Phosphodiesterase-2 inhibitor reverses corticosterone-induced neurotoxicity and related behavioural changes via cGMP/PKG dependent pathway

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Abstract
Phosphodiesterase 2 (PDE2) is an enzyme responsible for hydrolysis of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) to restrict intracellular signalling of these second messenger molecules. This study investigated how PDE2 inhibitor Bay 60-7550 affects the dysregulated glucocorticoid signalling in neuronal cells and regulates depressive behaviours after chronic stress in mice. We found that exposure of hippocampal neurons to corticosterone resulted in time- and concentration-dependent increases in PDE2 expression. These intriguing findings were confirmed in the hippocampal cell line HT-22. After corticosterone exposure for 24 h, HT-22 cells showed a concentration-dependent increase in mRNA levels for PDE2 subtypes, PDE2A1 and 2A3, as well as for the total PDE2A protein expression. Bay 60-7550 was found to reverse the cell lesion induced by corticosterone (50 μM). This neuroprotective effect was blocked by pretreatment with protein kinase G inhibitor KT5823, but not protein kinase A inhibitor H89, suggesting the involvement of cGMP-dependent signalling. Although Bay 60-7550 treatment for 24 h did not change the levels of phosphorylated mitogen-activated protein kinases ERK1/2 (pERK) and phosphorylated cAMP response element-binding protein (pCREB), it down-regulated pERK at 2 h and up-regulated a CREB co-activator, CREB-binding protein, at 24 h. Both of these effects were blocked by KT 5823. Furthermore, Bay 60-7550 reversed corticosterone-induced down-regulation of brain-derived neurotrophic factor protein levels 24 h after corticosterone exposure. In behavioural testing, Bay 60-7550 produced antidepressant-like effects and reduced corticosterone levels in stressed mice, further supporting the involvement of a PDE2-dependent pathway in mediating Bay 60-7550’s effect during stress hormone insults.

Key words: Bay 60-7550, cAMP, cGMP, CREB, ERK, phosphodiesterase-2.

Introduction
Depression is a chronic, recurring and potentially life-threatening illness that is a major cause of morbidity and mortality (Murray & Lopez, 1996). Stressful events can precipitate factors in the onset of major depression and stress paradigms have long been used to model depressive status (Bartolomucci & Leopardo, 2009; Mitra & Sapolsky, 2008). In response to sustained stress or excessive stress hormone secretion, the brain undergoes a complex array of cellular and molecular changes that lead to maladaptive remodelling and behavioural abnormalities. These result in increased susceptibility to depression, anxiety and related cognitive deficits. The link between genetic pre-disposition and life stressors in the aetiology of depression remains unknown because the mode of transmission in mood disorders is most likely complex. Recent studies report the existence of reciprocal interactions between cyclic adenosine monophosphate (cAMP) or cyclic guanosine monophosphate (cGMP)
and their respective effector enzymes [protein kinase A (PKA) and protein kinase G (PKG)] and stress-related emotional disorders (Berton & Nestler, 2006; Masood et al. 2008; Zhou et al. 2011). Stress induces a profound increase in glucocorticoid, with an associated acceleration of the degradation of cAMP and/or cGMP, which may result in depressive or anxiety-like behaviours and defective neuronal structure and function (Chen et al. 2010; Johnson et al. 2009).

cAMP and cGMP are primary regulators for intracellular communication in the brain, which has made it an attractive target for therapeutic intervention in stress-related emotional disorders. Activation of cAMP/cGMP signalling by means of phosphodiesterase (PDE) inhibition appears to be a viable and tractable means of enhancing neuronal communication since these enzymes catalyse the hydrolysis of the second messengers (Schmidt, 2010). The high expression of PDE2 in limbic structures that are closely associated with mood and cognitive function, such as the hippocampus and amygdala, suggests its possible role in the treatment of affective disorders and related cognitive symptoms (Xu et al. 2011b). In support of this, the selective PDE2 inhibitor Bay 60-7550 reverses the deficit in object recognition induced by tryptophan depletion (van Donkelaar et al. 2008). In a subsequent study, it was found to be effective against oxidative stress-induced anxiety-like behaviours in elevated plus maze and open field tests (Masood et al. 2008). Following these observations, our laboratory aimed to explore the causal links between excessive stress hormones and the activity of PDE2 enzyme. We also investigated how Bay 60-7550 affected intracellular signalling when neuronal cells were treated with corticosterone and regulated the depressive-like behaviours in stressed mice. Our results showed that excessive stress hormone led to an increase in PDE2 expression and hippocampal cells’ lesion, as well as depressive-like behaviours in mice. These effects were reversed by PDE2 inhibition, possibly through regulating cGMP/PKG signalling processes.

**Materials and method**

**Animals**

Male Imprinting Control Region mice weighing 22–25 g were obtained from the Animal Center at the West Virginia University. Mice were kept in a temperature-controlled room under standard laboratory conditions, with a 12 h light/12 h dark cycle (lights on 06:00 hours). All animals were allowed at least 1 wk habituation before any treatments. Water and food were freely available in their home cages. All the experiments were carried out from 09:30 to 16:30 hours in a quiet room.

Foetal pups from 18-d pregnant Sprague–Dawley rats (500–550 g) were used to prepare neuronal cultures as described previously (Xu et al. 2009). All experiments using animals were carried out according to the ‘NIH Guide for the Care and Use of Laboratory Animals’ (NIH Publications No. 80-23, revised 1996) and were approved by the Institutional Animal Care and Use Committee of West Virginia University (USA).

**Surgery for brain cannula implantation**

Animals were anaesthetized with ketamine and xylazine (100 and 10 mg/kg i.p., respectively) and placed in a stereotaxic frame with flat-skull position. The stainless steel guide cannulae were implanted bilaterally into the CA1 of hippocampus (anterior–posterior −1.7 mm from bregma, mediolateral ±0.8 mm from midline and dorsoventral −2.0 mm from dura). The cannulae were anchored to the skull with dental cement and then stainless steel stylets were inserted into the guide cannulae to maintain patency for micro-injections. All mice were treated with penicillin after surgery to prevent infection. The mice were allowed to recover for 7–10 d and were handled every other day to reduce stress associated with handling at the time of testing.

**Drugs and treatments**

Corticosterone and mifepristone [RU486, 11β-(4-dimethyl-amino)-phenyl-17β-hydroxy-17-(1-propynyl)estradiol, 9-dien-3-one] were purchased from Sigma Chemical Co. (USA). KT5823 (2,3,9,10,11,12-hexahydro-10H-diindolo[1,2,3-fg:3’,2’,1’-kl][1,6]benzodiazocine-10-carboxylic acid, methyl ester) and Bay 60-7550 [2-[[3,4-dimethoxyphenyl]methyl]-7-[[IR]-1-hydroxyethyl]4-phenylbutyl]-5-methyl-imidazo[5,1-f][1,2,4]triazin-4(1H)-one] were obtained from Cayman Chemical (USA).

Corticosterone, KT5823, H89, RU486, Bay 60-7550 and ND7001 were dissolved in 0.5% dimethyl sulfoxide (DMSO). Animals were given bilateral micro-injections of 2 μl KT5823 (1 μl/side) into the CA1 of the hippocampus. A 27-gauge injection cannula was lowered to extend 1 mm beyond the tip of the guide cannulae. The solution was then administered at the rate of 0.5 μl/min over 1 min, with the injector cannula remaining in the guide cannula for an additional minute to prevent backflow. RU486 (2.5 μM), KT5823
RNA (800 ng) was reverse transcribed using MJ Mini (Invitrogen) according to the manufacturer’s protocol. Extraction was performed using TRIzol Reagent. HT-22 cells were washed and the total cellular mRNA was isolated before tests. Real-time polymerase chain reaction before tests and were treated with different drugs for 24 h before Bay 60-7550 and ND7001 were chosen to elucidate the roles of PDE2 in corticosterone-induced neurotoxicity and related behavioural changes.

**Cell cultures**

**Primary hippocampal neuron cultures**

Primary hippocampal cultures were prepared as described previously (Xu et al. 2009). Pregnant rats (18 d) were anaesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine. The uterus was carefully separated from the abdominal viscera and rat pups were decapitated and their hippocampi dissected with the aid of a light microscope. Cells were harvested from a homogenized pool of 10 hippocampi and were seeded at a density of 2 x 10^5 cells/ml on poly-γ-lysine-coated six-well plates (Nunc A/S, Denmark). Cultures were maintained in DMEM in a humidified incubator at 37 °C. The culture medium was replaced with warm fresh DMEM containing 1% cytosine-1-beta-D-arabinofurcnoside after 3 d, followed by fresh DMEM containing horse serum after 2 d. The cells were cultured for another 7 d before use.

**HT-22 cell cultures**

HT-22 cells were a generous gift from Dr David Schubert (The Salk Institute for Biological Studies, USA; Li et al. 1997). Foetal bovine serum (FBS) and N₂ nutrient supplement were obtained from Invitrogen (USA). The HT-22 cells were maintained in DMEM, supplemented with 10% FBS, cultured at 37 °C in 5% CO₂. Cells were plated at 1 x 10^5 cells/ml for all the tests and were treated with different drugs for 24 h before tests.

**Real-time polymerase chain reaction**

HT-22 cells were washed and the total cellular mRNA extraction was performed using TRizol Reagent (Invitrogen) according to the manufacturer’s protocol. RNA (800 ng) was reverse transcribed using MJ Mini TM Gradient Thermal Cycler (Bio-Rad, USA). The polymerase chain reaction (PCR) was performed using an iCycler Real-Time PCR machine (Bio-Rad). SYBR Green (iQ SYBR Green supermix reagent; Bio-Rad) was added to each sample at a concentration of 50 nmol/l. The specific primers were designed for each reaction: PDE2A1: (5′-GGAGGAGATTTCCGACTTC-3′), 5′-ATCTCTGCGTGTGAGGT-3′); PDE2A2: (5′-GGAGTCCAGACTGTGTGT-3′), 5′-CAGGGGT-ATGACCACACTT-3′); PDE2A3: (5′-TCAGGCAGTGACCTGAAG-3′), 5′-TGATATGGCGCTTGAAGG-3′); and β-actin: (5′-CGTCCGTGACATTAAAGAG-3′), 5′-GCCACAGGATTCCATAC-3′). PCR products were amplified in the iCycler real-time PCR machine followed by melt curve analysis and gel electrophoresis to verify specificity and purity of the measured product. The values were normalized against the housekeeping gene β-actin.

**Immunoblot analysis**

Hippocampal neurons and HT-22 cells were lysed with RIPA lysis buffer (Upstate Chemicon, USA) containing protease and phosphatase inhibitors (Pierce Biotechnology, USA) and centrifuged at 4000 g for 30 min at 4 °C. The supernatant was assayed for total protein concentrations using BCA assay kit (Thermo Scientific, USA). Samples were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and the separated proteins were transferred onto polyvinylidene difluoride membranes. Blots were then incubated in blocking buffer (phosphate buffered saline containing 0.1% sodium azide) for 2 h at room temperature, washed in tris-buffered saline with 0.1% Tween 20 and incubated with the appropriate primary antibodies overnight at 4 °C [anti-pERK1/2, 1:200; anti-ERK1/2, 1:200; anti-phosphorylated cAMP response element-binding protein (pCREB), 1:1000; anti-CREB binding protein (CBP), 1:200; anti-brain-derived neurotrophic factor (BDNF), 1:200; Santa Cruz, CA; anti-β-actin, 1:1000; Abcam, USA]. After washing, the blots were incubated with the secondary antibodies [IRDye 800CW Gt anti-mouse IgG (H + L) or IRDye 700CW Gt anti-rabbit IgG (H + L), 1:10000] for 1 h at room temperature. The detection quantification of specific bands was carried out using a fluorescence scanner (Odyssey Infrared Imaging System; LI-COR Biotechnology, USA) at 700 and 800 nm wavelengths.

**Cell viability assay (MTT assay)**

Cells were plated onto 96-well plates at 1 x 10⁵ cells/well density. Twenty-four hours after drug treatment,
cell viability was assessed using the MTT method according to a previous description (Xu et al. 2011a). MTT solution (10 μl) was added into each well and cells were incubated at 37 °C for an additional 4 h. Culture medium was then replaced with 200 μl DMSO in each well to dissolve the formazan by pipetting up and down several times. Absorbance at 570 nm was measured using a microplate reader (BioTek, USA).

**cAMP and cGMP analyses**

cAMP and cGMP levels were measured using enzyme-linked immunosorbent assay (ELISA). Cells were plated onto 96-well plates at 1 × 10^4 cells/well density. Twenty-four hours after treatment with drugs, cells were treated with 0.1 M HCl containing 0.5% Triton X-100, followed by 10 min incubation at room temperature. Samples were then centrifuged to remove cellular debris. The supernatant was assessed using ELISA kits (Enzo Life Sciences, USA). Optional densities were measured at 405 nm using a microplate reader.

**Behavioural and biochemical tests**

**Repeated unpredictable stress procedure**

Mice were subjected to chronic unpredictable stress for 10 d. They were exposed to two different stressors each day, which included 2-h restraint, damp bedding, tilted cage, 10-min forced-forced swim and overnight illumination, according to the procedure described earlier (Perrotti et al. 2004). This protocol has been shown to cause significant effects on behavioural, biochemical and neurochemical parameters characteristic of depressive/anxiogenic behaviours (Ortiz et al. 1996; Perrotti et al. 2004; Willner et al. 1992).

**Tail suspension test**

Mice were suspended from a bar 50 cm above the floor by means of an adhesive tape, placed approximately 1 cm from the tip of the tail, as described previously (Xu et al. 2005). The time mice remained immobile during the 6-min test was recorded. Mice were only considered immobile when they hung passively and completely motionless.

**Novelty suppressed feeding test**

Novelty suppressed feeding was performed as described previously (Li et al. 2009) with minor modification. Animals were deprived of food and water 24 h before testing. A single pellet of food was placed at the centre of a 33 × 33 × 30 cm box during testing. Each animal was placed at a corner of the box and the latency for the animal to chew the pellet was recorded during a 3-min test period.

**Serum corticosterone measurement**

Following behavioural testing, rats were killed by decapitation and trunk blood was collected. Plasma was removed by centrifuging at 1500 g for 10 min (4 °C). Serum corticosterone was assessed by ELISA (Enzo Life Sciences, USA) according to the manufacturer’s instructions. Optional densities were measured at 405 nm using a microplate reader.

**Statistical analysis**

The data are expressed as means ± S.E.M. and statistical significance was assessed by Student’s t test or one-way analysis of variance followed by least significant difference post-hoc tests. A value of p < 0.05 was considered statistically significant.

**Results**

**Corticosterone increases PDE2A protein levels in a concentration- and time-dependent manner in primary hippocampal neurons**

To determine whether corticosterone induces PDE2A expression, we analysed PDE2A protein levels by immunoblot analysis after hippocampal neurons were exposed to corticosterone at different concentrations and for different durations. As shown in Fig. 1a, b, corticosterone caused concentration- and time-dependent increases in PDE2A expression. Significant increases in PDE2A protein were found at 25 and 50 μM corticosterone treatment (F<sub>2,23</sub> = 5.143, p < 0.05 and p < 0.01), reaching a maximum at 50 μM. PDE2A expression peaked at 24 h after 50 μM corticosterone treatment (F<sub>7,31</sub> = 11.25, p < 0.001) and remained at significantly higher levels than the normal control up to 48 h. This corticosterone-induced PDE2A expression was reversed by the glucocorticoid receptor antagonist RU486 at 2.5 μM (p < 0.01, Fig. 1c).

**Corticosterone up-regulates PDE2A variants mRNA and protein levels in HT-22 cells**

To confirm the relationship between stress hormone and PDE2A expression, three variants of PDE2A mRNA were measured in the hippocampus-derived cell line HT-22 (Li et al. 1997; Fig. 2a–c). Concentration-dependent changes in PDE2A1 and PDE2A3 mRNA were found by real-time PCR when HT-22 cells were treated with corticosterone. Cycle threshold values...
(Cts) of 33, 32 and 24 were needed to induce PDE2A1, 2A2 and 2A3 mRNA expression, respectively, when 800 ng DNA templates were added to 25 μl reaction buffer. Since Cts are usually inversely proportional to the amount of target nucleic acid in the sample, PDE2A3 (with the lowest Ct) might be transcribed the most after treatment with 50 μM corticosterone.

Subsequent immunoblot analyses found a concentration-dependent increase in PDE2A protein level (Fig. 2d), further supporting the relationship between corticosterone exposure and PDE2A expression. This corticosterone-induced increase in PDE2A expression was reversed by pretreatment with RU486.

The protective effect of Bay 60-7550 against corticosterone-induced cell lesion

When HT-22 cells were exposed to stress-appropriate concentrations of corticosterone for 24 h (1–50 μM), a significant, concentration-dependent, decrease in cell viability was observed. The greatest cytotoxicity was observed when cells were treated with 50 μM corticosterone ($F_{3,23}=4.866, p<0.001$); this effect was antagonized by 2.5 μM RU486 ($p<0.001$, Fig. 3a).

As shown in Fig. 3b, 1 μM Bay 60-7550 rescued HT-22 cells from 50 μM corticosterone-induced cytotoxicity ($F_{3,11}=8.921, p<0.05$). Pretreatment with the selective PKG inhibitor KT5823 (1 μM) reversed Bay 60-7550’s protective effect against corticosterone ($p<0.05$), whereas the selective PKA inhibitor H89 (1 μM) did not show a similar effect.

The effects of Bay 60-7550 on cAMP and cGMP levels in corticosterone-treated HT-22 cells

The effects of Bay 60-7550 on cAMP and cGMP in corticosterone-treated HT-22 cells are shown in Fig. 4. The results revealed a significant decrease in cAMP and cGMP when HT-22 cells were treated with 50 μM corticosterone ($t$ test, $p<0.01$ and $p<0.001$, respectively). The decreased cGMP was reversed by pretreatment with Bay 60-7550 (1 μM; $F_{3,11}=36.94, p<0.001$); Bay 60-7550, however, did not induce a significant increase in cAMP.
Bay 60-7550 reversed corticosterone-induced transient increase in ratio of phospho-ERK1/2 and ERK1/2 (pERK/ERK)

Figure 5 shows that ERK phosphorylation did not increase significantly when HT-22 cells were exposed to 50 μM corticosterone for 24 h. Pretreatment with Bay 60-7550 also did not affect the ratio of pERK/ERK at this time-point. However, a transient response was found at 1, 2 and 4 h after treatment with corticosterone, among which the highest ratio was detected at 2 h. The ratio decreased shortly afterwards, returning

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### Table 1: PDE2A variants mRNA expression

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<th>Cort (μM)</th>
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<th>10</th>
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<td>PDE2A2</td>
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<tr>
<td>PDE2A3</td>
<td>% of control</td>
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### Table 2: PDE2A expression

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<tr>
<td>PDE2A</td>
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**Fig. 2.** Corticosterone (Cort) induced increases in PDE2A variants mRNA and protein expression in a concentration-dependent manner in HT-22 cells. (a)–(c) PDE2A1, PDE2A2 and PDE2A3 mRNA levels after HT-22 cells were treated with various concentrations of Cort for 24 h. (d) PDE2A protein expression after treatment with various concentrations of Cort for 24 h. Results are expressed as mean ± S.E.M. from at least three independent experiments. ** p < 0.01 and *** p < 0.001 vs. vehicle-treated control; **** p < 0.001 vs. 50 μM Cort-treated group.

**Fig. 3.** Corticosterone (Cort) induced HT-22 cells lesion in a concentration-dependent manner, which was reversed by Bay 60-7550 (Bay), as measured by MTT assay. (a) Various concentrations of Cort induced cells lesion. (b) The effect of Bay (0.5 and 1 μM) on Cort-induced cells lesion was reversed by KT5823 (20 μM), but not H89 (1 μM). OD, Optical density. Results are expressed as mean ± S.E.M. from at least three independent experiments. * p < 0.05 and *** p < 0.001 vs. vehicle-treated control; # p < 0.05 and ### p < 0.001 vs. 50 μM Cort-treated group; $ p < 0.05 vs. Bay (1 μM)-treated group.
close to normal by 24 h. This increase of pERK/ERK at 2 h was reversed by pretreatment with Bay 60-7550 ($F_{2,11} = 26.31$, $p < 0.01$). The PKG inhibitor KT5823 (1 μM) blocked the effect of Bay 60-7550 on the ratio of pERK/ERK ($p < 0.05$).

The effects of Bay 60-7550 on corticosterone-induced changes in pCREB/CREB, CBP and BDNF expression

The ratio of pCREB/CREB was reduced by treatment of HT-22 cells with corticosterone for 24 h ($p < 0.01$). Bay 60-7550 and KT5823 did not affect the reduced ratio of pCREB/CREB (Fig. 6a). However, CBP, a histone acetyltransferase that serves as a co-activator of CREB, was affected during stress hormone insults and drug treatment. The down-regulation of CBP proteins induced by corticosterone was reversed by pretreatment with 1 μM Bay 60-7550 ($F_{3,39} = 16.68$, $p < 0.001$); KT5823 inhibited the effect of Bay 60-7550 on CBP ($p < 0.01$) (Fig. 6b).

In order to verify the association between PDE2A and downstream BDNF after corticosterone exposure, we analysed BDNF levels with and without Bay 60-7550 treatment in HT-22 cells (Fig. 6c). The results revealed a significant decrease in BDNF expression after corticosterone exposure ($p < 0.001$), which was reversed by 1 μM Bay 60-7550 treatment ($F_{2,11} = 5.466$, $p < 0.01$); KT5823 inhibited Bay 60-7550’s effect ($p < 0.001$).

The effects of Bay 60-7550 on chronic stress-induced depressant-like behaviours and related corticosterone secretion

To extend the in vitro results, we examined the involvement of PDE2A in chronic stress-induced behavioural changes. As shown in Fig. 7a, chronic stress induced a significant increase in immobility time of mice in the tail suspension test ($p < 0.001$). Similar to the classical antidepressant desipramine, Bay 60-7550 at doses of 1 and 3 mg/kg (i.p.) significantly reduced immobility time ($F_{3,39} = 13.51$, $p < 0.01$ and $p < 0.001$). Pretreatment of animals with the PKG inhibitor KT5823 (20 μmol i.c.v.) prevented the anti-immobility effect of Bay 60-7550 ($p < 0.05$). Similar findings were obtained in the novelty suppressed feeding test ($F_{3,39} = 21.71$, $p < 0.05$), which indicate an antidepressant-like effect of Bay 60-7550 on behaviours induced by chronic stress (Fig. 7b).

In a subsequent experiment, we found that the chronic stress paradigm caused a significant elevation of serum corticosterone relative to the non-stressed control group ($p < 0.001$). Bay 60-7550 (3 mg/kg i.p.) significantly reduced stress-induced increase in corticosterone ($F_{3,39} = 61.64$, $p < 0.001$). Although robust, the Bay 60-7550-induced reduction was not sufficient to bring the hormone level back to non-stressed baseline levels (Fig. 7c). Pretreatment with KT5823 (i.c.v.) did not block the effect of Bay 60-7550 on serum corticosterone.

Discussion

The cGMP-specific PDE targeted by Bay 60-7550, PDE2, is found in the brain in areas known to maintain balance between pro- and antidepressant signalling and to regulate emotional status, such as the cerebral cortex, hypothalamus, hippocampus and amygdala (Reyes-Irisarri et al. 2007; Xu et al. 2006). Based on the electrophysiological response that inhibition of PDE activity is effective against stress-induced dysfunction of hippocampal plasticity and related behaviours.
(Chen et al. 2010), we tried to address the relationship between stress hormone exposure and the action of PDE2 inhibitors on this dysregulated signal pathway and related behavioural change. The current study demonstrated for the first time that PDE2 plays an important role in the pathology of stress-related emotional disorders, as evidenced by: (1) the increases in PDE2 expression under corticosterone exposure in primary hippocampal neurons and hippocampal cell lines; (2) the neuroprotective action of PDE2 inhibitor Bay 60-7550 against corticosterone-induced dysregulated glucocorticoid signalling in hippocampal cells; (3) the antidepressant-like effects of Bay 60-7550 against chronic stress-induced corticosterone secretion and depressive-like behaviours.

Three PDE2 variants (PDE2A1, PDE2A2 and PDE2A3) have been cloned from different tissues, such as bovine adrenal (PDE2A1), rat brain (PDE2A2) and human brain (PDE2A3; Rosman et al. 1997; Sonnenburg et al. 1991). The enriched PDE2A expression in the hippocampus, amygdala, hypothalamus and adrenal cortex has been reported, which may implicate its role in negative feedback inhibition of the limbic-hypothalamus-pituitary-adrenal gland axis in stress-induced depression and anxiety (Rosman et al. 1997; Xu et al. 2011a). In the first set of the present experiments, we investigated the effects of corticosterone exposure at different concentrations and durations, characteristic of stress on PDE2A protein levels in hippocampal neurons. Hippocampal neurons were used because their involvement in emotional, motivational and mnemonic processes may be associated with stress events and excess stress hormone secretion (Xu et al. 2011c). The results showed a concentration-dependent PDE2A protein expression when neurons were exposed to 1–50 μM corticosterone for 24 h. Particularly, 25 and 50 μM corticosterone were found to significantly increase the PDE2A protein expression, which peaked at 50 μM (Fig. 1a). PDE2A protein level also showed a time-dependent effect,
reaching maximum at 24 h after corticosterone exposure (Fig. 1b). Since corticosterone can bind to corticosterone-binding globulin in the cell culture media and subsequently have its activity blocked (Sapolsky et al. 1995), we used a concentration of corticosterone in vitro (50 μM) in our study that is marginally higher than a moderate stress level in vivo (Xu et al. 2009, 2011a). Next, the glucocorticoid receptor antagonist RU486 was used to determine whether the observed effects of corticosterone were specifically through the glucocorticoid receptor pathway. The fact that RU486 (2.5 μM) reversed corticosterone-induced PDE2A expression suggests that corticosterone acts through the glucocorticoid receptor pathway to elicit its effects on PDE2A levels (Fig. 1c).

HT-22 cells are immortalized mouse hippocampal-neuronal precursor cells, which have characteristics that are similar to primary neurons isolated from hippocampus (Liu et al. 2009). These cells serve as valuable models to better understand the cellular and molecular processes relevant to the hippocampus. To reveal whether the alterations in PDE2A protein levels are in part due to its synthesis, mRNA levels of three PDE2A variants were measured within these cells. The analyses by real-time PCR revealed concentration-dependent PDE2A variants mRNA changes. A significant increase in the three PDE2A variants was found after treatment with 50 μM corticosterone (Fig. 2a–c). Since the lowest Ct was needed to induce PDE2A3 mRNA change, it is possible that PDE2A3 is synthesized the most in face of corticosterone insults. Subsequent immunoblot analyses confirmed that PDE2A protein levels are up-regulated concentration-dependently after treatment with corticosterone (Fig. 2d). These results were reversed by pretreatment with RU486, which further supports that corticosterone-induced PDE2A up-regulation in HT-22 cells may be glucocorticoid-dependent.

To explore if the deleterious effect of corticosterone is relevant to PDE2 and the dysregulated-PDE2-dependent pathway, cell viability was investigated by MTT assay. The results suggested that corticosterone started to have significant neurotoxic effects on HT-22 cells at 25 μM and reached maximum toxicity at 50 μM. This effect was reversed by RU 486, which further confirms that corticosterone induces cell lesion through a glucocorticoid-dependent pathway. In addition, the effect of Bay 60-7550 (1 μM) on 50 μM corticosterone-induced cell lesion was reversed by a PKG inhibitor KT 5823, but not a PKA inhibitor H89 (Fig. 3b). First,
these observations and the supplementary data (Supplementary Figs. S1 and S2) suggest that direct inhibition of PDE2 is beneficial in reducing corticosterone-induced neurotoxicity. Second, they, together with the subsequent ELISA data (Fig. 4a, b) and our previous studies in vivo (Masood et al. 2008), indicate that cGMP/PKG signalling is involved in the protective effects of Bay 60-7550 against stress hormone insults.

ERK 1 and 2, members of the mitogen-activated protein kinase (MAPK) family, are functionally versatile intracellular signalling molecules and play an important role in the regulation of cellular growth and neurodevelopment (Martinez-Finley et al. 2011; Osterlund et al. 2011; Roberson et al. 1999). Growing evidence suggests that the intracellular ERK-associated MAPK pathway is responsible for the optimal stress response that is essential for maintenance of homeostasis (Roberson et al. 1999; Trentani et al. 2002). Phospho-ERK may mediate cellular stress by interacting with nuclear targets involved in neuronal survival and plasticity, which include the transcription factor CREB and its co-activator CBP (Finkbeiner et al. 1997; Martinez-Finley et al. 2011). To explore whether the protective effect of Bay 60-7550 on corticosterone-induced neurotoxicity is associated with regulation of ERK, the phosphorylation levels of ERK were analysed by immuno-blot analysis in the present study. Although the results did not show any change in ratio of pERK/ERK at 24 h after treatment with corticosterone or Bay 60-7550 (Fig. 5a), a time-dependent increase of ERK phosphorylation was found up to 2 h after corticosterone treatment (Fig. 5b). This rapid increase in pERK1/2 in neuronal cells is supported by other studies, which suggest that acute stress or corticosterone-induced pERK changes were found within 2 h in neurons and neuronal cell lines (Gao et al. 2009; Kwon et al. 2006; Osterlund et al. 2011). Further studies reveal that rapid increase in pERK1/2 in the hippocampus may be mediated by the mineralocorticoid receptor (MR) in addition to the glucocorticoid receptor (Groeneweg et al. 2011; Karst et al. 2005, 2010). Interestingly, the present study showed that this transient increase in pERK/ERK at 2 h was significantly reversed by pretreatment with Bay 60-7550, whose effect was abolished by KT5823 (Fig. 5c). The rapid effect of Bay 60-7550 on pERK/ERK may involve the indirect regulation on MR. Further studies are needed.
to investigate the exact role of Bay 60-7550 in the rapid regulation of pERK/ERK after corticosterone exposure.

A previous study suggested that the transient activation of ERK might represent an early mechanism of pathology of cell stress, while permanent activation might result in neuronal susceptibility to stress insults and subsequent cell death (Trentani et al. 2002). Moreover, prolonged pERK activation is usually associated with a decrease in CREB phosphorylation. There are at least three potential phosphorylation sites in the human CREB protein related to stress (Sakamoto et al. 2010). The present study detected activated CREB only when phosphorylated at Ser133, the main site of regulation. Moreover, it was found that corticosterone exposure brought about only the short-term alteration of pERK (1–4 h) together with a delayed decrease in pCREB at Ser133 (24 h). These results may reflect corticosterone-induced signalling dysfunction or defect. Bay 60-7550 did not show any effect on the decreased ratio of pCREB/CREB (Fig. 6a). Since Ser133 is primarily regulated by cAMP-dependent PKA and related kinases (Sakamoto et al. 2010), a possible explanation for Bay 60-7550’s lack of effect is that it may only be affecting cGMP- but not cAMP-dependent signalling. This appears to be supported by the results that the effects of Bay 60-7550 on the hippocampal cells were blocked by KT5823, but not H89. Another possibility is that its effect during stress hormone insults may involve the other CREB phosphorylation sites, such as Ser121,143.

CBP usually acts as a scaffold protein by interacting with several transcription factors, including CREB, and components of the RNA polymerase II, which initiates the transcription of various downstream genes, such as those triggered by neurotrophic factors and G-protein-coupled enzymes and receptors (Maurice et al. 2008). The effect of Bay 60-7550 on CBP levels (Fig. 6b) implies that CBP-related histone acetyltransferases might specifically be involved in the stress-induced cell lesion and treatment with PDE2 inhibitors (Milara et al. 2011). Stress hormone may affect neuronal growth and survival through its deleterious effects on the function and expression of neurotrophins, such as BDNF. In this study, we found that corticosterone induced a significant decrease in BDNF expression, which may involve, at least in part, the up-regulation of PDE2 and the dysregulation of glucocorticoid-related signalling cascade. Selective PDE2 inhibitors prevent the breakdown of cAMP and/or cGMP, which could have a potential effect on BDNF expression. Our findings support this chain of events, suggesting that Bay 60-755 is acting mainly through its inhibitory action on PDE2 to protect against corticosterone-induced cytotoxicity and BDNF abnormalities.

Interestingly, two classical depression models, the tail suspension and novelty suppressed feeding tasks, confirmed the in vitro results, which suggest that the antidepressant-like effects of Bay 60-7550 involves modulating the chronic stress-induced excess corticosterone secretion.

In summary, the PDE2 enzyme is involved in corticosterone-induced neurotoxicity in hippocampal neurons. The fact that Bay 60-7550 reversed corticosterone-induced hippocampal cell lesion and abnormalities of cGMP, pERK, CBP and BDNF levels implies that Bay 60-7550 in part uses the cGMP/ PKG–ERK–BDNF pathway to protect against corticosterone insults. It also reversed depressant-like behaviours and corticosterone secretion in stressed mice, further supporting the involvement of a PDE2-dependent pathway in mediating Bay 60-7550’s effects during stress hormone insults.

Supplementary material
For supplementary material accompanying this paper, visit http://dx.doi.org/10.1017/S146114571200065X

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Statement of Interest
None

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