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European Journal of Neuroscience 2005;21(11):3089-96

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Running title: Role of calcineurin in the effects of drugs of abuse

ABSTRACT

It has recently emerged that there is a commonality in the molecular mechanisms underlying long-term neuronal changes in drug addiction and those mediating synaptic plasticity associated with learning and memory. In the hippocampus, the calcium/calmodulin-dependent protein phosphatase calcineurin plays a pivotal role in the molecular mechanisms that underlie learning and memory functions. Transgenic mice that express an active form of calcineurin specifically in forebrain structures have previously been shown to have a deficit in the transition from short- to long-term memory. Here, we investigated the involvement of calcineurin in the motivational effects of amphetamine and morphine using this line of transgenic mice (CN98). Our results showed that amphetamine and morphine did not induce conditioned place preference in calcineurin mutant mice, whereas food remained an efficient reinforcer. In addition, behavioural sensitisation to these two drugs, as measured by horizontal locomotion, was disturbed in the transgenic mice. In contrast, neither the horizontal locomotion in response to acute d-amphetamine or morphine nor the somatic signs of morphine withdrawal were affected in calcineurin mutant mice compared to their wild-type littermates. Our data indicate that calcineurin-mediated protein dephosphorylation in the hippocampus is involved in the long-term effects of drugs of abuse without influencing the motivational response to a natural reward or the physical component of opioid withdrawal. The present results emphasise the essential role of hippocampal-dependent learning and memory in the development of drug addiction.

Keywords: conditioned place preference, locomotor activity, morphine withdrawal, hippocampus, transgenic mice

INTRODUCTION

Calcineurin, a calcium(Ca^{++})/calmodulin-dependent phosphatase abundant in the hippocampus and enriched at synapses (Yakel, 1997), plays a major role in signal transduction pathways (Rusnak & Mertz, 2000). In the presynaptic compartment, Ca^{++} entry activates calcineurin, which coordinates the dephosphorylation of proteins involved in synaptic vesicle endocytosis (Cousin & Robinson, 2001). At the postsynaptic level, calcineurin is the first component of a cascade initiated by Ca^{++} influx through the N-methyl-D-aspartate (NMDA) receptor, giving rise to long-term depression (LTD) of synaptic transmission when low-frequency stimulation is applied for prolonged time (Mulkey *et al.*, 1994). In contrast, brief stimulation at high frequency activates kinases such as Ca^{++}/calmodulin-dependent protein kinase II (CaMKII) and protein kinase A (PKA), resulting in long-term potentiation (LTP) (Malenka, 1994). The dynamic balance between phosphorylation and dephosphorylation of specific target proteins mediated by kinases and phosphatases, respectively, determines the long-term processes of memory formation (Malenka & Nicoll, 1999).

In order to further examine the role of phosphatases in synaptic plasticity and memory, transgenic mice expressing a truncated and active form of calcineurin under the control of the CaMKIIα promoter were generated (Winder *et al.*, 1998). In this line of mice, referred to as CN98, the enzymatic activity of calcineurin is increased by about 75% in the hippocampus. They have normal short-term memory but exhibit a profound and specific defect in long-term memory on the spatial version of the Barnes maze and on a novel object recognition task (Mansuy *et al.*, 1998). This deficit resides in the transition from short- to long-term memory and can be partially compensated by increasing the number of training trials.

Several lines of evidence indicate that the brain regions involved in learning and memory and those underlying drug addiction may overlap. It is well known that the hippocampus is crucial for declarative and spatial learning (Squire, 1992), whereas the mesolimbic dopaminergic system (i.e., the ventral tegmental area, VTA, and its forebrain targets such as the nucleus accumbens) is essential for addiction (Di Chiara & Imperato, 1988). However, recent studies have shown that the hippocampus is also important for the rewarding effects of drugs (Fan *et al.*, 1999; Vorel *et al.*, 2001), and that the nucleus accumbens can modulate the strength of memories encoded in the hippocampus (Nestler, 2001). The hippocampus also projects directly to the nucleus accumbens (Floresco *et al.*, 2001) and can activate dopaminergic neurones in the VTA (Legault *et al.*, 2000).

Learning and memory processes and drug addiction also share intracellular signalling cascades and are associated with similar changes in synaptic plasticity, including LTP and LTD (Nestler, 2002). LTP is considered an experimental model of the synaptic changes underlying learning and memory formation (Bliss & Collingridge, 1993) and the development of addiction (Malenka & Nicoll, 1999). In addition, LTD can be most reliably generated in brain regions involved in learning and memory storage, like the CA1 region of the hippocampus, but also in the mesolimbic pathways involved in addiction (Jones *et al.*, 2000; Thomas *et al.*, 2001).

In the present study we used memory-impaired CN98 mutant mice overexpressing calcineurin in the hippocampus to examine the role of this phosphatase in the long-term and acute behavioural responses to drugs of abuse. We used two experimental paradigms, behavioural sensitisation and conditioned place preference, and two drugs, d-amphetamine and morphine, which belong to two different classes of addictive drugs. Moreover, we examined the effect of a natural rewarding stimulus, food, in the conditioned place preference paradigm. The acute horizontal locomotion response to

increasing doses of d-amphetamine and morphine was also measured. Finally, we investigated the implication of calcineurin in the physical dependence to morphine in order to compare the influence of this phosphatase in the motivational and physical components of addiction.

MATERIALS AND METHODS

Animals

Transgenic mice overexpressing a dominant-positive Ca^{++}-insensitive form of calcineurin in forebrain structures were obtained by genetic manipulation, as previously described (Winder *et al.*, 1998). Briefly, a cDNA encoding a truncated form of the murine calcineurin catalytic subunit Aα (ΔCaM-AI) (O'Keefe *et al.*, 1992) under the control of the CaMKIIα promoter was used to construct the expression vector for the generation of CN98 mice. The transgenic mice were generated by microinjection of the linear constructs into fertilized eggs collected from BL6/CBA F1/J females mated with BL6/CBA F1 males. Afterwards, the two-cell embryos were transferred into pseudopregnant BL6/CBA F1/J females. After the integration of the transgene was verified by Southern blotting and PCR, founder mice were backcrossed to C57BL6 F1/J mice to generate the transgenic line CN98 (Winder *et al.*, 1998). Overexpression of the transgene results in a 76% increase in phosphatase activity in the hippocampus of CN98 mutant mice compared to wild-type (WT) mice. The study of the brain distribution of the transgene mRNA by *in situ* hybridization using a radiolabeled oligonucleotide showed that calcineurin overexpression is primarily restricted to the hippocampus and dentate gyrus (Winder *et al.*, 1998). Heterozygous (CN98+/-) were backcrossed to C57Bl6/J background for more than ten generations. These CN98+/- mice were then mated with WT C57Bl6/J mice and the offspring, CN98+/- (50%) and CN98-/- (50%), were used for our experiments. Ten breeding couples were routinely established and were renewed after the second litter in order to avoid genetic drift. Genotypes were determined by PCR analysis as described previously (Mansuy *et al.*, 1998). Mice were maintained under standard laboratory conditions (12-h light/dark cycle with lights on at 07:30; room temperature 21 ± 1°C), with food and water provided ad libitum. Mice were 8-10 weeks old at the time of the experiments and drug-naive. Each experimental group consisted of 8-14 animals. All experiments were conducted in accordance with standard ethical guidelines (European Communities Council Directive for the Care and Use of Laboratory Animals, 86/609/EEC) and approved by the local ethical committee.

Drugs

D-amphetamine sulphate (Sigma, St. Louis, MO, USA), morphine sulphate (Francopia, Paris, France) and naloxone-HCl (Sigma) were dissolved in saline (0.9% NaCl). Amphetamine and morphine were administered intraperitoneally (i.p.) and naloxone was administered subcutaneously (s.c.), in a volume of 0.01 ml/g.

Behavioural analysis

Locomotor activity

Locomotion was evaluated in activity boxes (20 x 15 x 25 cm) connected by an interface to a computer (Imetronic, Bordeaux, France) and located in a sound-attenuated experimental room. Boxes were equipped with two rows of four photocell beams located along their lengths, 15 mm (horizontal

activity) and 30 mm (vertical activity) above the floor. The level of horizontal locomotion, hereafter referred to as locomotor response, was defined as the number of photocell beams from the lower row (15 mm) broken per unit of time. Locomotor activity tests were performed between 10:00 and 15:00 h.

The locomotor response to acute injection of each drug was evaluated following administration of d-amphetamine (1, 2, 5, and 10 mg/kg, i.p.), morphine (2, 5, 10, and 20 mg/kg, i.p.), or saline. Mice were weighed, injected and individually placed in the activity boxes for a 1-h session.

Evaluation of the locomotor response to repeated administration of d-amphetamine or morphine was performed as follows: 1) during the preconditioning phase (day 0), the basal locomotor activity was evaluated by directly introducing each mouse in an activity box for a 1-h session without any preliminary injection; 2) during the pairing phase, mice received one daily injection of d-amphetamine (2 mg/kg, i.p.), morphine (10 mg/kg, i.p.) or saline at three-day intervals (on days 1, 4, 7, 10, and 13) and were directly placed in the activity boxes for a 1-h session; 3) during the test phase, occurring seven days after the last injection of the pairing phase (day 20), both saline- and drug-pretreated animals were challenged with an acute injection of d-amphetamine (2 mg/kg, i.p.) or morphine (10 mg/kg, i.p.) before their introduction in the activity boxes and their locomotor activity was measured for 1 h.

Conditioned place preference test

Place preference was evaluated in a Plexiglas Y-shaped apparatus and two out of the three compartments were used for the conditioning. The apparatus was located in a soundproof testing room with white noise and low luminosity. Three distinctive sensory cues differentiated the compartments: the wall and floor colouring (black or white), the floor texture (rough or smooth), and the odour (wood or nothing). The combination was as follows: a) black walls, smooth floor, no smell added or b) white walls, rough floor, wooden smell. The place preference conditioning schedule consisted of three phases: 1) during the preconditioning phase (day 1), mice were given free access to both compartments for a period of 15 min; the animals were then randomly assigned to a drug-treatment group and the administration of the drugs was counterbalanced between the two compartments; 2) during the conditioning phase, mice were treated for eight consecutive days with alternate injections of drug (amphetamine 2 mg/kg or morphine 5 mg/kg, i.p.), or saline before being confined for 30 min in one of the compartments; mice received the drug on days 2, 4, 6, and 8, and saline on days 3, 5, 7, and 9; control animals received saline every day; 3) the test (or postconditioning) phase (day 10), was conducted exactly as the preconditioning phase (free access to each compartment for 15 min in the absence of drug administration).

To evaluate the reinforcing effect of a natural rewarding stimulus, the administration of drug was substituted by the presence of food in the testing compartment. Five days before the beginning of the experiment, a group of drug-naive mice was submitted to a daily food restriction regimen (2 g of standard chow per mouse per day), which was maintained until the end of the experiment. Mice had free access to food (standard laboratory food pellets) in the confined compartment on days 2, 4, 6, and 8, and were given no food in the other compartment on days 3, 5, 7, and 9. Control animals had no access to food during the experiment.

Morphine-induced physical dependence

Opiate dependence was induced by repeated i.p. injections of morphine, twice daily at an interval of

12 h, for 6 days. The dose of morphine was progressively increased as follows: day 1, 10 mg/kg; day 2, 20 mg/kg; day 3, 30 mg/kg; day 4, 40 mg/kg; day 5, 50 mg /kg, day 6, 50 mg/kg (only one injection in the morning). The control groups were treated with saline following the same schedule. Withdrawal was precipitated by an injection of naloxone (a mu-opioid antagonist, 1 mg/kg, s.c.) 2 h after the last morphine administration. Thirty minutes before naloxone injection, mice were placed individually into transparent round plastic cylinders (diameter 20 cm, height 30 cm). The somatic signs of withdrawal were evaluated over six periods of 5 min for 30 min before and 30 min after the injection of the opiate antagonist. The behavioural observations before the injection of naloxone are not presented. The number of wet-dog shakes, jumping and paw tremor was counted. Tremor, ptosis and teeth chattering were evaluated and one score point given per period for the presence of each sign. The number of periods showing the sign was then counted (maximum score of 6). Body weight was measured before the naloxone injection and 30 min after.

Statistical analysis

Data were analysed using three-way analyses of variance (ANOVA) for acute locomotor activity (treatment x genotype x dose) and behavioural sensitisation (treatment x genotype x time) or two-way ANOVAs (treatment x genotype) for other experiments, followed by one-way ANOVAs and post-hoc tests (Fisher's Protected LSD test) after significant main effects. Data are presented as mean ± SEM.

RESULTS

Effects of acute injections of d-amphetamine and morphine on locomotor activity

To determine whether overexpression of hippocampal calcineurin could affect the acute locomotor effects of drugs, we administered amphetamine (1, 2, 5 and 10 mg/kg, i.p.) and morphine (2, 5, 10 and 20 mg/kg, i.p.) before introducing mice in the activity boxes for a 1-h session. The results of the locomotor dose-response to increasing doses of d-amphetamine did not show differences between genotypes (Figure 1). Accordingly, the ANOVA revealed a dose effect ($F_{4,80} = 16.63$, $p<0.0001$), but neither a genotype effect nor a genotype-dose interaction. Both WT and mutant mice showed an increase in locomotion at 2 and 5 mg/kg, while 1 mg/kg had no effect and 10 mg/kg did not enhance locomotor activity because of its effects on stereotypies.

Similarly, the locomotor stimulatory effects of morphine did not differ significantly between genotypes (Figure 1). Analysis of the acute locomotor response to increasing doses of morphine showed a dose effect ($F_{4,81} = 10.05$, $p<0.0001$), but neither a genotype effect nor a genotype-dose interaction. WT mice showed an increase in locomotor activity that reached significance at 10 mg/kg ($p<0.05$) but not at 20 mg/kg ($p<0.06$). In mutant mice, the dose-response curve to morphine showed a significant increase in locomotion both at 10 mg/kg and 20 mg/kg. The doses of 2 mg/kg and 5 mg/kg did not produce any significant locomotor effects in WT or mutant mice. These results indicate that altered hippocampal calcineurin expression does not disturb the acute response to drug administration, when measured by horizontal activity.

Effects of repeated administration of d-amphetamine and morphine on locomotor activity

Given the deficit in long-term memory observed in CN98 mutant mice, we sought to determine the role of calcineurin in drug-induced behavioural sensitisation. We chose this animal model because it

reflects the long-lived behavioural abnormalities induced by chronic drug exposure and the underlying changes in synaptic plasticity at the molecular and cellular levels (Robinson & Berridge, 1993). For this purpose, mice were given five injections of d-amphetamine (2 mg/kg, i.p.) or morphine (10 mg/kg, i.p.) at 3-days intervals. Seven days after the cessation of treatment, they were challenged with the same dose of d-amphetamine or morphine.

Analysis of the locomotor response to repeated administration of d-amphetamine or saline revealed a genotype effect ($F_{1,333} = 13.95$, $p<0.0002$), a treatment effect ($F_{1,333} = 94.28$, $p<0.0001$), a day effect ($F_{6,333} = 15.72$, $p<0.0001$), a genotype-treatment interaction ($F_{1,333} = 4.41$, $p<0.04$), and a treatment-day interaction ($F_{6,333} = 5.52$, $p<0.0001$), but neither genotype-day nor genotype-treatment-day interactions. In WT mice, repeated d-amphetamine exposure during the pairing phase produced a progressive increment in the locomotor activity, indicating the development of sensitisation to the hyperlocomotor action of this drug (Figure 2). In these mice, the ANOVA indicated a treatment effect ($F_{1,165} = 55.40$, $p<0.0001$), a day effect ($F_{6,165} = 8.75$, $p<0.0001$) and treatment-day interaction ($F_{6,165} = 3.70$, $p<0.002$). In drug-treated WT mice, within group one-way ANOVA showed a significant day effect ($F_{6,88} = 6.86$, $p<0.0001$), and the response to amphetamine on the last injection of the pairing phase (day 13) was significantly higher compared to the first amphetamine injection. As expected, after the drug challenge (day 20), WT mice pretreated with d-amphetamine displayed higher locomotor activity than saline-pretreated mice of the same genotype, indicating the expression of amphetamine-induced locomotor sensitisation (Figure 2). In mutant mice, analysis of the locomotor activity during repeated amphetamine or saline administration revealed a treatment effect ($F_{1,168} = 38.64$, $p<0.0001$), a day effect ($F_{6,168} = 7.04$, $p<0.0001$) and a treatment-day interaction ($F_{6,168} = 2.86$, $p<0.01$). In amphetamine-treated mutant mice, the locomotor activity also increased during repeated drug injections (day effect, $F_{6,91} = 3.31$, $p<0.006$), indicating that overexpression of hippocampal calcineurin does not prevent the development of amphetamine-induced behavioural sensitisation. However, as shown in Figure 2, the increase in locomotor activity observed in mutant mice during repeated amphetamine administration was significantly lower than that observed in WT animals (genotype effect, $F_{1,110} = 7.66$, $p=0.0066$). On the day of the drug challenge, the response of CN98 mutants to the acute administration of amphetamine was higher compared to the first amphetamine injection (Figure 2). Interestingly, unlike WT littermates, mutant mice did not display a significant locomotor stimulant effect upon a drug challenge compared to saline-treated mice, and their response was lower compared to WT animals.

Analysis of the locomotor response to repeated administration of morphine or saline revealed a genotype effect ($F_{1,328} = 14.32$, $p<0.0002$), a treatment effect ($F_{1,328} = 98.98$, $p<0.0001$), a day effect ($F_{6,328} = 12.23$, $p<0.0001$), a genotype-treatment interaction ($F_{1,328} = 9.89$, $p<0.002$), and a treatment-day interaction ($F_{6,328} = 3.70$, $p<0.001$), but neither genotype-day nor genotype-treatment-day interactions. In WT mice, we observed a progressive enhancement of locomotor activity after repeated administration of morphine (Figure 2). In these mice, the ANOVA showed a treatment effect ($F_{1,161} = 60.91$, $p<0.0001$), a day effect ($F_{6,161} = 6.30$, $p<0.0001$), and treatment-day interaction ($F_{6,161} = 2.92$, $p<0.01$). In drug-treated WT mice, within group one-way ANOVA showed a significant effect of day ($F_{6,77} = 3.91$, $p<0.002$) and higher response to morphine on day 13 compared to the first drug injection, indicating the expression of sensitisation to morphine (Figure 2). The mutant mice, like their WT littermates, developed sensitisation to the hyperlocomotor effects of repeated morphine administration (Figure 2). In these mice, analysis of the locomotor activity during the pairing phase revealed a

treatment effect ($F_{1,167}$ = 37.34, p<0.0001) and a day effect ($F_{6,167}$ = 7.22, p<0.0001), but no treatment-day interaction. In drug-treated mutant mice, within group one-way ANOVA indicated that locomotor activity increased during daily injections (day effect, $F_{6,83}$ = 2.68, p<0.02). As observed for amphetamine, the progressive enhancement of locomotor activity after repeated exposure to morphine was significantly lower in mutant mice compared to WT littermates (genotype effect, $F_{1,110}$ = 11.12, p=0.0012). The response of mutant mice to the acute administration of morphine on the day of drug challenge (day 20) was also higher compared with the first injection on day 1. However, these mutant mice did not display a significant response upon a drug challenge compared to saline-treated mice of the same genotype.

Taken together, these findings indicate that an excess of hippocampal calcineurin modifies the acquisition and the expression of drug-induced behavioural sensitisation. Thus, the change in calcineurin signalling, already shown to be responsible for a deficit in long-term memory, alters the sensitised response to chronic drugs of abuse.

Conditioned place preference

The rewarding properties elicited by d-amphetamine or morphine administration were analysed using the conditioned place preference paradigm. During the preconditioning phase, no differences were observed between WT and mutant mice in the time spent in each compartment of the place preference apparatus. Amphetamine (2 mg/kg, i.p.) induced a clear conditioned place preference in WT mice, indicated by a significant increase in the time spent in the drug-associated compartment during the postconditioning phase (Figure 3). Strikingly, amphetamine-induced conditioned place preference was completely absent in CN98 mutant mice, which spent the same time in the drug-associated compartment during the preconditioning and the postconditoning phases. These observations were confirmed by the two-way ANOVA, which indicated no genotype effect ($F_{1,49}$ = 1.14, p=0.29), a treatment effect ($F_{1,49}$ = 17.99, p<0.0001), and a genotype-treatment interaction ($F_{1,49}$ = 5.42, p<0.024). Similarly, after morphine administration (5 mg/kg, i.p.) conditioned place preference was observed in WT mice, but not in CN98 mutant mice (Figure 3). The ANOVA revealed no genotype effect ($F_{1,49}$ = 1.77, p=0.19), a treatment effect ($F_{1,49}$ = 6.52, p<0.014), and genotype-treatment interaction ($F_{1,49}$ = 3.96, p<0.05).

Remarkably, food, as a natural reward, produced the same conditioned place preference in both lines of food-restricted mice, as indicated by a significant increase in the time spent in the food-associated compartment during the postconditioning phase compared to a control group that didn't have any access to food during conditioning (Figure 3). Indeed, the two-way ANOVA indicated a significant effect of food ($F_{1,44}$ = 28.14, p<0.0001), but neither a genotype effect ($F_{1,44}$ = 1.81, p=0.19) nor an interaction. These data provide the first evidence for the specific involvement of calcineurin-mediated protein dephosphorylation in the long-term motivational component of drug dependence without influencing the motivational response to a natural reward.

Naloxone-precipitated morphine withdrawal

Given the deficit in the long-term conditioned effects of drugs in calcineurin-mutant mice, we evaluated the involvement of calcineurin in the development of the physical component of opioid dependence, i.e., manifestation of somatic symptoms of morphine withdrawal precipitated by naloxone. Opiate dependence was induced by repeated i.p. injection of increasing doses of morphine

(10, 20, 30, 40 and 50 mg/kg), twice daily, for 6 days. Withdrawal was precipitated by an injection of naloxone (1 mg/kg, s.c.). Our results show that none of the classical signs of opioid physical dependence were altered in mutant animals (Figure 4). The ANOVA showed a significant treatment effect for tremor ($F_{1,20} = 39.10$, $p<0.0001$), ptosis ($F_{1,20} = 24.70$, $p<0.0001$), teeth chattering ($F_{1,20} = 52.55$, $p<0.0001$), jumping ($F_{1,20} = 32.58$, $p<0.0001$), wet dog shakes ($F_{1,20} = 20.91$, $p<0.0002$), and paw tremor ($F_{1,20} = 12.00$, $p<0.003$), but neither a genotype effect nor a genotype-treatment interaction for any of these measures. These findings suggest that morphine withdrawal symptoms are expressed independently of calcineurin.

DISCUSSION

Temporality is a fundamental dimension in drug addiction, when recreational drug use progressively evolves into compulsive intake associated with withdrawal discomfort in the absence of drug, craving and relapse even after years of abstinence. Based on the hypothesis that learning and memory are essential for the development of addiction, we used a transgenic mouse line overexpressing the protein phosphatase calcineurin in the forebrain (line CN98), previously shown to have a deficit in hippocampal-dependent long-term memory (Mansuy *et al.*, 1998). We showed here that calcineurin overexpression suppresses a major long-term behavioural response induced by drugs of abuse. D-amphetamine and morphine-induced conditioned place preference was absent in CN98 mutant mice, whereas the same drugs elicited a clear conditioned response in WT littermates. This effect was specific to addictive substances as the natural reward food produced similar conditioned place preference in WT and transgenic mice. These data thus provide direct evidence for the specific involvement of calcineurin in the motivational component of drug dependence, and extends the previous demonstration of its pivotal role in memory formation.

In addition, our results showed that repeated d-amphetamine or morphine exposure produced a progressive increase in the locomotor response of WT and mutant mice, indicating the development of locomotor sensitisation to the effects of these drugs. Nevertheless, mutant mice exhibited a reduced degree of sensitisation to both drugs when compared to WT animals. Furthermore, the expression of locomotor sensitisation, tested during a drug challenge performed seven days after the end of drug treatment, was blocked in mutant mice, indicating that forebrain-restricted overexpression of calcineurin can modify the acquisition and the expression of d-amphetamine- or morphine-induced sensitisation.

The interpretation of the present findings is limited by the fact that we only used a single line of transgenic mice, and the possibility that the phenotype may be due to alterations in genes other than the one targeted because of an insertion effect cannot be completely ruled out. However, two arguments strongly suggest that the effects we observed in the CN98 mouse line are most likely a direct consequence of calcineurin overexpression: 1) The deficits in LTP and long-term memory previously shown in CN98 mice (Mansuy *et al.*, 1998; Winder *et al.*, 1998) were also observed in an additional line of mice (Tet-CN279) expressing the same active calcineurin mutant in the hippocampus under the regulation of a tetracycline-controlled transactivator system (Mansuy *et al.*, 1998; Winder *et al.*, 1998). 2) The spatial memory defect in the Tet-CN279 line was reversed when the expression of the transgene was repressed by doxycyline administration (Mansuy *et al.*, 1998). In order to exclude an effect of the insertion site of the transgene, future studies should examine the role of calcineurin in the behavioural responses to drugs of abuse in mice having a constitutive regulation

of the transgene expression, such as the Tet-CN279 line (Mansuy *et al.*, 1998), and assess whether the defects observed can be reversed by repression of the calcineurin transgene with doxycycline. Variegation effects (i.e. mosaicism of cellular expression) may also be of concern when using transgenic mice, but such effect is unlikely here since a strong phenotype was consistently observed throughout our experiments, suggesting sufficient expression levels.

There is a growing body of evidence showing a critical role for calcineurin as a negative regulator of several forms of synaptic plasticity, learning and memory. Genetic inhibition of calcineurin in mice prolongs LTP in the hippocampus, an effect correlated with memory improvement in mutant mice (Malleret *et al.*, 2001). Similarly, inhibition of calcineurin activity using either FK506 or cyclosporin A prevents the extinction of conditioned fear memory in rats (Lin *et al.*, 2003). These observations are in agreement with the direct role of calcineurin in the transition from short- to long-term memory (Mansuy *et al.*, 1998; Winder *et al.*, 1998; Malleret *et al.*, 2001). In addition, inactivation of protein phosphatase 1 (PP1) by expression of a constitutively active inhibitor (I1, which is dephosphorylated by calcineurin) improves learning and memory (Genoux *et al.*, 2002). These findings suggest that decreased calcineurin activity promotes learning, whereas increased calcineurin activity might privilege forgetting, causing a deficit in the long-term effects of drugs.

Besides its role in memory consolidation, the kinases/phosphatases cascade has also been implicated in addiction. Inhibition of CaMKII expression in the hippocampus strongly attenuates the development of tolerance and physical dependence in response to chronic morphine treatment (Fan *et al.*, 1999). Moreover, an intra-hippocampal injection of a specific CaMKII inhibitor abolishes the development of amphetamine-induced conditioned place preference in rats (Tan *et al.*, 2002), suggesting that activation of hippocampal CaMKII is necessary for amphetamine-induced place conditioning as well as behavioural learning. Stimulation of PKA activity in the amygdala has been reported to facilitate reward-related learning and to contribute to drug-induced neuroadaptation, including opioid tolerance and dependence (Jentsch *et al.*, 2002; Liu & Anand, 2001). Extracellular signal-regulated kinases (ERK1 and 2) have also been described as synaptic signalling components necessary for several forms of learning and addiction. For instance, mice lacking ERK1 exhibit a significant enhancement of striatum-dependent long-term memory, correlated with a facilitation of LTP in the nucleus accumbens; these plasticity changes result in an hypersensitivity to the reinforcing properties of morphine (Mazzucchelli *et al.*, 2002). In view of our results with calcineurin, it is possible that both down-regulation of kinases as well as up-regulation of phosphatases prevent some of the neuronal changes induced by chronic drug treatment.

Different calcineurin substrates have been identified, including the glutamate receptors NMDA, α-amino-3-hydroxy-5-methyl-isoxazole proprionic acid (AMPA) and metabotropic receptors. NMDA receptors are essential for the induction of LTP or LTD depending on the degree of Ca^{++} influx, and play an important role in the long-term changes in synaptic plasticity in different brain regions, including the CA1 area of the hippocampus (Malenka & Nicoll, 1993). Induction of hippocampal LTP, mediated predominantly by CaMKII, and LTD, mediated by calcineurin and PP1, require activation of NMDA receptors and Ca^{++} channels following high or low frequency stimulation, respectively. Low level of Ca^{++} entry via NMDA receptors preferentially activates Ca^{++}-dependent protein phosphatases, like calcineurin, and provokes the activation of PP1 and dephosphorylation of receptors including $GABA_A$ and AMPA receptors as well as the metabotropic glutamate receptor 1 (mGluR1) subunit (Malenka, 1994). These receptor changes may underlie the induction of LTD

mediated by calcineurin, leading to a deficit in memory storage. Similar mechanisms may come into play in the long-term conditioned effects of drugs observed in our study, since long-term changes in synaptic strength at excitatory synapses seem to be particularly important for the development of drug-induced sensitization and reward (Koob & Bloom, 1988; Vezina & Queen, 2000).

Dopamine release and dopamine receptors also constitute possible targets of calcineurin in the motivational effects of amphetamine and morphine investigated in our study. As already mentioned, the ability of drugs of abuse to increase the concentration of dopamine in the nucleus accumbens is considered to be crucial for their reinforcing and stimulant effects (Di Chiara & Imperato, 1988; Koob & Bloom 1988). Moreover, the nucleus accumbens has been directly linked with associative learning for drug-related stimuli (Di Chiara, 1999). Calcineurin is known to inhibit dopamine release at presynaptic sites by inactivating synapsin I, a calmodulin-binding protein involved in exocytosis and neurotransmitter release from synaptosomes (Iwata *et al.*, 1997). However, using *in situ* hybridization, we did not observe any significant expression of the calcineurin transgene in dopamine cell bodies within the substantia nigra *pars compacta* or the VTA in CN98 mutant mice (Claire Crozatier & BG, unpublished results), ruling out the possibility for such changes. At the postsynaptic level, dopamine receptors are involved in calcineurin-mediated signalling through their action on DARPP-32, a dopamine- and cAMP-regulated phosphoprotein, involved in dopamine function in brain areas thought to be important for drug addiction (Nishi *et al.*, 1997). DARPP-32 can be phosphorylated by PKA and dephosphorylated by calcineurin. Dopamine D1 receptor activation results in DARPP-32 phosphorylation, whereas the activation of D2 receptors decreases basal and agonist-stimulated phosphorylation of DARPP-32, probably by increasing intracellular Ca^{++} followed by calcineurin activation (Nishi *et al.*, 1997). With respect to synaptic plasticity and memory consolidation related to addiction, dopamine pathways in forebrain structures are considered as possible substrates (Jay, 2003; Setlow & McGaugh 1999). A potential relationship between hippocampal LTP or LTD and dopamine pathways has been suggested previously. For example, mesoaccumbens dopamine transmission is modulated by excitatory glutamatergic input from the CA1 region of the hippocampus to the nucleus accumbens (Legault *et al.*, 2000; Vorel *et al.*, 2001). Moreover, intrahippocampal injections of dopamine agonists improve spatial working memory in the radial maze paradigm (Packard & White 1991). The hippocampus may thus play a role in the contextual learning of a drug experience and may integrate a rewarding and memory-related amphetamine and morphine effects (Fan *et al.*, 1999; present study). Because calcineurin is involved in both glutamatergic and dopaminergic signalling pathways, it is possible that its overexpression disrupts dopamine/glutamate interconnections in the nucleus accumbens, amygdala or hippocampus, brain areas relevant for learning and memory formation, and for drug-induced long-term conditioned responses.

Another calcineurin target shown to be involved in addiction and memory processes is the transcription factor CREB (cAMP-responsive element binding protein) (Bito *et al.*, 1996). Several studies indicated that learning and long-term memory consolidation in the hippocampus require CREB activation. Conditional knock-out mice with inactivated CREB isoforms in the hippocampus show impaired conditioned taste aversion learning when tested for hippocampus-dependent memory (Balschun *et al.*, 2003). CREB is critically involved in the conversion from short-term to long-term memory, as multiple training sessions are known to increase CREB phosphorylation and activate CREB-mediated gene transcription (Genoux *et al.*, 2002). In the context of addiction, it has been shown that acute administration of opioids inhibits CREB phosphorylation, which recovers after

chronic treatment and increases further during withdrawal (Guitart *et al.*, 1992). Therefore, we suggest that the dephosphorylation of phospho-CREB by calcineurin via PP1, may participate in the regulation of the long-term motivational effects of drugs described in our study.

Taken together, our data suggest that hippocampal calcineurin signalling is involved in the long-term adaptation after chronic drug treatment, in a way that may parallel its role during memory formation. Such an effect is coherent with the common molecular mechanisms shared by addiction and memory processes discussed above. These findings indicate that activation of calcineurin, by decreasing the conditioning effect of drugs, may represent a potential therapeutic pathway to prevent the long-term neuroadaptive changes elicited by chronic drug exposure. Given the well-established role of the hippocampus in associative learning and memory, our results further emphasize the important role of this forebrain structure in controlling long-lasting features of addiction. Knowledge about the mechanisms involved in calcineurin regulation of drug-induced synaptic modifications should be extended by the analysis of additional mouse models with regulated modulation of calcineurin-mediated signalling.

Acknowledgements

This work was supported by grants from INSERM and MILDT to BG. G.B. was supported by a fellowship from INSERM. I.M.M. is supported by the Swiss Federal Institute of Technology, the University of Zürich, the Swiss National Science Foundation, the National Center for Competence in Research 'Neural Plasticity and Repair', Human Frontier Science Program, EMBO. The authors thank Cécile Spielewoy for critical reading of the manuscript and Marika Nosten-Bertrand for her involvement during the early stage of this work.

Abbreviations

CaMKII, calcium/calmodulin-dependent protein kinase II; i.p., intraperitoneally; LTD, long term depression; LTP, long term potentiation; NMDA, N-methyl-D-aspartate; PKA, protein kinase A; PP1, protein phosphatase 1; s.c., subcutaneously; WT, wild-type.

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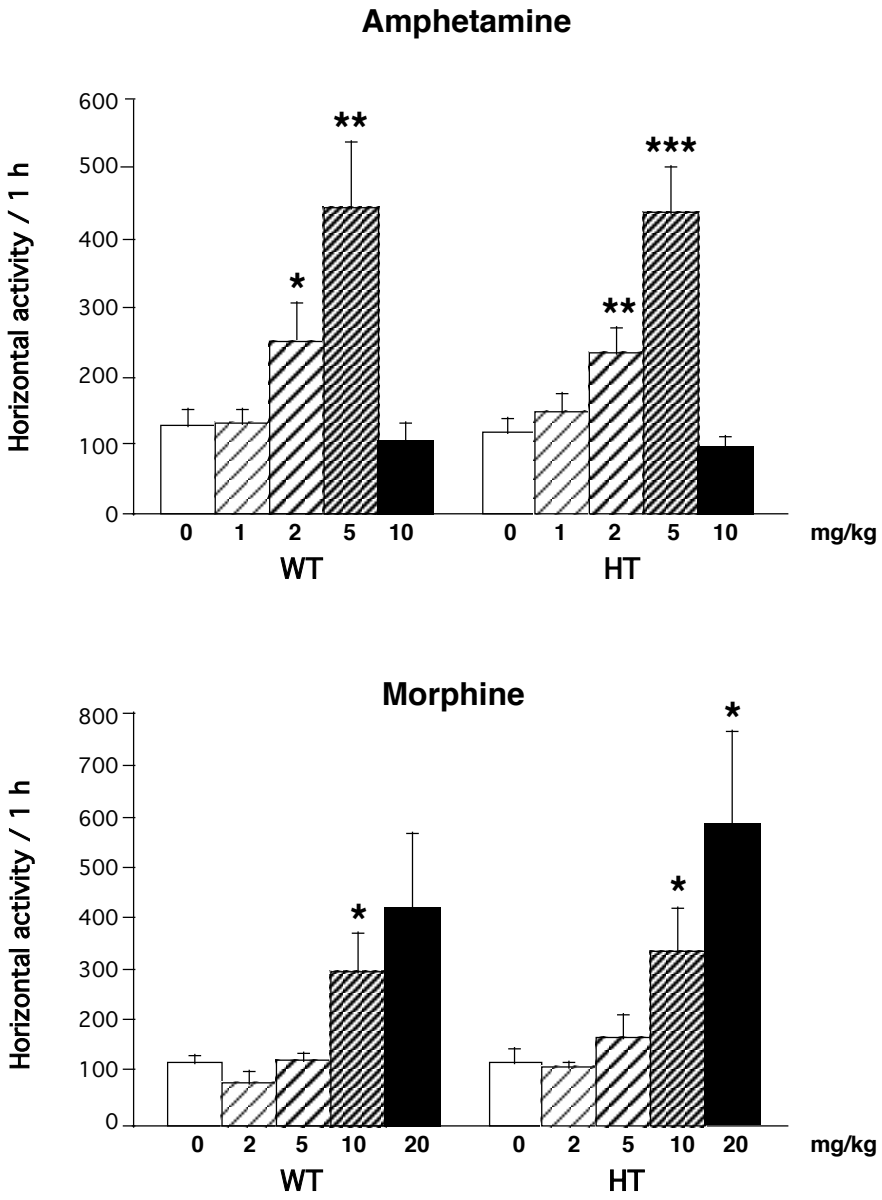

Figure 1. Effects of acute d-amphetamine and morphine in CN98 WT and mutant mice. Values represent mean ± SEM (n = 8-10). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, vs. saline-treated mice of the same genotype (ANOVA followed by Fisher's test).

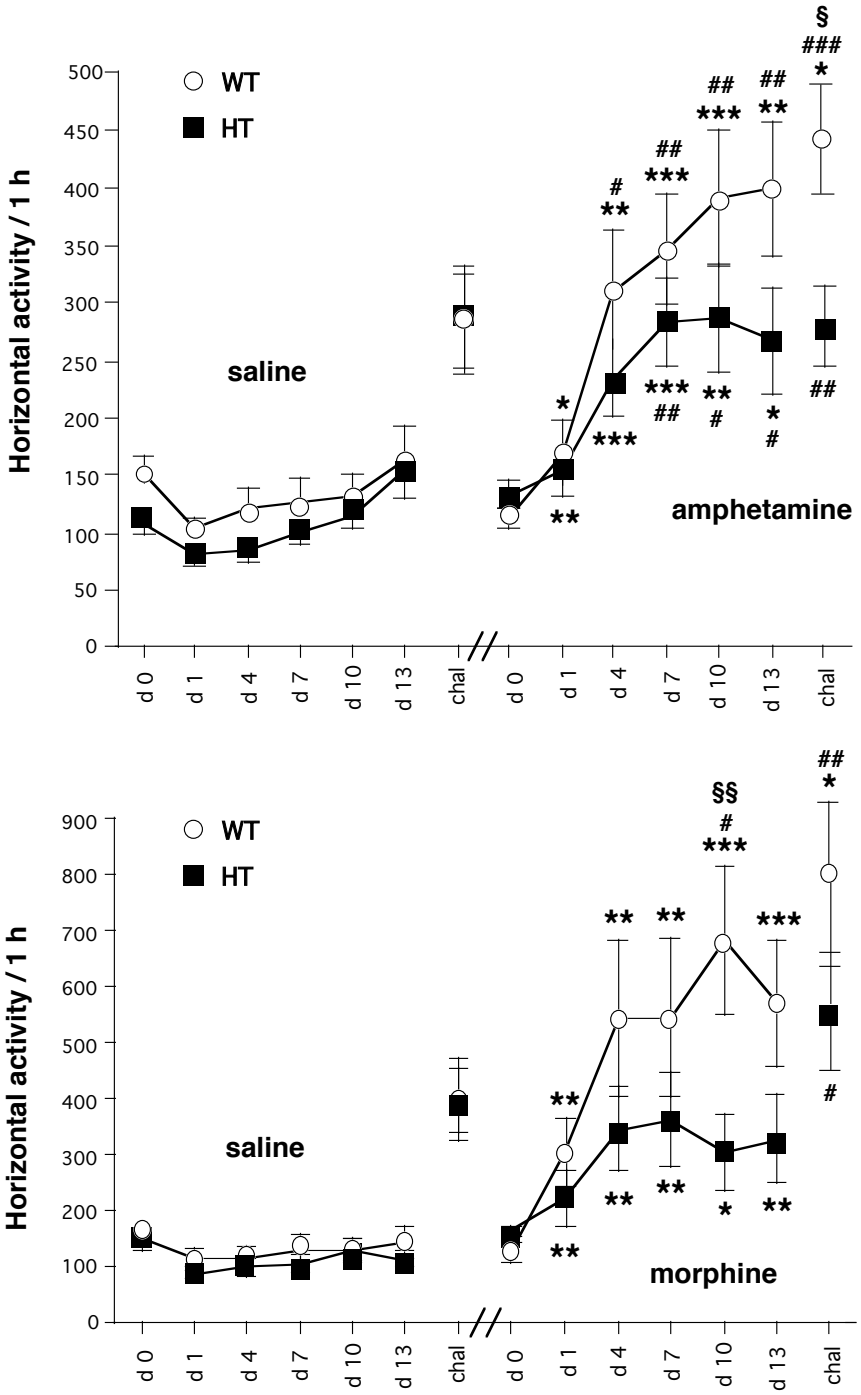

Figure 2. Effects of repeated administration of d-amphetamine and morphine in CN98 WT and mutant mice. Values represent mean ± SEM (n = 12-14). **P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs. saline-treated mice of the same genotype at the same time point; #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001 vs. the first pairing day within the same treatment group; § *P* < 0.05, §§*P* < 0.01, WT vs. mutant mice at the same time point (ANOVA followed by Fisher's test).

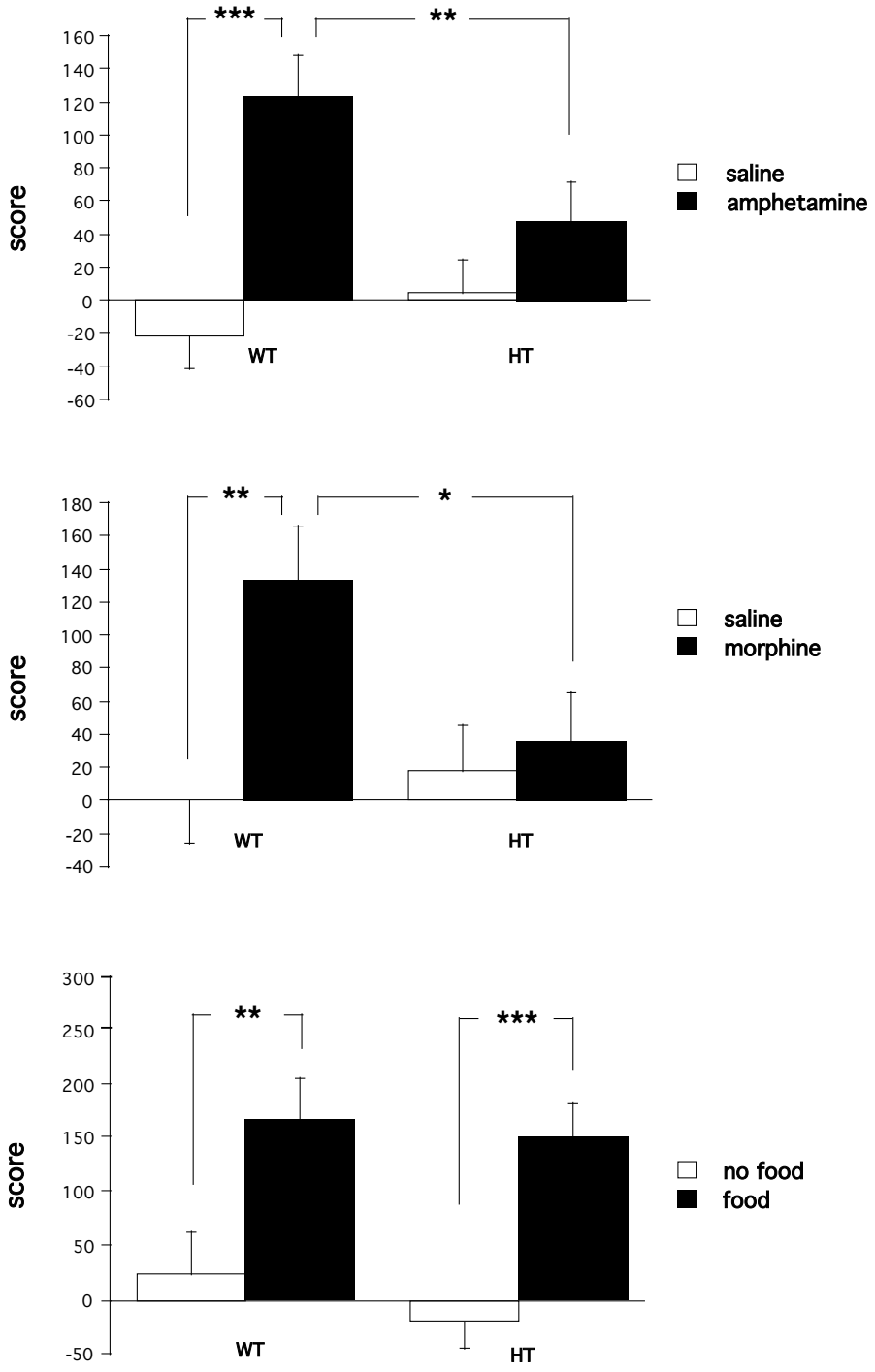

Figure 3. Conditioned place preference in CN98 WT and mutant mice. Scores represent the difference in seconds between the post- and pre-conditioning time spent in the drug-associated compartment. Values represent mean ± SEM (n = 12-14). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. saline-treated mice of the same genotype (ANOVA followed by Fisher's test).

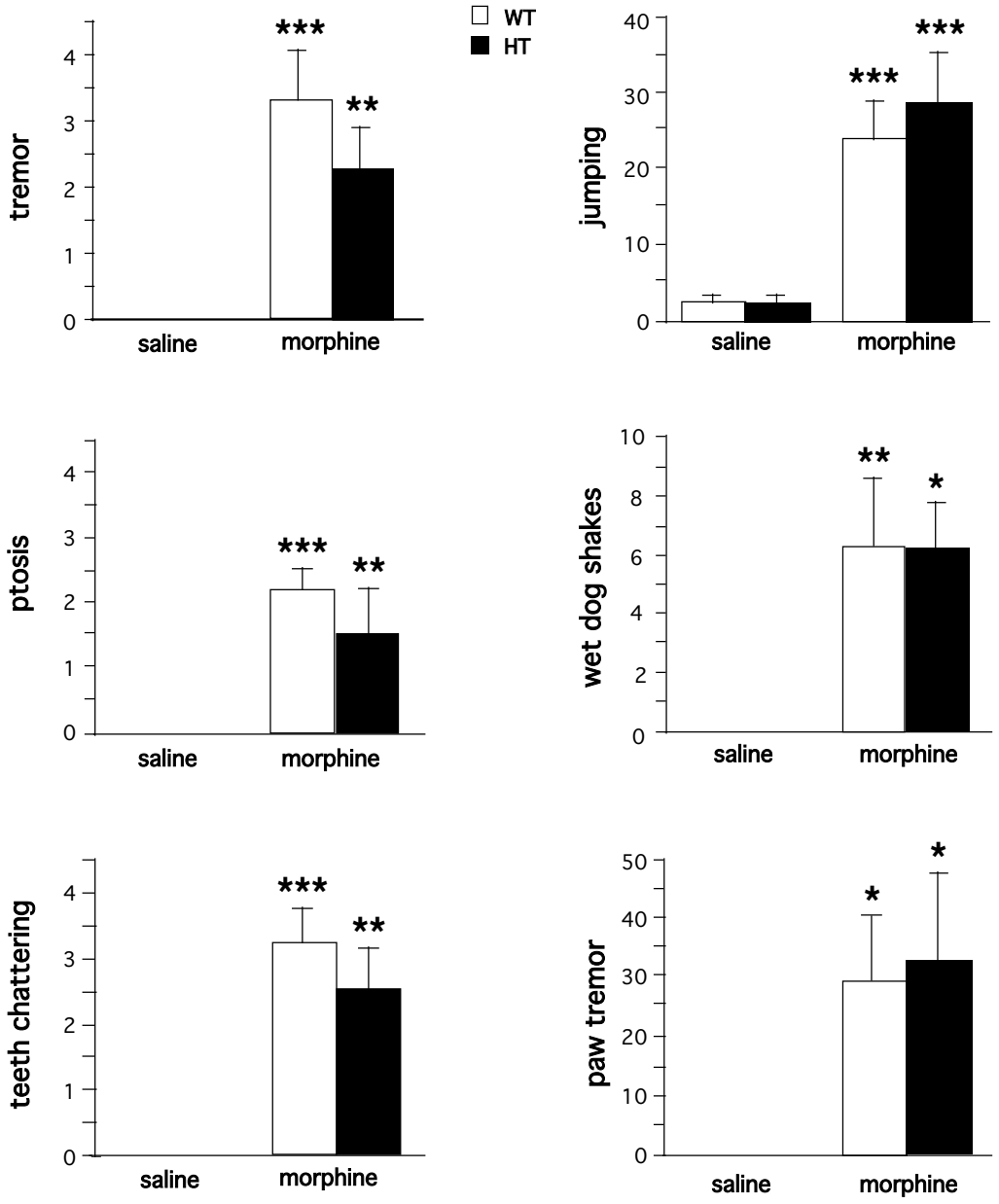

Figure 4. Naloxone-precipitated morphine withdrawal syndrome in CN98 WT and mutant mice. Values represent mean ± SEM (n = 6). **P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs. saline-treated mice of the same genotype (ANOVA followed by Fisher's test).