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CART Peptides Modulate the Locomotor and Motivational Properties of Psychostimulants

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Running Title: Attenuated Psychostimulant Effects in Cart KO mice

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Abbreviations: ANOVA, analysis of variance; CART, Cocaine- and Amphetamine-Regulated Transcript; CPP, conditioned place preference; i.p., intraperitoneal; i.v., intravenous; KO, knockout; nAcc, nucleus accumbens; PCR, polymerase chain reaction; SN, substantia nigra; VTA, ventral tegmental area; WT, wild-type

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Abstract

Drug addiction results from a subversion of neural circuits that control motivation. Although the hedonic and addictive properties of psychostimulants and drugs of abuse are predominantly attributed to dopamine and glutamate, it is appreciated that other signaling molecules in the brain are important. This study suggests that CART (Cocaine- and Amphetamine-Regulated Transcript) peptides modulate the locomotor and motivational properties of psychostimulants. The behavioral effects of cocaine and amphetamine were examined in Cart^{tm1Amgen} knockout (Cart KO) and wild-type (WT) mice. Acute amphetamine administration increased in locomotor activity in WT mice, but this response was attenuated in Cart KO mice. Repeated amphetamine produced locomotor sensitization WT mice, but hardly any in Cart KO mice. Amphetamine elicited conditioned place preference in both genotypes, but amphetamine's potency was reduced in the Cart KO mice. Intravenous cocaine self-administration was observed in both genotypes, but Cart KO mice consumed less cocaine and responded less for cocaine than WT mice. The behavioral effects of psychostimulants were reduced in the mutant Cart KO mice. By contrast, open field activity and sucrose preference of drug-naïve mice WT and Cart KO mice was not significantly different. The attenuated effects of amphetamine and cocaine in Cart KO mice suggests a positive neuromodulatory role for CART peptides in the locomotor and motivational properties of psychostimulants, and implicates CART peptides in psychostimulant addiction.

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Introduction

Drug addiction remains one of the most pervasive and stigmatized diseases (Leshner, 1997). Understanding of how drugs of abuse engage the brain to alter motivation and cause addiction is necessary for developing effective treatments. Drugs of abuse elicit drug seeking, drug taking, hedonia and addiction by altering dopaminergic and glutaminergic neurotransmission in neural circuits of motivation (Koob, 1992; Kelley, 2004). We suspected CART peptides were important for the behavioral actions and addictive properties of psychostimulants after finding that its cDNA was up-regulated by acute cocaine and amphetamine administration in the striatum, but not elsewhere in the brain where it is expressed (Douglass et al., 1995). Based on these initial findings, it was speculated that psychostimulants use CART peptides in the brain to produce their behavioral effects.

CART peptides and mRNA are found in brain areas involved in locomotor activity and motivation (Koob, 1992; Kelley, 2004). In the rat, these areas are the nucleus accumbens (nAcc), ventral pallidum, ventral tegmentum (VTA), substantia nigra (SN), infralimbic prefrontal cortex, anterior cingulate cortex, basolateral and central nucleus of the amygdala, the Bed nucleus of the stria terminalis and dentate gyrus of the hippocampus; the VTA lacks CART, but contains CART peptides (Douglass et al., 1995; Couceyro et al., 1997; Koylu et al., 1997; Koylu et al., 1998). CART peptides and mRNA are also present in the hypothalamus, sensorimotor cortex, brain stem, and pituitary to mention a few other areas (Douglass et al., 1995; Couceyro et al., 1997; Koylu et al., 1997; Koylu et al., 1998). Interestingly, CART expression in the human brain is similar to that of the rodent, but more extensive in the neocortex (Douglass et al., 1995; Hurd and Fagergren, 2000). The neuroanatomical distribution of CART peptides suggests that they participate locomotor activity, the appraisal of rewarding/reinforcing stimuli, and in the

learning associated with habit formation that is critical in the transition from drug use to drug addiction (Kelley, 2004).

Behavioral and neurochemical studies support a role for CART peptides in the locomotor activity and motivation. Exogenous and endogenous CART peptides suppress food intake much like psychostimulants (Kristensen et al., 1998; Lambert et al., 1998). This anorectic action coupled and the psychostimulant regulation of CART expression in the striatum, which occurs specifically within the nAcc (Hurd et al., 1999), first suggested that CART peptides are endogenous stimulant-like compounds. Subsequent studies showed that injection of CART peptide 55-102 into the VTA increased locomotor activity and produced conditioned place preference in rats (Kimmel et al., 2000); other CART peptides have similar effects on locomotion (Bannon et al., 2001; Kimmel et al., 2002). Intracerebroventricular injection of CART peptide 55-102 increased dopamine turnover in dopaminergic terminal fields including the nAcc and dorsal striatum (Yang et al., 2004). Thus, CART peptides affect locomotion and motivation on their own or in concert with other transmitter systems. CART peptides are also important in anxiety, pain, arousal, startle response, regulation of calcium channels, and neuroendocrine hormone secretion (Kask et al., 2000; Bannon et al., 2001; Yermolaieva et al., 2001; Chaki et al., 2003; Smith et al., 2004). Thus, the molecular, anatomical and behavioral data show a role for CART peptides in motivation and implicate them in the behavioral properties of psychostimulants.

In order to explore the role of CART peptides in actions of psychostimulants, we compared the behavioral effects of psychostimulants in adult, male wild-type (WT) and $Cart^{tm1Amgen}$ knockout (Cart KO) mice. The lack of pharmacological agents, or antagonists, to block CART peptide activity prompted the use of mutant mice lacking CART peptides. These

studies show that psychostimulant mediated increases in locomotor activity, locomotor sensitization, conditioned place preference and intravenous drug self-administration are attenuated in Cart KO mice as compared to WT mice. They suggest the behavioral effects of psychostimulants are modulated by CART peptides. This data is the first to show a causal link between CART peptides and the locomotor and motivational effects of psychostimulants.

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Methods and Materials

All animal procedures were done in accordance with the rules established by the NIH Guide for the Care and Use of Laboratory Animals and with the approval of the Institutional Animal Care and Use Committee of the Rosalind Franklin University of Medicine and Science/Chicago Medical School.

Materials. Oligonucleotides were purchase from Sigma-Genosys (Woodlands, TX). D-amphetamine, cocaine hydrochloride, heparin and sucrose were purchase from Sigma-Aldrich (St. Louis, MO). Ketamine and xylazine were purchase from Burns Veterinary Supply, Inc. (Elk Grove, IL). Gentamicin was purchased from ICN Biomedicals (Aurora, OH). Bullet cameras and video splitters were purchased from Security Depot (Miami, FL). The digital video device was a Philips Progressive Scan DVDR75. The intravenous silastic catheter was purchased from Fisher Scientific (Pittsburg, PA). Operant activity boxes were purchased from Med Associates (Medford, VT).

Nomenclature. CART refers to the mRNA or gene whereas the encoded protein/peptide products are referred to as CART peptide. Cart is used to denote the mouse mRNA or gene. The CART peptide numbering system is based on the102 amino acid pro-peptide or the long variant in rat as originally described by Douglass et. al. (1994).

Cart knockout mice. Cart^{tm1Amgen} knockout (Cart KO) mice with a deletion of all three exons and two introns of the mouse Cart gene were constructed as described elsewhere (Wierup et al., 2005). Agouti chimeric mice were crossed to outbred Black Swiss mice and the resulting agouti

mice genotyped for the presence of the targeting allele. Cart heterozygous animals were intercrossed to establish background control homozygous Cart wild-type (WT) and Cart KO mice. WT and Cart KO mouse colonies were maintained separately by random breeding at Charles River Laboratories (Wilmington, MA). Mice were genotyped by PCR analysis of genomic DNA with two different reactions to identify the wild-type gene (with 5'-CCATTCGAGGCATTCTCCTTC -3', UTR and 5' -GGAACTTCCTGCAATTCTTTC- 3' primers) or the deletion mutant construct (with UTR and 5'- CTTCGTTTATCTTGCCTGCTC-3' primers). The PCR products for the wild-type and deletion mutant construct were 230 bp and 450bp, respectively (data not shown).

Behavioral testing. All studies were performed in adult male mice (7-9 weeks old at the start of the study). Mice were single housed in 12 hr. dark-12 hr. light cycle (lights on at 0700) unless otherwise stated. Mice were housed for 5-7 days after arriving at the University before any tests were conducted. All testing was performed in rooms other than the housing room. Housing and test rooms had low ambient lighting (1.5-2.5 ft. candles). Mice were habituated to handling and intraperitoneal (i.p.) saline injections in the housing room. The number of mice used in each experiment is given in the figure legends.

Open field test. Mice were handled (i.e., held) daily for 1-2 min. three days prior to testing. On the test day, mice were transferred to the test room and immediately placed in the center of an open field (60 X 60 X 40 cm, LWH) made of an aluminum floor, black Plexiglas walls and an open top. Their behavior over 60 min. was videotaped. One mouse was tested at a time and the

order of testing alternated between genotypes. The floor and walls of the test box were cleaned with hot water and dried in between tests. The test was conducted between 0900 and 1500 hrs.

Open field activity was measured by counting the number of entries or crosses made into different areas or quadrants of the field, the number of rears, and the time spent in along the wall (i.e., thigmotaxis) and in the center of the field. The open field was divided into nine equal quadrants. Thigmotaxis was counted only when the mouse was within one body width of the wall. Locomotor activity over the first 10 min. and then for the first 2 min. of ever 10 min. bin was assessed and is reported. Two independent investigators blind to genotype analyzed the videotapes.

Drug induced locomotion studies. Amphetamine was selected for the locomotor studies because of the extensive literature and history on its effects, especially in mice, where there is often variability among the strains (Shuster et al., 1977; Phillips et al., 1994). Mice were given amphetamine in a novel environment because psychostimulants and drugs of abuse produce more robust locomotor responses in novel environment rather than familiar ones such as the home cage (Badiani et al., 1995).

Mice were handled (i.e., held) for 1-2 min. daily for the three days prior to testing. Mice received one i.p. saline injection two days before testing between 1500-1600, a second injection one day before testing between 0900-1000 and a third injection one day before testing between 1500-1600. On the test day, mice were placed in Plexiglas cages of the same size (28 X 17 X 12 cm, LWH) as the home cage with bedding. This box was located inside a photobeam activity system with two photobeams 3 cm from the floor from which ambulation was calculated and six beams located 6 cm from the floor from which vertical activity score was calculated (San Diego

Instruments, San Diego, CA). Mouse activity was monitored daily with this photobeam system for the 30 min. of habituation or time before receiving an injection, and for 2 hr. after an i.p. injection of saline or D-amphetamine dissolved in saline (1 ml/kg). Mouse activity was digitally recorded with cameras every other day onto DVDs with a digital recorder device. Locomotor activity was defined as ambulation, vertical activity, stereotypic grooming and stereotypic head-bobs. Ambulation and vertical activity were measurements were derived from photobeam activity system. Stereotypies quantified from visual inspection of the video records. Stereotypies were defined as continuous, repeated movements produced in one location. The duration, and not the number of bouts, of each stereotypy were scored for the first minute of every 10 min. over 2 hrs. after each injection. Three independent investigators blind to genotype scored the stereotypies. The scores represent the mean of three the independent investigator's scores. There was over a 90% concordance between investigator reported scores.

Conditioned Place Preference (CPP). Amphetamine was chosen for this study, in part, because it reliably elicits CPP, even in mice where strain variability is prevalent (Belzung and Barreau, 2000). CPP was conducted with the biased method for drug conditioning (i.e., drug was paired with least preferred pre-conditioning environment). A three-chambered Plexiglas box (22 X 14 X 15 cm, LWH, per chamber) with different texture floors was used. The textured floors were made of rods (4 mm diameter stainless steel rods spaced 8 mm apart) or mesh (1 mm diameter stainless steel thread weaved into 4 mm X 4 mm squares). The textured floor chambers were positioned on either side of a smooth, white Plexiglas floored chamber and sliding doors separated each. Three of the four walls of the textured floor chambers were black, and one was transparent through which the mice were video taped. The top of the chamber had a transparent

Plexiglas ceiling. Two separate CPP boxes were placed in an open test room with low ambient lighting (1.5 – 2.5 ft. candles); the room did not house any animals when these experiments were conducted. Mice were first habituated to handling and i.p. saline injections as follows. Mice were held daily for 1-2 min. for three days between 0900-1000. On the second and third day of handling, mice were given saline i.p. four to six hrs. after being handled. On day four, an initial assessment of chamber preference was conducted. Mice were given access to all three chambers for 20 min. Their activity was video taped and the time spent in each of the chamber was measured and reported as the pre-conditioning chamber preference. Mice that spent greater than 15% more time in any one chamber compared to the other two were eliminated from the study. Of the remaining mice, those with the strongest pre-conditioning chamber bias were used in the saline control group. The chamber walls were cleaned with hot water, and the removable floors and underlying waste bin were cleaned with soap and water after each session. On day five, mice were exposed to all three chambers as one the previous day, but the activity was not monitored. On days 6-9, drug conditioning was performed. On each day between 0900-1200, mice were injected with saline i.p., and confined for 45 min. in the most preferred textured floor chambers and access to the other chambers was blocked. Between 1200-1500, mice were injected with D-amphetamine i.p., and confined for 45 min. to least preferred textured floor chamber and access to the other chambers was blocked. A minimum of 3 hrs. was allowed between saline and drug conditioning. On day 11, place preference was determined between 1200-1500 by giving mice access to all three chambers for 20 min. and video taping the activity. No injections were given prior to this test. Place preference was determined by subtracting the amount of time spent in the drug-paired compartment before drug conditioning (on Day 4) from the time spent there after drug conditioning (on Day 11).

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In this study, CPP was produced with the biased method (i.e., drug conditioning is performed in the least preferred pre-conditioning compartment) in order to enhance the possibility of observing place preference. This biased protocol is questioned because it may measure the removal of aversion to an environment or reveal an anxiolytic effect of the drug, instead of measuring a positive hedonic (Carr et al., 1989); however, this criticism is now being questioned (Cunningham et al., 2003). Interestingly, the unbiased CPP method must contend with this issue when an animal is assigned, albeit randomly, to the least preferred environment for drug conditioning. A ceiling effect is also a problem for the unbiased method when animals are randomly assigned to the most-preferred pre-conditioning chamber for drug conditioning. Although neither method is ideal, these problems are resolved by using a CPP box with minimal chamber bias (Bardo and Bevins, 2000). The 3-chambered CPP box used in these experiments produced a small pre-conditioning chamber bias (Figure 1). The middle chamber with the smooth floor (31.3 % ± 0.9 preference) was preferred less than the mesh floor (34.0 % ± 0.9 preference) or rod floor (34.7 % ± 0.9 preference) (two-factor ANOVA: floor preference, $F_{2, 129} = 4.16$, $p < 0.05$; floor preference X genotype, $F_{2, 129} = 10.31$, $p < 0.001$) during the pre-conditioning test (Figure 1A). The bias against the smooth floor chamber resided in the WT mice, and not the Cart KO mice, which showed no statistically significant pre-conditioning chamber bias (Figure 1B). Thus, the problems associated with the biased CPP method were minimized by using a CPP box with a small pre-conditioning chamber bias, eliminating mice with the strong pre-conditioning chamber bias and parceling the remaining mice with the strongest pre-conditioning chamber bias into saline control groups.

Intravenous cocaine self-administration. Cocaine was chosen because it is a reliable

reinforcer and its use would allow us to generalize these results to psychostimulants. Mice were housed in a reverse 12 hr. light-12 hr. dark cycle with lights on at 2000. After seven days, mice were implanted with an indwelling silastic catheter (2 French) in the external jugular under ketamine (100 mg/kg) and xylazine (20 mg/kg) anesthesia. A 1.2 cm segment of the catheter was inserted into the vein and the distal end of the catheter exited the midcapsular region in between the shoulder blade. Mice were allowed 4-5 days to recover. Catheters were flushed daily with 25-50 µl each of saline, gentamicin (0.8 mg/ml) and heparin (70 U/ml). Gentamicin was given only 4 days post-operatively. Mice were allowed to self-administer cocaine in sound attenuated operant boxes 4-5 days after surgery. Unlimited cocaine was available daily for 2 hrs. between 1000 and 1400 hrs. Mice could nose-poke for cocaine from either of two holes on one wall under fixed ratio-1 schedule of reinforcement. A nose-poke in one hole assigned as the "active hole" resulted in one intravenous (i.v.) infusion of cocaine (1 mg/kg/injection, 120 µl infused over 4 sec.), activation of a light inside the nose-poke hole for 20 seconds and no scheduled consequences of further nose-pokes during this 20 sec. time period. The number of injections and nose-pokes in the active and inactive hole were recorded throughout the entire session. A cocaine dose-response curve was generated for each mouse by serially lowering the dose of cocaine once stable operant responding was observed at each cocaine dose. Stable responding was defined as three consecutive days or sessions with cocaine intake that varied < 10%, active hole nose-pokes that varied < 20% and nose-poke accuracy or the active/inactive nose-poke ratio that varied < 20%. Catheter patency was checked every 5-6 days by infusing 20-25 µl of ketamine/xylazine anesthetic i.v. at least 3 hrs. after a self-administration session. Mice were removed from the study if loss of righting reflex and consciousness was not observed within 5 and 10-15 seconds, respectively.

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Sucrose Preference. A two-bottle, unlimited access preference test was used to measure sucrose preference in the home cages. Upon arriving in the University animal care facility, mice were given access to two identical drinking bottles of tap water for several days to establish a baseline. Fluid intake was measured daily between 1000 and 1200. After drinking preferences for water stabilized, 0.5%, 1% or 2% sucrose dissolved in tap water was placed in one of the two drinking bottle for each mouse. Fluid intake was measured daily for four days and the position of the bottles was switched after two days. The values reported represent an average of the last two days of intake.

Statistical Analysis. All data are reported as the mean ± standard error. The open field data was analyzed with a Student's *t*-test (unpaired, two-tailed). All other data was analyzed using an analysis of variance (ANOVA). Statistically significant effects in ANOVAs were followed with post-hoc tests. Within group drug to saline control comparisons were performed with Dunnett's test. Within group comparisons with repeated measures were made with Bonferroni/Dunn's test. Between groups comparisons were made with Tukey's test. Acute amphetamine locomotor activity was analyzed with a two-factor ANOVA with genotype and dose as between factors. Repeated amphetamine data were analyzed with a mixed-factor ANOVA with genotype and dose as between factors and days as the within factor. Significant interactions were further examined with two and one factor ANOVAs. Conditioned place preference and sucrose preference data were analyzed with a two-factor ANOVA with genotype and dose as between factors. Self-administration data was analyzed with a two-factor ANOVA with genotype as the between factor and dose as the within factor. Data were analyzed with

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StatView (version 5.0.1, Cary, NC). The ED_{50} and p values for the cocaine self-administration dose-intake curve were determined with GraphPad Prism (version 4, San Diego, CA). Statistical significance was set at $p < 0.05$.

Results

Cart KO and WT mice

Adult male mice (7-9 weeks old at the start of the study) with a deletion of the entire coding region of Cart gene ($Cart^{tm1Amgn}$, or Cart KO) were utilized in these studies. This eliminated the possibility that any bioactive CART peptides could be generated from the Cart locus (Wierup et al., 2005). Body weight, longevity and fecundity did not differ between WT and Cart KO mice (data not shown). The breeding background had little effect on body weight in young adult male mice as those used here that are on a Black Swiss background, or whether the Cart gene deletion is on a C57BL/6 background (Asnicar et al., 2001; Wierup et al., 2005); only later in life does body weight differ between genotypes.

Exploratory activity in a novel open field

Locomotor activity in a novel, open field was similar between Cart KO and WT mice (Table 1). Crosses into different quadrants of the field or ambulation, and rears did not differ between genotypes over the first 10 min. or the entire 60 min. test period (p= 0.47 - 0.97, *t*-test, two-tailed). The number of entries into the center of the field, the time spent in the center of the field and the time along the walls (i.e., thigmotaxis) did not differ between genotypes over the first 10 min or the entire 60 min. test period (p= 0.14 – 0.84, *t*-test, two-tailed). Thus, the anxiety produced by the center of the open field (Britton and Britton, 1981) did not differ between Cart KO and WT mice. This finding contrasts with an anxiogenic effect of CART peptides in rats (Kask et al., 2000; Chaki et al., 2003). Failure to observe reduced anxiety in the Cart KO mice may reflect compensatory changes that obscured this phenotype, or it may suggest

that CART peptides are relevant in selective states of anxiety. The open field data suggests that exploratory behavior and motor function of WT and Cart KO mice were similar.

Reduced amphetamine-induced locomotor activity in Cart KO mice

Four distinct psychostimulant-induced behaviors including hyperlocomotion, locomotor sensitization, conditioned place preference and drug self-administration were examined in WT and Cart KO mice. Psychostimulants and many drugs of abuse increase locomotor activity (Wise and Bozarth, 1987). In this study, locomotor activity was defined as ambulation, vertical activity, stereotypic grooming and stereotypic head bobs.

Acute amphetamine administration increased locomotor activity in WT and Cart KO mice (Figure 2A). Amphetamine produced a dose-dependent increase in ambulation in both genotypes (two-factor ANOVA: dose, $F_{3, 50} = 13.72$, $p < 0.001$). The two highest amphetamine doses were significantly greater than saline and 1 mg/kg amphetamine in both genotypes ($p < 0.05$). Cart KO mice exhibited more ambulation than in WT mice at 6 mg/kg amphetamine ($p < 0.05$). However, WT mice were engaged in greater vertical activity at this dose instead (see below). Amphetamine produced a dose-dependent increase in vertical activity in WT mice only (two-factor ANOVA: genotype, $F_{1,50} = 4.29$, $p < 0.05$; dose, $F_{3,50} = 3.22$, $p < 0.05$; genotype X dose, $F_{3,50} = 4.45$, $p < 0.01$) and stereotypic grooming (two-factor ANOVA: genotype, $F_{1,50} = 8.04$, $p < 0.01$; dose, $F_{3,50} = 10.99$, $p < 0.0001$; genotype X dose, $F_{3,50} = 6.37$, $p < 0.001$), and at 6 mg/kg amphetamine ($p < 0.05$). Thus, vertical activity and stereotypic grooming were greater in WT mice than in Cart KO mice ($p < 0.05$). Acute amphetamine induced stereotypic head bobs in WT mice in a dose-dependent trend that never reached statistical significance; these were rarely seen in Cart KO mice after acute amphetamine administration. Ambulation and vertical activity

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during the 30 min. habituation period prior to receiving amphetamine did not differ between WT and Cart KO mice (data not shown). In summary, acute amphetamine increased ambulation, vertical activity and stereotypic grooming in WT mice, but only ambulation in Cart KO mice. The effects of acute amphetamine on locomotor activity were reduced in Cart KO mice compared to WT mice.

Repeated amphetamine administration produced different effects on locomotor activity in WT and Cart KO mice (Figure 2B). Locomotor sensitization was observed in WT mice, but rarely in Cart KO mice. Repeated amphetamine sensitized ambulation in dose-dependent manner in WT mice only (mixed-factor ANOVA, day as the repeated measure: dose, $F_{3,49}$ = 22.03, $p< 0.0001$; genotype X dose, $F_{3,49}$ = 2.75, $p< 0.05$; day, $F_{13,637}$ = 4.76, $p< 0.0001$; day X genotype, $F_{13,637}$ = 4.24, $p< 0.0001$; day X dose, $F_{39,637}$ = 2.25, $p< 0.0001$; day X genotype X dose, $F_{39,637}$ = 1.85, $p< 0.005$), and at the two highest amphetamine doses ($p< 0.05$). WT mice exhibited greater ambulation than Cart KO mice on days 8-14 at 6 mg/kg amphetamine ($p< 0.05$). Repeated amphetamine sensitized vertical activity in dose-dependent manner in WT mice only (mixed-factor ANOVA, day as the repeated measure: dose, $F_{3,49}$ = 9.26, $p< 0.0001$) at 3 mg/kg amphetamine ($p< 0.05$). WT mice exhibited more vertical activity than Cart KO mice between days 11-14 at 3 mg/kg amphetamine, and on days 1, 4 and 14 at 6 mg/kg amphetamine ($p< 0.05$). In summary, repeated amphetamine elicited robust sensitization of ambulation and vertical activity in WT mice, but not in Cart KO mice.

Stereotypic grooming and head bobs failed to sensitize with repeated amphetamine administration except in one case (Figure 2B). Stereotypic grooming increased in a dose-dependent manner in Cart KO mice only (mixed-factor ANOVA, day as the repeated measure: genotype effect, $F_{1,49}$ = 6.74, $p< 0.01$; dose effect, $F_{3,49}$ = 24.33, $p< 0.0001$; genotype X dose

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interaction, $F_{3,49} = 8.67$, $p < 0.0001$), and at 3 mg/kg amphetamine ($p < 0.05$). In Cart KO mice, the 6 mg/kg amphetamine dose showed a trend towards sensitization, but never reached statistical significance. Repeated amphetamine was unable to sensitize head bobs in either genotype. Although stereotypic behaviors generally failed to sensitize with repeated amphetamine administration, they were greater in WT mice than in Cart KO mice. Stereotypic grooming was significantly greater in WT mice than Cart KO mice at 6 mg/kg amphetamine on all days measured except day 5 ($p < 0.05$). Stereotypic head bobs were greater in WT mice than Cart KO mice at 6 mg/kg amphetamine on day 5 ($p < 0.05$); stereotypic head bobs were rare in Cart KO mice. In summary, repeated amphetamine administration sensitized stereotypic grooming in Cart KO mice only, but stereotypies were more pronounced in WT mice than Cart KO mice. Taken together, the ability of amphetamine to increase locomotor activity and produce locomotor sensitization was considerably attenuated in Cart KO mice by comparison with WT mice.

Reduced amphetamine conditioned place preference in Cart KO mice

The conditioned place preference (CPP) paradigm was used to measure the ability of amphetamine to produce a positive association with an environment. This effect is characteristic of rewarding/reinforcing stimuli like as drugs of abuse (Carr et al., 1989; Bardo and Bevins, 2000). Five mice from each genotype were not included in the study because they exhibited a strong pre-conditioning chamber bias. One Cart KO mouse died after an i.p. injection of saline. WT mice, but not Cart KO mice, exhibited an aversion to the smooth floor of the CPP apparatus during pre-conditioning test (Figure 1). The smooth floor was white, a color that may produce aversion in rodents. The aversion of WT mice to the smooth floor should have a small impact on

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the conditioning properties of amphetamine because this chamber was not used for drug-conditioning. Amphetamine produced CPP in dose-dependent manner (two-factor ANOVA: dose, $F_{1,43} = 18.05$, $p < 0.0001$) (Figure 3). Both amphetamine doses elicited CPP in WT mice, but only the higher dose was effective in Cart KO mice ($p < 0.01$). In Cart KO mice, 0.3 mg/kg amphetamine was as ineffective as saline in producing CPP, and was significantly less than the CPP produced in WT mice ($p < 0.05$). It is noteworthy that equal amounts of CPP were produced by 1 mg/kg amphetamine in both Cart KO and WT mice. Amphetamine never elicited place aversion in any mouse. In conclusion, amphetamine produced place preference in Cart KO and WT mice, but its conditioning effects were attenuated in Cart KO mice.

Reduced cocaine self-administration in Cart KO mice

One of the main characteristics of drugs of abuse is that they are voluntary consumed or self-administered (Schuster and Thompson, 1969). Drug self-administration is one of the most powerful measures of a drug's reinforcing effect (Shippenberg and Koob, 2002). Intravenous cocaine self-administration was used to examine the reinforcing effects of cocaine in Cart KO and WT mice. Mice were trained to respond for cocaine at 1 mg/kg/infusion under a fixed ratio-1 schedule of reinforcement. WT and Cart KO mice acquired cocaine self-administration at similar rates. Stable responding (see Methods and Materials for definition) at 1 mg/kg cocaine was attained at similar rates, by day 6.3 ± 0.5 for WT mice and by day 7.8 ± 0.9 for Cart KO mice ($p = 0.15$, *t*-test, two-tailed). Thereafter, a dose-response curved was generated by serially decreasing the cocaine dose. Cocaine self-administration behavior diverged between the genotypes once the cocaine dose was lowered (Figure 4). Cocaine intake (two-factor ANOVA, dose as repeated measure: genotype, $F_{1,20} = 5.58$, $p < 0.05$; dose, $F_{4,80} = 75.90$, $p < 0.0001$;

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genotype X dose, $F_{4,80} = 2.53$, $p < 0.05$) and responding for cocaine (two-factor ANOVA, dose as repeated measure: genotype, $F_{1,20} = 8.70$, $p < 0.01$; dose, $F_{4,80} = 6.56$, $p < 0.0001$; genotype X dose, $F_{4,80} = 2.82$, $p < 0.05$) was greater in WT mice than in Cart KO mice. WT mice exhibited greater cocaine intake at 0.125 mg/kg , 0.25 mg/kg and 0.5 mg/kg cocaine ($p < 0.05$), and greater response rates at 0.125 mg/kg and 0.25 mg/kg cocaine than Cart KO mice ($p < 0.05$). The right shift in the cocaine intake curve of Cart KO mice compared to the WT mice curve (ED_{50}= 0.20 and 0.38 mg/kg, WT and KO, respectively; $p < 0.005$) represented a 2-fold reduction in the potency of amphetamine without a change in its efficacy (Figure 4A). By contrast, the dose-response curve of Cart KO mice shifted down compared to that of the WT mice (Figure 4B). The efficacy of cocaine as a reinforcer in Cart KO mice appeared reduced, while its potency remained unaltered.

The difference in cocaine intake between WT and Cart KO mice was reflected by the activity in the nose-poke holes (Figure 4C). Responding for cocaine depended on the hole, cocaine dose, and genotype (mixed-factor ANOVA, dose as repeated measure: genotype, $F_{1,40} = 4.05$, $p < 0.05$; hole, $F_{1,40} = 136.26$, $p < 0.0001$; dose, $F_{4,200} = 7.25$, $p < 0.0001$; genotype X dose, $F_{4,200} = 3.15$, $p < 0.05$; hole X dose, $F_{4,200} = 4.93$, $p < 0.001$). Simplified analysis of the nose-poke activity in the active and inactive holes showed differential responding based on hole and/or genotype at 0.5 mg/kg cocaine (two-factor ANOVA: hole, $F_{1,38} = 91.33$, $p < 0.0001$), at 0.25 mg/kg cocaine (two-factor ANOVA: genotype, $F_{1,40} = 7.67$, $p < 0.01$; hole, $F_{1,38} = 67.13$, $p < 0.0001$; genotype X hole, $F_{1,40} = 7.40$, $p < 0.01$) and at 0.125 mg/kg cocaine (two-factor ANOVA: genotype, $F_{1,40} = 7.59$, $p < 0.01$; hole, $F_{1,40} = 50.66$, $p < 0.0001$; genotype X hole, $F_{1,40} = 6.05$, $p < 0.05$). At all cocaine doses, WT and Cart KO mice showed significantly greater activity in the active than in the inactive hole ($p < 0.01$) and significant differences in the active holes

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between genotypes at 0.125 mg/kg and 0.25 mg/kg cocaine ($p < 0.01$). In the course of this study, one mouse from each genotype was eliminated from this study because of a blocked i.v. catheter. In summary, WT and Cart KO self-administered cocaine, but the amount of cocaine consumed and responding for cocaine was attenuated in Cart KO mice.

To evaluate the possibility of a biased assessment of the reinforcing effects of cocaine from the serial dilution dose-response data, separate groups of mice were trained with a single, modest cocaine dose (i.e., 0.5 mg/kg/infusion). Cocaine self-administration was acquired at similar rates between Cart KO and WT mice. Stable responding for cocaine was observed at 5.4 ± 0.2 days for WT mice (n= 8) and 5.3 ± 0.2 days for Cart KO mice (n= 7) ($p = 0.99$, *t*-test, two-tailed). However, cocaine intake (12.3 ± 1.6 mg/kg, WT; 7.6 ± 1.0 mg/kg, Cart KO; $p < 0.05$, *t*-test, two-tailed) and responding for cocaine (active and inactive hole nose-pokes: 27.3 ± 4.4 and 1.8 ± 0.2, WT; 18.7 ± 3.2 and 7.9 ± 4.0, Cart KO; $p = 0.15$, *t*-test (active hole responses, WT vs. KO), two-tailed) differed between genotypes and was 68% in Cart KO mice compared to WT mice. In summary, a modest dose of cocaine supported self-administration in WT and Cart KO mice, but again, there was an attenuation in the amount of cocaine consumed and responding for cocaine in the Cart KO mice.

Sucrose preference is similar between Cart KO and WT mice

To determine if the diminished motivational response in Cart KO mice could result from a wide-ranging alteration in motivation, the motivational value of a natural reward was assessed. A sucrose preference test was conducted to address this possibility in WT and Cart KO mice (Catalanotto and Lacy, 1977). In a two-bottle, 24 hr. unlimited access test, a sucrose solution was preferentially consumed over water in a dose-dependent manner by WT and Cart KO mice

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(two-factor ANOVA: concentration, $F_{1,36} = 8.29$, $p = 0.001$; genotype, $F_{1,36} = 0.005$, $p = 0.94$) (Figure 5). The 2% sucrose solution was preferred over water by both genotypes ($p < 0.01$), but there was no difference between Cart KO and WT mice sucrose preferences at any sucrose concentration ($p > 0.05$). The amount of sucrose solution, water and total fluid consumed did not differ between genotypes (data not shown). In summary, sucrose preference did not differ between Cart KO and WT mice.

Discussion

We show that CART peptides are important for psychostimulant induced locomotor activity, locomotor sensitization, conditioned place preference and self-administration. These psychostimulant mediated behaviors were attenuated in Cart KO mice by comparison with WT mice. The data suggest a causal link between CART peptides and psychostimulant effects.

Studies with knockout mice must be interpreted with caution because deletion of the gene and breeding strategies may inadvertently alter the phenotype under investigation. The reduced responses to psychostimulants of Cart KO mice were not due to sensorimotor or cognitive impairments. First, locomotor activity in a novel open field was indistinguishable between WT and Cart KO mice. Second, Cart KO mice developed similar levels of CPP as WT mice at 1 mg/kg amphetamine, thereby suggesting similar capacities for forming contextual associations. Moreover, cocaine self-administration acquisition rates and discrimination between active and inactive nose-poke holes did not differ between genotypes. Third, fecundity, which requires partner recognition and receptivity was similar between genotypes. These data suggest there were no overt sensorimotor or cognitive impairments in Cart KO mice compared to WT mice. A potential limitation of these studies is variation in the background of the WT and Cart KO mice because each genotype was maintained as a separate colony without backcrossing onto the Black Swiss background. However, there was no drift in body weight or litter size within genotype throughout the course of these studies. Moreover, the amphetamine locomotor studies took over 10 months to perform, but variation in the responses to amphetamine within genotypes never emerged. Differences between WT and Cart KO mice in open field activity and sucrose preference might be expected if their backgrounds differed, but this was not observed.

Therefore, the attenuated psychostimulant responses of Cart KO mice were more likely to result from changes in the properties of these drugs in these mutant mice.

Locomotor responses to acute amphetamine were attenuated in Cart KO mice. Ambulation was the only locomotor response enhanced by acute amphetamine in Cart KO mice. By contrast, WT mice exhibited increases in ambulation, vertical activity and stereotypic grooming. Perhaps higher amphetamine doses would have elicited greater locomotor responses in Cart KO mice, but the similarity in the responses to two high amphetamine doses is one argument against this possibility. It is possible that CART peptides may be important for vertical activity and stereotypies, and less so for ambulation. The reduced potency, and possible efficacy of amphetamine in Cart KO mice may reflect a decreased ability to engage central motor systems. The inability of psychostimulants to stimulate CART peptide release in Cart KO mice may be important because CART peptides stimulate locomotor activity on their own. Injection of CART peptide 55-102 into the VTA was shown to increase locomotor activity in rats (Kimmel et al., 2000). Systemic haloperidol blocked this effect, thus suggesting dopaminergic regulation of CART peptide effects. Moreover, CART peptides modulate dopaminergic activity. Injection of CART peptide 55-102 into the lateral ventricles was shown to increase dopamine turnover in the nAcc and dorsal striatum (Yang et al., 2004). CART peptide modulation of psychostimulant locomotor activity is more complex, however. Injection of CART peptide 55-102 into the nAcc blocked cocaine and amphetamine induced locomotor activity (Jaworski et al., 2003; Kim et al., 2003). This inhibitory action of CART peptides may reflect a local negative feedback within the nAcc. Therefore, psychostimulants may induce increases in extracellular dopamine within the nAcc that stimulates CART peptide release at distal sites such as the VTA, medial SN and ventral pallidum as well as locally in the nAcc. CART peptides released in the

ventral midbrain probably modulate feedback to the nAcc perhaps by further increasing dopamine release as well as transfer of information from the limbic to motor pathways in the striatum, or translate motivation into action. The data suggests that CART peptides modulate psychostimulant induced locomotor activity presumably by regulating dopaminergic activity within mesolimbic and mesostriatal pathways.

Repeated amphetamine produced little locomotor sensitization in Cart KO mice compared to WT mice. The development of locomotor sensitization was delayed and the magnitude of the response in Cart KO mice was a fraction of that observed in WT mice. This data contrasts with a lack of locomotor sensitization produced by repeated injections of CART peptide 55-102 into the VTA of rats (Kimmel et al., 2000). Whether this disparity arises from methodological issues, or whether CART peptides alone are insufficient for sensitization to develop requires further research. CART peptides in the VTA are probably important for psychostimulant sensitization because this brain area is central to the development amphetamine locomotor sensitization (Dougherty and Ellinwood, 1981; Vezina and Stewart, 1990). Sensitization is considered important for understanding drug addiction because it reflects a long-lasting behavioral alteration that persists even after drug intake ceases in animals (Robinson and Berridge, 1993). The data suggests a role for CART peptides in the initiation, and perhaps in the expression of psychostimulant locomotor sensitization, and subsequently implicates them in psychostimulant addiction.

Amphetamine's ability to produce conditioned place preference was attenuated in Cart KO mice. Although Cart KO mice did not exhibit major sensorimotor and cognitive deficiencies, pre-drug conditioning tests showed that Cart KO mice failed to find the smooth, white floor chamber of the CPP box an aversive environment, as the WT mice did. This may

reflect an inability of Cart KO mice to perceive color, light intensity or both because CART peptides are normally found in ganglion neurons of the retina (Couceyro et al., 1997). However, this could not account for the attenuation of amphetamine induced CPP in Cart KO mice because the smooth, white floor chamber was never used for drug conditioning. Thus, the decreased potency, and possibly the efficacy of amphetamine in Cart KO mice may reflect a reduction amphetamine's associative or conditioning property. The ability of amphetamine to produce CPP relies, in part, on the ability of CART peptides to elicit CPP on their own. Intra-VTA injections of CART peptide 55-102 produced CPP in rats (Kimmel et al., 2000). CART peptides in the nAcc, VTA, basolateral and central nucleus of the amygdala, and dentate gyrus of the hippocampus may contribute to psychostimulant induced CPP because these brain areas important for CPP especially in the formation and retrieval of associative memories (Carr and White, 1986; Olmstead and Franklin, 1997). The data suggests that CART peptides modulate psychostimulant induced contextual associations and they may be important for habit learning and drug relapse.

Cocaine self-administration was attenuated in Cart KO mice compared to WT mice. The amount of cocaine consumed as well as responding for cocaine was reduced in Cart KO mice compared to WT mice. Yet, Cart KO mice learned to respond for cocaine at the same rate as WT mice. The Cart KO mice cocaine dose-response curve shifted down compared to that of WT mice. This vertical shift in the dose-response curve is consistent with a change in the efficacy of cocaine for maintaining response rates. By contrast, the cocaine dose-intake curve of Cart KO mice shifted to the right relative to the WT mice curve. This horizontal shift represented a change in the potency of cocaine to support intake. The decrease in cocaine intake and

responding for cocaine in Cart KO mice most likely reflected a change in the reinforcing property of cocaine.

The cocaine self-administration data also suggest a possible role of CART peptides in the susceptibility to psychostimulant self-administration. Downward vertical shifts in the dose-response curves as those observed for Cart KO mice is associated with decreased vulnerability to self-administer drugs of abuse (Piazza et al., 2000). The horizontal shift of Cart KO dose-intake curve is incongruent with this vulnerability model, but it does not preclude reduced vulnerability. This data predicted that cocaine would be less reinforcing in Cart deficient mice at low to moderate doses. In fact, when drug-naïve mice had access to a modest dose of cocaine (i.e., 0.5 mg/kg/infusion), the Cart KO consumed less and responded less for cocaine than WT mice; cocaine was less reinforcing in Cart KO mice. CART peptides in brain areas that control motivation support a role for CART peptides in the reinforcing effects of psychostimulants. In summary, these data suggest that CART peptides are important for the reinforcing properties of psychostimulants as well as the susceptibility to psychostimulant self-administration.

Deletion of the Cart gene failed to elicit generalized changes in motivation. Body weight and sucrose preference was similar between Cart KO and WT mice. Differences in body weight between WT and Cart KO mice appear in older (i.e., 40 week old) male mice and in heterozygous female mice fed a high fat diet (Asnicar et al., 2001; Wierup et al., 2005), but not in 7-9 week old male mice used in these studies. Either CART peptides do not modulate food preference or intake in young, sated mice, or compensatory changes have obscured this phenotype. The similarity in sucrose preference between WT and Cart KO mice suggest that CART peptides may modulate stimuli with relatively high values, or in conditions where a

stimulus assumes a high rewarding/reinforcing value. Whether CART peptides help to discriminate the value of the stimuli requires further investigation.

This study demonstrates a causal link between CART peptides and the behavioral effects of psychostimulants. In the absence of CART peptides, psychostimulant mediated hyperlocomotion, locomotor sensitization, conditioned place preference and drug self-administration are considerably attenuated. The data suggests that CART peptides are neuromodulators of the behavioral properties of psychostimulants, and are important for psychostimulant addiction. Unless CART peptides are selective modulators of psychostimulants, they may be important for drug addiction in general, but this warrants further research.

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Footnotes

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Legends for Figures

Figure 1. Chamber bias of the 3-chamber conditioned place preference box. (A) The percentage of time spent in each chamber over 20 min. during the pre-conditioning test phase. Floor texture (i.e., wire mesh, rod or smooth floor) differentiated the chambers. A modest bias against the smooth floor chamber is evident. Data represents all the mice tested irrespective of genotype (n= 49). (*, $p< 0.05$, Tukey's test) **(B)** Segregating mice by genotype showed that the WT mice, but not the Cart KO mice, exhibited an aversion to the smooth floor chamber. (means ± SEM are reported; n= 25, WT and n= 24, KO)

Figure 2. Amphetamine induced locomotor activity in WT and Cart KO mice. Locomotor activity was measured and reported here for the 2 hrs. after amphetamine or saline administration. Ambulation and vertical activity were measured daily, and stereotypies were measured on alternate days. **(A)** Acute amphetamine administration increased ambulation, vertical activity, and stereotypic grooming in WT mice, and ambulation in Cart KO mice. Significant differences between drug and saline within genotype (*, $p< 0.05$, Dunnett's test) are noted as well as between genotypes within a drug dose (‡, $p<0.05$, Tukey's test). **(B)** Repeated amphetamine administration increased ambulation in WT mice (*, $p< 0.05$, Bonferroni/Dunn, 3 mg/kg: days 1, 2 vs. 6-14; *days 3-5 vs. 9-14, not show*n, and 6 mg/kg: day 1 vs. 10-14; *days 2, 3 vs. 8-14, not shown*), vertical activity in WT mice (3 mg/kg: days 1, 2 vs. 14), and stereotypic grooming in Cart KO mice (3 mg/kg: days 1, 3, 5 vs. 13). At individual doses, WT mice exhibited more activity than Cart KO mice in ambulation, vertical activity (*6 mg/kg: days 6, 14, not shown*), stereotypic grooming and head bobs (‡, $p< 0.05$, Tukey's test). The repeated 1 mg/kg amphetamine data for both genotypes was omitted because it was not different than

saline. (means ± SEM are reported; ◊, saline; □, 3 mg/kg; O, 6 mg/kg; WT, open symbols; KO filled symbols; n= 7-10/dose/genotype, except n=3/genotype at 1 mg/kg amphetamine).

Figure 3. Amphetamine induced conditioned place preference (CPP) paradigm in wild-type and Cart KO mice. A three-chamber box with different textured floors was used for CPP. Place preference was established if the mice spent more time in a chamber previously associated with amphetamine. Preference was calculated by subtracting the time spent in the amphetamine-paired chamber before drug conditioning from the time spent there after drug conditioning. Amphetamine elicited place preference in WT and KO mice (*, $p< 0.01$, Dunnett's test). However, the low dose of amphetamine was ineffective in producing CPP in KO mice when compared to saline, or to WT mice given the same dose of amphetamine (‡, $p< 0.05$, Tukey's test). (means ± SEM are reported; n= 5, saline, WT and KO; n= 11, 0.3 mg/kg amphetamine, WT and KO; n= 9 and n=8, 1 mg/kg amphetamine, WT and KO respectively)

Figure 4. Intravenous cocaine self-administration in WT and Cart KO mice. Both WT and Cart KO mice were given daily, unrestricted access to cocaine for 2 hrs. on a fixed ratio 1 schedule of reinforcement. (**A** & **B**) A dose-intake and dose-response curve was generated for each animal by training at 1 mg/kg/infusion and serially lowering the dose once stable responding was established at each dose. Group means reflect averages of three days of stable intake for each animal at each dose. Stable responding was defined as cocaine intake that varied < 10% and active hole nose-pokes that varied < 20%. Cocaine intake, cocaine responding and active hole nose-poke behavior were significantly reduced at several cocaine doses in the KO mice compared to WT mice (‡, $p< 0.05$, Tukey's test). **C**. Nose poke behavior in the active and

inactive hole at each cocaine dose. At all cocaine doses, activity in the active hole was significantly greater than in the inactive hole in both genotypes (*, $p < 0.05$, Bonferroni-Dunn). A significance difference in active hole nose-pokes between genotypes was observed at two amphetamine doses (‡, $p < 0.01$, Tukey's test). (means ± SEM are reported; n= 11/genotype)

Figure 5. The reinforcing effects of a natural reward in WT and Cart KO mice. A two-bottle preference test was used to evaluate the reinforcing properties of sucrose. Different concentrations of sucrose were tested in separate groups of mice from each genotype. The average of two daily readings is reported. Both WT and KO mice showed concentration-dependent sucrose preferences over water (*, $p < 0.01$, Dunnett's test). However, there were no differences in sucrose preference between genotypes across all sucrose concentrations tested. (means ± SEM are reported, n= 6-8/dose)

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Table 1. Exploratory behavior of WT and Cart KO mice in a novel open field. Mice were exposed to a large open field for 1 hr. in low ambient lighting. Their activity over the entire first 10 min. and the total 60 min. (i.e., first two min. of every ten min.) is reported. No statistically significant differences were found between genotypes (p< 0.8-0.9, t-test, two tailed) (n= 8/genotype).

	10 min.		60 min.	
	WT	KO	WT	KO
crosses	174 ± 14	189 ± 28	120 ± 15	122 ± 21
crosses (middle)	19 ± 3	21 ± 5	15 ± 2	14 ± 4
rears	30 ± 5	39 ± 11	34 ± 8	34 ± 9
time in middle (sec.)	27 ± 5	25 ± 2	36 ± 5	25 ± 5
time along walls (sec.)	408 ± 30	449 ± 14	523 ± 30	581 ± 21

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Figure 1

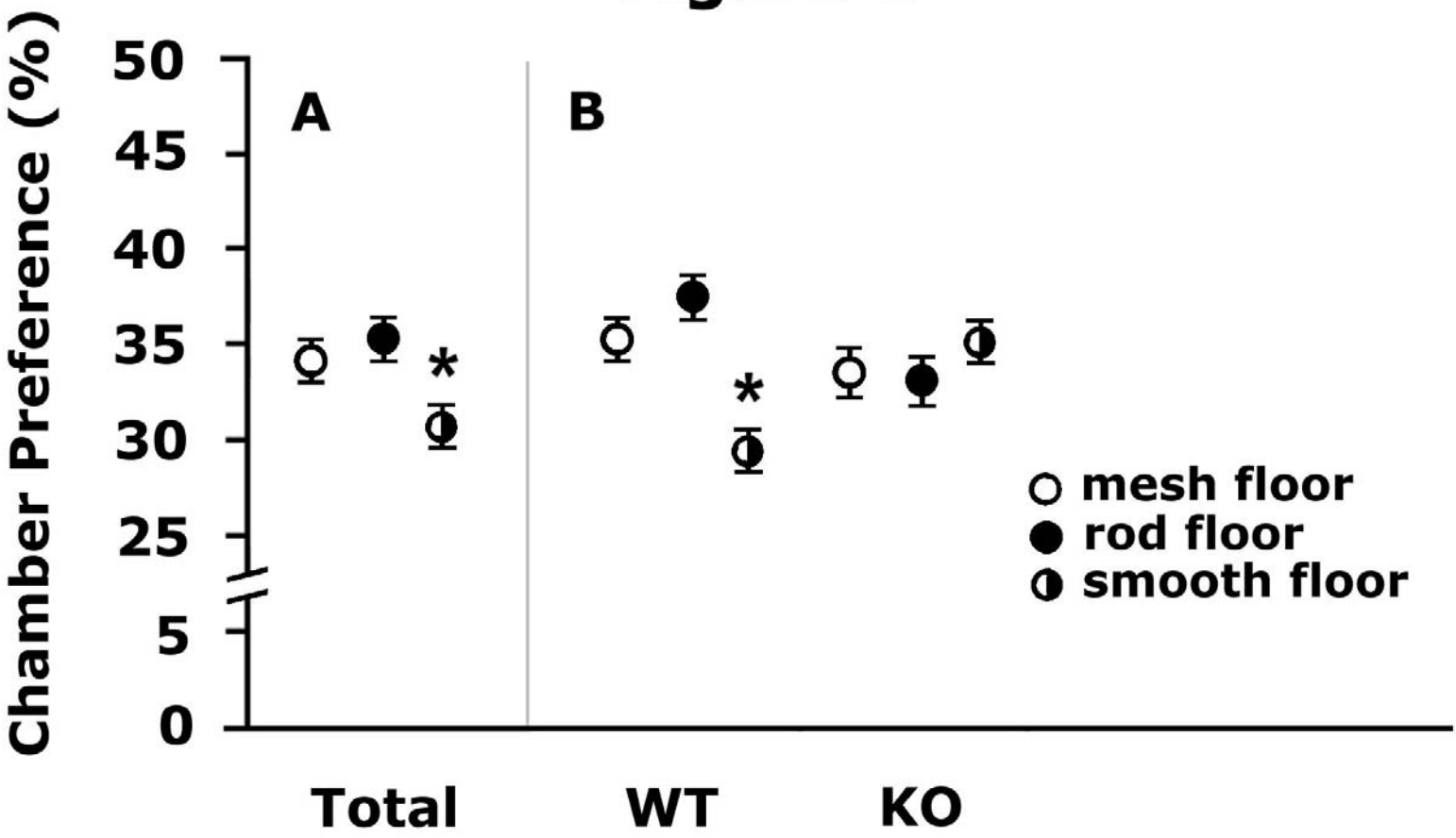

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Figure 2

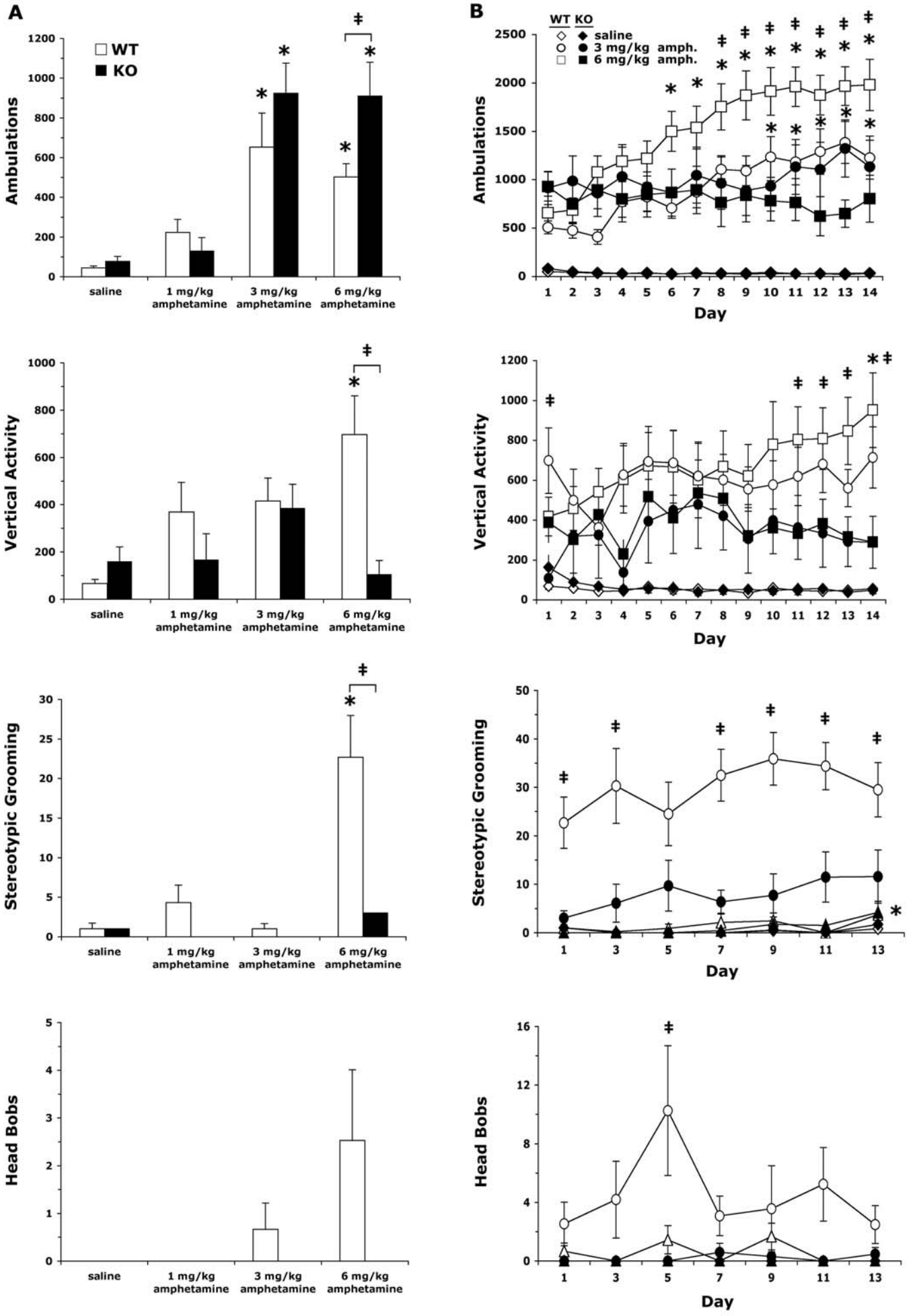

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Figure 3

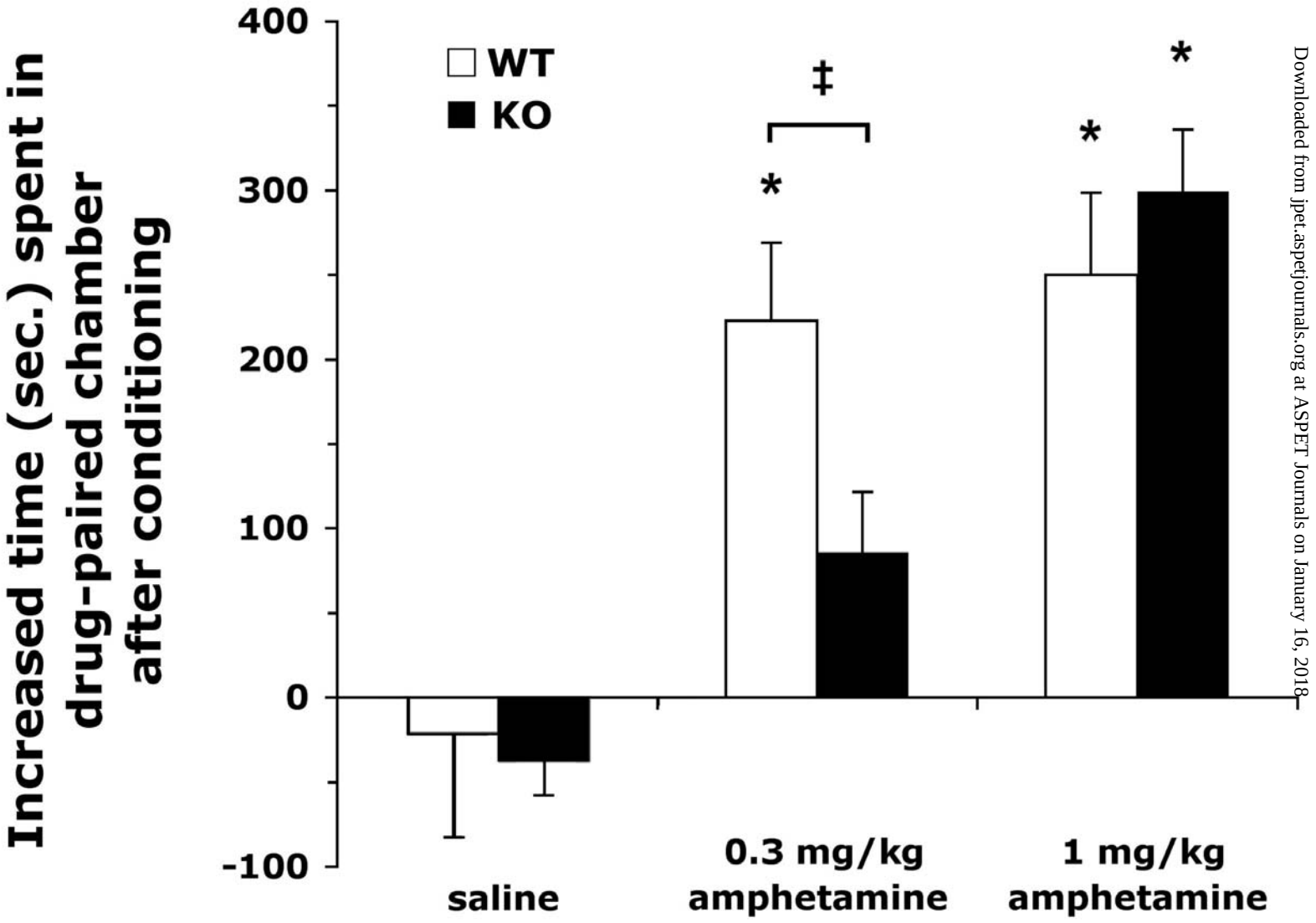

Figure 4

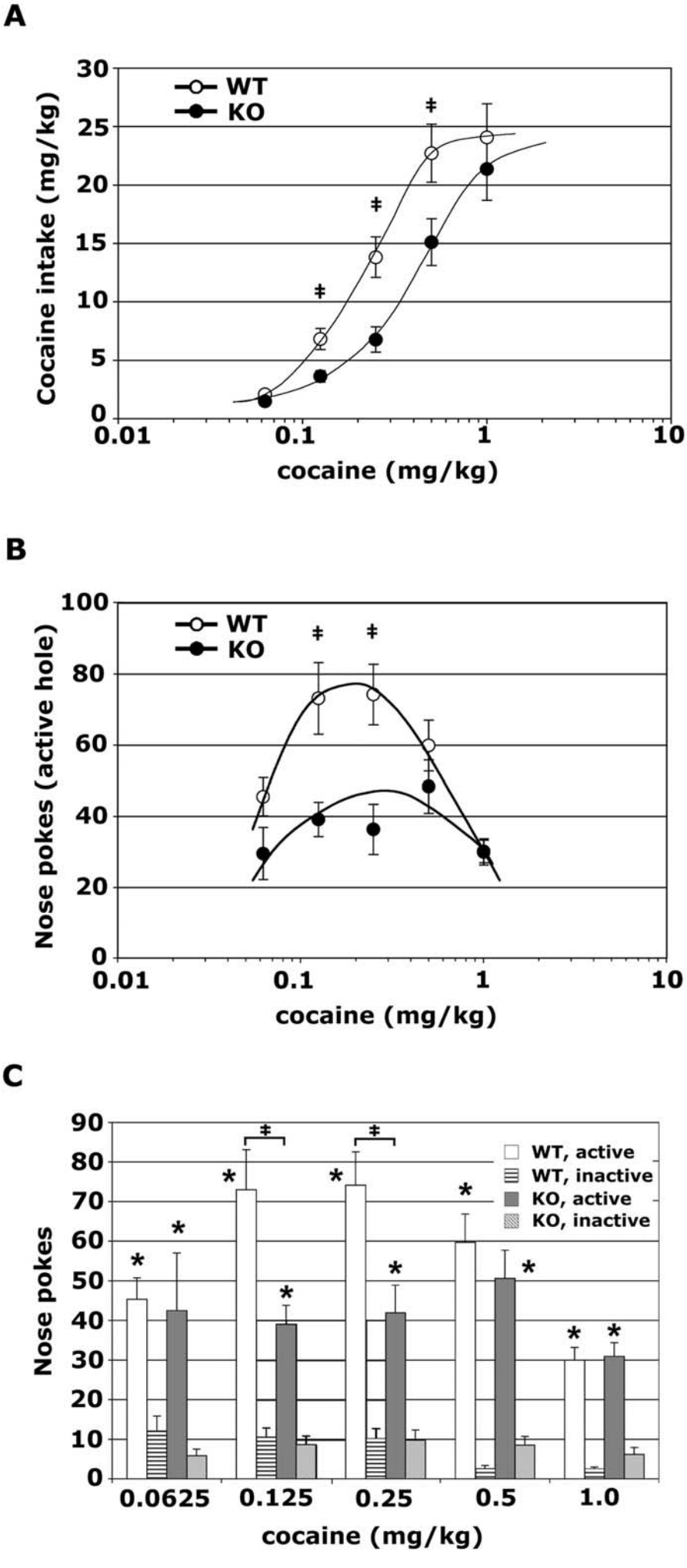

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Figure 5