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Genome-edited skin epidermal stem cells protect mice from cocaine-seeking behaviour and cocaine overdose

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Abstract

Cocaine addiction is associated with compulsive drug-seeking, and exposure to the drug or to drug-associated cues leads to relapse, even after long periods of abstention. A variety of pharmacological targets and behavioral interventions have been explored to counteract cocaine addiction, but to date no market-approved medications for treating cocaine addiction or relapse exist, and effective interventions for acute emergencies resulting from cocaine overdose are lacking. We recently demonstrated that skin epidermal stem cells can be readily edited by using CRISPR (clustered regularly interspaced short palindromic repeats) and then transplanted back into the donor mice. Here, we show that the transplantation, into mice, of skin cells modified to express an enhanced form of butyrylcholinesterase, an enzyme that hydrolyzes cocaine, enables the long-term release of the enzyme and efficiently protects the mice from cocaine-seeking behavior and cocaine overdose. Cutaneous gene therapy through skin transplants that elicit drug elimination may offer a therapeutic option to address drug abuse.

Introduction

Drug addiction is a brain disorder that is characterized by compulsive drug-seeking and taking and a high likelihood of relapse when exposed to drugs or drug-associated cues[1–3]. Cocaine is a commonly abused drug, and current medications do not meet the urgent needs for treating ongoing cocaine use, relapse, or acute emergencies that result from cocaine

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AUTHOR CONTRIBUTIONS

XW and MX designed the experiments. YL, QK, JY, and XG performed the experiments. YL, QK, JY, MX, and XW analyzed the data. XW and MX wrote the manuscript. All authors edited the manuscript.

DATA AVAILABILITY

The authors declare that all data supporting the findings of this study are available within the paper and its supplementary information. Source data for Figures 2 and 3 are available in Figshare at https://figshare.com/s/898c3ab26b10a3d08b13 (ref. 63).

COMPETING INTERESTS

The authors declare no competing interests.

overdose[4,5]. BChE (butyrylcholinesterase) is a natural enzyme that is present in hepatocytes and plasma and it hydrolyzes its normal substrate acetylcholine[6–8]. BChE can also hydrolyze cocaine at low catalytic efficiency into benzoic acid and ecogonine methylester, which are low in toxicity and rewarding properties[9]. Protein engineering has greatly enhanced the catalytic potency and substrate specificity of human BChE (hBChE) for cocaine hydrolysis[10–13]. The modified hBChE has more than 4400 times higher catalytic efficiency than wild-type (WT) enzyme with significantly reduced activity for acetylcholine[14]. However, the recombinant hBChE protein has to be delivered via a parenteral route and has a very short half-life *in vivo*[6,8,15], making it potentially useful only for acute treatment of cocaine overdose. In recent clinical trials, an hBChE-albumin fusion protein TV-1380 was ineffective in facilitating cocaine-abstinence in dependent individuals, most likely due to the short half-life of the protein and also the inefficient intramuscular route of injection[16,17]. Development of an ability to stably deliver the engineered hBChE *in vivo* to allow continuous expression will be highly desirable. Although viral systems have been used for somatic gene delivery, the potential clinical applicability for this approach is greatly limited by drawbacks of viral vectors including: strong inflammatory response and tissue toxicity upon viral infection, genotoxicity and oncogenic risk of different viral vectors, limited packaging capacity for most viral vectors as well as extremely high cost for preparation of viral stocks at therapeutic grade and quantity[18,19].

As the rationale of this study is similar to that in our previous work in ref. 50, for clarity and thoroughness the text in this paragraph has been adapted from that publication. Our *ex vivo* system built upon genome-edited skin epidermal stem cells has several unique advantages[20–22], making it particularly suitable for long-term delivery of hBChE and other effector proteins[50]: ***i)*** as the most accessible organ in the body, the human skin is readily available for extraction of epidermal stem cells through well-established protocols [23–25]; skin transplants are also easily monitored for potential off-target effects of gene targeting, and removal of skin implants is relatively uncomplicated; ***ii)*** epidermal stem cells can be readily engineered *in vitro*, and standard procedures exist for transplantation of the differentiated and stratified skin tissue back onto donor patients[26,27]. Compared to viral packaging, autologous skin grafts are inexpensive; skin transplantation is minimally invasive and has been used for treating burn patients for decades [28,29]; ***iii)*** skin epidermal keratinocytes have low immunogenicity, as Langerhans cells are the only cell subset that expresses *major histocompatibility complex* (*MHC*) class II for antigen presentation antigens in healthy skin[30]; ***iv)*** other examples of therapeutic large proteins secreted by skin epidermal cells have been reported (for example, apolipoprotein E and the blood clotting Factor VIII and Factor IX), can cross the dermal barrier and enter the blood circulation for a systemic therapeutic effect[31–36]. Thus, cutaneous gene therapy can be used as a safe and effective way for treatment of non-skin diseases, including drug abuse, a scenario that has not been explored before. In this report, we demonstrated key evidence that engineered skin transplants can efficiently deliver BChE *in vivo* and protect against cocaine-seeking and overdose.

Results

CRISPR-edited epidermal stem cells can express engineered *hBChE* for cocaine hydrolysis.

As the methodology of this study is similar to that in our previous work in ref. 50, text in this section has been adapted from that publication. To carry out CRISPR-mediated genome editing in mouse epidermal stem cells, we developed DNA vectors encoding the D10A mutant of *Cas9* (CRISPR associated protein 9)[37], two gRNAs (guide RNA) targeting the mouse *Rosa26* locus, and a *Rosa26*-targeting vector. The targeting vector contains two homology arms for the *Rosa26* locus, flanking an expression cassette that encodes the modified *hBChE* gene (Fig. 1A). Primary epidermal basal cells were isolated from newborn mice, and electroporated with the *Rosa26* targeting vector together with plasmids encoding *Cas9* and *Rosa26*-specific gRNAs. Clones were isolated upon selection and the correct integration to the *Rosa26* locus was confirmed by both PCR screening and southern blotting analysis (Fig. 1B). Engineered epidermal cells exhibited robust expression and secretion of hBChE as shown by immunoblots and ELISA (enzyme-linked immunosorbent assay) (Fig. 1C and D). The secreted hBChE protein was functional as the conditioned medium collected from *hBChE*-expressing cells but not the control cells significantly induced degradation of cocaine *in vitro* (Fig. 1E). Consistent with previous reports, similar mutations in *mBChE* (mouse *BChE*) lead to only residual activity in cocaine hydrolysis[38] (Fig. 1E). Expression of *hBChE* in epidermal stem cells did not significantly change cell proliferation (Fig. 1F and Supplementary Fig. 1A) or differentiation (Supplementary Fig. 1B) *in vitro.* To confirm that modified epidermal cells are not tumorigenic, we examined the potential anchorage-independent growth of cells. Our results indicated that epidermal stem cells with *hBChE* targeting cannot grow in suspension. By contrast, cancer initiating cells[39] isolated from mouse SCC (squamous cell carcinoma) exhibited robust colony forming efficiency in soft agar medium (Supplementary Fig. 1C).

Engraftment of engineered epidermal stem cells can protect mice from cocaine-seeking behavior and cocaine overdose.

To efficiently transplant mouse epidermal stem cells, we developed an organotypic culture model with mouse epidermal stem cells *in vitro* by culturing the cells on top of acellularized mouse dermis[20–22]. Exposure to the air/liquid interface can induce stratification of cultured cells to generate a skin-like organoid *in vitro.* Expression of *hBChE* did not change the ability of epidermal stem cells to stratify (Supplementary Fig. 2A). To investigate the potential therapeutic effect of *hBChE* expression *in vivo*, we transplant the organoids to isogenic host animals (CD1 and C57BL/6) (Fig. 2A and B). No significant rejection of the skin grafts was observed for at least 5 months after transplantation, suggesting that the targeted epidermal stem cells are well tolerated immunologically *in vivo.* Grafted skin exhibited normal epidermal stratification, proliferation and cell death (Fig. 2C, and supplementary Fig. 2B and C). The mice that were grafted with *hBChE*-expressing cells displayed significant levels of human BChE in the blood (Fig. 2D). Expression of *hBChE* in grafted animals was stable for more than 10 weeks (Fig. 2D). Consistent with previous observations [31–36,40], our results confirm that a skin-derived therapeutic protein can cross the basement membrane barrier and enter circulation *in vivo.*

Cocaine can block dopamine (DA) reuptake and elevate extracellular levels of DA, resulting in locomotor stimulation and reward-related behaviors[1,3,41]. Expression of *BChE* in GhBChE mice presumably removes cocaine much more quickly *in vivo*, leading to reduced extracellular DA and locomotor activity. We measured DA and cocaine levels in both GhBChE and GWT mice after an acute cocaine injection. We performed microdialysis in the NAc (nucleus accumbens) of freely moving mice (Supplementary Fig. 2D), and the dialysates collected were quantified using the liquid chromatography–mass spectrometry (LC-MS) method[42]. As expected, GhBChE mice exhibited a much faster cocaine clearance (Fig. 2E) and less extracellular DA (Fig. 2F) in the NAc than those in WT mice. Consistently, LC-MS analysis demonstrated a significantly faster clearance of BE (benzoylecgonine), a major cocaine metabolite, in the plasma of GhBChE animals (Supplementary Fig. 2E). Engineered hBChE has very low activity for acetylcholine[14]. We did not detect significant differences in plasma acetylcholine levels in GBChE and control mice (Supplementary Fig. 2F). To further assess the pharmacodynamics property of cocaine in GhBChE mice, we monitored acute cocaine-induced locomotor behavior. Both GhBChE and GWT mice exhibited dose-response relationships, as measured by distance traveled and stereotypic counts, after cocaine administration (Fig. 2G-H). GhBChE mice showed, however, significantly less distance traveled and stereotypic counts than GWT mice as revealed by two-way ANOVA. Together, our data strongly suggest that skin-derived hBChE can effectively hydrolyze cocaine and reduce extracellular levels of DA in the grafted mice without significant effects on acetylcholine.

To determine whether engrafting hBChE-expressing cells protects mice from acute systemic toxicity of cocaine, we delivered different doses of cocaine to grafted mice and calculated the lethality rates of cocaine. 40, 80, 120, 160 mg/kg of cocaine had nearly 0 lethality in mice grafted with *hBChE*-expressing cells (GhBChE), whereas 80 mg/kg of cocaine induced roughly 50% lethality and 120 and 160 mg/kg cocaine induced 100% lethality in control mice grafted with WT epidermal cells (GWT) (Fig. 2I, Supplementary Video). Parallel test was conducted to test the toxicity of a related stimulant METH (methamphetamine) in GhBChE and GWT mice. There was no difference in lethality induced by varies doses of METH between GhBChE and GWT animals (Supplementary Fig. 2G). This finding suggests that engraftment of *hBChE*-expressing cells can protect mice from toxicity of cocaine overdose.

We then assessed protection against development of cocaine-seeking using the conditioned place preference (CPP)[43,44] paradigm which is thought to model reward learning and seeking because experimental animals approach and remain in contact with cues that have been paired with the effects of the reward. We grafted *hBChE*-expressing cells to cocaine-naïve mice and used GWT animals as controls. After 4 days of place conditioning, GWT mice spent significantly more time in environments previously associated with cocaine, whereas GhBChE mice showed no such preference (Fig. 3A). As an additional control, ethanol CPP was measured after 4 days of conditioning in GhBChE and GWT mice. In contrast, both GhBChE and GWT mice spent significantly more time in ethanol-paired environments (Fig. 3B). This finding indicates that engraftment of hBChE-expressing cells efficiently and specifically attenuates cocaine-induced rewarding effect.

To determine whether engrafting hBChE-expressing cells affect cocaine-induced reinstatement of drug-seeking, we engrafted *hBChE*-expressing cells in mice that previously acquired cocaine CPP. Following 10 days of recovery, we performed extinction training and drug-elicited reinstatement[43,44]. After a priming dose of cocaine injection, the preference for the previously cocaine-associated environment was restored in the GWT mice but not in the GhBChE mice (Fig. 3C). Because *hBChE*-expression did not prevent CPP induction by ethanol (Fig 3B), we used these GhBChE and GWT mice and performed extinction training followed by reinstatement. In contrast to those induced by cocaine, ethanol CPP was similarly reinstated in both GhBChE and GWT mice (Fig. 3D). These results suggest that skin-derived hBChE efficiently and specifically disrupts cocaine-elicited reinstatement.

Engineered human epidermal stem cells can deliver hBChE *in vivo*.

To test the feasibility of cutaneous gene therapy with human epidermal stem cells, we cultured human skin organoids from primary epidermal keratinocytes isolated from human newborn foreskin. To perform CIRPSR-mediated genome editing in human cells, we developed vectors encoding two gRNAs targeting human *AAVS1* (adeno-associated virus integration site 1) locus, and an *AAVS1*-targeting vector (Fig. 4A) that harbors the expression cassette encoding engineered *hBChE*. Human epidermal keratinocytes were electroporated with the targeting vector together with plasmids encoding *Cas9* and the gRNAs. Clones were isolated and correct integration confirmed by southern blotting analysis (Fig. 4B). Like mouse cells, engineered human epidermal cells exhibited strong hBChE production as determined by immunoblots and ELISA (Fig. 4C and D). Expression of the *hBChE* protein in human cells did not significantly change cell proliferation (Supplementary Fig. 3A) or differentiation (Supplementary Fig. 3B) *in vitro.* The engineered cells stratified and formed skin organoids *in vitro,* which were transplanted to *nude* host (Fig. 4E). Grafted skin exhibited normal epidermal stratification, proliferation, and apoptosis *in vivo* (Fig. 4F and Supplementary Fig. 3C-D). Together, these results indicate that CRISPR editing of human epidermal stem cells does not significantly alter cellular dynamics and persistence *in vivo.* ELISA confirmed that the mice with engraftment of *hBChE*-expressing cells had significantly levels of human BChE in the blood, whose expression was stable for more than 8 weeks *in vivo* (Fig. 4G). Our results suggest the potential clinical relevance of cutaneous gene delivery for treatment of cocaine abuse and overdose in the future.

Discussion

Our study demonstrates that transplantation of genome-edited skin stem cells can be used to deliver an active cocaine hydrolase long-term *in vivo.* Skin epidermal stem cells can be successfully employed for *ex vivo* gene therapy, as efficient genetic manipulation is possible with minimal risk of tumorigenesis or other adverse events *in vivo*[31,32]. Skin transplantation protocols, including procedures based on the use of human epidermal stem cells to generate cultured epidermal autografts (CEAs), have been in clinical use for decades in the treatment of burn wounds[28,29,45]. Engineered skin stem cells and CEA have also been used to treat other skin diseases, including vitiligo and skin genetic disorders, such as epidermolysis bullosa[46,47]. These regenerated skin grafts are stable and have been shown to survive long-term in clinical follow-up studies[29,45–47]. As such, the cutaneous gene therapy is long-

lasting, minimally invasive and safe. For cocaine addicts and individuals with potentially high risk of cocaine abuse who seek help or treatment, the cutaneous gene therapy approach with *hBChE* expression can address several key aspects of drug abuse, including reducing development of cocaine-seeking, preventing cocaine-induced reinstatement of drug-seeking, and protecting against cocaine overdose after skin transplantation, potentially making them "immune" to further cocaine abuse. Because of the extremely high catalytic efficiency and high levels of hBChE present, this approach can be highly efficient in protecting a wide range of cocaine doses with little individual variation. It remains possible that the protective effect of *hBChE*-expressing skin grafts can be surmountable if extremely high doses of cocaine are used, or other psychostimulants are used. For instance, it has been shown that the longer acting mutant form of bacteria cocaine esterase can prevent cocaine-induced toxicity and the ongoing intravenous self-administration in rodents, but the effects are diminished when high doses of cocaine are used[48]. Additionally, development of drug abuse is accompanied by learned association between drug effects and environmental cues, which plays a significant role for cocaine craving and relapse[1,3,41]. Although hBChE is a highly potent cocaine hydrolase, it will unlikely affect cue-induced relapse. It is also noteworthy that cocaine intake is not under the control of the test animals in our CPP model. Thus, future studies will be essential to confirm and determine the protective effect of hBChE, particularly in response to extremely high doses of cocaine and test prevention of cue-induced relapse.

Cutaneous gene delivery with engineered epidermal stem cells may provide therapeutic opportunities for drug abuse or co-abuse beyond cocaine. For instance, glucagon-like peptide 1 (GLP1) is a major physiological incretin that controls food intake and glucose homeostasis[49]. Several GLP1 receptor agonists have been approved by the FDA to treat type II diabetes. Our recent study indicates that skin-derived expression of *GLP1* can effectively correct diet-induced obesity and diabetes in mice[50]. Interestingly, GLP1 receptor agonists can also attenuate the reinforcing properties of cocaine, alcohol, and nicotine in rodents[51–56]. Thus, future studies will determine whether expression of *GLP1* from skin transplants can reduce cocaine, alcohol, and nicotine use and relapse in patients with cocaine abuse, alcohol use disorder, and nicotine dependence. Additionally, it will be important to investigate whether co-expression of *hBChE* and *GLP1* in skin can be used for treatment of alcohol and/or nicotine co-abuse with cocaine, which occurs with high frequency and significantly increases the risk of drug-related morbidity and mortality.

The immune system recognizes and reacts against foreign antigens, including those arising from gene therapy-derived products. If an immune response to these products is triggered, neutralizing antibodies are produced that prevent the function of the therapeutic molecules and may induce the rejection of the genetically modified cells[57]. Our skin transplantation model built with WT isogenic animals provides a unique approach to examine this process *in vivo.* Skin epidermal keratinocytes have low immunogenicity[30]. In skin, Langerhans cells are the only cell type expressesing *MHC* class II for antigen presentation[30], as epidermal keratinocytes are considered as 'non-professional' antigen-presenting cells. Transplanted skin tissues generated from epidermal stem cells are devoid of Langerhans cells or other leukocytes with antigen presentation capabilities; therefore, the risk of antigenicity is significantly reduced. Consequently, engineered skin grafts are generally well taken and

immunologically tolerated in WT isogenic animals[20–22]. Indeed, skin-derived expression of *hBChE* in host mice with intact immune systems can be stable for more than 10 weeks without significant decrease in the serum level of engineered enzyme, strongly suggesting that the low immunogenicity of skin environment may help to reduce the antigenicity and immune reaction toward *hBChE*. Moreover, the oldest GhBChE mice are 6 months old and healthy with no tissue rejections, lending further support for the long-lasting feasibility of cutaneous gene therapy targeting cocaine abuse. Taken together, our results suggest that cutaneous gene therapy could become a potentially safe and cost-effective therapeutic option for cocaine abuse.

METHODS

As the methodology of this study is similar to that in our previous work in ref. 50, text in the sections “Reagents and plasmid DNA constructions”, “Skin organoid culture and transplantation”, “Histology and immunofluorescence”, “Cell cycle analysis” and “Protein biochemical analysis” has been adapted from that publication.

Reagents and Plasmid DNA Constructions

Guinea pig anti K5, rabbit anti K14, rabbit anti K10 and Loricrin antibodies were generous gifts from Dr. Elaine Fuchs at the Rockefeller University. Rat monoclonal β4-integrin (CD104, BD 553745) was obtained from BD Pharmingen (Franklin lakes, NJ). Ser10 pho-histone antibody was obtained from EMD Millipore (Billerica, MA). Cleaved caspase-3 antibody was obtained from Cell Signaling Technology (Danvers, MA). Rabbit polyclonal BChE antibody was obtained from Proteintech (Rosemont, IL). Human BChE quantikine ELISA kit was obtained from R&D systems (Minneapolis, MN). Other chemicals or reagents were obtained from Sigma, unless otherwise indicated.

Lentiviral vector encoding *Luciferase* has been described before[21,58]. Plasmid encoding *hCas9*-D10A mutant was a gift from George Church, obtained from Addgene (plasmid #41816). Plasmid encoding gRNA expression cassette was constructed with primers: AAG GAA AAA AGC GGC CGC TGT ACA AAA AAG CAG G; and gGA ATT CTA ATG CCA ACT TTG TAC, using gBlock as a template. *Rosa26* –targeting gRNA is constructed with primers: ACA CCG GCA GGC TTA AAG GCT AAC CG, AAA ACG GTT AGC CTT TAA GCC TGC CG, ACA CCG AGG ACA ACG CCC ACA CAC Cg, AAA ACG GTG TGT GGG CGT TGT CCT CG. *AAVS1*–targeting gRNA is constructed with primers: ACA CCG TCA CCA ATC CTG TCC CTA GG, AAA ACC TAG GGA CAG GAT TGG TGA CG, ACA CCG CCC CAC AGT GGG GCC ACT AG, AAA ACT AGT GGC CCC ACT GTG GGG CG. *Rosa26* targeting vector is constructed with pRosa26-GT as template (a gift from Liqun Luo, addgene plasmid 40025) using primers: GAC TAG TGA ATT CGG ATC CTT AAT TAA GGC CTC CGC GCC GGG TTT TGG CG, GAC TAG TCC CGG GGG ATC CAC CGG TCA GGA ACA GGT GGT GGC GGC CC, CGG GAT CCA CCG GTG AGG GCA GAG GAA GCC TTC TAA C, TCC CCC GGG TAC AAA ATC AGA AGG ACA GGG AAG, GGA ATT CAA TAA AAT ATC TTT ATT TTC ATT ACA TC, CCT TAA TTA AGG ATC CAC GCG TGT TTA AAC ACC GGT TTT ACG AGG GTA GGA AGT GGT AC. *AAVS1* targeting vector was constructed with AAVS1 hPGK-PuroR-pA

donor (a gift from Rudolf Jaenisch, addgene plasmid 22072) as template using primers: CCC AAG CTT CTC GAG TTG GGG TTG CGC CTT TTC CAA G, CCC AAG CTT CCA TAG AGC CCA CCG CAT CCC C, CAG GGT CTA GAC GCC GGA TCC GGT ACC CTG TGC CTT CTA GTT GC, GGA TCC GGC GTC TAG ACC CTG GGG AGA GAG GTC GGT G, CCG CTC GAG AAT AAA ATA TCT TTA TTT TCA TTA CAT C, GCT CTA GAC CAA GTG ACG ATC ACA GCG ATC. Genotyping primers for CIRPSR mediated knockin: GAG CTG GGA CCA CCT TAT ATT C, GGT GCA TGA CCC GCA AG, GAG AGA TGG CTC CAG GAA ATG. Human and mouse *BChE* with point mutations [14] were codon-optimized and synthesized from IDT (Integrated DNA Technology, Coralville, IA), and PCR amplified with primers: GCT CTA GAG CCA CCA TGC AGA CTC AGC ATA CCA AGG, CGG GAT CCA CCG GTT TAG AGA GCT GTA CAA GAT TCT TTC TTG, CCC AAG CTT GCC ACC ATG CAT AGC AAA GTC ACA ATC, ACG CGT CGA CTT AGA GAC CCA CAC AAC TTT CTT TCT TG.

Skin organoid culture and transplantation

Decelluralized dermis (circular shape with 1cm diameter) was prepared by EDTA treatment of newborn mouse skin [59]. 1.5×10^6 cultured keratinocytes were seeded onto the dermis in cell culture insert. After overnight attachment, the skin culture was exposed to air/liquid interface.

For grafting with skin organoids, CD1 or C57BL/6 males with the ages of 6–8 weeks were anesthetized. A silicone chamber bottom with the interior diameter of 0.8cm and the exterior diameter of 1.5cm was implanted on its shaved dorsal mid-line skin, which was used to hold the skin graft. A chamber cap was installed to seal the chamber right after a piece of graft was implanted. About one week later, the chamber cap was removed to expose the graft to air. A single dose of 0.2mg α-CD4 (GK1.5) and 0.2mg α-CD8 (2.43.1) antibodies was administered intraperitoneally for skin grafting.

Histology and Immunofluorescence

Skin or wound samples were embedded in OCT, frozen, sectioned, and fixed in 4% formaldehyde. For paraffin sections, samples were incubated in 4% formaldehyde at 4°C overnight, dehydrated with a series of increasing concentrations of ethanol and xylene, and then embedded in paraffin. Paraffin sections were rehydrated in decreasing concentrations of ethanol and subjected to antigen unmasking in 10 mM Citrate, pH 6.0. Sections were subjected to hematoxylin and eosin staining or immunofluorescence staining as described[60]. Antibodies were diluted according to manufacturer's instruction, unless indicated.

Cell Cycle Analysis

Propidium Iodide (PI) staining followed by Flow Cytometry Assay were used to determine the effect of cell cycle profiles. Mouse and human epidermal cells were cultured in two 6cm cell culture dish for 24 hours, respectively. Cells were trypsinized and 1×10^5 cells from each dish were collected, followed by one PBS wash. Fixation of cells was carried out using 70% (v/v) ice cold ethanol for 1 hour. Then, the fixed cells were centrifuged at 500g at 4°C for 10 minutes, followed by PBS wash for two times. The cells were then treated with 75μg RNAse A in 100μl PBS and incubated at 37°C for 1 hour. After incubation, the cells were collected

by centrifuging at 500g at 4°C for 10 minutes, followed by another PBS wash. The cell pellet was re-suspended in 200μl PBS, in addition of PI solution at a final concentration of 25ng/μl. After staining, the cells were analyzed immediately using flow cytometer BD FACSCanto™ II (BD Biosciences, San Jose, CA) with an excitation wavelength at 488 nm and emission at 585 nm. DNA content and histograms of cell cycle distribution were analyzed using FlowJo software, version 10 (FLOWJO LLC, OR).

Protein biochemical analysis

Western blotting was performed as described previously [61]. Briefly, equal amounts of the cell lysates were separated on a SDS-polyacrylamide gel electrophoresis (PAGE) and electroblotted onto a NC membrane. The immunoblot was incubated with Odyssey blocking buffer (Li-Cor) at room temperature for 1 h, followed by an overnight incubation with primary antibody. Blots were washed three times with Tween 20/Tris-buffered saline (TBST) and incubated with a 1:10000 dilution of secondary antibody for 1 h at room temperature. Blots were washed three times with TBST again. Visualization and quantification was carried out with the LI-COR Odyssey scanner and software (LI-COR Biosciences).

Cocaine-induced behaviors

For all behavioral experiments except where noted, C57BL/6J mice were used. Roughly equal numbers of adult male and female mice were group-housed until surgery. Mice were maintained under controlled temperature and humidity conditions on a 12 h:12 h light:dark cycle (lights on at 7:00). Water and food were available ad libitum. Mice weighed around 25–30 g at the beginning of the experiments. All procedures followed National Institutes of Health Guide for the Care and Use of Laboratory Animal and were approved by the University of Chicago Institutional Animal Care and Use Committee.

Drug: Cocaine HCl and Methamphetamine HCl (Sigma-Aldrich, Saint Louis, MO) were dissolved in sterile saline and delivered intraperitoneally at appropriates doses in a volume of 10 ml/kg. Ethanol (Sigma-Aldrich, Saint Louis, MO, 95%, density=0.816) was prepared in 20% (v/v) diluted in sterile saline and delivered intraperitoneally at appropriate doses. Vehicle (sterile saline) was intraperitoneally administered at 10 ml/kg as a control.

Microdialysis: GhBChE and GWT mice were anesthetized with isofluorane and placed in a flat skull position in a Kopf stereotaxic frame. Guide cannula was secured with dental cement on mouse skulls above the NAc using the anterior-posterior +1.2 mm, medial-lateral 1.0 mm coordinates and dorsal-ventral −5.0 mm from bregma. 24 h after implantation, a microdialysis probe (2 mm dialyzing membrane, CMA-7, Harvard Apparatus, Cambridge, MA) was inserted in to the guide cannula and lowered down to NAc region in freely moving animals. The microdialysis probe was flushed with artificial cerebrospinal fluid (aCSF) (KCl 2.5 mM, NaCl 125 mM,$CaCl_2$ 1.26 mM, $MgCl_2$ 1.18 mM, Na_2HPO_4 2 mM, pH 7.4) at a flow rate of 1 μL/min using a UMP3 UltraMicroPump (WPI, Sarasota, FL). Once the probe was positioned, the probe was flushed at 1 μL/min for 140 min. Dialysates were collected at 20 min intervals. 10 μL dialysate was derivatized using BzCl for analysis of extracellular dopamine. Another 10μL dialysate without derivatization were used for analysis of cocaine.

Dopamine-1,1,2,2-d4 and Cocaine-d3 were used as internal standards. At the completion of the experiment, animals were euthanized and probe placement was confirmed with histology (Supplementary Fig. 2D).

LC-MS analysis: We used a method by *Wong* et al[42]. Derivatized dialysate samples were analyzed by LC–MS using Agilent 1290 UHPLC coupled to a 6460 triple quadrupole mass spectrometer (Santa Clara, CA) in multiple reaction monitoring (MRM) mode. Five μLof samples were injected onto an InfinityLab Porshell 120EC-C18 (2.1 mm×100 mm, 4 μm, 100 Å pore size). Samples were analyzed in triplicate. Electrospray ionization was used in positive mode. Automated peak integration was performed using Agilent MassHunter Workstation Quantitative Analysis for QQQ. All peaks were visually inspected to ensure proper integration.

Acetylcholine (Ach) Assay: The base line level of Ach in GhBChE and GWT mice blood serum was quantified using QuickDetect™ Acetylcholine (Mouse) ELISA kit. Standard curve was generated using known concentrations of mouse Ach standard and its corresponding OD value. 10ul of blood serum collected before cocaine injection was diluted with 40ul sample dilution buffer (a dilution factor of 5). Ach concentration was determined with manufacturer recommended protocol.

Acute locomotor activity: Baseline motor activity and locomotor responses to cocaine were assessed using horizontal arenas equipped with infrared detectors[62]. Mice were habituated in the test chambers for 1 h before testing. Mice were then given an intraperitoneal injection of cocaine at three different doses (10, 20, 40 mg/kg) or saline. Their locomotor responses (distance traveled and stereotypic behaviors) were recorded for another hour after the injections.

CPP apparatus: The CPP apparatus (Med Associates, E. Fairfield, VT, USA) consisted of two larger chambers (16.8×12.7×12.7 cm), which were separated by a smaller chamber (7.2×12.7×12.7 cm) as previously described (Yan et al, 2013). Each chamber had a unique combination of visual and tactile properties (one large chamber had black walls and a rod floor, the other larger chamber had white walls with a mesh floor, whereas the middle chamber had gray walls and a solid gray floor). Each compartment had a light embedded in a clear, Plexiglas hinged lid. Time spent in each chamber was measure via photobeam breaks and recorded. CPP was determined on testing days via time spent in the drug-paired side minus time spent in the saline-paired side.

Acquisition of CPP: We used a biased CPP procedure similar to that from a previous study in our laboratory (Yan et al, 2013). Acquisition of CPP consisted of three sequential procedures—pretest, conditioning and test. After 7–12 days of recovery from engrafting surgeries, mice underwent pretest on Day 1, where mice were allowed to freely explore the entire chamber for 20 min once daily. Mice that spent more than 500 s in the gray compartment or more than 800 s in either of the large compartments were excluded from the study. Following the pretest day, mice underwent conditioning and testing on Day 2–5. Starting on Day 2, mice received an i.p. injection of drug (10 mg/kg cocaine in cocaine CPP or 2g/kg ethanol in ethanol CPP) and were confined to the white chamber for 30 min. At

least 5 hours after in the same day, mice received an i.p. injection of saline and were confined to the black compartment for 30 min. On Day 6 (test day) mice were allowed to explore the entire chambers for 20 min and time spent in each area was recorded.

Extinction and reinstatement of CPP: Following CPP acquisition, mice underwent extinction, in which the procedure was identical to that in the test day. In each extinction day, mice were allowed to explore the entire chambers for 20 min and time spent in each area was recorded. Extinction was performed until the CPP decreased to a level that was not different from that of the pretest in consecutive two days. On the following day of the last extinction, mice underwent reinstatement procedures, in which mice that were trained for cocaine CPP received an i.p. injection of 15 mg/kg cocaine, and mice that were trained for ethanol CPP received an i.p. injection of 1g/kg ethanol. Immediate after injection, mice were allowed to explore the entire chambers for 20 min and time spent in each area was recorded.

Acute drug overdose test: Two weeks after grafting surgery, 4 groups of GhBChE and 4 groups of GWT mice (n=8 each in each group) received i.p. injections of cocaine at 40, 80, 120 and 160 mg/kg. As a control, 4 groups of GhBChE and 4 groups of GWT mice (n=8 each) received i.p. injections of methamphetamine (METH) at 34, 68 (LD50), 100, and 160 mg/kg. Two each of GhBChE and GWT mice with CD1 mice as hosts were also used to videotape acute cocaine (80 mg/kg) induced behaviors. Mice were monitored for 2h following injection and percent of cocaine- and METH-induced lethality was calculated.

Specific methods: Fig. 3A: One group of GhBChE and one group of GWT mice (n=9 in each group) were trained for cocaine CPP 7 days after engraftment. Mice underwent pretest on Day 1, four days of cocaine conditioning day 2 to Day 5, and CPP test on Day 6. Fig. 3B: One group of GhBChE and one group of GWT mice (n=8 in each group) were trained for ethanol CPP 7 days after engraftment. Mice underwent pretest on Day 1, four ethanol conditioning day 2 to Day 5, and CPP test on Day 6. Fig. 3C: Two groups of drug-naïve wildtype mice (n=8 in each group) were trained for cocaine CPP from day 1 to Day 6. On the following day, mice underwent engrafting surgery. The behavioral procedure resumed after engraftment surgery from Day 18. Extinction was performed from Day 18 to Day 31. On Day 32, mice underwent reinstatement induced by i.p. injection of cocaine. Fig. 3D: One group of GhBChE and one group of GWT mice (n=8 in each group) were trained for ethanol CPP from day 1 to Day 6. Extinction was performed from Day 7 to Day 20. On Day 21, mice underwent reinstatement procedure induced by i.p. injection of ethanol.

Statistical analysis

Statistical analysis was performed using Excel or OriginLab software. Box plots are used to describe the entire population without assumptions on the statistical distribution. A student *t* test was used to assess the statistical significance (P value) of differences between two experimental conditions. For cocaine behavioral analysis, CPP results were analysed using repeated-measures ANOVA with within factor time (testing days) and between factor treatment (engraftment). Significant effects were further analyzed with Fisher's t-tests.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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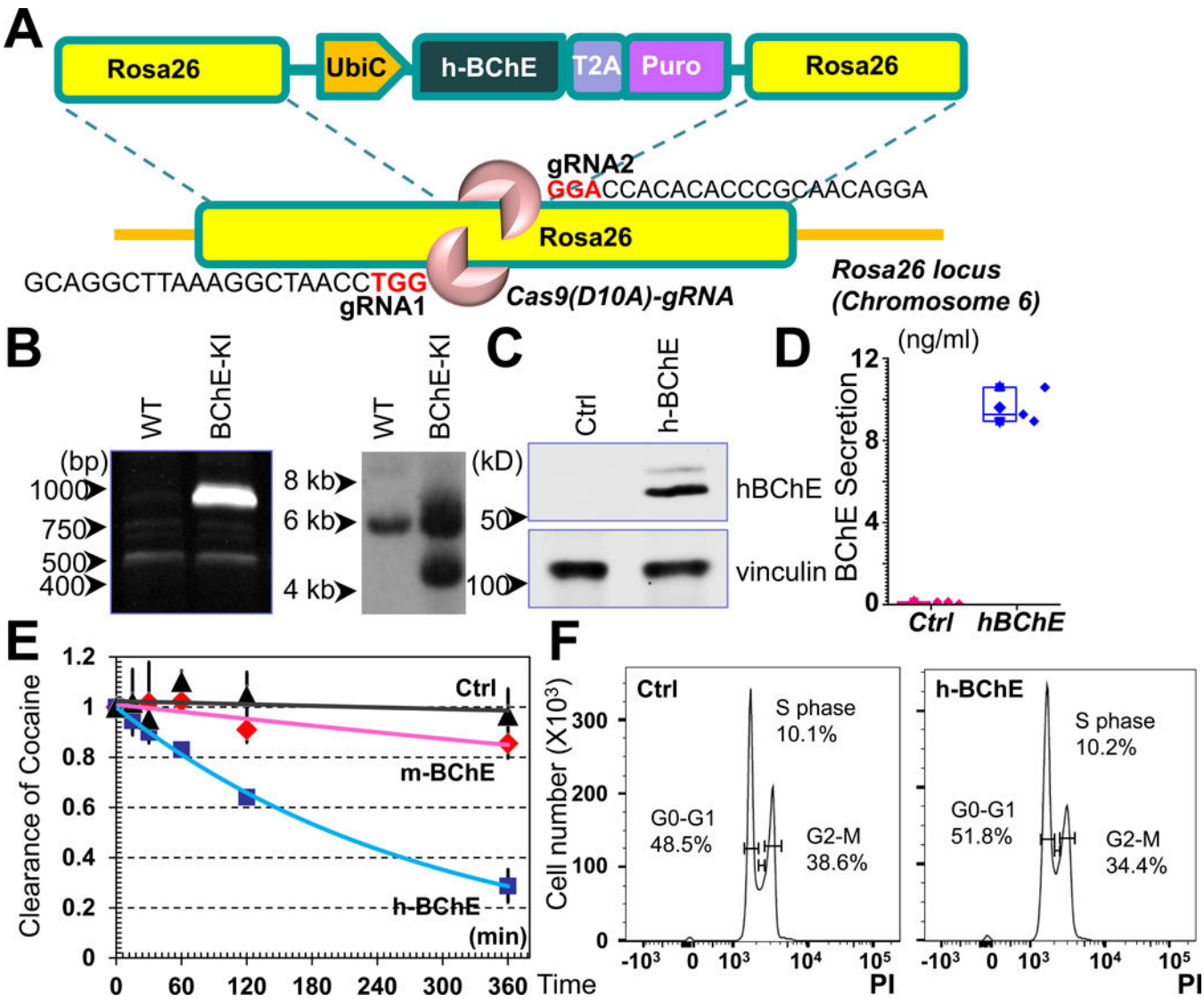

Figure 1. Expression of engineered *hBChE* via genome editing in skin epidermal stem cells.

(A) Targeting strategy for the expression of engineered *hBChE.* The targeting vector contains two *Rosa26* homology arms, flanking the expression cassette for *hBChE* and a selection marker (puromycin resistant gene, Puro) driven by a constitutive promoter UbiC (Ubiquitin C promoter). hBChE and Puro are separated by a self-cleavable peptide *T2A.* gRNA: guide RNA. **(B)** Integration of the targeting vector into *Rosa26* locus is verified by PCR (left panel) and southern blotting (right panel). Positive clones display an additional band of the expected size. Three independent experiments were performed with similar results. **(C)** Confirmation of *hBChE* expression in targeted cells by immunoblots with different antibodies. Numbers on left side indicate molecular weight markers. kD: kilodalton. Full scans of the western blots at Supplementary Fig. 1D. Three independent experiments were performed with similar results. **(D)** Confirmation of secretion of engineered hBChE in the culture media by ELISA. The box plot indicates the mean (solid diamond within the box), 25th percentile (bottom line of the box), median (middle line of the box), 75th percentile (top line of the box), 5th and 95th percentile (whiskers), 1st and

99th percentile (solid triangles) and minimum and maximum measurements (solid squares). N=number of independent experiments. **(E)** Cocaine hydrolysis activity *in vitro*. Cell culture supernatants were collected from cells targeted by *hBChE* or *mBChE.* Cocaine hydrolysis activity was examined by a clearance assay *in vitro*. N=3 (independent experiments), error bars represent standard deviation, and the measure of center represents the average. **(F)** Cell cycle profiles. FACS (fluorescence activated cell sorting) of control (Ctrl) and *hBChE*-expressing epidermal stem cells. PI: propidium iodine. Three independent experiments were performed with similar results. Text in this legend has been adapted from our previous work in ref. 50.

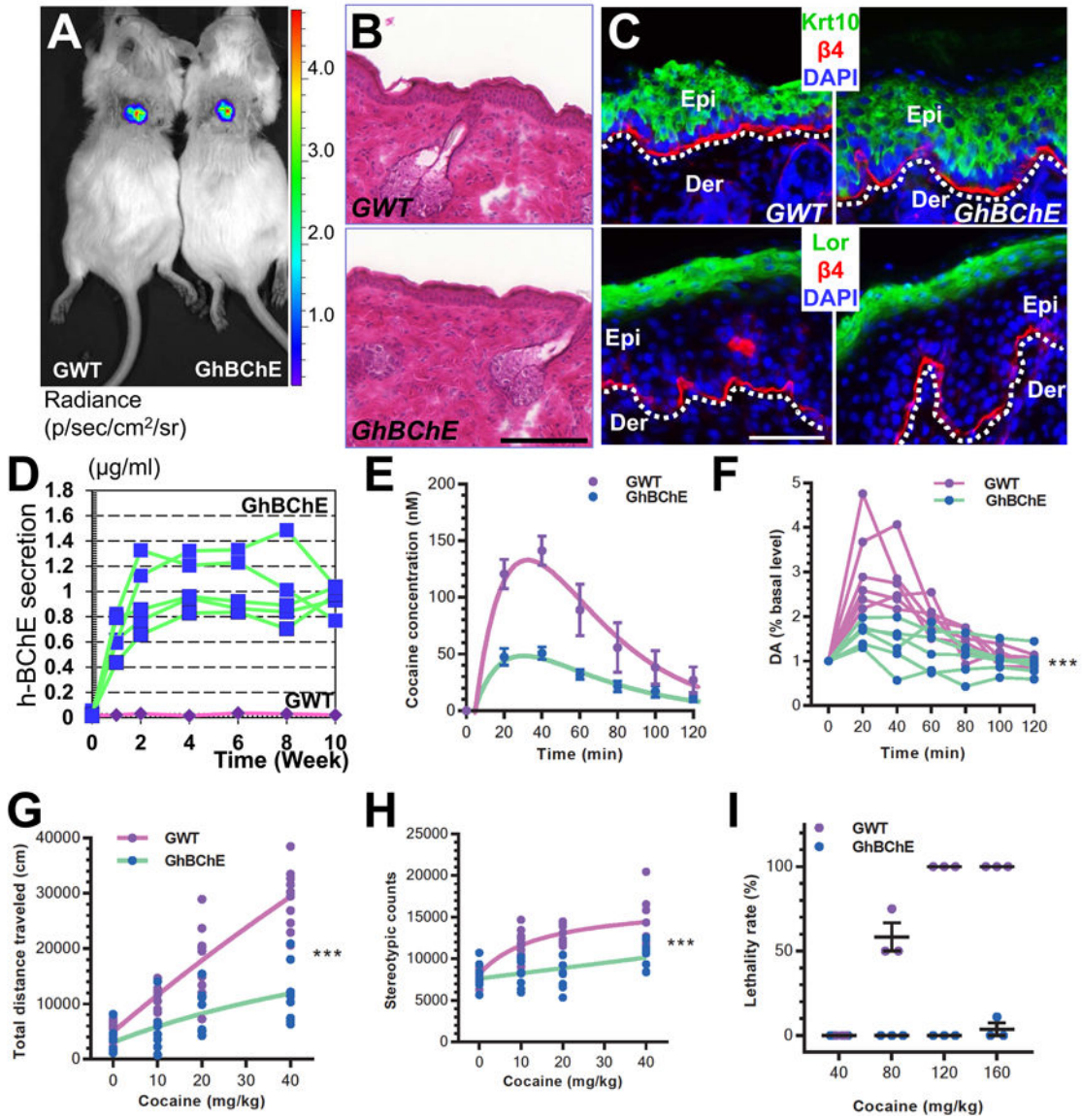

Figure 2. Engraftment of *hBChE*-expressing cells can reduce cocaine-induced locomotion and protect against cocaine overdose.

(A) Skin organoids are developed from control or *hBChE*-producing cells, and transplanted to the host mice. Cells were infected with lentivirus encoding *firefly luciferase* before engraftment to allow intravital imaging of the skin grafts. **(B)** Histological examination of grafted skin collected from mice grafted with control (GWT) or *hBChE* skin organoids (GhBChE). Scale bar=50 μm. **(C)** Sections of grafted skin were immunostained with different antibodies as indicated (Krt10: keratin 10, a marker for early epidermal differentiation, Lor: Loricrin, a marker for early epidermal differentiation, β4: β4-integrin, CD104, a marker for skin basement membrane). Dashed lines denote the basement of skin. Epi: epidermis, Der: dermis. Scale bar=50 μm. **(D)** Mice are grafted with control or *hBChE* skin organoids. Presence of hBChE in blood was determined by ELISA for 10 weeks after engraftment (n=5 mice in each group). **(E)** Cocaine pharmacokinetics in the NAc (nucleus accumbens) after an i.p. administration of 10 mg/kg cocaine in GhBChE and GWT mice (n

= 6 for each group). Data are plotted as mean±SEM (standard error of the mean). A two-compartment *in vivo* pharmacokinetic model was built to represent the cocaine concentration-time profile in the NAc. **(F)** Changes in DA (dopamine) level in the NAc after an i.p. administration of 10 mg/kg cocaine in GhBChE and GWT mice (n = 6 for each group). Individual lines for each animal were plotted. Treatment×time interaction: $F_{6, 70}$=6.549, P ＜ 0.0001, two-way ANOVA. **(G-H)** Cocaine-induced locomotor activity in in GhBChE and GWT mice (n = 11 for GWT, n=8 for GhBChE). Non-linear dose versus response curve was simulated to represent cocaine-induced dose-response locomotor activity. (G) Total distance traveled after 0, 10, 20, 40 mg/kg cocaine i.p. injection. Treatment×doses interaction: $F_{3, 68}$=11.83, P ＜ 0.0001, two-way ANOVA. (H) Stereotypic counts after 0, 10, 20, 40 mg/kg cocaine i.p. injection. Treatment×doses interaction: $F_{3, 65}$=6.223, P=0.0009, two-way ANOVA. **(I)** Lethality rates after injection of 40, 80, 120, 160 mg/kg cocaine in GhBChE and GWT mice (n=3 independent experiments, for each experiment, 8 animals were examined in each test group). Individual data point represents the result from each experiment. Error bars represent SEM, and the measure of center represents the average. Text in this legend has been adapted from our previous work in ref. 50.

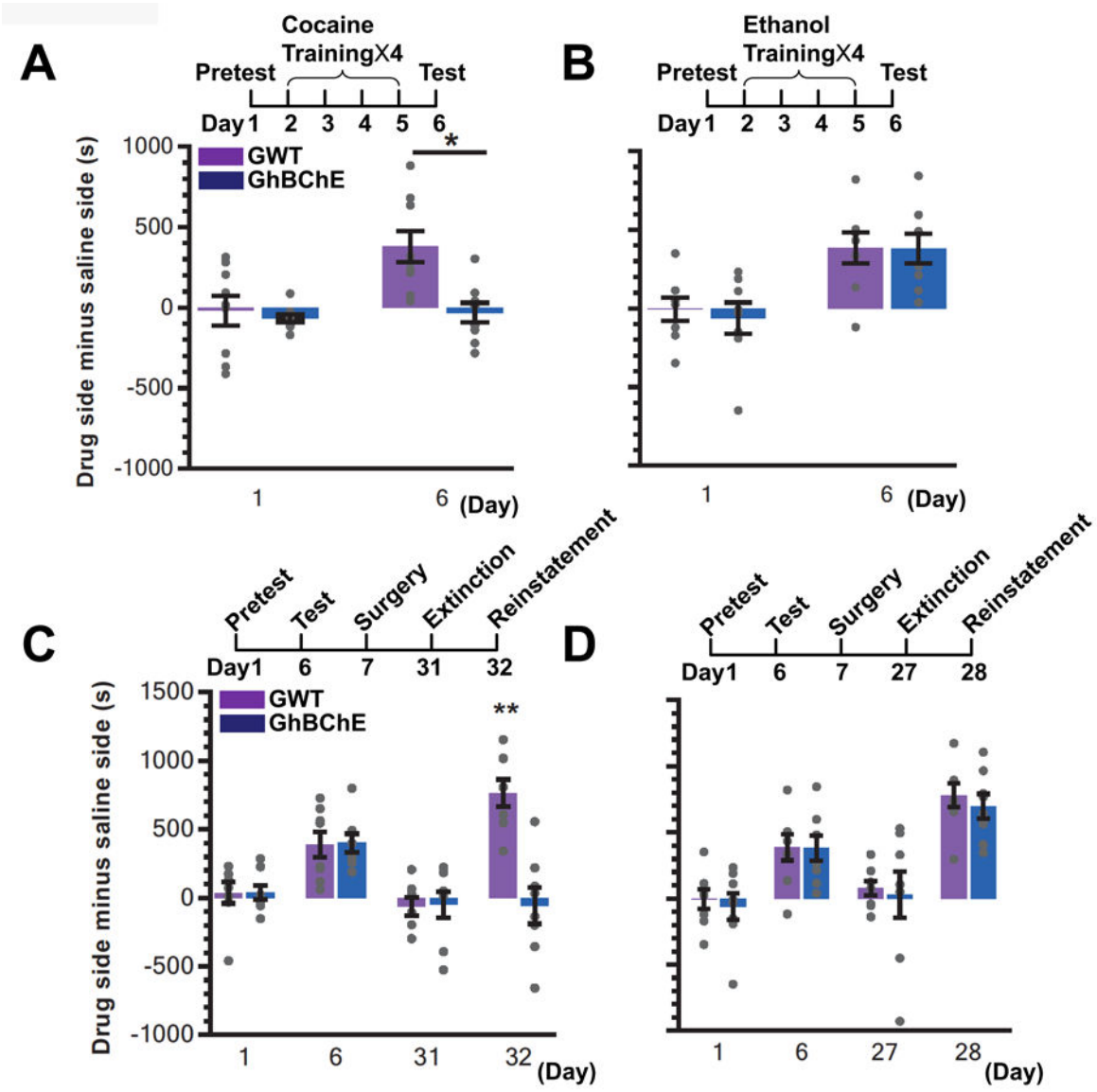

Figure 3. Engraftment of *hBChE*-expressing cells can attenuate CPP acquisition and reinstatement induced by cocaine.

(A) After engraftment, GhBChE and GWT mice underwent pretest (Day 1), cocaine conditioning (Day 2 to Day 5) and CPP expression test (Day 6). Data represent mean±SEM (n=9 in each group, treatment×days interaction by repeated measures ANOVA: $F_{1,16}$=5.04, P=0.039). Significance was tested by Fisher's Least Significance Difference (LSD) post hoc test. * P=0.016 between pretest and expression test. **(B)** After engraftment, GhBChE and GWT mice underwent pretest (Day 1), ethanol conditioning (Day 2 to Day 5) and CPP testing (day 6). Data represent mean±SEM (n=8 in each group, treatment×days interaction by repeated measures ANOVA: $F_{1,14}$=0.084, P=0.76). **(C)** Mice acquired similar levels of cocaine CPP after pretest, cocaine conditioning and test and underwent engrafting surgery on Day 7. Following 10 days of recovery, GhBChE and GWT mice underwent extinction till Day 31. During reinstatement on Day 32, GhBChE and GWT mice were given a cocaine injection and CPP was measured again. Data show mean±SEM (n=8 in each group,

treatment×days interaction by repeated measures ANOVA: $F_{3, 42}$=12.45, $P = 6 \times 10^{-6}$). Significance was tested by LSD post hoc test, ** P=0.00013 between last extinction and reinstatement. **(D)** Mice acquired similar levels of ethanol CPP after pretest, ethanol conditioning and test from Day 1 to Day 6, and underwent skin grafting surgery and extinction till Day 27. During reinstatement on Day 28, were given an ethanol injection and CPP was recorded (mean±SEM) (n=8 in each group, treatment×days interaction by repeated measures ANOVA: $F_{3, 42}$=0.0574, P=0.98).

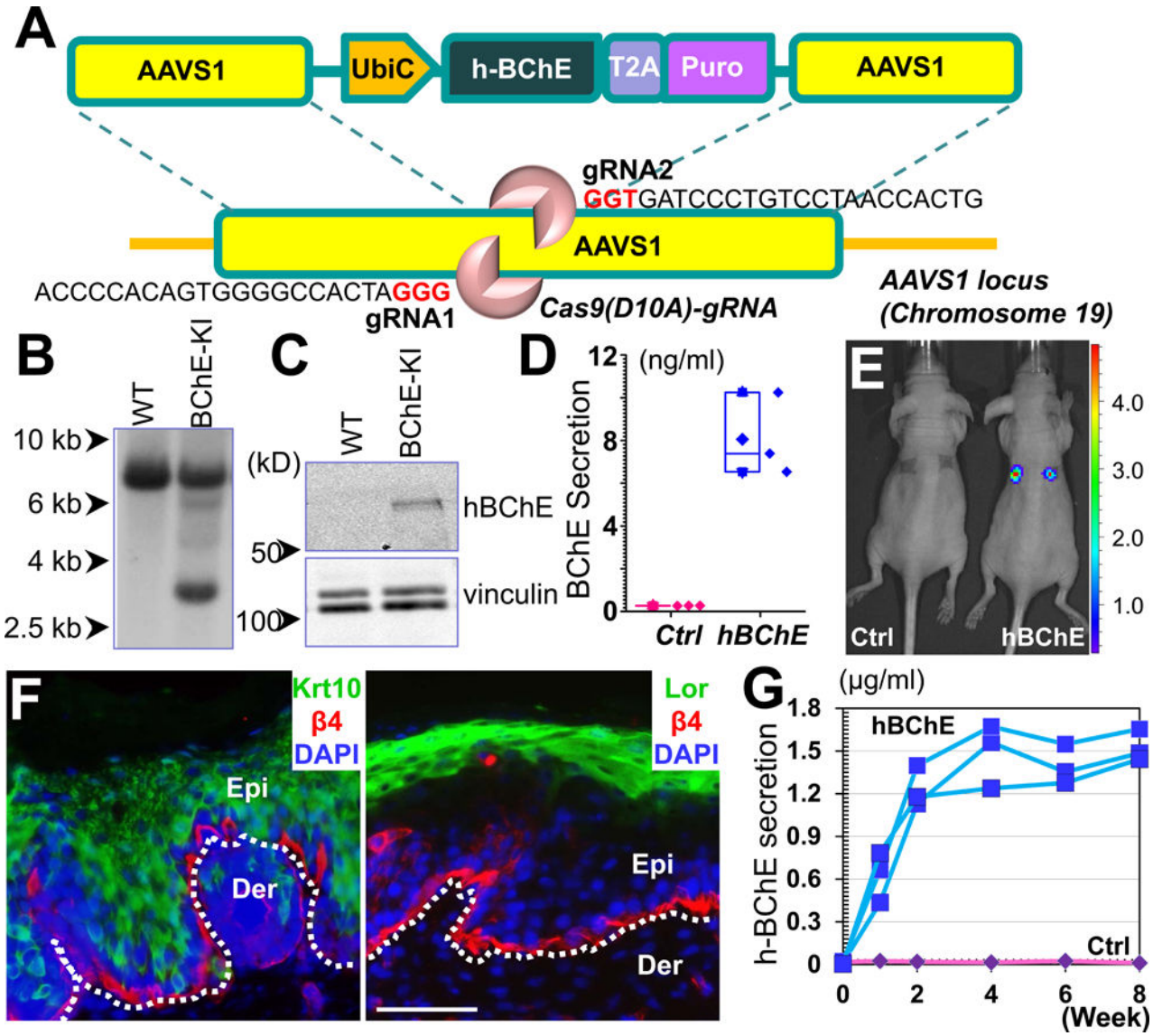

Figure 4. Expression of *hBChE* in human epidermal stem cells with CRISPR.

(A) The *AAVS1* targeting strategy for expression of engineered *hBChE.* The targeting vector contains two *AAVS1* homology arms, flanking the expression cassette for *hBChE* and a selection marker (puromycin resistant gene, Puro) driven by a constitutive promoter UbiC (Ubiquitin C promoter). hBChE and Puro are separated by a self-cleavable peptide *T2A.* **(B)** Integration of the targeting vector into *AAVS1* locus is verified by southern blotting. Positive clones display an additional band of the expected size. Three independent experiments were performed with similar results. **(C)** Expression of *hBChE* is confirmed in targeted cells by immunoblots with different antibodies as indicated. Full scans of the western blots at Supplementary Fig. 3E. Three independent experiments were performed with similar results. **(D)** Secretion of engineered hBChE in the culture media is confirmed by ELISA. N=3 (independent experiments). Box plots were generated with the same elements as described in Fig. 1D. **(E)** Image of nude mouse grafted with organotypic human skin culture. Intravital imaging shows efficient incorporation of grafted cells expressing *luciferase* (right side) or

control cells (left side) upon engraftment. **(F)** Sections of grafted skin were immunostained with different antibodies as indicated. Dashed lines denote the basement of skin. Scale bar=50 μm. **(G)** Mice are grafted with control or *hBChE* skin organoids. Presence of hBChE in blood was determined by ELISA for 8 weeks after engraftment (n=3 mice in each group). Text in this legend has been adapted from our previous work in ref. 50.