higher levels of phosphorylated $\beta$CaMKII was observed in the hippocampus. Both in hippocampus and ventral striatum, the animals conditioned with morphine showed statistically significant higher phosphorylation of $\beta$CaMKII. After the sub-chronic opioid administration, phosphorylation of $\beta$CaMKII was mostly reduced in the three areas studied, being statistically significant ($p = 0.010$) in the dorsal striatum, especially in the animals treated with M6G.

$\beta$-Actin levels (Fig. 8) after the acute treatment were generally elevated, principally in the dorsal ($p = 0.002$) and ventral ($p = 0.006$) striatum, while only a tendency was seen in hippocampus ($p = 0.094$). After the CPP procedure, the levels of $\beta$-actin were statistically significantly elevated in the dorsal striatum ($p = 0.035$) and the hippocampus ($p = 0.020$). Despite the levels of $\beta$-actin were also elevated in the ventral striatum, it was far from statistical significant ($p = 0.190$). No differences in the levels of $\beta$-actin were observed after the sub-chronic treatment.

Fig. 4. Levels of $\alpha$CaMKII, standardised to the saline control of the corresponding blot (mean ± s.e.m., arbitrary units), in samples from dorsal and ventral striatum, and hippocampus from mice taken 30 min after a single administration (Acute), after the test session of the CPP procedure, or after the last drug administration following the CPP pharmacological treatment protocol (Sub-chronic). Mice were treated with saline (black bars), or 10 (hatched bars) or 30 (no hatched bars) μmol/kg morphine (grey bars) or M6G (white bars). * $0.05 < p \leq 0.1$ drug type (saline vs morphine or M6G), linear mixed models; # $p \leq 0.05$ drug type (saline vs morphine or M6G), linear mixed models; * $p \leq 0.05$ against saline, linear mixed models. $p = 0.XXX$ at the up left corner shows significance of fixed effects drug factor (saline vs opioid), linear mixed models.
3.2. CPP in autophosphorylation-deficient αCaMKII<sub>T286A</sub> mice

Treatment with both doses of morphine, but not saline, induced a significant CPP in both mutant αCaMKII<sub>T286A</sub> and wild type WT mice (Fig. 9). There were no statistically significant differences between mutant αCaMKII<sub>T286A</sub> and wild type WT mice in the magnitude of the CPP.

The αCaMKII<sub>T286A</sub> animals treated with saline showed a statistically significant higher locomotion than the wild type WT mice during the two first conditioning sessions (Fig. 10), as well as the mice treated with 30 μmol/kg in the second conditioning session. None of the groups tested showed any significant sensitization of the locomotor activity.

4. Discussion

In the present study, CPP induced by morphine or M6G was accompanied by changes in CaMKII and β-actin in selected areas of the mouse brain. Increases in protein levels and Thr286 phosphorylation of both CaMKII isoforms were observed in dorsal and ventral striatum, and hippocampus from mice taken 30 min after a single administration (Acute), after the test session of the CPP procedure, or after the last drug administration following the CPP pharmacological treatment protocol (Sub-chronic). Mice were treated with saline (black bars), or 10 (hatched bars) or 30 (no hatched bars) μmol/kg morphine (grey bars) or M6G (white bars). * 0.05 < p ≤ 0.1 drug type (saline vs morphine or M6G), linear mixed models; # p ≤ 0.05 drug type (saline vs morphine or M6G), linear mixed models; * p ≤ 0.05 against saline, linear mixed models. p = 0.XXX at the up left corner shows significance of fixed effects drug factor (saline vs opioid), linear mixed models.
Chronic pharmacological treatment showed no major changes in the levels of β-actin, but showed marked decreases in Thr286 phosphorylation of both CaMKII isoforms in dorsal striatum. This last would imply that the changes observed after CPP are due to the exposition of the opioid treated animals to the CPP procedure and not to the pharmacological treatment itself. Despite the changes observed in phosphorylation at Thr286 in both CaMKII isoforms, phosphorylation of α-CaMKII is not necessary for the acquisition of CPP induced by morphine.

The observed changes in CaMKII and β-actin associated with the CPP suggest that synaptic plasticity occurs during the development of CPP induced by these opioids. This interpretation is endorsed by the increases in β-actin and CaMKII protein levels detected in the animals subjected to the CPP procedure, but not in the ones only subjected to the sub-chronic treatment. CPP is a procedure where the environmental setting and associated stimuli are coupled with the subjective and behavioural effects of a drug [10]. CPP would thus model the learning which mediates the approach to drug conditioned stimuli which triggers new intake of the drug, one of the elements sustaining acquisition, maintenance [32], and relapse [33] of drug consumption. As has been observed for other types of learning, the formation of memories linked to CPP should be accompanied by changes in the functionality and anatomy of synapses [34]. Activation of CaMKII by autophosphorylation at Thr286, particularly the α-isofrom, has been shown to be central for the synaptic plasticity associated with learning processes (see i.e. [35, 36]), and would play a key role in the development of psychoactive drug consumption, abuse, and addiction [13,19]. CaMKII can interact with β-actin [4], inducing the bundling of β-actin.
filaments, hence coupling the functional activation of the synapse with the structural synaptic plasticity.

CaMKII phosphorylation was observed primarily after the acute administration. This suggests that CaMKII phosphorylation can be important for engaging the learning process associated to opioid conditioning. Phosphorylation of CaMKII at Thr286 appears to be important for the initiation of synaptic plasticity, namely memory formation, but not for its maintenance and long-term memory storage [37,38], whereas the binding of CaMKII to the NMDA receptor has been shown to be important for the latter [39]. Thus, the present results can indicate that a single opioid administration is able to activate the intraneuronal mechanisms responsible for initiation of synaptic plasticity, mainly in ventral striatum, as reflected in the increased levels of phosphorylated CaMKII and β-actin. After additional administration coupled to a new environment and establishment of CPP, the changes observed most probably reflect formed drug memories. Indeed, once these memories are consolidated, their maintenance would not further depend on phosphorylated CaMKII but on CaMKII/NMDA complexes, which would explain the relative low phosphorylated CaMKII and the yet high CaMKII protein levels detected after CPP. In the situation where repeated administrations are not connected to a learning procedure, subsequent administration of the opioids appears to have a negative effect on CaMKII activation, with a decline in the levels of the Thr286 phosphorylated form especially in the dorsal striatum.

The results also suggest a different progression in the activation of the three areas studied during time. Thus, whereas after the acute administration changes were observed mainly in the ventral and, in a lesser extent, dorsal striatum, after CPP they were detected in the dorsal

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**Fig. 7.** Levels of phosphorylated βCaMKII at Thr286, standardised to the saline control of the corresponding blot (mean ± s.e.m., arbitrary units), in samples from dorsal and ventral striatum, and hippocampus from mice taken 30 min after a single administration (Acute), after the test session of the CPP procedure, or after the last drug administration following the CPP pharmacological treatment protocol (Sub-chronic). Mice were treated with saline (black bars), or 10 (hatched bars) or 30 (no hatched bars) μmol/kg morphine (grey bars) or M6G (white bars). *p < 0.05 < p ≤ 0.1 drug type (saline vs morphine or M6G), linear mixed models; # p ≤ 0.05 drug type (saline vs morphine or M6G), linear mixed models; * p ≤ 0.05 against saline, linear mixed models. p = 0.XXX at the up left corner shows significance of fixed effects drug factor (saline vs opioid), linear mixed models.
striatum and hippocampus. These results would suggest that while synaptic plasticity in the striatum, especially in its ventral area, is robustly initiated by acute opioids, they only slightly activate plasticity processes in the hippocampus. On the other hand, when the opioid administration is concurrent with establishment of drug memories, like in CPP, the hippocampus is also recruited during the learning process, while the activation of the dorsal striatum is maintained, and declines in the ventral striatum. This last development would correspond with the notion that a shift of behavioural control from ventral to dorsal striatum underlies progression from initial to compulsive drug intake [40].

Autophosphorylation of αCaMKII at Thr286 has been shown to be indispensable for the establishment of cocaine-induced CPP, as it is blocked in gene modified αCaMKII<sup>T286A</sup> mice with impaired Thr286 phosphorylation of the αCaMKII [17]. Thus, the changes in CaMKII phosphorylation detected after the acute administration suggest that CaMKII phosphorylation could also be crucial for opioid-induced CPP. However, morphine was able to induce a similar CPP in these autophosphorylation deficient αCaMKII<sup>T286A</sup> mice as in the wild type mice. This implies that αCaMKII Thr286 autophosphorylation is not specifically required for the development of CPP induced by opioids. This obvious contrast between stimulants such as cocaine [17] and opioids such as morphine (present study) would indicate that, despite similar final behavioural effects, the underlying mechanism differs between these two classes of drugs. Differences between stimulants and opioids in their effects on behavioural models of reward and addiction and on synaptic plasticity have already been described [41]. The results from the present study add to a corpus of evidence suggesting that different

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**Fig. 8.** Levels of β-actin, standardised to the saline control of the corresponding blot (mean + s.e.m., arbitrary units), in samples from dorsal and ventral striatum, and hippocampus from mice taken 30 min after a single administration (Acute), after the test session of the CPP procedure, or after the last drug administration following the CPP pharmacological treatment protocol (Sub-chronic). Mice were treated with saline (black bars), or 10 (hatched bars) or 30 (no hatched bars) μmol/kg morphine (grey bars) or M6G (white bars). * 0.05 < p ≤ 0.1 drug type (saline vs morphine or M6G), linear mixed models; # p ≤ 0.05 drug type (saline vs morphine or M6G), linear mixed models; * p ≤ 0.05 against saline, linear mixed models. p = 0.XXX at the up left corner shows significance of fixed effects drug factor (saline vs opioid), linear mixed models.
neural mechanisms, even if overlapping [42], might lie behind the differences observed between opioid and psychostimulant addiction [41]. The involvement of αCaMKII Thr286 phosphorylation in the conditioning learning seems to be complex and depending on the class of substance administered, since alcohol induced CPP is not reduced but actually enhanced in αCaMKII(T286A) mice [43], despite a lower motivation for alcohol consumption [44]. It is also possible that different conditioning processes [10] contribute to CPP induced by stimulants, opioids, or alcohol, engaging learning mechanisms supported by distinct underlying neuronal processes [13]. An increasing body of studies already shows that environment and non-pharmacological factors determine the differences in the final pharmacological effects observed between different types of addictive drugs [45]. This evidence, together with the present study, suggests that the addictive effect of different drugs would be mediated by different neuropsychological mechanisms, most likely subsystems integrated in the neural reward/reinforcement circuits, a possibility that call for a greater examination.

The study of Easton et al. [17] aimed to identify putative differences in CPP learning/acquisition curves between mice lacking αCaMKII autophosphorylation and WT, whereas the present study aimed only at end point differences. Thus, while the test design in the former study introduced several test trials, which would also work as extinction trial during the CPP establishment phase, ours used a classical design with only one test trial. These differences do not allow drawing conclusions about the effect of αCaMKII autophosphorylation on the learning speed of CPP conditioning induced by the two drugs. However, it allows the comparison on the endpoint, that is, on the achievement (or not) of learning, as determined by acquisition of CPP in wild type mice. Thus, despite the differences in the CPP procedures used, we still can assume that, unlike with cocaine, αCaMKII phosphorylation at Thr286 is not an essential element in the learning process of morphine-induced CPP. It has been shown that long-term potentiation or long-term memory formation can also be established when αCaMKII autophosphorylation is disabled [46,47], indicating that synaptic plasticity can also be engaged by other intraneuronal mechanisms. It is worth noting that phosphorylation of the β-isoform in the ventral striatum was increased both after acute administration and after CPP development. It has been shown that both isoforms not only have different roles during synaptic plasticity [35,48], but their activation can have different effects on the final outcome of the plastic process involved [49-51]. Whether the pivotal task phosphorylation of αCaMKII has in cocaine-induced CPP can be played by the β-isoform for opioids is a question that ought to be further explored.

The effects on the three proteins studied were not restricted to the classic opioid morphine, but were shared by its metabolite M6G. In humans, morphine is conjugated in the liver by UDP-glucuronyltransferase to morphine-3-glucuronide and M6G, which is present in the blood long after morphine has disappeared from circulation. M6G is not synthesised in rodents, which facilitates its study in the most common animal model [52]. M6G is known to be active at the opioid receptor and has analgesic activity equipotent with morphine [21]. Studies in our lab have already shown in behavioural [23,53] and neurochemical [22] animal models that M6G is rewarding and has abuse potential, a property which is further confirmed by the present results. Besides, taking into account the effects of M6G on CaMKII and that this metabolite is present in blood longer than morphine, M6G may prolong and enhance morphine effects after intake of this drug or heroin [54,55].

In conclusion, the present study shows that an acute administration of morphine or M6G and acquisition of a CPP induced by these opioids, are able to generate changes in the phosphorylation at Thr286 and/or levels of α- and βCaMKII, as well as in the levels of β-actin, in striatal and hippocampal regions in mice. An initial administration of these opioids would engage the mechanisms allowing synaptic plasticity to occur if new stimuli are present, like in conditioning during a CPP procedure. These changes are compatible with a putative functional and morphological synaptic plasticity accompanying the learning process associating environmental stimuli with the subjective experience of the drug, a characteristic of the establishment of CPP. However, and in contrast to what has been observed in cocaine induced CPP, phosphorylation at Thr286 of αCaMKII is not essential for the establishment of CPP induced by morphine, indicating that opioids and stimulants would engage different learning mechanisms related to drug addiction. In this regard, the present results suggest a possible involvement of βCaMKII in this process. Administration of the morphine metabolite M6G induced similar effects as morphine, corroborating that this metabolite is also active [53] and can contribute to morphine effects and have abuse potential.
CRediT authorship contribution statement

Jannike M. Andersen: Conceptualization, Methodology, Investigation, Formal analysis, Writing - review & editing. Siri H. Opdal: Methodology, Investigation, Writing - original draft. Christian P. Müller: Conceptualization, Methodology, Writing - review & editing. Fernando Boix: Conceptualization, Formal analysis, Investigation, Project administration, Resources, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

None.

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