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Abstract

Rationale Major depressive disorder is a leading cause of suicide and disability. Despite this, current antidepressants provide insufficient efficacy in more than 60% of patients. Most current antidepressants are pre-synaptic reuptake inhibitors; postsynaptic signal regulation has not received as much attention as potential treatment targets.

Objectives We examined the effects of disruption of the postsynaptic cyclic nucleotide hydrolyzing enzyme, phosphodiesterase (PDE) 1b, on depressive-like behavior and the effects on PDE1B protein in wild-type (WT) mice following stress.

Methods Littermate knockout (KO) and WT mice were in locomotor activity, tail suspension (TST), and forced swim tests (FST). FST was also used to compare the effects of two antidepressants, fluoxetine and bupropion, in KO versus WT mice. mRNA expression changes were also determined. WT mice underwent acute or chronic stress and markers of stress and PDE1B expression examined.

Results *Pde1b* KO mice exhibited decreased TST and FST immobility. When treated with antidepressants, both WT and KO mice showed decreased FST immobility and the effect additive in KO mice. Mice lacking *Pde1b* had increased striatal *Pde10a* mRNA expression. In WT mice, acute and chronic stress upregulated PDE1B expression while PDE10A expression was downregulated after chronic but not acute stress.

Conclusions PDE1B is a potential therapeutic target for depression treatment because of the antidepressant-like phenotype seen in *Pde1b* KO mice.

Key words: Phosphodiesterase; PDE1B; Pde1b knockout mice; stress resistance; forced swim test; Pde brain gene expression

Introduction

Depression is a leading cause of disability, with a lifetime prevalence of 16% (Kennedy 2013). The Centers for Disease Control and Prevention report that two-thirds of suicides are depression-related (Cassano and Fava 2002). In the United States, 83.1 billion dollars is spent annually treating depression, yet current treatments are often not effective (Greenberg et al. 2003).

Most antidepressants target presynaptic neurotransmitter reuptake transporters; postsynaptic targets have received less attention. A potential postsynaptic site for modulating neuronal activity is through influencing the duration of action of second messengers (cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP)). Increased levels of cGMP have been associated with antidepressant efficacy by increasing neuronal activity (Reierson et al. 2011). This may contribute to secondary effects, such as promoting progenitor cell proliferation in the subventricular and subgranular zones (Gómez-Pinedo et al. 2010; Reierson et al. 2011).

Phosphodiesterases (PDEs) hydrolyze the phosphodiester bond of cAMP and/or cGMP. There are 11 PDE families composed of 21 isoforms each with a different specificity for cAMP, cGMP, or both. Most PDEs have distinct tissue distributions (Maurice et al. 2014). The rate of hydrolysis determines the duration of cyclic nucleotide signaling on downstream effectors such as protein kinase A (PKA), protein kinase G, exchange protein activated by cAMP, and cyclic nucleotide gated channels (Conti and Beavo 2007).

Human and animal studies have linked other PDEs (e.g., PDE4) to depression (O'Donnell and Zhang 2004). Patients with major depressive disorder have decreased positron emission tomography binding of ^{11}C-(R)-rolipram, a PDE4 inhibitor, (Fujita et al. 2012). Chronic exposure to antidepressants, including rolipram, increase levels of brain-derived neurotropic factor and neurogenesis through the activation of the PKA and phosphorylated cAMP-response

binding protein (Duman et al. 1999). Rodents treated with etazolate, another PDE4 inhibitor, exhibit antidepressive-like changes on tests of locomotor activity, tail suspension test (TST), and forced swim test (FST); it is also effective at blocking the induction of depressive-like behaviors caused by chronic mild stress (CMS) (Jindal et al. 2013; Jindal et al. 2012). RNA interference or knockout (KO) of *Pde4d* increases cAMP signaling and decreases immobility in the TST and the FST in mice and in the FST in rats (Schaefer et al. 2012; Wang et al. 2013; Zhang et al. 2002). In humans, PDE4 inhibitors have antidepressant effects, however they also cause unacceptable gastrointestinal side-effects (Hansen and Zhang 2015).

An alternate PDE target for depression is PDE1. Vinpocetine, a PDE1 inhibitor, produced enhancement of long-term potentiation (LTP) and increased dendritic spine density in rats, suggesting that PDE1 inhibitors have neurotrophic effects (Filgueiras et al. 2010). There are three PDE1 subtypes: A, B, and C. PDE1A is in brain, heart, lung, and testis and is involved in regulating vascular smooth muscle (Kim et al. 2001). PDE1C is found in brain, heart, and testis and promotes arterial smooth muscle cell proliferation and down-regulation of glucose-induced insulin secretion (Han et al. 1999; Rybalkin et al. 2002). PDE1 is a dual substrate for cAMP and cGMP and is found in areas rich in dopamine (DA) (Essayan 2001), including the caudate-putamen, nucleus accumbens, dentate gyrus, and substania nigra, areas linked to mood and other functions (Lakics et al. 2010; Polli and Kincaid 1994). The described neurotrophic effects of PDE1 inhibitors and the localization of PDE1B suggests it might be promising in relation to depression. We created a constitutive *Pde1b* KO mouse (Reed 2000). These mice exhibit minor increases in locomotor activity (Reed 2000), differential responses to stimulants, but in one report, no change in FST behavior (Siuciak et al. 2007). However, in the latter study the mice were on a mixed background, whereas our mice were back-crossed 10 generations. Previously, we crossed *Pde1b* KO mice with *Darpp32* KO mice [dopamine and cyclic-adenosine 5'-phosphate (cAMP)-regulated phosphoprotein, M_r 32 kDa that plays a role in

dopaminergic and serotonergic pathways]. *Pde1b-Darpp32* double KO (dKO) mice exhibited increased DA turnover in striatum (Ehrman et al. 2006) compared with single KO and WT mice (Ehrman et al. 2006; Fienberg et al. 1998; Reed et al. 2002; Svenningsson et al. 2000; Svenningsson et al. 2004; Svenningsson et al. 2003; Svenningsson et al. 2002). These data suggest that PDE1B may be involved in DA signaling, and DA has been implicated in depression (Chaudhury et al., 2013). Accordingly, we hypothesized that *Pde1b* disruption would result in a stress/depressive-resistant phenotype.

Methods

Animals and Husbandry

Mice used for experiment 1 were congenic C57BL/6N KO mice bred in house from heterozygous (*Pde1b*$^{+/-}$ x *Pde1b*$^{+/-}$) parents to obtain litters containing WT, KO, and heterozygous littermates (Reed et al. 2002). Mice were tested as adults (postnatal day (P) 60 or later) with not more than one mouse per genotype per litter used where possible to control for litter effects. Offspring were housed 2-4 per cage after weaning. All mice were housed in polysulfone cages in a pathogen free vivarium using Modular Animal Caging System (Alternative Design, Siloam Spring, AR) with HEPA filtered air (Alternative Design, Siloam Spring, AR) at 30 air changes/h. Water was provided ad libitum using an automated reverse-osmosis filtering system (SE Lab Group, Napa, CA). Cages had ad libitum food, corncob bedding, and cotton nest material. Mice were maintained on a 14 h light-10 h dark cycle (lights on at 600 h) that is standard in our institution's vivarium. Protocols were approved by the Institutional Animal Care and Use Committee. The vivarium is accredited by AAALAC International. Wild-type C57BL/6J male mice used in experiment 2 were purchased from Jackson Laboratories, randomly assigned to treatment groups, given one week to acclimate before experiments, and housed four per cage. All behavioral testing was done blind to the

genotype, and all behavioral testing was done in the Animal Behavioral Core at Cincinnati Children's with the exception of those mice tested at Mount St. Joseph University (see below). Sample sizes for each experiment are given in figure legends and **Table 1**.

Experiment 1: KO phenotype and mRNA Pde isoform expression

Reverse Transcription-qPCR

RNA was isolated from the striatum and cerebellum of 4 KO and 5 WT mice using the RNeasy kit (Qiagen) according to manufacturer's instructions. The striatum was chosen because it is the region of highest Pde1b expression; the cerebellum was chosen as a negative control region. The RNA was treated with TURBO DNase (Ambion), quantified by Nanodrop (Thermo Scientific), and integrity measured on an Agilent 2100 Bioanalyzer using an RNA Nano 6000 Labchip (Agilent). The RNA integrity number ranged from 8.3 to 9.5. Reverse transcription (RT) reactions were performed using 1 x reaction buffer, 2.5 mM $MgCl_2$, 1 μg of RNA template, 2.5 μM random hexamers, 0.25 mM of each dNTP, 40 U RNase inhibitor, 150 U MMLV-RT (Applied Biosystems) in a final volume of 100 μL. Reactions lacking the RT enzyme (RT-) were used as negative controls. Reactions were carried out in a PCR machine using the following program: 10 min at 25 ^{o}C, 60 min at 37 ^{o}C, and 5 min at 75 ^{o}C. Quantitative PCR (qPCR) contained 80 ng of cDNA, 300 nM of each primer (forward and reverse), and 1x SYBR Green Master Mix (Qiagen) in a 40 μL volume. Four 5 μL aliquots of the mix were placed in a 384-well plate and the qPCR was performed on an ABI Prism 7900HT (Applied Biosystems) using the following cycling conditions: 50 ^{o}C for 2 min, 95 ^{o}C for 10 min, and 40 cycles at 95 ^{o}C for 15 s and 60 ^{o}C for 1 min. Primers were synthesized by Eurofins Genomics (Ebersberg, Germany) and selected for this study based on primer efficiency, empirically determined to be 95 - 100%. Mouse primer sequences are listed in **Table 1**. Negative controls included qPCR with RT- samples or in the absence of template. Ct values were determined by the SDS 2.4 software after manually setting the threshold to 0.5. The denaturation curve showed a single

peak, representative of a single PCR product. The average Ct values from quadruplicate repeats were calculated. These were then averaged with values obtained from 2 independent qPCR experiments. Changes in *Pde* mRNA levels were measured with the ΔΔCt method, using PSMB2 (proteasome subunit beta type 2) as the housekeeping reference and the *Pde1b* WT striatum sample as calibrator (set at 100%).

Open-Field Locomotor Activity

One set of mice was given the following tests: Open-field, TST, and FST with sample sizes of 8-25 mice per genotype. Activity was assessed in 40 x 40 cm automated locomotor activity chambers (PAS System, San Diego Instruments, San Diego, CA) as described (Hautman et al. 2014). Mice were placed in test chambers for 1 h, and data were collected every 5 min. The total number of infrared beam interruptions was analyzed.

Tail Suspension Test

TST followed the method of Cryan et al. (Cryan et al. 2005a). The apparatus allowed the mouse's tail to be inserted through a hole in a transparent horizontal acrylic plate mounted on four legs. The tail was pulled snuggly against the underneath surface so that no space remained between the base of the tail and the plate. The test was scored manually in 1 min intervals during the 5 min test. Immobility time and latency to the first immobile event were scored. Immobility was defined as the absence of movement except minor paw or nose movements.

Forced Swim Test

Mice were placed in a transparent glass cylindrical vessel 10 cm in diameter (i.d.) and 25 cm tall filled to a depth of 6 cm with 22 ± 1 ºC water. Two procedures were used. The first group was given a single 6 min trial with minutes 2-6 scored for immobility. Later groups were tested using the 2 day method (Cryan et al. 2005b; Porsolt et al. 1979). On day 1, mice were placed in the vessel for 15 min. On day 2, mice were given a second trial for 5 min and scored

for immobility, latency to immobility, and active swimming. Immobility was defined as minimal movement sufficient to keep the mouse's nose above water. The 2 day procedure was used on two sets of mice at two separate institutions to verify the phenotype: Cincinnati Children's Research Foundation (**Fig. 2D**) and Mount St. Joseph University (**Fig. 2E**).

Antidepressant Treatment

A different set of mice was used for the antidepressant experiment, and these mice also received the FST with sample sizes of 8-14 per group. Antidepressant effects in WT and Pde1b KO mice were assessed using the FST. Different antidepressants show efficacy with different doses and dosing regimens, therefore, we used procedures previously found to be effective. We chose one SSRI (fluoxetine) and one non-SSRI (bupropion) for comparison. Drugs were given subcutaneously in a volume of 10 mL/kg. Fluoxetine (20 mg/kg; Sigma-Aldrich, St. Louis, MO) was administered three times at 23.5, 5, and 1 h prior to day-2 of FST as per (Mason et al. 2009). Bupropion (20 mg/kg; Toronto Research Chemicals, Toronto, Ontario, Canada) was administered 30 min before day-2 of FST as per (Dhir and Kulkarni 2008).

Experiment 2: Effects of stress on PDE1B protein

Acute Stress

Adult male WT mice were rehoused four times in random combinations to normalize the gut microbiota between treatment groups (Stappenbeck and Virgin 2016), and cages were randomly assigned to stress or non-stress groups (8 mice/group for corticosterone and 12 mice/group for Western blots). Mice had a submandibular blood sample taken 48 h before day-1 of FST or handling in the no-stress group. Mice in both groups had blood drawn after day-1 FST or handling. FST mice were given day-2 of the FST and both groups sacrificed 24 h later. Mice were decapitated, blood collected, and brain collected (brain was cut along the midline and the striatum, cerebellum, or whole brain collected) and frozen at –80 ºC.

Chronic Mild Stress

Adult male WT mice were housed 4/cage for one week prior to the start of the chronic mild stress (CMS) exposure. Each cage was randomly assigned to CMS or no-stress groups (12/group). Mice were weighed every third day. No-stress mice were handled daily to control for being removed from cages. Submandibular blood samples were taken before the first and after the last stressor. For the next three days all mice were tested in the morning in TST and in the afternoon in FST, albeit no differences were detected in the behavior of the stressed and non-stressed groups on any day. Twenty-four hours after the final FST mice were euthanized and blood, thymus, spleen, adrenal, and brain were collected. CMS used two stressors per day for 21 days (**Table 2)** (Castaneda et al. 2011). Stressors were: Tilted cage (tilted 45° with no bedding); restraint: mice placed in 50 mL conical centrifuge tubes with holes for air circulation; shaker: mice were restrained in 50 mL tubes attached to a shaker plate and rotated at 200 rpm; predator: mice were placed in 50 mL tubes and placed in an F344 male rat's cage; standing water: 500 mL of water in cage; dirty rat cage: mice placed in a soiled rat cage; grid floor: mice housed in a cage with a wire floor; hypoxia: mice placed in a hypoxia chamber (Biospherix Lacona, NY) and exposed to 8% oxygen and 92% nitrogen; cold: mice were placed in boxes in a 4 °C room.

Corticosterone Assay

Collected blood was placed in micro-centrifuge tubes with 2% ethylene diamine tetra acetic acid as anticoagulant. Samples were stored on ice and later spun at 610 RCF for 15 min at 4 °C. Plasma was transferred to clean micro-centrifuge tubes and stored at -80 °C. Plasma samples were assayed using a single lot of Enzo Life Sciences® Corticosterone EIA Kits and run in duplicate following the manufacturer's instructions.

Western Blot

Frozen brain tissue was homogenized in radioimmuno-precipitation assay buffer with protease inhibitors. Protein was quantified using the BCA™ Protein Assay Kit and diluted to 3

μg/μL. Western blots were performed using LI-COR Odyssey® procedures. Primary antibodies from Abcam and their dilutions were: rabbit anti-PDE1B C-terminal (Ab170441 or Ab182565) at 1:500 or 1:5000 respectively, rabbit anti-PDE10A (Ab177933) at 1:1000, and mouse anti-actin (Ab3280) at 1:2000 as a loading control. Odyssey IRDye 680 and 800 secondary antibodies were used at a 1:15,000 dilution. Relative protein levels are quantified using the LI-COR Odyssey® scanner and Image Studio analysis software that reads fluorescent intensity of the sample normalized to actin.

Data Analysis

Data were analyzed using SAS (v9.3, SAS Institute, Cary, NC), where $p \leq 0.05$ was the threshold for significance. To control for litter effects only one mouse per treatment group per genotype per litter was used. T-tests were used when there was only two levels of an independent variable, i.e., genotype (KO vs WT) or stress (stress vs no-stress). In these cases dependent variables were immobility time, protein level, or organ weight. Results from t-tests are presented as ordinary means ± standard error of the mean (SEM). Where there were more than two factors, mixed linear model ANOVAs were used. In these analyses data are presented as least square mean ± SEMs. Two way ANOVAs were used when between subject factors were genotype (KO vs WT) and drug (saline, fluoxetine, or bupropion) or genotype (KO vs HET vs WT) and sex. A three way ANOVA was used when the factors were genotype, gene (Pde 1A, 1B, 1C, 2, 4A, 4B, 4D, 10), and brain region (striatum, cerebellum, or whole brain). Repeated measure ANOVA was used for body weight, blood samples, and locomotor activity interval. Mixed models used the autoregressive-1 covariance matrix and Kenward-Roger first order adjusted degrees of freedom. Litter was a random factor in ANOVA models.

Results

Experiment 1: KO mice phenotype and mRNA Pde isoform expression

RT-qPCR

Expression of mRNA was assessed by RT-qPCR for eight *Pde* isoforms in the striatum and cerebellum (control region) of WT and *Pde1b* KO mice to confirm complete KO of *Pde1b* and test for compensation by other *Pde* isoforms. As shown in **Fig. 1A**, *Pde2* and *Pde10a* expression levels were highest in the striatum, followed by *Pde4b* and *Pde1b*. Expression of all *Pde* isoforms were low in the cerebellum (**Fig. 1B**) compared with striatum [$F(1,112)=2103.7$, $p<0.001$]. *Pde1b* mRNA was abolished in striatum and cerebellum of KO mice [$F(1,112)=48.8$, $p<0.001$]. Apart from *Pde1b*, no differences were seen between WT and *Pde1b* KO mice for *Pde1a* or *Pde1c* isoforms or for *Pde2 or Pde4* mRNA. There was a significant upregulation of *Pde10a* in KO mice compared with WT mice in the striatum [$F(1,112)=58.1$, $p<0.001$], but this was not observed in the cerebellum.

General characteristics and Locomotor Activity

The appearance and overall behavior of KO mice showed no differences compared with WT mice. No mortality was observed. KO mice were well groomed and of comparable body weight as WT mice. KO mice were modestly more active in the open-field than WT littermates (**Fig. 2A;** [$F(1,21.3)=5.1$, $p<0.05$]) but the effect was not overtly observable.

Tail Suspension and Forced Swim Tests

In order to assess acute stress-depressive related behavior, two tests were used: FST and TST. **Fig. 2B** shows that *Pde1b* KO mice had reduced immobility in the TST compared with WT mice [$t(22)=-4.8$, $p<0.001$]. Similarly, *Pde1b* KO mice, regardless of FST method, showed reduced immobility using a one-day, **Fig. 2C** [$t(14)= 6.3$, $p<0.001$], or two-day procedure, **Fig. 2D** [$t(13.8)=-3.1$, $p<0.01$], compared with WT mice. This was confirmed by collaborators in which *Pde1b* KO mice showed reduced immobility compared with heterozygous ($p<0.001$) and WT littermates ($p<0.001$), the heterozygous and WT mice did not differ from one

another [main effect: $F(2,52)=8.2$, $p<0.001$, **Fig. 2E**]. In addition, no sex differences ($p>0.8$) were found, i.e., Pde1b KO females showed similar reductions in immobility as males. This being the case, only males were used in subsequent experiments.

Antidepressant treatment in Pde1b KO mice

In order to determine if *Pde1b* deletion is efficacious independent of mechanisms of current antidepressants, we tested two drugs from different classes: a selective serotonin reuptake inhibitor, fluoxetine, and a norepinephrine-DA reuptake inhibitor, bupropion. Analysis of time spent immobile in the two day FST following treatment with fluoxetine showed significant genotype and drug effects [Genotype: $F(1,27)=13.1$, $p<0.01$, Drug: $F(1,27)=25.0$, $p<0.001$]; the interaction was not significant. KO mice showed a decrease in immobility independent of drug compared with WT littermates (**Fig. 3A**). Mice given fluoxetine had reduced immobility compared with those given saline. The data suggest that the effects of the KO and fluoxetine were additive but not synergistic.

A similar effect was seen with bupropion. There were significant main effects of genotype and drug on FST immobility [Genotype: $F(1,48)= 20.9$, $p<0.001$, Drug: $F(1,48)= 102.2$, $p<0.001$] but no interaction. The KO mice had reduced immobility compared with the WT mice regardless of drug treatment: mice treated with bupropion had decreased immobility compared with saline treated mice; hence, the effects were additive but not synergistic (**Fig. 3B**).

Experiment 2: Effects of acute and chronic stress on PDE1B protein expression

Acute stress

If PDE1B is involved in stress-induced immobility responses, we reasoned that it should change in WT mice subjected to acute stress. Accordingly, we measured corticosterone and PDE1B following forced swim stress. As shown in **Fig. 4A,** corticosterone levels did not differ prior to FST. After FST, there was the predicted increase in corticosterone [$F(2,30.2)=25.2$, $p<0.001$] compared with non-stressed controls. Corticosterone levels returned to baseline

levels 48 h following FST. For PDE1B, there was a stress-induced increase in whole brain [$F(1,61)=6.3$, $p<0.05$] and in striatum [$F(1,61)=4.4$, $p<0.05$] but no change in cerebellum as shown in **Fig 4B**. There was no change in PDE10A (**Fig 4C**) in these regions.

Chronic mild stress

We next asked, if PDE1B is sensitive to acute stress, would it also be responsive to chronic stress? One common method of inducing chronic stress is CMS. We therefore tested the effect of CMS in WT mice. No differences in body weight were found prior to CMS, however stress decreased body weight after 21 days of CMS [Stress x Day: $F(7,139)=11.3$, $p<0.001$] in the CMS stressed mice [23.8 ± 0.6 g] compared with non-stressed WT mice [27.2 ± 0.6 g]. As expected, CMS-exposed mice had increased corticosterone compared with non-stressed mice ($p<0.001$) after the last stressor, but no differences were noted prior to CMS or after 3 days of repeated daily TST and FST testing [CMS X Day: $F(2,17.2)=9.5$, $p<0.01$, **Fig. 5A**]. There was also a decrease in thymus weight in relation to body weight in stressed vs. non-stressed mice [$t(20)=8.0$, $p<0.001$; Thymus: Control=0.128 ± 0.004% Stress=0.060 ± 0.007%] but no change in adrenal (Control=0.024 ± 0.003% Stress=0.022 ± 0.002%) or spleen weight (Control=0.316 ± 0.01% Stress=0.292 ± 0.026%). PDE1B expression was increased after CMS (**Fig 5B**) whereas PDE10A (**Fig 5C**) was decreased [PDE1B: $t(21)=-3.1$, $p<0.01$, PDE10A: $t(19)=2.5$, $p<0.05$]. Hence, acute and chronic stress increased PDE1B whereas acute stress had no effect on PDE10A and chronic stress decreased PDE10A. Since PDE1B is not present in *Pde1b* KO mice, there could be no PDE1B changes from stress, therefore, we did not test PDE1B KO mice with the CMS procedure.

Discussion

The phenotype of *Pde1b* deficient mice on a C57BL6/129svj x C57BL/6N F1 mixed hybrid background was reported previously (Reed et al. 2002; Siuciak et al. 2007). *Pde1b* KO

mice showed several effects, including a probe trial deficit in the Morris water maze (Reed et al. 2002) and modest hyperactivity in an open-field (Siuciak et al. 2007). There were no alterations in conditioned avoidance learning, elevated zero maze, FST, passive avoidance, hot plate, or olfactory orientation (Reed et al. 2002; Siuciak et al. 2007). However, the breeding strategy used in the Siuciak et al. (2007) study was not optimal. By comparison, we used het x het crosses and drew not more than one KO and one WT mouse from any given litter to control genetic background and litter effects, whereas Siuciak et al. used KO x KO and WT x WT mice from separate lines and did not control for litter. In order to ensure that our FST results were sound, we replicated the finding multiple times using different experiments over a period of several years; we also had a collaborator test the mice at another university, and we used the TST to confirm our FST phenotype. The KO immobility effect is not likely to be attributable to simple activity differences since we previously showed that KO mice are not different in other swimming tests, including the Morris water maze and straight swimming channel. In these tests, KO and WT mice show comparable swim speeds, indicating that KO mice do not differ in swimming even if they do show small open-field activity differences (Ehrman et al. 2006; Reed 2000; Reed et al. 2002). This is also in agreement with other studies that show that spontaneous locomotor activity and swimming are not predictive of one another (Cravens 1974). Moreover, by using the two day FST method we reduced the influence of novelty since the mice habituate to the forced swim environment on day-1 and immobility is assessed on day-2. For these reasons, we suggest that the present findings are more reliable compared with those of Siuciak et al. (2007). Interestingly though, Siuciak et al. did report increased DA turnover in the striatum, which may be involved in the mechanism behind the TST and FST phenotype (Siuciak et al. 2007).

We show that *Pde1b* KO mice have complete deletion of DNA and RNA of the catalytic region of the *Pde1b* gene by Southern and Northern blot analyses (Reed et al. 2002).

Furthermore, *Pde1b* KO mice have increased DA, DOPAC, and DA utilization compared with WT mice in striatum and hippocampus and reduced 5-HT in striatum and cerebellum (Siuciak et al. 2007). Here we add a comparison by qPCR of expression of mRNA of *Pde* isoforms in KO mice relative to WT mice in striatum and cerebellum. The relative expression profile in C57BL mice is similar to that reported for human and BALB/c mice (Kelly et al. 2014; Lakics et al. 2010). However, exceptions exist in human caudate and nucleus accumbens where *PDE1B* expression is the highest isoform expressed, whereas it is higher for *Pde2*, *4b*, and *10a* in C57BL mice; BALB/c mice show similar expression patterns of *Pde1b and 10a* (Kelly et al. 2014; Lakics et al. 2010). The differences between the expression patterns in different mice may be attributable to genetic strain effects, because of primers targeted to different exon regions, use of different reference genes, or different methods of normalization. Regardless of these differences, in each case *Pde1b* and *Pde10a* are the highest expressing *Pde* isoforms in the striatum and much lower expression in cerebellum that we used as a control region. As expected, *Pde1b* mRNA was not detected in KO mice. We found that *Pde1b* is highly expressed in the striatum of WT mice at levels comparable to *Pde4b* that is known to be involved with anxiety and depression (Siuciak et al. 2008; Zhang et al. 2008). There were no differences between WT and *Pde1b* KO mice in the expression of other *Pde1* isoforms (*Pde1a* or *1c*). Thus, no other *Pde1* isotype showed compensatory changes in the *Pde1b* KO mice. There were elevated levels of *Pde10a* in the striatum of KO mice, a region where *Pde10a* is expressed at higher levels than *Pde1b.* Interestingly, Schmidt et al. 2010 showed an upregulation in striatal *Pde1c* after administration of the *Pde10a* inhibitor TP-10 compared with controls. This suggests an inverse relationship between *Pde1* and *Pde10a* (Kleiman et al. 2011), but our acute vs. chronic stress data suggest that this relationship depends on the stimulus and its duration.

To date, the phenotype of *Pde10a* overexpression has not been investigated in relation to depression but *Pde10a* downregulation has been investigated in relation to schizophrenia. Genetic or biochemical inhibition of *Pde10a* in mouse and rat models, respectively, shows a decrease in locomotor activity and in stimulant-induced (PCP, amphetamine, MK-801) hyperactivity (Schmidt et al. 2008; Siuciak et al. 2006b). Other characteristics in *Pde10a* inhibitor-treated or KO rodents include blockade of apomorphine-induced climbing, inhibited conditioned avoidance (rats and mice), blockade of NMDA antagonist-induced deficits in acoustic startle (rats), improved sensorimotor gating, increased sociability and social odor recognition, reversal of stereotypy, and improved novel object recognition (mice) (Grauer et al. 2009; Höfgen et al. 2010; Schmidt et al. 2008; Siuciak et al. 2006a; Siuciak et al. 2006b). Chronic exposure to antipsychotics (haloperidol and clozapine) increases *Pde10a* (Xu et al. 2013), suggesting an interaction between *Pde10a* and the positive symptoms of schizophrenia (Dlaboga et al. 2008; Hebb and Robertson 2007; Natesan et al. 2014; Xu et al. 2011). Siuciak et al. 2006a also showed no differences between *Pde10a* KO and WT mice in the elevated plus maze.

We also tested FST responses after antidepressant treatment to a prototypical selective serotonin reuptake inhibitor (fluoxetine) and a prototypical norepinephrine-DA reuptake inhibitor (bupropion). Both WT and KO mice showed reduced immobility from the drugs compared with vehicle-treated mice. Interestingly, the effects of the antidepressants added to the immobility of *Pde1b* KO mice. The efficacy of the antidepressants independent of the *Pde1b* deletion suggests that the mechanism of immobility induced by the drugs and gene deletion are different. This supports the idea that PDE1B may be a useful target for drug development.

Forced swimming itself increases corticosterone levels, and we used the FST to test for changes in PDE1B protein levels in WT mice. FST caused a significant increase in PDE1B protein in striatum and whole brain. While forced-swim stress causes many changes, the

increase in PDE1B is consistent with a role for this enzyme in stress and depression. CMS was used to test chronic stress in WT mice. After 21 days of CMS, mice showed reduced weight gain, elevated plasma corticosterone, and decreased thymus weight all of which are hallmarks of stress; they also showed increased PDE1B levels in whole brain, accompanied by decreased PDE10A levels. These data suggest that PDE1B responds to acute and chronic stress while PDE10A responds to chronic but not acute stress. Xu et al. 2013 showed that corticosterone exposure increased *Pde2* expression in the hippocampus with a peak 24 h later (Xu et al. 2013). A similar phenomenon is seen with PDE1B between acute and chronic stress. In this case, the acute stress increases PDE1B expression more significantly than chronic stress, perhaps because of the prolonged exposure to heightened corticosterone.

Our data and those of Kleiman et al. suggest a relationship between *Pde1(b and c)* and *Pde10a* in the striatum (Kleiman et al. 2011). The phenotype of *Pde1b* KO mice appears specific to the reduction of acute stress-induced depressive-like behavior while the phenotype of *Pde10a* KO mice appears to be specific to the reduction of positive symptom-related behaviors (Höfgen et al. 2010).

We recognize that constitutive KO mice have limitations compared to the use of pharmacological inhibitors. Specifically, constitutive genetic KO models have ablated gene and protein from conception and this can result in compensatory changes during development that can be difficult to estimate. Pharmacological inhibitors can cause changes in neuronal activity when applied, whereas genetic KO models may not induce any change in neuronal function. Although the current data suggest PDE1B to be a potential target for stress resistant depressive-like effects, further research is necessary to establish this association. Spatially and temporally targeted reductions of PDE1B is another way to estimate the suitability of this model.

Despite the indirect relationship between genetic deletion and a pharmacological treatment, our data suggest that changes to *Pde1b* may open a new avenue for research into depression.

PDE1 inhibitors have received less consideration for involvement in anxiety and depression; however, there are PDE1 inhibitors in clinical trials for other indications (Li et al. 2016; Snyder et al. 2016). Given that PDE1B is expressed in high abundance in regions known to be involved in anxiety, depression, and other behaviors (Lakics et al. 2010), more research on PDE1B is warranted.

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Tables

Table 1. Description of mice used in experiments			
Experiment	Mice Used	Assays	Figure
1	Males WT: n=5 KO: n=4	RT-qPCR	1
1	Males WT: n=12 KO: n=12	Locomotor Activity, TST, and FST (2 Day)	2 A,B,D
1	Males WT: n=8 WT: n=8	FST (1 Day)	2 C
1	Males & Females WT: n=15 HET: n=25 KO: n=20	FST (2 Day)	2 E
1	Males WT-SAL: n=8 WT-FLX: n=8 KO-SAL: n=8 KO-FLX: n=7	FST (2 Day)	3 A
1	Males WT-SAL: n=13 WT-BUP: n=14 KO-SAL: n=14 KO-BUP: n=11	FST (2 Day)	3 B
2	WT Males Control: n=8 Acute Stress: n=8	Plasma Corticosterone and Western Blots	4
2	WT Males Control: n=12 Chronic Stress: n=12	Plasma Corticosterone and Western Blots	5

Table 2. Primer sequences	
Gene	Primer Sequence (5'->3')
PDE1A	GAAGCAAGCGGGGAGCATAG
	AAAGGCAATTAGGCAAGAAACAGG
PDE1B	TTATCAATCTCACCAAGGATG
	GCTGTCTTCATAGTCTTCAC
PDE1C	TTGGTTATTGAGATGGTAATGG
	ATGAGGGATAAGGCTTTCG
PDE4A	CCGTATCCAGGTCCTCAG
	ATGCGATCAGTCCATTGT
PDE4B	CCAGCAGGGAGACAAAGAAC
	ACAATGTAGTCAATGAAACCAACC
PDE4D	GCTTCATAGACTATATCGTTCATC
	GTCCTCCAAAGTGTCCAAG
PDE2	CACATTGCCATGCCTATCTAC
	CCTTGGTCCAGTGCTCAC
PDE10A	CACTTTGACATTGGTCCTTTCG
	TTCTTCACAGACATGATAAAACGG
PSMB2	AAATGCGCAATGGATATGAATTG
	GAAGACAGTCAGCCAGGTT

Table 3. Chronic Variable Stress Paradigm	
AM (8:00-12:00)	PM (13:00-17:00)
Restraint (2 h)	Tilted Cage (24 h)
Shaker (1 h)	Flooded Cage (18 h)
Predator-Restraint (30 min)	Dirty Rat Cage (18 h)
Cold Room (1 h)	Grid Floor (24 h)
Hypoxia (30 min)	Dirty Rat Cage (18 h)
Restraint (2 h)	Flooded Cage (18 h)
Shaker (1 h)	Grid Floor (24 h)
Hypoxia (30 min)	Tilted Cage (24 h)
No Test	
Restraint (2 h)	Tilted Cage (24 h)
Predator-Restraint (30 min)	Dirty Rat Cage (18 h)
Hypoxia (30 min)	Cold Room (1 h)
Predator-Restraint (30 min)	Flooded Cage (18 h)
Cold Room (1 h)	Dirty Rat Cage (18 h)
Shaker (1 h)	Grid Floor (24 h)
Predator-Restraint (30 min)	Hypoxia (30 min)
No Test	
Restraint (2 h)	Flooded Cage (18 h)
Cold Room (1 h)	Grid Floor (24 h)
Hypoxia (30 min)	Tilted Cage (24 h)
Cold Room (1 h)	Shaker (1 h)

Figure Captions

Figure 1 *Pde* expression in WT and KO mice striatum and cerebellum. *Pde1a*, *Pde1b*, *Pde1c*, *Pde2*, *Pde4a*, *Pde4b*, *Pde4d*, and *Pde10a* mRNA expression levels were measured by RT-qPCR in the striatum (**A)** and cerebellum (**B)** in WT and KO *Pde1b* mice. Percent mRNA expression was normalized to *Pde1b* WT striatum, set at 100%. Data are represented as LS Mean ± SEM (WT n=5, KO n=4). ***p ≤ 0.001.

Figure 2 *Pde1b* KO produced resistance to induced immobility in FST and TST compared with WT littermates. **A,** KO mice have increased locomotor activity (WT n=11, KO n=12). **B,** TST (WT n=12, KO n=12). **C,** 1-day 6 min FST method (WT n=8, cKO n=8). **D,** 2 day FST method with 5 min on day 2 (WT n=10, KO n=12). **E,** 2 day FST method with 5 min on day 2 (WT n=15, Het n=25, KO n=20). KO mice differ from both the Het and WT littermates. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

Figure 3 *Pde1b* KO mice have a similar antidepressant-like phenotype as currently marketed antidepressants when compared with WT littermates. Note that antidepressant efficacy occurred independent of the genotype. **A,** FST 2-day method, day for day-2 (5 min) (WT-Saline n=8 WT-Fluoxetine n=8 KO-Saline n=8 KO-Fluoxetine n=7). **B,** FST 2 day method 5 min (WT-Saline n=13; WT-Bupropion n=14; KO-Saline n=14; KO-Bupropion n=11). **p ≤ 0.01, ***p ≤ 0.001.

Figure 4 PDE1B is elevated by acute stress. **A,** Plasma corticosterone was collected 48 h prior to stress, right after day-1 FST, and again at sacrifice 24 h later. **B,** Fluorescent intensity of PDE1B normalized to actin (ab182565, ab3280). **C,** Fluorescent intensity of PDE10A normalized to actin (ab177933, ab3280). Tissue was collected 24 h after completion of day-2 of the FST. *p ≤ 0.05, ***p ≤ 0.001 (Control n=8, Stress n=8).

Figure 5 PDE1B is elevated while PDE10A is reduced in chronically stressed mice. **A.** Plasma for corticosterone was collected 24 h prior to stress, after the 21st day of stress, and upon sacrifice. **B,** Fluorescent intensity of PDE1B normalized to actin (ab170441, ab3280). **C,** Fluorescent intensity of PDE10A normalized to actin (ab177933, ab3280). Tissue and blood was collected 24 h after the 3 days of TST and FST in both the stressed and control mice. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 (Control n=12, Stress n=12).

Fig. 1

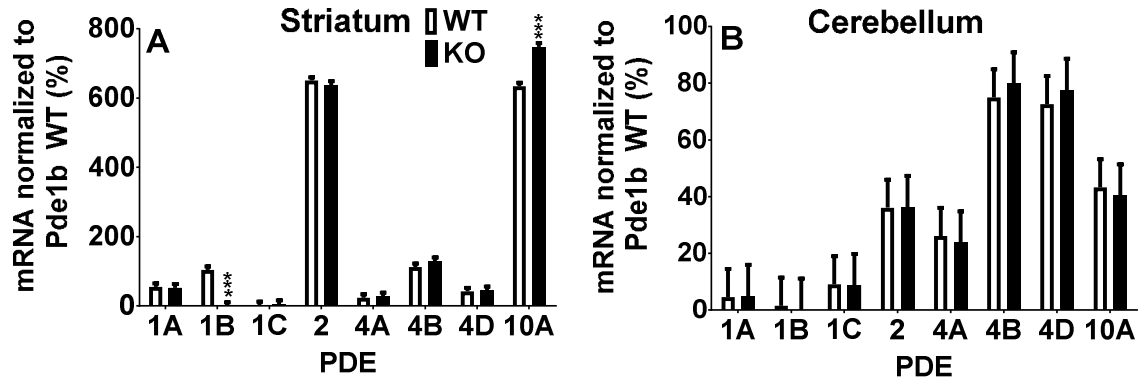
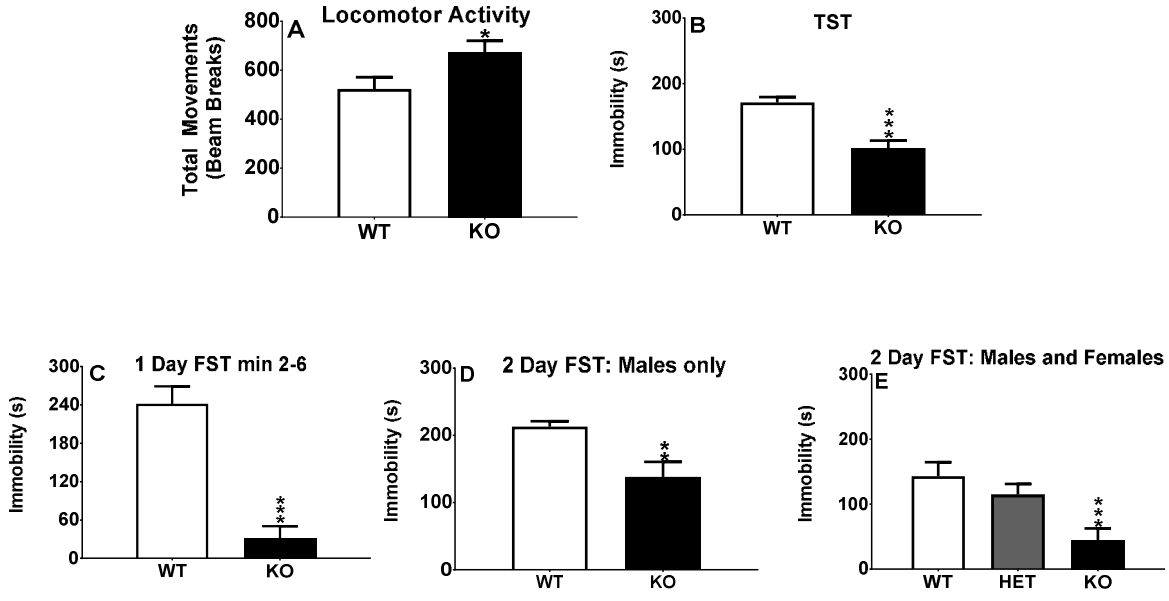

A
Striatum
WT
KO
mRNA normalized to Pde1b WT (%)
800
600
400
200
0
***
***
1A 1B 1C 2 4A 4B 4D 10A
PDE
B
Cerebellum
mRNA normalized to Pde1b WT (%)
100
80
60
40
20
0
1A 1B 1C 2 4A 4B 4D 10A
PDE

Fig. 2


A
Locomotor Activity
Total Movements (Beam Breaks)
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600
400
200
0
*
WT
KO
B
TST
Immobility (s)
300
200
100
0
***
WT
KO
C
1 Day FST min 2-6
Immobility (s)
300
240
180
120
60
0
***
WT
KO
D
2 Day FST: Males only
Immobility (s)
300
200
100
0
**
WT
KO
E
2 Day FST: Males and Females
Immobility (s)
300
200
100
0
***
WT
HET
KO

Fig. 3

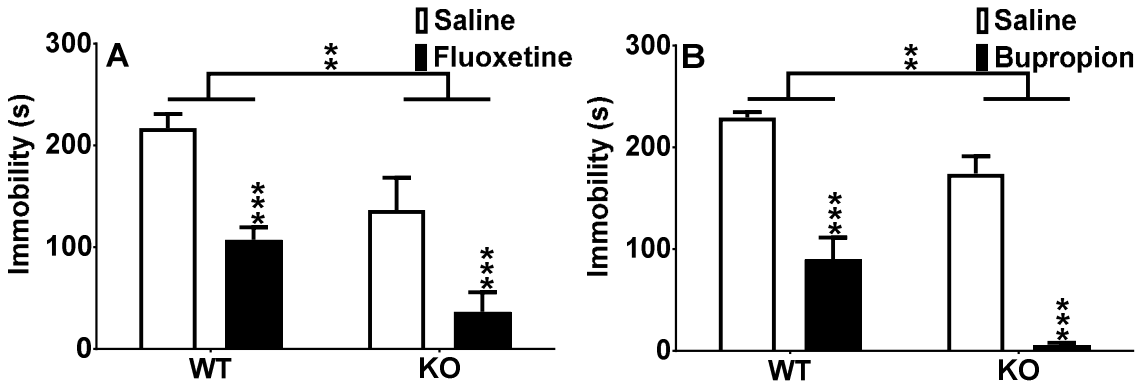


Fig. 4

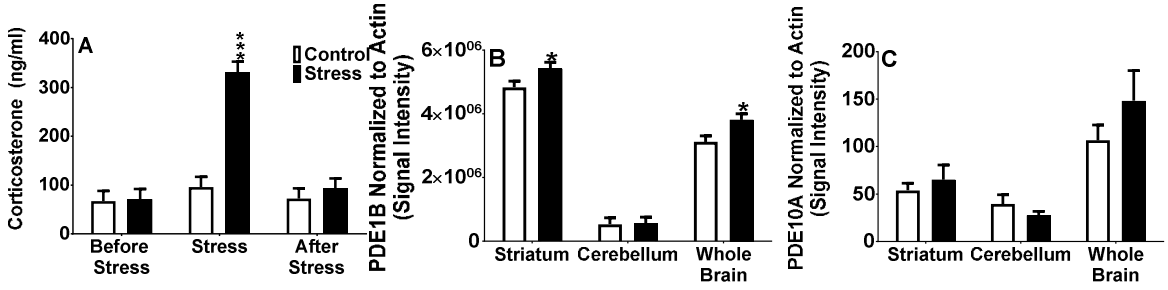
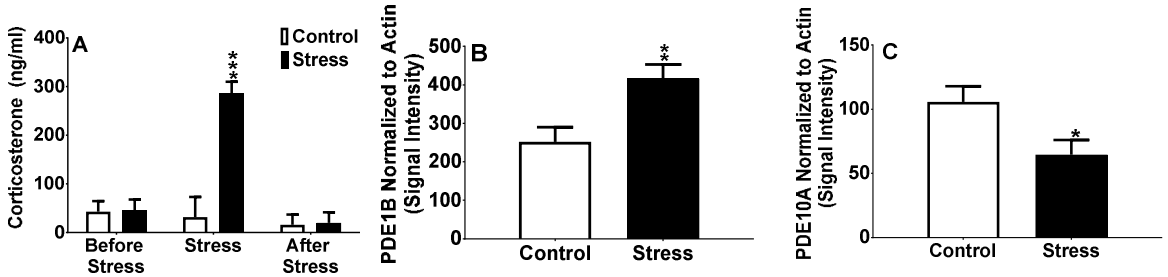


Fig. 5



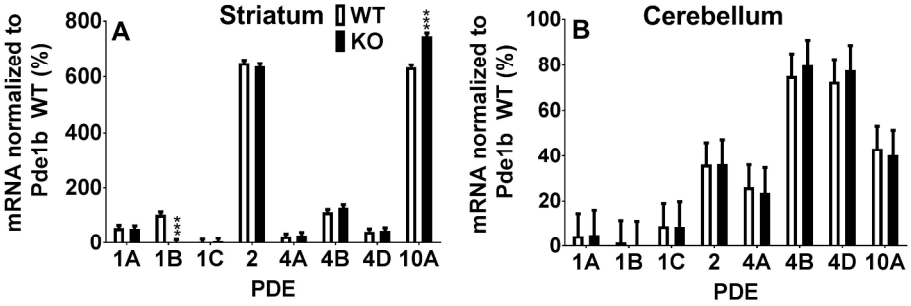


Fig-1

283x98mm (300 x 300 DPI)

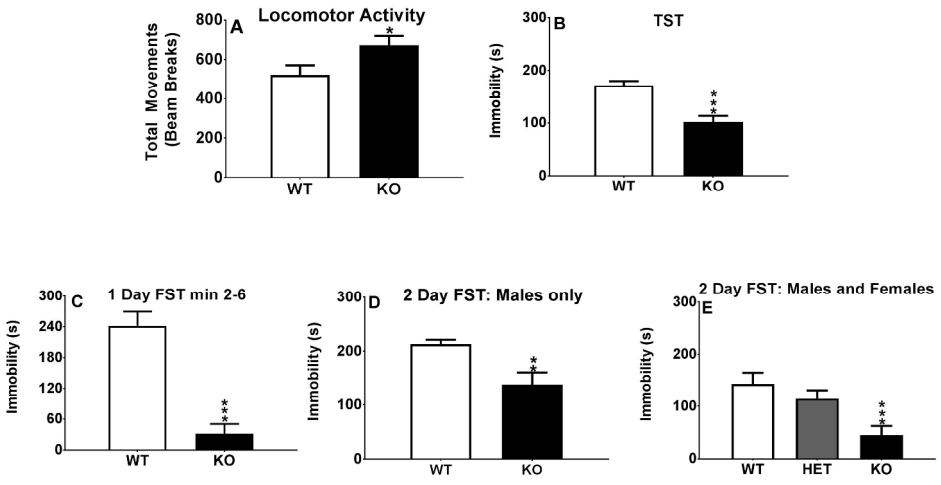


Fig-2

274x141mm (300 x 300 DPI)

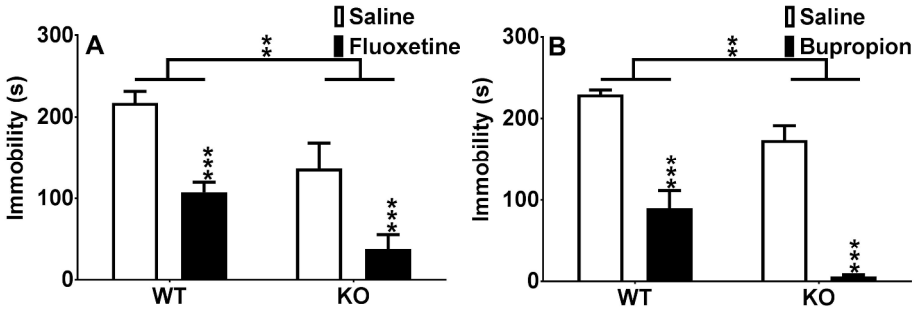


Fig-3

284x99mm (300 x 300 DPI)

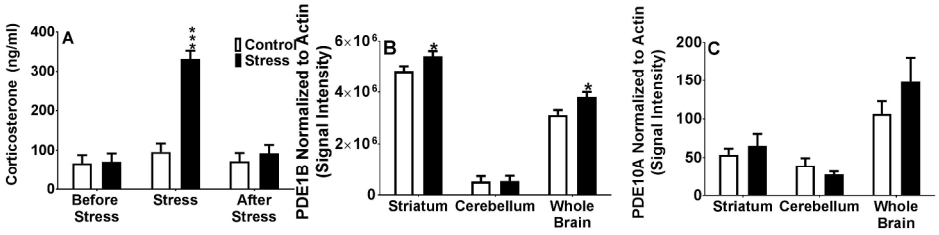


Fig-4

281x76mm (300 x 300 DPI)

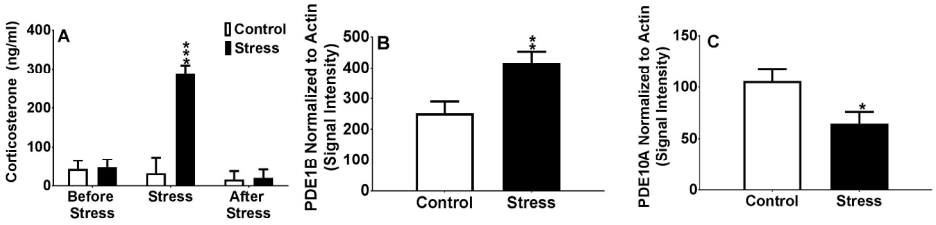


Fig-5

283x73mm (300 x 300 DPI)