

JPET #188235

Activation of PKC α or PKC ϵ as an approach to increase morphine tolerance in respiratory depression and lethal overdose

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Running Title: PKC α and ϵ in morphine respiration and overdose tolerance

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Number of text Pages- 31

Figures – 6

Abstract words – 250

Introduction words – 736

Discussion words – 1203

References-40

List of Abbreviation: CAPKC α , constitutively active PKC alpha; CAPKC ϵ , constitutively active PKC epsilon; WTPKC ϵ , wild type PKC epsilon; preBötC, pre-Bötzinger complex; vIPAG, ventrolateral periaqueductal gray; Hb Sat., hemoglobin oxygen saturation; VRG, ventral respiratory group; pFRG/RTN, parafacial respiratory group/retrotrapezoid nucleus; BötC, Bötzing complex; MOR, μ -opioid receptor; WT, wild type; CA, constitutively active

Recommended section assignment: Neuropharmacology

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Abstract

Long-term use of opioids is hindered by respiratory depression and the possibility for fatal overdose in drug abusers. This is due to higher levels of tolerance that develops against antinociception than to respiratory depression. Identifying important mechanisms that would increase morphine respiratory depression and overdose tolerance could lead to the safer use of opioids. Since PKC activity mediates the development and maintenance of morphine antinociceptive tolerance, we hypothesized that activating PKC α or ϵ at the pre-Bötzinger complex (preBötC) can increase morphine tolerance in respiration and overdose. Laser microdissection and qRT-PCR were used to compare the relative mRNA abundances of PKC α , γ , and ϵ between vIPAG vs. preBötC. To test whether PKC α or ϵ could enhance morphine tolerance in respiratory depression and overdose, lentivirus carrying the wild type (WT), constitutively activated mutants (CA), and siRNA against PKC α or ϵ were stereotaxically injected into the preBötC. Expressing CAPKC α or ϵ , but not WTPKC α or ϵ , at the preBötC allowed rats to develop tolerance to morphine respiratory depression. In terms of lethality, expressing either WTPKC ϵ , CAPKC α , or CAPKC ϵ at preBötC increased morphine tolerance to lethal overdose. CAPKC ϵ expressing rats developed the highest level of respiratory depression tolerance. Furthermore, when CAPKC ϵ lentivirus was injected into the vIPAG, rats were able to develop significant antinociceptive tolerance at low doses of morphine that normally do not cause tolerance. The approach of increasing morphine respiratory depression and lethality tolerance by increasing PKC α or ϵ activity at preBötC could be used to make opioids safer for chronic use.

Introduction

Opioids are the most effective analgesics for the treatment of moderate to severe pain, and morphine is the prototypical opiate. The long-term use of morphine is hampered both by the development of analgesic tolerance and the relative lack of tolerance to side effects like respiratory depression (Marks CE Jr, 1973; Ling et al., 1989; Paronis and Woods, 1997; Athanasos et al., 2006). Moreover, morphine is thought to cause lethality via respiratory depression (White and Irvine, 1999). With chronic use, differential tolerance to morphine analgesia and respiratory depression would decrease the therapeutic index, the ratio between LD₅₀ and ED₅₀ (White and Irvine, 1999). Therefore, morphine would be less lethal if tolerance to respiratory depression can be increased relative to analgesic tolerance. More than 10,000 people died of unintentional non-heroin prescription opioid overdose in the United States in 2007, and the majority of deaths occurred in chronic opioid users (Hall et al., 2008; Okie, 2010). Thus, there is the need to develop a safer opioid that has similar levels of tolerance to analgesia, respiratory depression, and lethal overdose.

The preBötC initiates respiration rhythm and also mediates opioid-induced respiratory depression (Janczewski and Feldman, 2006; Montandon et al., 2011). Somatostatin is both a functional and anatomical marker for preBötC since abrupt silencing of somatostatin-expressing neurons at the preBötC results in apnea and death (Ruth et al., 2003; Tan et al., 2008). preBötC also stains positive for the μ -opioid receptor (MOR), and microinjection of DAMGO, a MOR agonist, slows down the respiration rate (Gray et al., 1999). Additionally, preBötC is involved in mediating an enhanced respiratory drive in response to hypoxic or acidotic conditions (Solomon et al., 2000; Krause et al., 2009). preBötC is sandwiched by Böttinger complex (BötC) and parafacial respiratory group/retrotrapezoid nucleus (pFRG/RTN) rostrally and the ventral respiratory group (VRG) caudally—all of them involved in respiration. Morphine is a well known respiratory depressant, and it is thought to cause lethality by depressing

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respiration (Pattinson, 2008). If cellular responses to morphine at preBötC can be altered, it might be possible to increase overdose and respiratory depression tolerance during chronic morphine treatment. One such cellular response is the μ -opioid receptor-mediated PKC activation.

PKC activity is required for both the initiation and maintenance of morphine antinociceptive tolerance. Intracerebroventricular (i.c.v.) injection of PKC inhibitors prior to chronic morphine administration prevented the development of antinociceptive tolerance (Gabra et al., 2008). After tolerance has developed, i.c.v. injection of either non-specific PKC inhibitor or subtype specific inhibitors of α , γ , and ϵ each reversed morphine antinociceptive tolerance (Javed et al., 2004). Inhibiting PKC α has been shown to block MOR desensitization in locus coeruleus neurons while inhibiting PKC ϵ blocked MOR desensitization in HEK293 cells expressing MOR (Bailey et al., 2009; Chu et al., 2010). Furthermore, development of morphine tolerance was attenuated in PKC γ or ϵ knockout mice (Zeitz et al., 2001; Newton et al., 2007). Therefore, due to preBötC's role in initiating respiration, mediating opioid respiratory depression, and chemoreception, we reasoned that if PKC activity at the preBötC is increased by expressing constitutively active PKC, tolerance to respiratory depression and lethality will be increased. Although several if not all of the classical or novel PKC subtypes could be involved, we decided to examine one classical subtype, PKC α , and one novel subtype, PKC ϵ , in controlling respiratory depression and lethality tolerance. The reason for choosing these two PKC subtype over others was also based on their relative abundance within the preBötC and ventrolateral periaqueductal gray (vlPAG) as demonstrated in our current study. By increasing morphine tolerance in respiratory depression and lethal overdose through PKC α or ϵ activation at the preBötC, the safety of morphine might be improved.

The effect of increasing PKC activity at preBötC on morphine respiratory depression and lethality tolerance was tested in this study. Lentivirus carrying wild type, constitutively active, or

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siRNA against either PKC α or PKC ϵ was stereotaxically microinjected into the preBötC bilaterally. Activation of PKC α or ϵ at preBötC increased tolerance to morphine respiratory depression and lethality. We observed that rats expressing constitutively activated PKC ϵ (CAPKC ϵ) developed the highest level of tolerance to morphine respiratory depression in terms of the time to onset of tolerance, level of hemoglobin saturation, and fold increase in ED₅₀. We also observed that wild type PKC ϵ (WTPKC ϵ) expressing rats developed overdose tolerance but not rats which received wild type PKC α (WTPKC α) lentivirus injections. Therefore, there might be PKC subtype selectivity in morphine respiratory depression and lethality tolerance development.

Methods

Animals

Male Sprague-Dawley rats between 250-275 grams were purchased from Harlan Laboratories (Madison, WI). All surgeries and behavioral tests were approved by the University of Minnesota IACUC (protocol # 0902A60283). Rats were housed in a 12h light/12h dark cycle with food and water ad libitum. Only well-healed rats were used in subsequent experiments as approximately 25% of rodents either died or suffered neurological deficits from stereotaxic brain surgery.

Chemicals

Morphine sulfate was obtained from NIDA (Bethesda, MD). Ketamine was purchased from Phoenix Pharmaceuticals (St. Joseph, MO), xylazine from Lloyd Laboratories (Shenandoah, IA), isoflurane from Phoenix Pharmaceuticals (St. Joseph, MO), and cefazolin from Apotex Corporation (Weston, FL). Triton X-100, bovine serum albumin, paraformaldehyde,

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and iodixanol were purchased from Sigma-Aldrich (St. Louis, MO). Toluidine blue was from Ricca Chemical (Arlington, TX). Advanced DMEM reduced serum medium, penicillin, and streptomycin were purchased from Invitrogen (Carlsbad, CA).

Cell culture

NS20Y neuroblastoma cells were grown in advanced DMEM reduced serum medium (Invitrogen, 12491) with 5% fetal calf serum, 100 units/ml penicillin, and 100 µg/ml streptomycin in a 10% CO₂ incubator. For lentiviral transductions, virus was applied at a multiplicity of infection of 500 when the cells were 25% confluent.

Lentivirus production

Plasmids containing constitutively active PKCα (CAPKCα; A25E point mutation), CAPKCε (pseudosubstrate inhibitory site amino acid 154–163 deletion), WTPKCα, and WTPKCε were generously provided by Drs. Allen Samarel and Jody Martin from Loyola University (Strait et al., 2001). Deleting or mutating the pseudosubstrate inhibitory site causes PKC to become constitutively activated (Pears et al., 1990). WTPKCα and CAPKCα were cloned into the EcoRI sites of the pCDHGFP vector to generate pCDHGFP-WTPKCα and pCDHGFP-CAPKCα (System Biosciences Cat. # CD511B-1). Correct gene orientation was confirmed by nucleotide sequencing. WTPKCε and CAPKCε plasmids were amplified with primers 5'-TCAAGCTAGCACCATGGTAGTGTTC AATGGCCT-3' and 5'-TCATGCGGCCGCTCAGGGCATCAGGTCTTACC-3' and cloned into the NheI and NotI sites of pCDHGFP to make pCDHGFP-WTPKCε and pCDHGFP-CAPKCε. Plasmids containing siRNA against PKCα were constructed using Invitrogen's Block-it Pol II RNAi expression kit (5'-AGGTGAAAGACCACAAATTCA-3'; K4935-00). Plasmids containing siRNA against PKCε were either purchased from Open Biosystems (TRCN22759 5'-CCCTTATCTAACCCAACTCTA-3'; TRCN22762 5'-CGTCACTTCGAGGACTGGATT-3') or constructed using Invitrogen's Block-it

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Pol II RNAi expression kit (5'-AAGATCGAGCTGGCTGTCTTT-3'; 5'-AGAGCCAATACTTACACTTGT-3'; 5'-GCAGATCAACCAGGAAGAATT-3'; K4935-00). Vector for scramble siRNA was obtained from Open Biosystems (pGIPZ Non-silencing negative control). HEK293T cells were purchased from Open Biosystems. 15 cm plates were coated with poly-lysine for 15 minutes at 37°C and washed thoroughly with distilled water. 20 million cells were plated on each 15 cm dish 24 hours before transfection. All plasmids were harvested using Qiagen's Endotoxin-free Plasmid Maxi kits and kept in 0.3M sodium acetate/ethanol until ready to use (Qiagen, 12362). On the day of transfection, plasmids were ethanol precipitated and dissolved in TE pH 8.0. Invitrogen's ViraPower Lentivirus Expression System was used to produce lentivirus (Invitrogen K4975-00). 3 µl of Lipofectamine 2000 was used to transfect each µg of the four plasmid mixture: pLP1, pLP2, pLP/VSVG, and pCDHGFP (ratio 3: 1: 1.5: 5). Supernatants were collected every 24 hours for 4 days and stored at 4°C. Cell debris were cleared by centrifuging at 3000 rpm for 5 minutes and then filtered through 0.45 µm filters (Millipore, SCHVU01RE). To concentrate lentivirus, 30 ml of supernatant were put into sterilized ultracentrifuge tubes and 200 µl of 60% iodixanol was layered on the bottom using a metal cannula (Beckman 358126; Sigma D1556-250mL). After ultracentrifugation for 2.5 hours 4°C at 55,000 x g, the virus appeared as a visible band between the clear iodixanol and red media. Afterwards, media was removed from the top until the last 3 ml. For larger volumes, ultracentrifugation was repeated until the suspension decreased to less than 30 ml, which underwent a last round of ultracentrifugation for 180 min 4°C at 75,000xg using only 30 µl of iodixanol underlayer. Very carefully, all the supernatant was removed until the last 200 µl (Coleman et al., 2003). The concentrated lentivirus solution was aliquoted and frozen at -80°C. Lentivirus was titered using flow cytometry with HT-1080 cells following manufacturer protocols (Invitrogen K4975-00). All lentivirus used had a titer between 2-3 X 10⁹ TU/ml.

Morphine respiratory depression and lethal overdose

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Dixon's up and down method was used to determine the morphine ED₅₀ dose for respiratory depression and lethal overdose (Dixon, 1965). The up and down method was used because it required less animals to determine ED₅₀ without compromising accuracy, and its use has been supported by other investigators (Lichtman, 1998; Kao et al., 2010). This method requires consecutive testing of rats using doses spaced at constant log intervals where a negative response elevates the dose for the next animal, but a positive result decreases the dose for the following rat. The log interval was 0.15 for respiration and 0.11 for lethality experiments. The initial dose of morphine used on the first rat in the sequence was an estimation of the ED₅₀, and the second dose would either be a log interval higher or lower than the first dose depending on the response of the first rodent. Each sequence required between 6-9 animals to assess ED₅₀. All rats were used only once in each up and down sequence. After completing the sequence with 6-9 animals, the equation $ED_{50} = X_f + K X d$ was used to calculate the ED₅₀, where X_f was the value of the last dose injected, K was a tabular number obtained from a table reported by Dixon, and d was the interval in log scale (Dixon, 1965).

To determine the ED₅₀ of morphine to cause respiratory depression, the baseline hemoglobin oxygen saturation (Hb Sat.) without any opioid treatment was measured for 5 minutes for all animals using the MouseOx (Starr Life Sciences, Oakmont, PA). The baseline Hb Sat. was consistently between 94%-98%. Since Dixon's up and down method required a cut off threshold to differentiate positive responses to morphine from negatives, a decrease in Hb Sat. to less than 85% was deemed to be a positive response for morphine respiratory depression. 85% was chosen as the threshold because it represented a significant and robust decrease in Hb Sat. from baseline ($P < 0.001$). After measuring the baseline Hb Sat., morphine was injected intraperitoneally, and the rats were returned to home cages for 25 minutes. Then, Hb Sat. was recorded for 3 minutes with 60 Hz data collection rate. Excluding motion artifacts, the average Hb Sat. during the 3 minute interval was recorded. If the average Hb Sat. was

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lower than 85%, then that rat exhibited a positive response to morphine respiratory depression, and the subsequent rodent would be tested with a smaller dose. The ED₅₀ of morphine to cause respiratory depression was determined for both morphine naïve rats and ones that received four daily injections of 20 mg/kg morphine. Morphine tolerance to respiratory depression is defined by two methods in this report: a significant increase in ED₅₀ or significant attenuation in the severity of respiratory depression in response to 20 mg/kg morphine as explained in the following paragraph.

In addition to measuring morphine respiratory depression ED₅₀ on the 1st and 5th day, rats' Hb Sat. after injection with 20 mg/kg morphine were measured every day for 5 days. Attenuation in the severity of Hb Sat. decrease compared to day 1 would also be considered as respiratory depression tolerance in addition to ED₅₀ changes. Hb Sat. was measured at 25 and 50 minutes after morphine injections, and the lower of the two time points was recorded. Two time points were taken (25 min and 50 min) instead of only one (25 min) because rats were measured in parallel instead of sequentially such as during ED₅₀ determination allowing more data acquisition time. Hb Sat. was used to monitor respiration as it integrates both the breathing frequency and amplitude.

To determine morphine LD₅₀, Dixon's up and down method was also used. Lethality was defined as death within 24 hours of morphine injection. 24 hours was chosen as the cut off for comprehensiveness even though most deaths occurred 1.5 to 5 hours after morphine injection. The LD₅₀ of morphine were determined for both morphine naïve rats and ones that have been chronically treated with 4 days b.i.d. morphine 5 mg/kg for a total of 8 injections.

Morphine antinociception

Dixon's up and down method was again used to determine the ED₅₀ of morphine to elicit antinociception because this approach required fewer animals. Antinociception was measured

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with the hot plate test. The hot plate was set to 55°C and the cut off time was 30 seconds. Thirty minutes after morphine injection, rats were tested on the hot plate. Licking of the hind paw was deemed a positive nociceptive response. % MPE was calculated according to the formula: $(\text{test latency} - \text{baseline latency}) / (\text{cut off} - \text{baseline latency}) \times 100$ where greater than 50% MPE was noted as a positive morphine antinociceptive response. This threshold was instituted because Dixon's up and down method to assess ED₅₀ required a binary outcome of positives and negatives. 50% MPE was chosen as the threshold because it represented robust morphine antinociception. The ED₅₀ of morphine to cause antinociception was determined for both morphine naïve rodents, and rats that were chronically treated with 4 days of b.i.d. morphine 5 mg/kg or 1.5 mg/kg. Morphine antinociception and respiratory depression were measured in different groups of animals.

Stereotaxic injections

For microinjections at the preBötC, rats between 250-275 grams were anesthetized with ketamine 90 mg/kg and xylazine 10 mg/kg. Depth of anesthesia was monitored every 15 minutes and additional doses given as needed. After mounting on a Stoelting stereotaxic frame (Stoelting, Wood Dale, IL), a mid sagittal incision was made to expose the bregma and lambda. Bregma was positioned 5 mm below lambda, and the muscles on the back of the head were removed to uncover the skull. The connective tissue was cut away with a scissor, and a dental drill cleared away the skull to expose the obex. With obex as zero, coordinates for preBötC were AP 0.9 mm, ML \pm 2 mm, and DV 2.7 mm (Tan et al., 2008). 1 μ l of the lentiviral solution was injected on each side over 5 minutes for a total of two injections, and the cannula was kept in place for another 5 minutes before withdrawn to prevent backflow (Stoelting Quintessential Stereotaxic Injector, Wood Dale, IL). Viral solution levels in the cannula tubing were marked before and after the injection to confirm virus delivery. For lentivirus microinjections at the vPAG, four injections of 1 μ l each (two injections/side) were made to each rat. Coordinates

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were AP -8.16 mm and AP -7.44 mm, ML \pm 0.8 mm, DV -6 mm with respect to bregma. Four injections were made in order to cover the entire rostrocaudal extent of vIPAG as the structure is longer in the anterior-posterior axis. Rats received i.p. ketoprofen 5 mg/kg b.i.d. for antinociception and i.p. cefazolin 20 mg/kg t.i.d. for antimicrobial prophylaxis after surgeries.

Immunohistochemistry

Rats were anesthetized with ketamine 90 mg/kg and xylazine 10 mg/kg, perfused with PBS, and then with 4% paraformaldehyde (Sigma, 158127). Brains were post-fixed in 4% paraformaldehyde overnight at 4°C, immersed in 30% sucrose until they sank to the bottom, frozen in OCT (Sakura Finetek, Japan), and 25 μ m thick sections were cut on a cryostat (Leica Microsystems, CM1950). Brains of rats that died from morphine overdose were removed from the skull, directly frozen in OCT, cut into 25 μ m thick sections with a cryostat, and fixed with 4% paraformaldehyde for 3 minutes before staining. Sections were blocked with 10% normal goat serum (Jackson ImmunoResearch, 005-000-121), 0.3% Triton X-100 (Sigma), and 0.5% BSA (Sigma) for 1 hour at room temp. All primary antibodies were dissolved in TBS (25mM Tris, 3mM KCl, 140mM NaCl, 0.05% Tween 20, pH 7.4) containing 0.3% Triton X-100 and 0.5% BSA. GFP was stained with mouse anti-GFP 1:1000 (A11120, Invitrogen), somatostatin with rabbit anti-somatostatin 1:600 (20067, Immunostar), and MOR with rabbit anti-MOR 1:10000 (custom antibody, GeneTex). To characterize the rabbit anti-MOR, rat and wild type mice cervical spinal cords served as the positive control while MOR knockout mice served as negative control (supplemental Fig. 1). Sections were incubated with primary antibodies with gentle agitation overnight at 4°C and washed three times 15 minutes each. Secondary antibodies were goat anti-mouse Alexa 488 1:200 (A11001, Invitrogen) and goat anti-rabbit Alexa 594 1:200 (A11012, Invitrogen) and were diluted in TBS with 0.3% Triton and 0.5% BSA. Tissue was incubated in secondary antibody for 1 hour at room temperature and then washed three times for 15 minutes each before mounting on slides using Fluoromount-G (0100-01, Southern Biotech).

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Fluorescence images were captured with a Leica DMIRE2 microscope connected to a BD CARVII confocal imager and a Hamamatsu EM CCD camera with laser light sources from Spectral Applied Research (green: 473 nm; red: 561 nm). Successful lentivirus stereotaxic microinjection to the preBötC or VIPAG was confirmed through immunohistochemical analyses after behavioral studies.

Laser microdissection and qRT-PCR

Rats were anesthetized with 5% isoflurane/95% O₂ and decapitated. The brains were immediately removed and frozen in OCT (Sakura Finetek). On the day of microdissection, 8 μm thick sections were cut with a cryostat, mounted on 2.0 μm thick PEN membrane coated slides (Cat. # 11505158, Leica), stained with 1% toluidine blue (8654-16, Ricca Chemical) for 5 seconds, fixed in acetone 4°C for 3 minutes, and stored on dry ice until microdissection. Laser microdissection was performed with a Leica AS LMD (Leica), and mRNA was isolated using TRI Reagent according to manufacturer's protocol (Molecular Research Center, Cincinnati OH). qRT-PCR was performed using Qiagen's Quantitect SYBR Green RT-PCR kit and the Biorad iCycler. qRT-PCR primers to amplify PKC α were 5'-GTTTACCCGGCCAACGACT-3' and 5'-GGGCGATGAATTTGTGGTCTT-3'; PKC γ were 5'-GACCCTCGCAACAAGCACAAG-3' and 5'-GATTTCCAGTTGCAGACGTCC-3'; PKC ϵ were 5'-AGCCGGCTTCTGGAACTCCC-3' and 5'-AGCTGCCTTTGCCTAACACCTTGAT-3'; β -actin were 5'-GACGATATGGAGAAGATTTGGCAC-3' and 5'-GAGGCATACAGGGACAACACAGC-3'. In order to translate Ct values into absolute copy numbers, standard curves were obtained for PKC α , γ , ϵ , and β -actin. Constructing the standard curve required a 10 fold serial dilution of templates. The templates were longer and contained the segments that were amplified in qRT-PCR analyses mentioned above. Since the templates were longer, a different set of primers were required first to make the template for each gene. To obtain the templates to make the standard curve, the following primers were used to amplify rat whole brain cDNA: PKC α 5'-

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GGGACCATGGCTGACGTTTAC-3' and 5'-CCCTCTTCTCTGTGTGATCCATTC-3'; PKC γ 5'-CCACAAGTTCACCGCTCGTTTC-3' and 5'-CATCCACAGGAGCCTTGAGTAGC-3'; PKC ϵ 5'-TACGAAGTGCGCTGGGCTAAAG-3' and 5'-GTCAGCCAGCTTGACAGTGACC-3'; β -actin 5'-GATGGTGGGTATGGGTCAGAAG-3' and 5'-ACGATTTCCCTCTCAGCTGTGG-3'. A 10 fold serial dilution of the templates was then amplified using the same primers for qRT-PCR PKC α , γ , ϵ , and β -actin mRNA abundance analysis. Using the standard curve, Ct values for each gene was translated into absolute copy numbers, and the copy number was then normalized to the absolute quantity of β -actin. Minimally, cryostat sections from 5 rats were laser dissected and used in quantification of the transcripts.

Statistical analysis

Results were analyzed with Graphpad Prism 5 (GraphPad Software Inc., La Jolla, CA). Data is expressed as mean \pm S.E.M. Two-way ANOVA with Bonferroni's post test was used when there were two factors (i.e. gene and nuclei). One-way ANOVA with Bonferroni's post test was used to compare one factor among three or more groups. Student's t-test was used to compare one factor between two groups. $P < 0.05$ was considered to be statistically significant.

Results

The relative level of PKC subtypes at the vIPAG vs. preBötC. Since PKC α , γ , and ϵ are the three subtypes involved in mediating morphine antinociceptive tolerance (Smith et al., 2007), we decided to compare their relative abundances in the vIPAG and preBötC. The vIPAG is an important center for morphine antinociception while the preBötC is involved in opioid respiratory depression (Siuciak and Advokat, 1987; Lane et al., 2005; Montandon et al., 2011). The relative abundance of the three subtypes at vIPAG vs. preBötC might provide some insight into why

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morphine develops more tolerance in antinociception than respiratory depression or lethal overdose. vIPAG or preBötC were laser microdissected and the relative quantities of PKC α , γ , ϵ , and β -actin mRNAs were determined via qRT-PCR (supplemental Fig. 2). Standard curves were constructed for each gene in order to translate Ct values into copy number. The copy numbers for the three PKC subtypes were normalized to that of β -actin. Two-way ANOVA analyses indicated that PKC abundances were significantly different [$F(6,41) = 84.21$; $P < 0.0001$](Fig. 1). Bonferroni's post tests were used after two-way ANOVA analysis. In vIPAG, PKC α and γ transcripts exist at comparable levels, which are significantly lower than the PKC ϵ abundance ($P < 0.001$). At the preBötC, PKC ϵ was again the highest followed by PKC α , and PKC γ is the least abundant subtype ($P < 0.001$). Furthermore, preBötC had more PKC α transcripts than vIPAG ($P < 0.01$) while preBötC had less PKC ϵ than vIPAG ($P < 0.001$). PKC γ transcripts were present at similar levels in the two nuclei (Fig. 1). Although all three PKC subtypes have been implicated in morphine antinociceptive tolerance development (Smith et al., 2007), we focused on the probable roles of PKC α and PKC ϵ in current studies because these two PKC subtypes represent the classical and novel PKC subtypes, respectively. Also, the higher PKC ϵ levels in vIPAG compared to preBötC ($P < 0.001$) might reflect PKC ϵ 's role on the observed greater morphine tolerance in antinociception than respiratory depression or lethal overdose (Fig. 1).

Lentiviral expression of PKC at the preBötC. Immunohistochemistry was carried out to confirm transduction of PKC in the preBötC. preBötC is located in the ventrolateral medulla, and somatostatin is both a functional and anatomical marker for preBötC neurons (Ruth et al., 2003; Tan et al., 2008). GFP delivered by the lentivirus confirms transgene expression in the ventrolateral medulla cells (Fig. 2A). Higher magnification colocalization studies indicated that a subset of GFP positive cells also expressed the μ -opioid receptor and somatostatin, markers for preBötC neurons (Fig. 2B, 2C). The intracellular staining of MOR is due to permeabilization of

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the plasma membrane with 0.3% Triton X-100, which was required in the co-staining with GFP and is in accord with earlier reports (Arvidsson et al., 1995). By the second week after lentivirus injection, robust GFP expression was readily observed. Such GFP expression continued for at least 6 months. All experiments were completed within the time frame between 2 weeks to 6 months after lentivirus injection.

The role of PKC α in tolerance to morphine-induced respiratory depression. PKC α is known to be important in maintaining morphine antinociceptive tolerance (Smith et al., 2007). Whether PKC α is involved in respiratory depression tolerance was investigated by expressing the wild type, constitutively active, or siRNA against PKC α at preBötC through lentivirus injection. siRNA knockdown of PKC α was confirmed by laser microdissecting rat preBötC, and its mRNA abundance quantitated via qRT-PCR. Lentiviral expressed PKC α siRNA knocked down PKC α by 85% relative to GFP ($P < 0.001$) and 80.4% relative to scramble siRNA ($P < 0.001$) [Fig. 3A and supplemental Fig. 2; $F(3,19) = 5314$; $P < 0.0001$]. Rats receiving GFP lentivirus, scramble siRNA, or siRNA against PKC α consistently had their Hb Sat. depressed to less than 80% after daily 20 mg/kg morphine injections indicating a lack of respiratory depression tolerance (Fig. 3B). Relative to day 1, rodents that received CAPKC α began to develop respiratory depression tolerance to 20 mg/kg morphine on the 4th day with Hb Sat. of 84.3% ($P < 0.05$), and 88.9% ($P < 0.001$) on the 5th day [Fig. 3C; $F(3,19) = 4.32$; $P < 0.05$]. As CAPKC α is a constitutively active mutant that by-pass the normal regulation for PKC activation, WTPKC α lentivirus was injected into preBötC in order to determine if over-expression of the PKC α level was enough to increase respiration tolerance. In contrast to CAPKC α , WTPKC α expressing rats did not develop tolerance to morphine respiratory depression throughout the five days of treatment (Fig. 3C). In addition to the onset of tolerance, the magnitude of tolerance was quantified by increases in ED₅₀ after chronic treatment. Rats that received CAPKC α increased their ED₅₀ for respiratory depression significantly from 14 ± 2.37 mg/kg to 29.9 ± 4.42 mg/kg ($P < 0.001$) after four daily injections of 20 mg/kg morphine [Fig. 3D; $F(10,61) = 9.04$; $P <$

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0.0001]. In contrast, GFP, scramble siRNA, PKC α siRNA, and WTPKC α rats did not have significantly increased respiratory depression ED₅₀ after 4 injections of 20 mg/kg morphine (Fig. 3D). These results indicate that increased activity of PKC α , and not the mere over-expression of WTPKC α are needed to enhance tolerance to morphine respiratory depression.

PKC ϵ activation increases morphine respiratory depression tolerance. To test whether activating PKC ϵ at the preBötC would increase respiratory depression tolerance, we expressed the constitutively active, wild type, or siRNA against PKC ϵ at preBötC by stereotaxically injecting lentivirus containing the respective genes. Since the siRNA sequences used matched both mouse and rat PKC ϵ mRNA, the ability of lentiviral delivered PKC ϵ siRNA to knockdown was confirmed both in mouse NS20Y neuroblastoma cells and rat preBötC. Knockdown in rat preBötC was 88.2% compared to GFP ($P < 0.001$) and 85.6% compared to scramble siRNA ($P < 0.01$)[Fig. 4A; $F(3,20) = 216.1$; $P < 0.0001$]. To study respiratory depression tolerance, 20 mg/kg morphine was injected intraperitoneally once a day for five days and the Hb Sat. was measured. In rats that received GFP, scramble siRNA, or PKC ϵ siRNA lentivirus at the preBötC, morphine 20 mg/kg consistently depressed Hb saturation below 79% throughout the five daily administrations indicating a lack of respiration tolerance (Fig. 4B). In contrast, CAPKC ϵ injected rats started to develop significant tolerance to morphine respiratory depression on the third day with Hb Sat. of 91.7% ($P < 0.01$)[Fig. 4C; $F(3,18) = 26.15$; $P < 0.0001$]. CAPKC ϵ also developed tolerance faster than CAPKC α as CAPKC α rats exhibited tolerance from the 4th day instead of the 3rd like CAPKC ϵ (Fig. 3C and 4C). Alternatively, tolerance can be quantified as increases in ED₅₀, and morphine naïve ED₅₀ for respiratory depression was compared to the ED₅₀ after four injections of 20 mg/kg morphine sulfate. Rats that received CAPKC ϵ at preBötC demonstrated a three-fold increase in respiratory depression ED₅₀ from 16.8 ± 1.81 mg/kg to 51.4 ± 8.55 mg/kg ($P < 0.001$)(Fig. 4D). When both were treated with chronic morphine, the ED₅₀ of CAPKC ϵ is significantly higher than that of WTPKC ϵ ($P < 0.01$)[Fig. 4D; $F(10,60) = 8.74$;

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$P < 0.01$]. Injection of GFP, scramble siRNA, PKC ϵ siRNA, and WTPKC ϵ into preBötC did not result in significant increases in the respiratory depression ED₅₀ after chronic morphine treatment (Fig. 4D). Thus, similar to PKC α , activation of PKC ϵ rather than simple over-expression is needed to cause morphine tolerance in respiratory depression.

PKC α and PKC ϵ increase morphine tolerance to lethal overdose. Lethal overdose is the most feared adverse effect during opioid treatment, and respiratory depression is thought to be the cause of morphine lethality (White and Irvine, 1999). Increasing tolerance to overdose would make morphine a safer drug for long-term use. Since activating PKC α and PKC ϵ increased tolerance to respiratory depression, tolerance to lethal overdose might also be increased. In rats that received GFP, scramble siRNA, PKC α siRNA, PKC ϵ siRNA, or WTPKC α lentivirus injections at preBötC bilaterally, LD₅₀ did not increase significantly after 4 days of b.i.d. morphine 5 mg/kg. When rats received siRNA against PKC ϵ at the preBötC, there was a non-statistically significant decrease in LD₅₀ after chronic morphine. With the same 4 day b.i.d. 5 mg/kg morphine, rats injected with WTPKC ϵ had their LD₅₀ value increased by 2.02 fold ($P < 0.01$) while those injected with CAPKC α and CAPKC ϵ had their LD₅₀ value increased by 2.12 fold ($P < 0.001$) and 2.76 fold ($P < 0.001$), respectively when compared to the morphine naïve LD₅₀ values within the same gene [Fig. 5A; $F(16,107) = 6.49$; $P < 0.001$]. Also, after chronic morphine treatment, the LD₅₀ of CAPKC α is significantly higher than WTPKC α and the LD₅₀ of CAPKC ϵ higher than WTPKC ϵ ($P < 0.05$)(Fig. 5A). To complement LD₅₀ quantifications, a Kaplan-Meier curve illustrates how GFP and CAPKC ϵ expressing rats survived when injected with increasing doses of morphine. GFP and CAPKC ϵ rodents were first treated with 5 mg/kg morphine b.i.d. for 4 days, and then both groups were challenged to increasing doses of morphine each day to measure the percentage survival. CAPKC ϵ expressing rats survived higher doses of morphine than GFP expressing rats (Fig. 5B). 78% of GFP virus injected rats died at 301 mg/kg, and the remaining 22% died when injected with 390 mg/kg the next day. In

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contrast, only 43% of CAPKC ϵ expressing rats died when injected with morphine 301 mg/kg, and the remaining 57% all survived 645 mg/kg with one rat able to survive 1071 mg/kg. When morphine caused lethality, most rats died between 1.5 to 5 hours after morphine injection.

Expressing CAPKC ϵ at the vIPAG decreased the dose required to develop morphine

antinociceptive tolerance. One possible explanation as to why expressing PKC at the preBötC increased tolerance to respiratory depression and lethal overdose is that PKC lowered the threshold to develop tolerance. The threshold required to develop morphine tolerance is different for antinociception versus lethality. In wild type rats treated with morphine b.i.d 5 mg/kg for 4 days, the ED₅₀ of morphine to cause antinociception increased three-fold from 4.04 mg/kg to 12.87 mg/kg ($t = 3.17$; $P < 0.01$), but their LD₅₀ did not change significantly (Fig. 6A and 6B). Thus, lethality has a higher threshold to develop tolerance than antinociception. Expressing CAPKC ϵ at preBötC might increase tolerance to respiratory depression and lethality by decreasing the threshold to develop tolerance. If this is the case, then injection of CAPKC ϵ could also alter tolerance development to morphine's other effects. Hence, in order to determine whether CAPKC ϵ is able to decrease the threshold for morphine antinociceptive tolerance development, we injected either saline or CAPKC ϵ lentivirus into the vIPAG (Fig. 6C). When both groups were treated with 1.5 mg/kg b.i.d. for 4 days, only the CAPKC ϵ expressing rats developed tolerance—ED₅₀ increased two-fold to 11.9 ± 1.73 mg/kg ($P < 0.01$)[Fig. 6D; $F(4,26) = 20.04$; $P < 0.001$]. Since CAPKC ϵ decreased the threshold to develop morphine tolerance, we sought to investigate whether CAPKC ϵ could further increase tolerance after morphine antinociceptive tolerance has developed. Rats injected with saline or CAPKC ϵ at the vIPAG were treated with morphine 5 mg/kg b.i.d. for 4 days, a regimen that results in significant antinociceptive tolerance. Both saline and CAPKC ϵ injected rats developed similar levels of antinociceptive tolerance ($P < 0.05$ for saline and $P < 0.01$ for CAPKC ϵ)[Fig. 6E; $F(4,26) = 22.48$; $P < 0.0001$]. Thus, CAPKC ϵ expression at the vIPAG allowed rats to develop significant

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antinociceptive tolerance when injected with morphine doses that normally do not cause tolerance, but CAPKC ϵ would not increase the degree of tolerance once antinociceptive tolerance has occurred.

Discussion

The hypothesis that activating PKC α or ϵ at the preBötC could lead to increased morphine tolerance in respiratory depression and lethal overdose was tested. Expression of CAPKC α and ϵ at preBötC increased morphine tolerance to respiratory depression and lethal overdose. The level of morphine tolerance in respiratory depression was higher in CAPKC ϵ rats than CAPKC α lentivirus injected rodents. Past studies on PKC's role in morphine tolerance have focused on inhibiting PKC in order to decrease antinociceptive tolerance. Our current study is the first report that has investigated PKC's ability to increase morphine tolerance in respiratory depression and lethal overdose. Since respiration and overdose tolerance is increased, augmenting PKC activity at the preBötC could be one approach to improve the safety of morphine in chronic use.

The goal of this study was to test whether enhancing PKC activity at an important respiration control center could increase tolerance to opioid lethal overdose and respiratory depression. While the stereotaxic coordinates targeted the preBötC, our lentivirus carried the CMV promoter, which meant that exogenous PKC expression was not limited to the preBötC. Although immunohistochemistry showed that some viral transduced GFP positive cells also stained for somatostatin and MOR, colocalization was not 100%. Caudal neighbors to preBötC include the rostral and caudal VRG. The rostral VRG is involved in inspiration by sending projections to the phrenic nucleus while caudal VRG is implicated in expiration by projecting to expiratory motor neurons (Kirkwood and Sears, 1973; Dobbins and Feldman, 1994). Rostral to

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preBötC are BötC and pFRG/RTN. BötC neurons are glycinergic and project to the VRC and phrenic nucleus to inhibit inspiratory neurons during expiration (Dobbins and Feldman, 1994; Ezure et al., 2003). pFRG/RTN is involved in both expiration and CO₂ chemosensation (Janczewski and Feldman, 2006). Dorsal to preBötC is the nucleus ambiguus, which control muscles of the larynx and pharynx as well as contain premotor parasympathetic neurons that regulate cardiac function (Dergacheva et al., 2010). Respiratory neurons in the ventrolateral medulla are known to project extensively to each other, and preBötC is known to have reciprocating projections with VRG, BötC, and pFRG/RTN (Ellenberger and Feldman, 1990; Tan et al., 2010). Since many adjacent neurons to preBötC are involved in respiration, unintended viral transduction of preBötC neighbors would still allow us to test the ability of PKC in enhancing morphine tolerance to respiratory depression and lethal overdose.

WTPKC α or ϵ rodents did not develop significant morphine tolerance in respiratory depression, but CAPKC α or ϵ expressing rats did. These results can be explained by the higher PKC activity of the constitutively active mutants. It is well known that deleting or mutating the PKC pseudosubstrate inhibitory region increased PKC activity both in vitro and in vivo as judged by increased translocation to the plasma membrane (Pears et al., 1990; Takeishi et al., 2000). Although μ -opioid receptor-mediated activation of PKC has been reported in cell models (Chu et al., 2010), our current result implies that lower PKC activation by morphine at preBötC could be one of the reasons for less morphine respiration and overdose tolerance than antinociception. At the preBötC, the lack of effect from PKC α and ϵ siRNA lentivirus indicated that PKC activation was minimal, and PKC might not be an intrinsic mechanism to regulate MOR (Fig. 3B, 3D, 4B, 4D). Both CAPKC α and CAPKC ϵ were able to increase respiration and overdose tolerance, and this is in agreement with both subtypes' role in mediating receptor desensitization after morphine treatment (Bailey et al., 2009; Chu et al., 2010). Whether other members of the classical and novel PKC subtypes will elicit similar responses remain to be demonstrated. In

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addition, CAPKC ϵ was able to increase respiratory depression tolerance to a higher extent than CAPKC α , which can be caused by differences in kinase activity levels or downstream phosphorylation targets. An alternative explanation for this difference could stem from the non-specific promoter, CMV, used to drive the expression of the transgenes causing the expression of the constitutively active PKC in non-opioid receptor and non-somatostatin expressing neurons (Fig. 2). Whether CAPKC α and CAPKC ϵ were expressed at identical neurons after injection was assumed and not determined.

Several mechanisms have been hypothesized as causing morphine tolerance, and this report investigated PKC because of the kinase's role in opioid receptor signaling when morphine is the agonist. Morphine is known to cause PKC activation, which in turn participates in opioid receptor desensitization and tolerance (Smith et al., 2007; Bailey et al., 2009; Chu et al., 2010). Besides PKC, β -arrestin mediated opioid receptor desensitization and opioid receptor recycling have been proposed as mechanisms to explain opioid antinociceptive tolerance (He and Whistler, 2005). β -arrestin 2 knockouts are known to experience less respiratory depression and constipation than wild type animals with single morphine injections, but it is not known whether the same applies to chronic treatment or respiratory depression tolerance (Raehal et al., 2005). More work needs to be done to determine the effects of β -arrestin and opioid receptor recycling on morphine tolerance in respiratory depression or lethal overdose.

There is correlation between the development of tolerance to morphine respiratory depression and lethality for CAPKC α and CAPKC ϵ . Conversely, when tolerance did not develop to respiratory depression, such as in WTPKC α , there was a corresponding lack of tolerance to morphine lethality. This correlation between respiratory depression and lethality agrees with the widely believed idea that respiratory depression is the main cause of morphine lethality (White and Irvine, 1999). The majority of morphine lethality occurred between 1.5 to 5 hours after injection, which is later than the morphine peak effect at 30 minute for intraperitoneal

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injections. This observation is consistent with the fact that morphine does not cause complete cessation of breathing even at lethal doses, but a precipitous reduction instead. While total respiratory arrest would likely cause death close to the peak effect of morphine at 30 minutes after injection, a longer time course is required to cause death when rats are breathing at less than adequate minute ventilation. The cumulated effect of depressed respiration such as respiratory acidosis did eventually cause lethality but with slower kinetics than complete respiratory arrest.

Although therapeutic applicability of the findings in this paper is limited by current technology, our results indicate that PKC ϵ activation could greatly narrow or equalize tolerance between morphine lethality and antinociception. Morphine lethality normally has a higher threshold to develop tolerance than antinociception (Fig. 6A and 6B), but expressing CAPKC ϵ would lower the threshold and allow morphine lethality tolerance to develop. On the other hand, morphine antinociceptive tolerance would be unchanged as antinociceptive tolerance easily develops, and CAPKC ϵ could not further increase the magnitude of tolerance after it has occurred (Fig. 6E). Equalizing antinociceptive and lethality tolerance would improve the safety of current opioids.

This paper tested the hypothesis that selective PKC activation in preBötC could increase tolerance to morphine respiratory depression and lethal overdose. Future opioid therapeutics that take advantage of differences in PKC subtype distribution and morphine-induced PKC activity between analgesic vs. respiratory centers could maintain or improve the therapeutic index of morphine during chronic pain treatment. As annual lethal overdoses from prescription opioid analgesics have increased to more than 10,000 in the US, developing approaches to improve the safety of opioids for chronic use is an important health care issue (Okie, 2010).

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Acknowledgements

We are grateful to Dr. Martin Wessendorf for the many discussions and recommendations that he has made to this project. We would also like to thank Drs. Allen Samarel and Jody Martin for kindly providing the PKC subtype plasmids. Dr. George Wilcox's generosity in loaning us the Mouseox is greatly appreciated. Jinghua Xi's assistance in producing the rabbit anti-MOR is much valued. We would like to thank Dr. Hui Zheng for technical assistance in siRNA lentivirus preparation.

Authorship Contributions

Participated in research design: Lin, Law, and Loh

Conducted experiments: Lin

Performed data analysis: Lin and Law

Wrote or contributed to the writing of the manuscript: Lin, Law, and Loh

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Footnotes

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Figure legends

Fig. 1. Relative abundance of PKC subtypes at the vIPAG and preBötC. vIPAG and preBötC were laser microdissected to compare the relative quantities of PKC α , γ , ϵ , and β -actin transcripts in the two nuclei. Copy numbers were normalized to that of β -actin, which was set as 1. *******, $P < 0.001$, compared with PKC α vIPAG, PKC γ vIPAG, and PKC ϵ preBötC. **†††**, $P < 0.001$, compared with PKC γ preBötC and PKC ϵ preBötC. ******, $P < 0.01$ compared to PKC α vIPAG. Data was analyzed with two-way ANOVA with Bonferroni's post test and presented as mean \pm S.E.M ($n \geq 5$).

Fig. 2. Lentiviral transduction of somatostatin and MOR positive preBötC neurons. Lentivirus was stereotaxically microinjected into the preBötC, and the animals were perfused for immunohistochemistry 9 weeks after surgery. A, GFP positive cells due to lentiviral transduction at the ventrolateral medulla corresponding to the location of the preBötC.

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Magnification is 100X and scale bar is 500 μm . B, Significant colocalization between lentiviral expressed GFP and endogenous somatostatin. Magnifications are 400X and 630X. Scale bars are 10 μm . C, Colocalization between exogenous GFP and endogenous MOR expressing neurons. Magnification is 400X and the scale bar is 10 μm .

Fig. 3. PKC α in morphine respiratory depression tolerance. Rats were injected with lentivirus carrying either GFP, scramble siRNA, PKC α siRNA, WTPKC α , or CAPKC α at the preBötC bilaterally. A, siRNA knockdown efficiency. After injection of PKC α siRNA lentivirus at the preBötC, PKC α knockdown was assessed through laser microdissection and qRT-PCR. β -actin was set as 1. ***, $P < 0.001$ compared to PKC α siRNA (one-way ANOVA). B and C, respiratory depression due to 20 mg/kg morphine. Data was analyzed using two-way ANOVA. *, $P < 0.05$ compared to day 1 CAPKC α . ***, $P < 0.001$ compared to day 1 CAPKC α . D, morphine respiratory depression ED₅₀. Respiratory depression ED₅₀ values was determined before and after chronic morphine treatment. ***, $P < 0.001$ compared to MS naïve CAPKC α (two-way ANOVA). Data is presented as mean \pm S.E.M. ($n \geq 6$).

Fig. 4. PKC ϵ in tolerance to morphine respiratory depression. Rats were microinjected bilaterally at the preBötC with either lentivirus carrying GFP, scramble siRNA, PKC ϵ siRNA, WTPKC ϵ , or CAPKC ϵ . A, PKC ϵ lentiviral siRNA knockdown. *In vitro*, NS20Y cells were transduced with PKC ϵ siRNA lentivirus. *In vivo*, rat preBötC injected with siRNA lentivirus was laser microdissected for qRT-PCR analysis. β -Actin was set as 1. *, $P < 0.05$ and ***, $P < 0.001$ compared to NS20Y PKC ϵ siRNA (one-way ANOVA). ††, $P < 0.01$ and †††, $P < 0.001$ compared to rat preBötC PKC ϵ siRNA (one-way ANOVA). B and C, respiratory depression due to 20 mg/kg morphine. **, $P < 0.01$ compared to day 1 CAPKC ϵ (two-way ANOVA). D, morphine respiratory depression ED₅₀. The ED₅₀ values to cause respiratory depression were determined before and after chronic morphine treatment. ***, $P < 0.001$ compared to naïve

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CAPKC ϵ and ††, $P < 0.01$ compared to chronic WTPKC ϵ using two-way ANOVA. Data is presented as mean \pm S.E.M. ($n \geq 6$).

Fig. 5. PKC α and ϵ increase morphine tolerance in lethal overdose. Rats were microinjected with lentivirus carrying GFP only, scramble siRNA, PKC α siRNA, PKC ϵ siRNA, WTPKC α , WTPKC ϵ , CAPKC α , or CAPKC ϵ at the preBötC bilaterally. A, morphine LD₅₀ for naïve and chronically treated rats. *, $P < 0.05$ compared to chronic WTPKC α . ***, $P < 0.001$ compared to naïve CAPKC α . **, $P < 0.01$ compared to naïve WTPKC ϵ . †, $P < 0.05$ compared to chronic WTPKC ϵ . †††, $P < 0.001$ compared to naïve CAPKC ϵ . Data was analyzed with two-way ANOVA and presented as mean \pm S.E.M. ($n \geq 6$). B, Survival to increasing doses of morphine. GFP and CAPKC ϵ expressing rats were treated with morphine 5 mg/kg b.i.d for 4 days before they were challenged to increasing doses of morphine each day. CAPKC ϵ ($n = 7$) and GFP ($n = 9$).

Fig. 6. PKC ϵ activation decreased the dose required to develop morphine antinociceptive tolerance. A and B, morphine differential tolerance. The ED₅₀ of morphine to cause antinociception (AD₅₀) or lethality (LD₅₀) was determined before and after chronic morphine. **, $P < 0.01$, compared to AD₅₀ MS naïve (t-test). C, lentiviral transduction. CAPKC ϵ lentivirus was injected into the vIPAG. Magnification is 100X and scale bar is 500 μ m. D, CAPKC ϵ rats became tolerant to 1.5 mg/kg of chronic morphine. ED₅₀ was determined in saline or CAPKC ϵ vIPAG microinjected rats. **, $P < 0.01$ compared to CAPKC ϵ MS Naïve (two-way ANOVA). E, CAPKC ϵ does not increase the magnitude of morphine tolerance. ED₅₀ values were determined before and after chronic 5 mg/kg morphine treatment in rats injected with either saline or CAPKC ϵ at the vIPAG. *, $P < 0.05$ compared to saline MS naïve. **, $P < 0.01$ compared to CAPKC ϵ MS naïve (two-way ANOVA). Data is presented as mean \pm S.E.M. ($n \geq 6$).

Figure 1

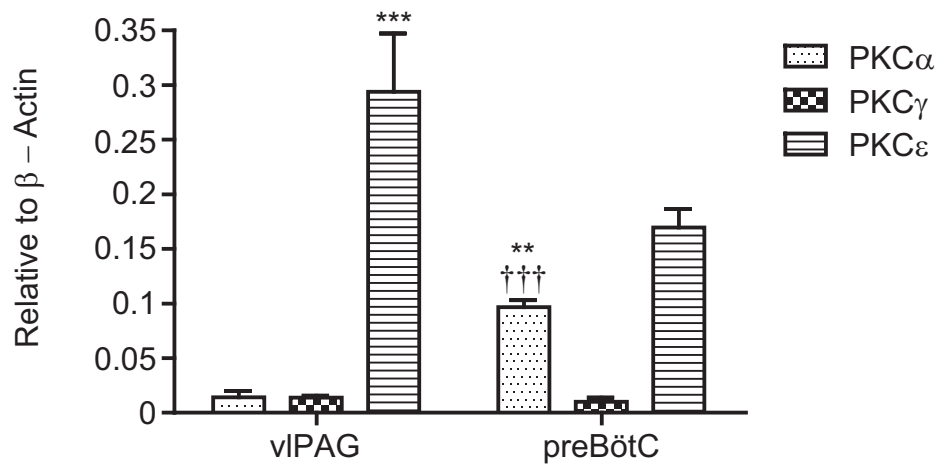


Figure 2A

A

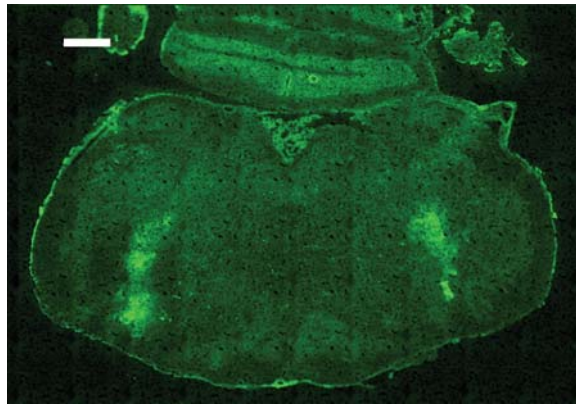
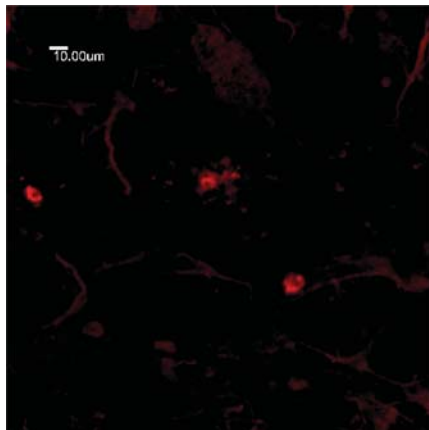
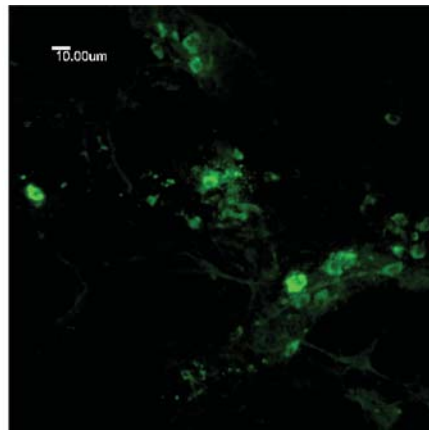


Figure 2B, 2C

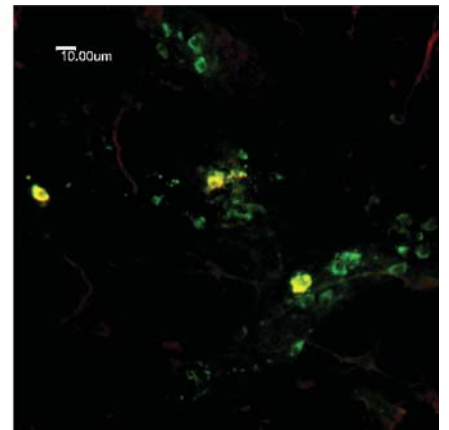
B



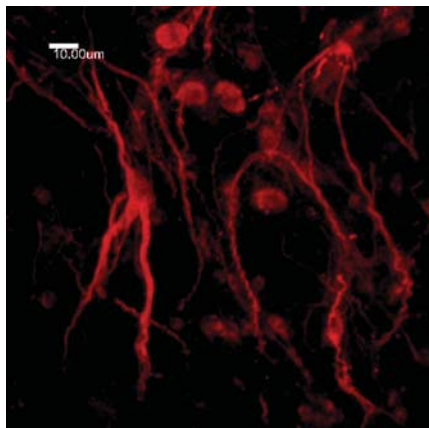
Somatostatin



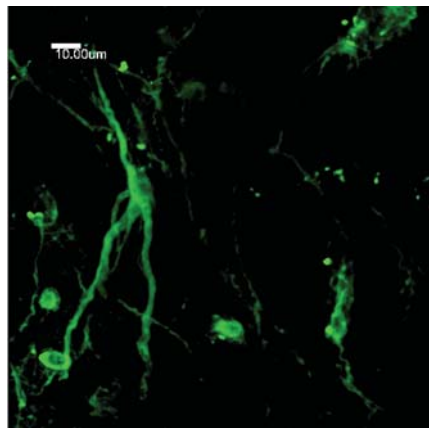
GFP



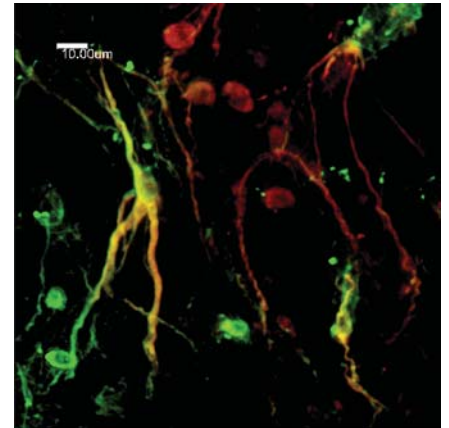
Merge



Somatostatin

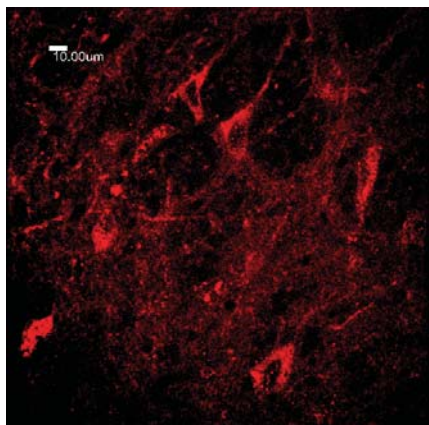


GFP

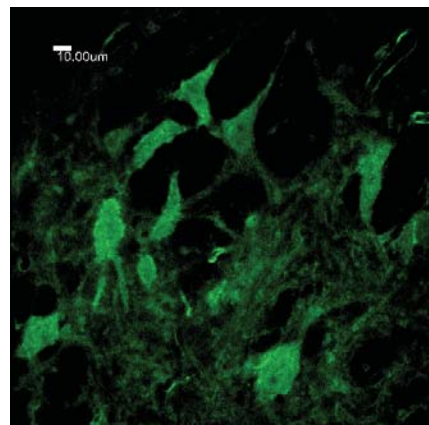


Merge

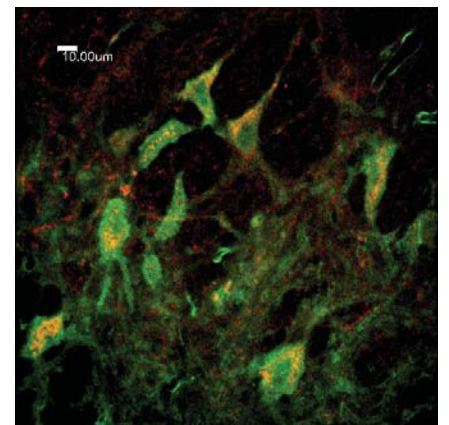
C



MOR



GFP



Merge

Figure 3

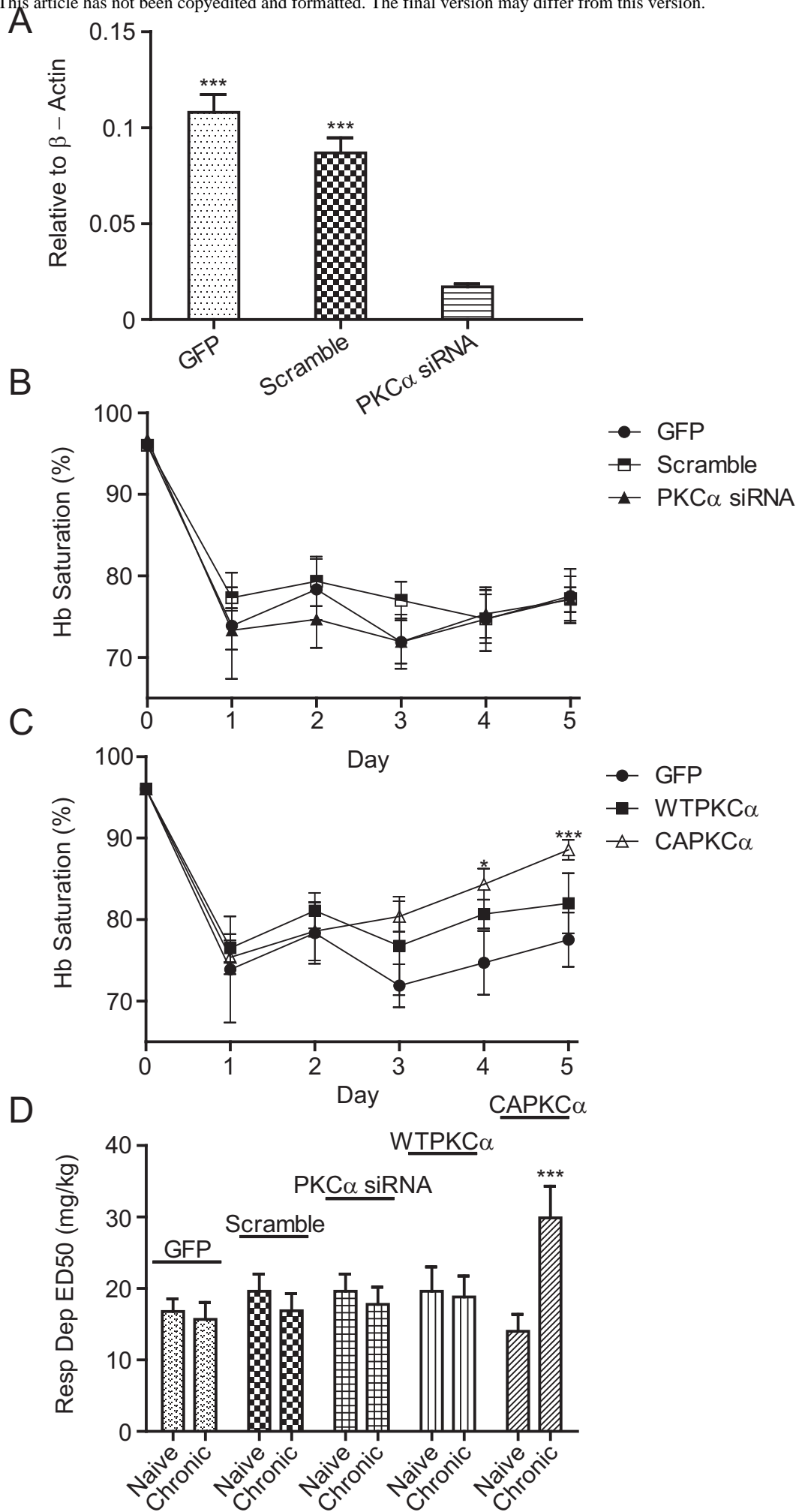


Figure 4

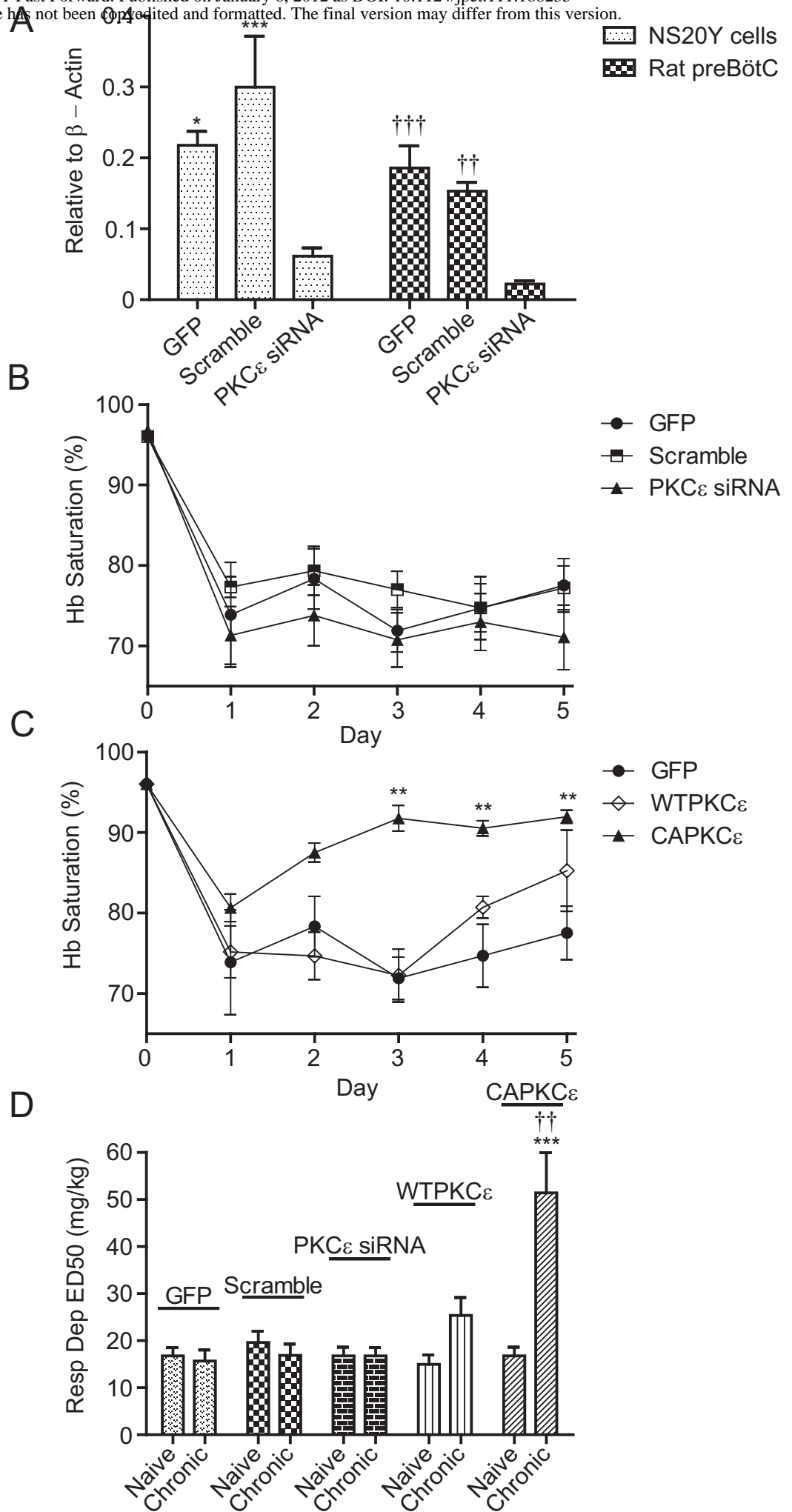
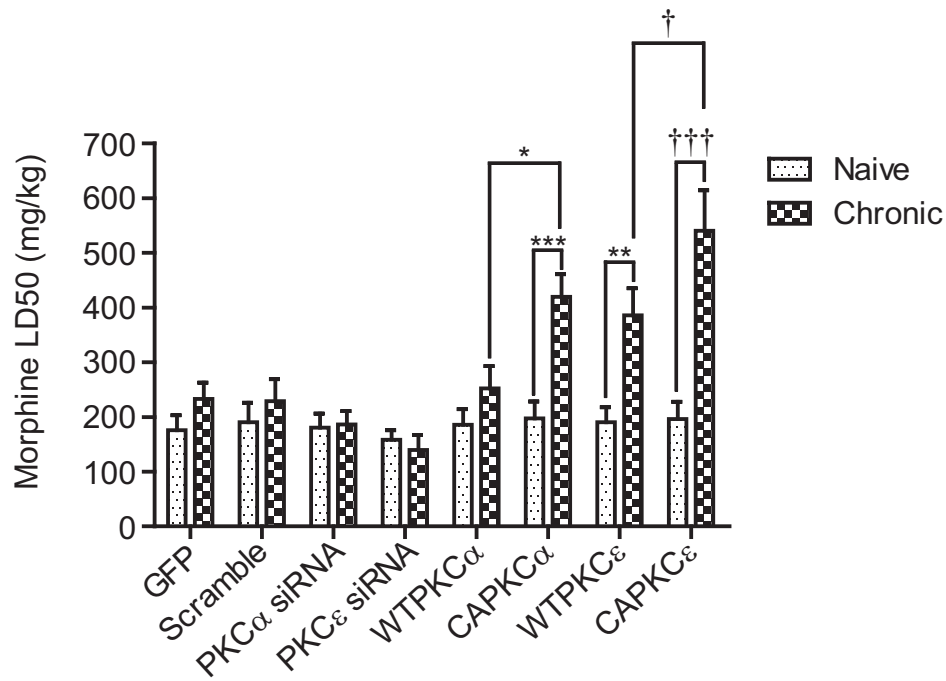


Figure 5

A



B

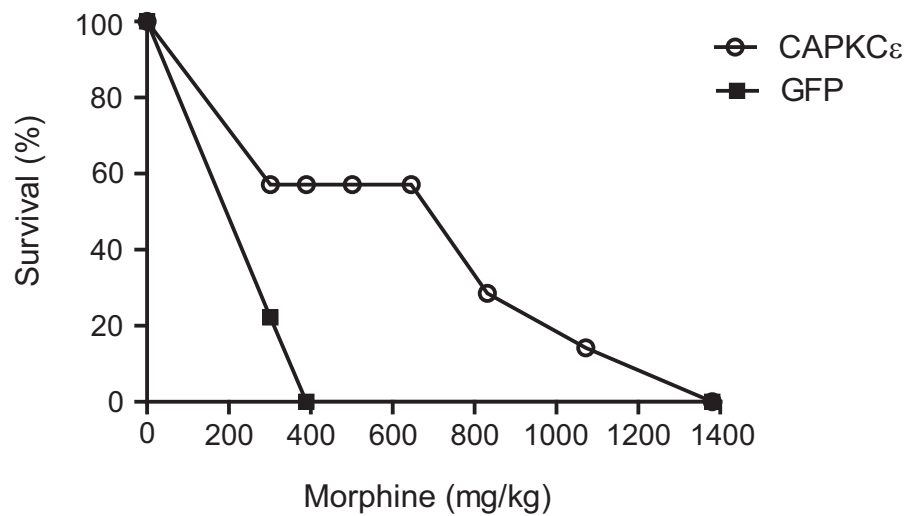
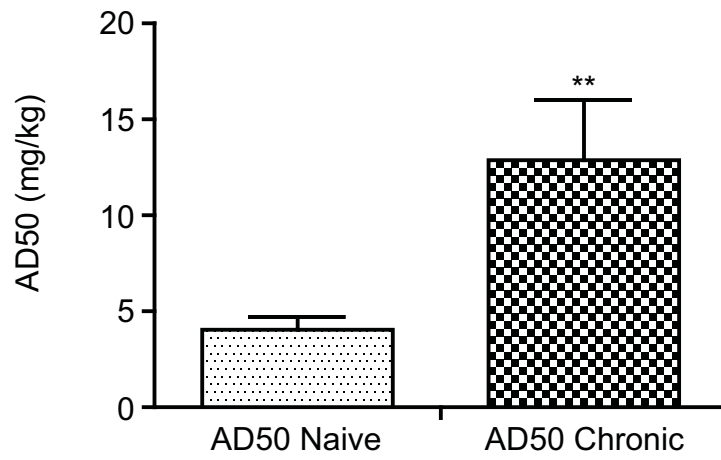
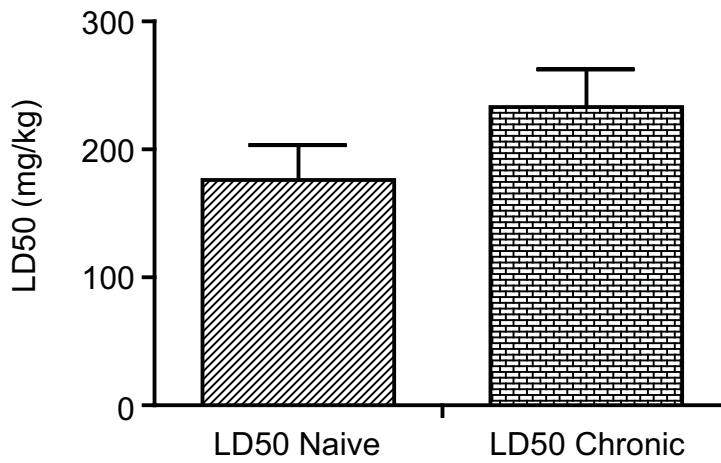


Figure 6A, 6B, 6C

A



B



C

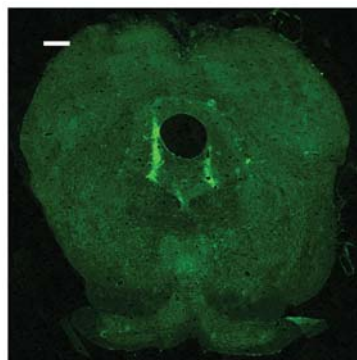
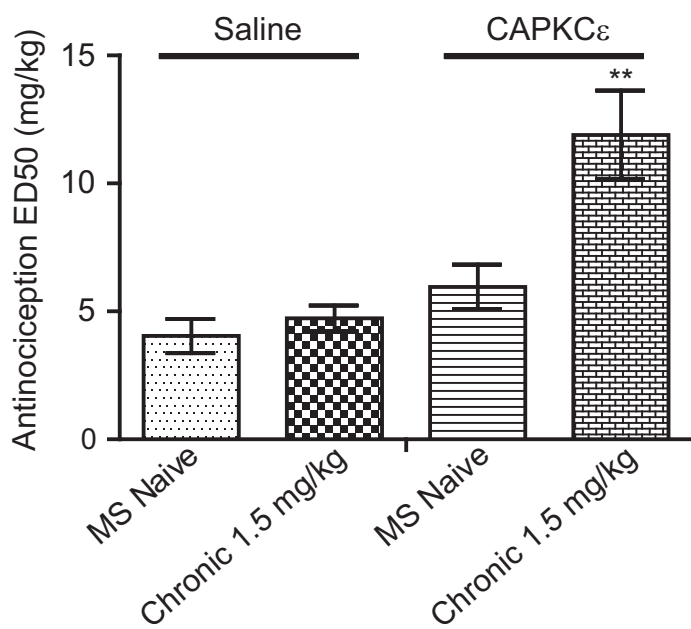


Figure 6D, 6E

D



E

