HHS Public Access

Author manuscript

Cell Mol Neurobiol. Author manuscript; available in PMC 2016 August 01.

Published in final edited form as:

Cell Mol Neurobiol. 2015 August ; 35(6): 819–826. doi:10.1007/s10571-015-0175-9.

Reward and Toxicity of Cocaine Metabolites Generated by Cocaine Hydrolase

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Abstract

Butyrylcholinesterase (BChE) gene therapy is emerging as a promising concept for treatment of cocaine addiction. BChE levels after gene transfer can rise 1000-fold above those in untreated mice, making this enzyme the second most abundant plasma protein. For months or years, gene transfer of a BChE mutated into a cocaine hydrolase (CocH) can maintain enzyme levels that destroy cocaine within seconds after appearance in the blood stream, allowing little to reach the brain. Rapid enzyme action causes a sharp rise in plasma levels of two cocaine metabolites, benzoic acid (BA) and ecgonine methyl ester (EME), a smooth muscle relaxant that is mildly hypotensive and weakly rewarding. The present study, utilizing Balb/c mice, tested reward effects and cardiovascular effects of administering EME and BA together at molar levels equivalent to those generated by a given dose of cocaine. Reward was evaluated by conditioned place preference. In this paradigm, cocaine (20 mg/kg) induced a robust positive response but the equivalent combined dose of EME + BA failed to induce either place preference or aversion. Likewise, mice that had undergone gene transfer with mouse CocH (mCocH) showed no place preference or aversion after repeated treatments with a near lethal 80 mg/kg cocaine dose. Furthermore, a single administration of that same high cocaine dose failed to affect blood pressure as measured using the non-invasive tail cuff method. These observations confirm that the drug metabolites generated after cocaine hydrolase gene transfer therapy are safe even after a dose of cocaine that would ordinarily be lethal.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

Keywords

Ecgonine methyl ester; Cocaine; Butyrylcholinesterase; Cocaine hydrolase; Addiction; Gene therapy; Adeno-associated viral vector; Place conditioning

Introduction

Cocaine addiction is a devastating condition that has evaded therapeutic relief for decades. Cocaine abuse occurs across all ages in both males and females, with severe adverse societal and medical consequences. A promising treatment strategy on the horizon is to administer enzymes that expedite cocaine metabolism in the bloodstream and prevent access to the brain. Over the past decade a highly effective cocaine hydrolase (CocH) has been derived by successive modifications of butyrylcholinesterase (Sun et al., 2001; Sun et al., 2002; Yang et al., 2010; Zheng et al., 2008), ultimately yielding a ~1500-fold increase in catalytic efficiency towards cocaine. We are actively exploring the therapeutic utility of gene transfer of CocH in mice and rats. Using adeno-associated viral vector (AAV) or helper-dependent adenoviral vector (hdAD) it has been proven possible to accelerate cocaine hydrolysis nearly 1,000,000-fold without apparent ill effects and keep high levels for months or years after a single treatment (Brimijoin et al., 2013; Gao et al., 2013; Geng et al., 2013). The result is near instantaneous hydrolysis that reduces brain cocaine uptake by 98% and eliminates many cocaine-induced effects, including ongoing operant responding for i.v. cocaine reward (Anker et al., 2012; Carroll et al., 2011; Carroll et al., 2012; Zlebnik et al., 2014).

The enzymatic attack on cocaine yields stoichiometric quantities of two products, ecgonine methyl ester (EME) and benzoic acid (BA), which are generally considered non-toxic. Consistent with that view, our preclinical studies encompassing a wide range of metabolic and physiologic functions found no observable adverse effects of high-dose vector treatment (Murthy et al., 2014a; Murthy et al., 2014b). It is conceivable, however, that a user taking larger cocaine doses to compensate for accelerated metabolism might encounter toxicity from a sudden release of EME and BA.

In fact, EME has mild rewarding effects but is also a potent vasodilator and has been suspected of cerebrovascular events associated with cocaine abuse (Kurth et al., 1993). BA, on the other hand, is not associated with rewarding effects and is not considered to be toxic. To our knowledge, there are no reports on the toxicity or reward value of these two compounds in equal part mixtures, and this information gap limits the ability to assess overall safety of CocH gene transfer. For that reason, the present study, based on gene transfer of a mouse CocH version, employed non-invasive paradigms to search for reward effects in conditioned place preference (CPP) tests, and cardiovascular effects detected by tail-cuff blood pressure determinations.

MATERIALS AND METHODS

Drug and biological sources

Cocaine HCl was obtained from the National Institute of Drug Abuse (Research Triangle Institute, Research Triangle Park, NC, USA) Nitroglycerin (NG) was obtained from

American Regent, Inc (Shirley, NY) through Mayo Medical Center Pharmacy Services. NG was diluted in 0.9% NaCl before i.p. injections.

Animals

Balb/c male mice (6–7 weeks old) from Harlan (Madison, WI) were housed in plastic cages with access to water and food (Purina Laboratory Chow, Purina Mills, Minneapolis, MN, USA) in rooms controlled for temperature (24 °C), humidity (40–50%), and illumination (light/dark, 12h/12h, lights on at 6:00 a.m.). The protocol (A59111) was approved by the Mayo Clinic Institutional Animal Care and Use Committee, and experiments were conducted in accordance with the Principles of Laboratory Animal Care in an AAALAC-accredited facility.

Viral gene transfer

AAV-8 vector incorporating cDNA for luciferase or mouse CocH with amino acid substitutions for improved cocaine hydrolysis (A199S/S227A/S287G/A328W/Y332G), was prepared as previously described (Balazs et al., 2012; Geng et al., 2013). The mCocH sequence was ligated into pAAV-VIP vector (Balazs et al., 2012) between Not I and BamH I. An AAV-VIP-mBChE mutant plasmid was co-transfected into HEK293T cells with pHelper and pAAV2/8, using **X-treme GENE HP** DNA Transfection Reagent (Roche). AAV virus was purified from cell lysates by ultracentrifugation against Optiprep Density Gradient Medium-Iodixanol (Sigma-Aldrich, St Louis MO). Vector (200 μl, 10^{13} viral particles) was delivered to 8 week old mice via tail-vein.

Blood collection

Blood (< 0.1 ml) was taken from the cheek with a 21-gauge mouse-lancet and bleeding was stopped with sterile gauze. Samples were centrifuged for 15 min (8000g) and plasma was stored at −20 °C. Cocaine hydrolase activity was assayed in a solvent-partitioning assay with ^{3}H-cocaine (50 nCi) and cold cocaine (18 μM) as described (Brimijoin et al., 2002). A related procedure determined levels of ^{3}H-cocaine and benzoic acid, with DFP added to halt enzymatic breakdown.

Conditioned Place Preference Apparatus

Procedures were conducted in a three chambered CPP apparatus within a sound-attenuating enclosure (MED-CPP-MSAT and ENV-016 MD), from Med Associates Inc, St Albans, VT USA. The two conditioning chambers, white on the left and black on the right, with dimensions of 16.8 × 12.7 cm, were separated by a central 7.2 × 12.7 cm gray chamber. The white chamber had a steel bar floor, the black chamber had a steel mesh floor, and the central gray chamber had a smooth PVC floor. The chambers were separated by automatic guillotine doors under computer control. Time spent in each chamber was automatically recorded in seconds by photo beams spaced 2.8 cm apart and 1 cm from the end wall. After each session the floors were removed, cleaned with 70% ethanol, and air dried, while the chambers were thoroughly wiped with 70% ethanol to eliminate waste and scent from the previous mouse.

Conditioned Place Preference Protocol

A standard “unbiased protocol” was adopted following published guidelines (Roux et al., 2003). This consisted of four phases conducted over 11 days: first, habituation to the experimental room (day 1); second, exclusion test (day 2); third, conditioning phase (days 3 – 10), fourth, place preference test (day 11). The habituation phase included general handling and acclimation to the experimental room with all CPP instruments running. For exclusion testing the mice were placed in the neutral gray central area and allowed free access to all three chambers for 20 min while time spent in each chamber was recorded. Mice were excluded from further study if they showed strong unconditioned aversion (< 25% of session time) or preference (> 75% of session time) for any compartment. The conditioning phase (days 3–10) consisted of 8 consecutive daily 20-min sessions alternating between drug and vehicle injection. Treatment groups were counterbalanced by assigning half to condition A (white chamber bar-floor + drug, black chamber mesh floor + saline) and half to condition B (black chamber mesh-floor + drug, white chamber bar-floor + saline). Overall, the mice received four stimulus trials (20 mg/kg cocaine or metabolite mixture paired with one color chamber and one floor cue) and four non-stimulus trials (saline injection paired with the other color chamber and floor cue). Trials occurred on alternate days at the same time for 8 consecutive days. Throughout the conditioning process, mice were returned to their assigned chambers immediately after receiving i.p. injections of drug or saline. During the conditioning phase, access to the central gray chamber was blocked. On day 11, place preference tests were run. As in the preconditioning test, each mouse received a vehicle injection and was then placed in the neutral gray chamber with free access to all chambers. Time spent in each chamber was recorded and factored into a place preference score (see Data Analysis).

Blood pressure

Systolic and diastolic pressure were measured non-invasively in conscious mice using a tail-cuff system (CODA; Kent Scientific). In brief, mice were acclimatized to the tail cuff system for four consecutive days. On the fifth day, data were collected over a 25 minute interval immediately following a given treatment (e.g., injection of cocaine or nitroglycerin).

Intraperitoneal Injections—For all the experiments, intended dose of cocaine was freshly dissolved in 0.9% NaCl before i.p., delivery. The volume was calculated according to the body weight of the subject (for 20 mg/kg cocaine dose, ~ 200 μl per subject was injected; for 80 mg/kg dose ~ 300 μl was injected). Control animals that received saline, were calculated accordingly and the volume of the injection was either 200 or 300 μl as required by the particular experiment.

The volume of nitroglycerin injections were calculated at 150 μl for i.p. delivery.

Data Analysis and Statistics

Place preference scores (PPS) were derived from the data obtained in the preconditioning phase and the final place preference test as described in a standard reference (Roux et al., 2003). PPS values indicate the relative proportion of time spent in the reward chamber vs. the non-reward chamber during a 20 min (1200 sec) session. These values were calculated

as follows: PPS = Tr × 1200/(1200 − Tg), where Tr is seconds in the reward chamber and Tg is seconds in the gray, non-reward chamber. This score accounts for 100% of the total time in each test by proportionally allocating time in the central gray compartment to the time value of each conditioned compartment.

A formal power analysis was not undertaken. However, with an intention to detect relatively small effects, group sizes were set at 8 for measurements of blood pressure and at a minimum of 8 to 12 for CPP behavioral tests. Data were analysed with Student's t-test using GraphPad Prism Statistical Software Version 6.0 (San Diego, CA, USA). Statistical significance was accepted if $p < 0.05$.

RESULTS

Experimental Sequence

After acclimation on arrival, mice were subjected to CocH gene transfer. At ~ 3 weeks, stable enzyme expression was confirmed and conditioned place preference studies began. On completion of CPP studies the mice were rested for two weeks, enzyme expression was reconfirmed, and blood pressure studies began (Flowchart, Fig. 1).

In Vivo Time Course of Cocaine Hydrolase

Initial experiments addressed the levels and duration of cocaine hydrolysing activity in plasma after BChE gene-transfer. As illustrated (Fig. 2), cocaine hydrolysis activity in plasma reached a level of 35 U/ml, 3 weeks after administration of AAV-CocH vector. At 7 and 12 weeks (on completion of CPP), the levels were ~ 45 U/ml and ~33 U/ml, indicating high and stable enzyme expression across the experimental cycle. Consistent with our earlier reports (Geng et al., 2013; Murthy et al., 2014a) the rise in cocaine hydrolysis activity was ~ 100,000 fold above the pre-treatment level driven by native BChE (0. 0004 U/ml).

Conditioned place preference

A standard CPP paradigm (schematic in Fig. 3A) was used to examine the "reward value" of cocaine and its metabolites. 30% of the mice were eliminated based on the exclusion analysis (preconditioned place preference) in untreated mice and mice subjected to vector treatment were not any different. In subjects with no vector treatment an initial positive control experiment with cocaine (20 mg/kg, i.p.) induced a robust CPP ($p = 0.001$) whereas a "saline reward" induced none (Fig. 3B). In comparison, no place preference, either positive or negative, arose in mice on a conditioning paradigm in which i.p. cocaine was replaced by an equimolar equivalent of its metabolites (13 mg/kg EME + 7 mg/kg BA). As an empty vector control, AAV Luciferase control mice (n =8) induced significant CPP ($p = 0.01$) at a dose of 20 mg/kg cocaine (data not shown).

Next we examined the rewarding effects of a much larger cocaine dose that was given after BChE gene transfer. For an initial safety test, two vector-treated mice were given cocaine at 80 mg/kg, i.p. (mouse LD_{50} is 95 mg/kg) and observed closely for one hour. As the mice gave no sign of behavioral disturbance or other overt reactions, this dose was selected for further CPP studies. We anticipated that the mice might show conditioned place *aversion*

(CPA). However, despite the large quantity of cocaine delivered, neither preference nor aversion for the drug-paired compartment arose (Fig. 3B).

Blood pressure

Several experiments were performed to characterize the effects of chronic mCocH expression as well as the acute pressor or depressor effects of cocaine administration in age-matched control and vector-treated mice.

Baseline measurements of systolic and diastolic blood pressure were taken from age-matched AAV-mCocH mice and controls (Fig. 4A). Mean systolic blood pressure at baseline was 132 ± 5 mmHg in controls and 141 ± 5 mmHg in AAV mCocH mice (p = N.S.), while mean diastolic pressure was 89 ± 5 mmHg in control mice and 101 ± 4 mmHg in AAV mCocH mice. The slight group difference in diastolic pressure was statistically significant ($p < 0.05$).

Once baseline blood pressure values were established, we attempted to determine whether mCocH-dependent metabolism of a large dose of cocaine (80 mg/kg) would generate sufficient EME and BA to elicit a sharp reduction in blood pressure. To test this possibility, control mice received saline (300 μl i.p.) and vector-treated mice received cocaine (80 mg/kg, i.p, ~ 300 μl; an otherwise lethal dose) and pressure measurements were made immediately afterward. AAV mCocH mice displayed a mean systolic pressure of 151 ± 2 mmHg and a diastolic pressure of 104 ± 3 mmHg (Fig. 4A). Corresponding values in controls were 140 ± 6 mmHg and 95 ± 4 mmHg. Blood pressure was slightly higher after saline injection in control mice (presumably due to the stress of injection) and did not decrease significantly over time. Similarly, and in contrast to the hypothesized depressor effects of EME and/or EME+BA, systolic and diastolic pressures in AAV-treated mice did not change significantly after the 80 mg/kg dose of cocaine.

Positive control for Blood Pressure—To validate the sensitivity and reliability of the system used in the previous experiment for detecting hypotensive events, we administered the potent vasodilator, nitroglycerin, to 8 previously untreated control mice. At baseline these mice recorded a systolic pressure of 155 ± 6 mmHg and diastolic pressure of 121 ± 5 mmHg (Fig. 4B). After 5 baseline sessions, the mice were given a 5 mg/kg dose of NG, which led to a systolic pressure of 148 ± 6 mmHg and a diastolic pressure of 114 ± 6 mmHg. This 7 mm Hg drop from baseline was not statistically significant. After a 3 hour rest, the mice received a 15 mg/kg dose of NG, which led to a mean systolic pressure of 132 ± 7 mmHg and a diastolic pressure of 98 ± 6 mmHg. The 23 mg Hg pressure drop was statistically significant (systolic $p = 0.01$* and diastolic $p = 0.005$**). Of note, these doses of NG are well below previously-reported depressor doses administered to mice. Thus we conclude that a clinically-relevant reduction in blood pressure caused by EME and BA would have been readily detected using our approach.

Discussion

The current experiments are part of ongoing preclinical testing for a proposed gene transfer therapy to treat cocaine addiction. We recently reported data indicating that the inherent risk

of this technology may be relatively low (Murthy et al, 2014a and b). A concern not previously addressed, however, was the potential for effects from cocaine metabolites suddenly generated in a treated user trying to overcome an enzymatic blockade of drug reward. Although neither of the main metabolites is considered highly toxic, EME does affect blood vessel smooth muscle and brain reward centers (Zakusov et al., 1978) while benzoic acid might evoke an array of unanticipated actions. Therefore, widening the scope of safety studies relevant to these two molecules, we explored effects of cocaine and metabolites with non-invasive CPP tests and tail-cuff blood pressure readings in vector-treated mice expressing high levels of CocH.

We chose the CPP paradigm over traditional reinstatement procedures to evaluate reward effect because of its operational ease and non-invasiveness (Cunningham et al., 2006). In light of published studies exploring phenotypic variability for place preference among inbred mouse strains (Eisener-Dorman et al., 2011), Balb/c were selected as promising responders. After successfully establishing CPP in cocaine-naïve mice with a drug dose of 20 mg/kg, we tested a “metabolite-equivalent,” which generated no CPP. This expected outcome essentially ruled out a significant central stimulatory effect from these two molecules in combination. In fact, the data were consistent with a conclusion that they might even generate a weak aversion, but the small trend was statistically insignificant.

Benzoic acid’s poor solubility prevented testing such mixtures at larger doses, but this limitation was overcome indirectly, by giving high-dose cocaine (80 mg/kg, i.p.) to vector-treated mice protected by CocH gene transfer. Our previous data report that such animals convert cocaine into metabolites within seconds (Brimijoin et al., 2013; Gao et al., 2010), and rats given CocH vector cease ongoing responding for i.v. cocaine reward (Zlebnik, 2014). Therefore we anticipated that the “therapeutically-relevant” levels of benzoic acid liberated from high-dose cocaine in this study would induce place aversion. In fact, the mice exhibited no immediate motor response to cocaine (e.g., hyper locomotion or ataxia) and they failed to exhibit CPP or CPA. We conclude that the metabolite mixture generated by CocH is, essentially, neither rewarding nor aversive.

The second issue investigated in this study was the possibility of cardiovascular side effects from metabolites generated after cocaine exposure (Kurth et al., 1993). A key question in regard to CocH safety, therefore, was whether the rapid increase in EME and BA generated from the near lethal 80 mg/kg dose of cocaine would affect blood pressure. Before cocaine administration we found no differences in systolic blood pressure between controls and vector-treated mice, but diastolic pressure was elevated by 10% in the latter ($p < 0.05$). In murine subjects, this elevation in diastolic pressure is not of great concern as alterations in systolic pressure are considered more important in any indications. Also, there is general trend in increase in blood pressure (Fig 4A) on acute treatment. As mentioned in the methods, acute treatment is immediately followed after a 25 min baseline session; the stress imparted on the mice on handling and injection could be a causal factor for the noted increase. In addition, even though utmost care is taken for minimal disturbance from external factors, it is not 100% controlled; minute disturbances such as someone walking beside the door, or an auto computer boot is enough to induce slight changes in the blood pressure. More importantly, there was a complete lack of effect on blood pressure when

CocH vector treated mice received high dose cocaine. The control experiment with the potent vasodilator NG confirmed that the instrumentation and procedures would have detected clinically-relevant reductions in blood pressure of 15 mm Hg or greater. Therefore our data suggest that there is little risk of acute and medically significant hypotension from rapid cocaine metabolism by CocH.

The information to date leads us to predict that only a small fraction of injected cocaine will reach the brain of rodents with high circulating levels of BChE-based cocaine hydrolase. In fact, we recently found that brain uptake of i.v. ^{3}H-cocaine was reduced 98% in rats treated months earlier with a single very large (10^{12} viral genomes) injection of CocH vector that appeared to obliterate cocaine reward (Zlebnik, 2014). It is highly unlikely that comparably dramatic protection can ever be obtained in human subjects, given the real and perceived risks of viral gene transfer. Bearing this in mind, one can imagine a treatment-seeking addict choosing a safe, low-dose vector therapy that will blunt but not eliminate drug reward. If he then relapses he might try to overcome the enzymatic effect with a larger than usual amount of cocaine, but over time, the increased cost, effort and risk in obtaining drug, combined with the reduced reward value, might increase the chances of returning to abstinence. Meanwhile, the present results encourage us to anticipate that the higher levels of metabolites produced by CocH in that setting do not by themselves appear to pose a substantial risk. On the contrary, as our prior studies of cocaine hepatotoxicity have indicated, this metabolic shift may offer substantial protection from liver damage. Overall, these findings lend support to the view that cocaine hydrolase gene transfer therapy for cocaine addiction does not in itself pose high risks of toxicity and might even lower the risk of adverse outcome if a user resorted to larger drug doses.

Acknowledgments

This work was supported by the National Institute on Drug Abuse at the National Institutes of Health (Grant numbers RO1DA23979 and D1DA31340).

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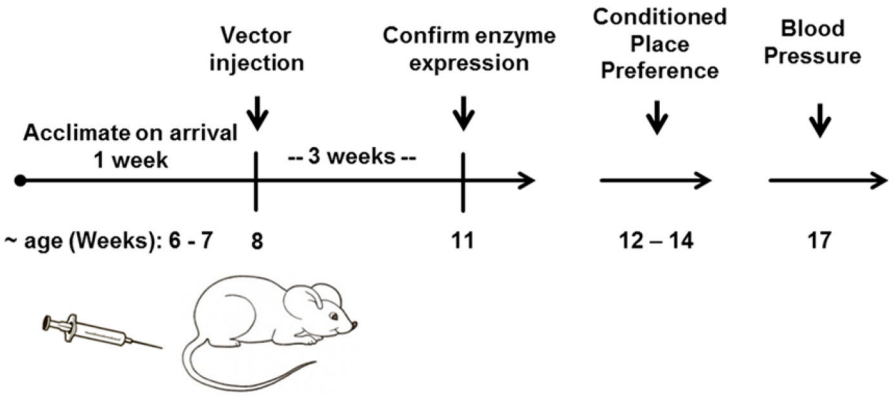

Figure 1.

Experimental flow chart. Mice were treated with vector soon after arrival. Place preference tests began 4 weeks later and were followed by blood pressure determinations as indicated.

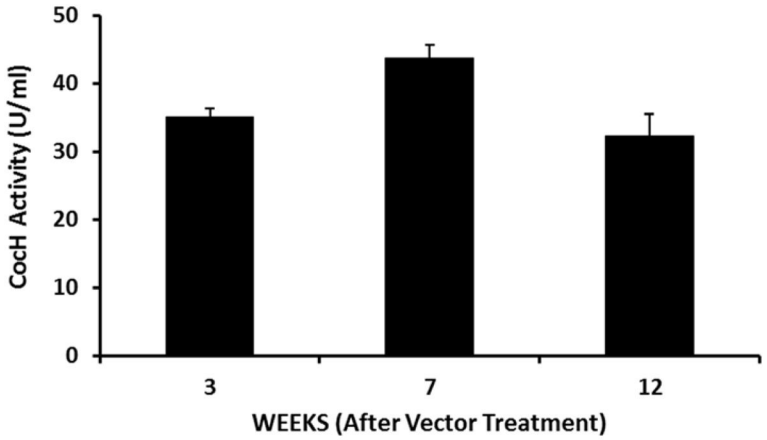

Figure 2. Cocaine hydrolase activity across time after vector transduction

Mice received AAV viral vector for mouse CocH (n = 10) at approximately 8 weeks of age and were followed for about 2 months. CocH activity in plasma samples is expressed in units (U) of micromoles cocaine hydrolysis per min. Baseline activity (pre-treatment levels) was typically less than 0.004 U/ml. Plasma CocH activity was measured at appropriate time points. Data are presented as mean ± standard error.

A. Scheme

Phase / Treatment Balb/c Mice	Acclimation to CPP Chambers Day 1	PRE-CPP (Exclusion) Day 2	CONDITIONING								POST-CPP TEST Day 11
			Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	
Positive Control (Cocaine) (20 mg/kg)	No Inj.	No Inj.	Coc	Sal	Coc	Sal	Coc	Sal	Coc	Sal	SALINE
Saline Control	No Inj.	No Inj.	Sal	Sal	Sal	Sal	Sal	Sal	Sal	Sal	SALINE
EME + BA (13 + 7) mg/Kg	No Inj.	No Inj.	EME + BA	Sal	EME + BA	Sal	EME + BA	Sal	EME + BA	Sal	SALINE
AAV mCocH (Cocaine) (80 mg/kg)	No Inj.	No Inj.	Coc	Sal	Coc	Sal	Coc	Sal	Coc	Sal	SALINE

B.

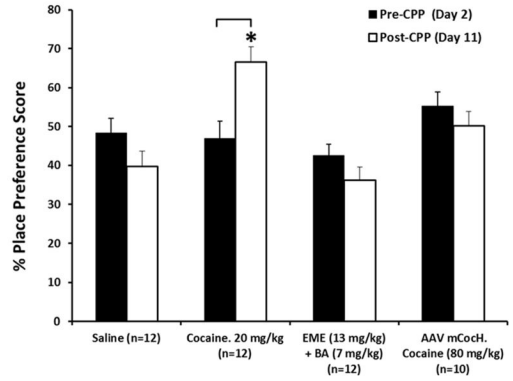

Figure 3. Conditioned Place Preference

(A). Schematic of the four-phase CPP paradigm: acclimation, Pre-CPP exclusion analysis, conditioning, and Post-CPP testing. Timeline of interventions showing alternate days of drug or saline treatments (totalling 4 of each). Saline group received only saline all 8 days, but, were identified to be paired with one compartment. (Sal: Saline and Coc: Cocaine) (B). Effect of cocaine (20 mg/kg) in positive control mice (n = 12), EME (13 mg/kg) and BA (7 mg/kg) in test group (n = 12), and cocaine (80 mg/kg) in test group AAV mCocH (n = 10) in CPP paradigm were evaluated. Positive control mice treated with 20 mg/kg showed significant preference for the paired compartment ($p = 0.001$). Mice that received combo treatment of EME + BA did not show any preference for the drug paired compartment similar to saline treated control mice. AAV mCocH mice that received a near lethal dose of 80 mg/kg cocaine did not show any preference for the drug paired compartment. Data are presented as mean ± standard error.

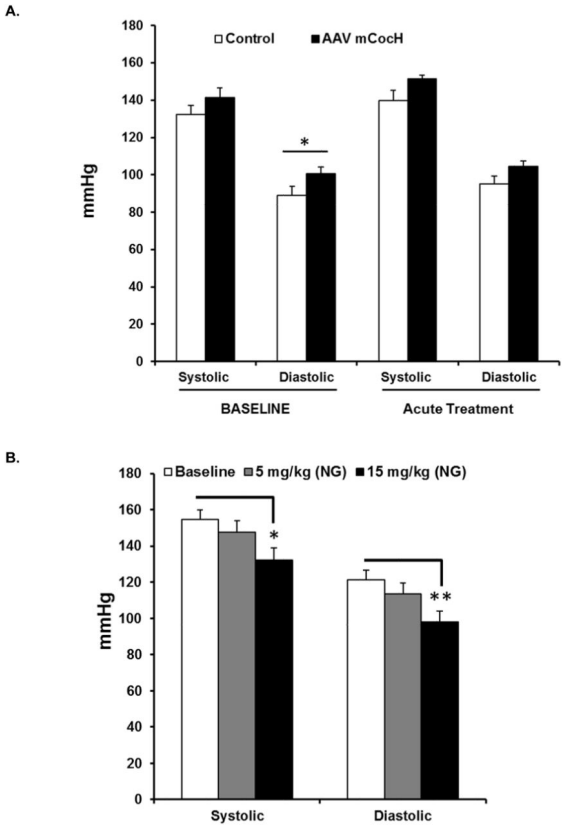

Figure 4. Blood pressure was analysed by a non-invasive tail cuff method

(A). AAV mCocH mice (n=8) and age-matched controls (n = 8). Baseline diastolic pressure was significantly higher in AAV treated group compared to control mice ($p = 0.03$). AAV treated mice then received high-dose cocaine (80 mg/kg i.p) while control mice received i.p. saline and immediately placed in the apparatus for pressure measurement. Data represent mean ± standard error of 5 baseline measurements from each mouse and one measurement after acute cocaine treatment.

(B). Untreated mice (n = 8) following baseline measurements were treated with the potent dilator, NG, at 5 and 15 mg/kg. After the higher dose, mice displayed a significant drop in both mean systolic pressure ($p = 0.01$*) and diastolic pressure $p = 0.005$**).