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Norepinephrine Induces BDNF and Activates CREB and Discriminates among Protein Kinase C Isoforms in Cultured Embryonic Hippocampal Neurons

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Abstract

Medications acting as mood stabilizers work by enhancing and maintaining the concentration of circulating synaptic neurotransmitters, which then activate a plethora of various intracellular signal transduction and second messenger cascades. Previously, we showed that two of these cascades, the PI-3K and MAPK pathways, are activated by cross-talk with the protein kinase A cAMP cascade and by brain-derived neurotrophic factor (BDNF), an immediate-early gene whose expression is the result of phosphorylation of the transcription factor, cAMP response element binding protein (CREB). In the current study, we extend these findings to include the protein kinase C (PKC) pathway. Western blotting studies show that application of norepinephrine to cultured hippocampal neurons leads to increased expression of BDNF, phosphorylation of CREB, activation of growth-associated protein-43 (GAP-43) and activation of PKCμ and PKCθ_{t538}. Because GAP-43 is a putative substrate for PKC, the results of this study lend further support of a G-protein coupled receptor cross-talking to an entirely distinct intracellular pathway that is known to be involved in neuritogenesis.

Keywords

BDNF, CREB, PKD, PKC, Hippocampus

1. Introduction

Norepinephrine is a key neurotransmitter, whose actions are putatively known to play a critical role in several types of mood disorders, such as major depres-

sion [1] [2] [3] [4] in which, it is presumed that anything that can boost the amount of circulating synaptic norepinephrine (and serotonin), such as physical exercise and antidepressant medications, will ameliorate depressive symptoms [5] [6] [7].

We had previously shown that application of norepinephrine to cultured hippocampal neurons results in increased expression of brain-derived neurotrophic factor (BDNF), phosphorylation of cAMP response element binding protein (CREB) and activation of the PI-3K and MAPK cascades, which are known to promote neuronal survival [8]. However, besides these two pro-survival cascades, other pathways also contribute to neuronal protection and survival by activating proteins, such as BDNF and CREB, which play key roles in plasticity.

The protein kinase C (PKC) family of structurally related phospholipid-dependent serine-threonine kinases comprises nearly a dozen different isoforms, most of which are involved in neural differentiation [9] and postnatal neural development [10], although their developmental profile differs among them [11] [12] [13]. Among this rather vast array of different isozymes, a related enzyme, protein kinase D (PKD) or PKCμ, displays multiple unique features that qualify it as a distant relative of the other PKC isozymes in both its regulation and substrate selectivity [14].

Moreover, there is now much compelling evidence that several PKCs, more specifically PKD, play a critical role in hippocampal neuronal plasticity [15] [16] by maintaining the structural integrity of the Golgi and trafficking proteins involved in dendritic arborization [17] and axonal elongation [18] and stabilizing cytoskeletal proteins in dendritic spines [19] in response to environmental or intracellular stimuli, such as learning [20] [21] [22] [23], memory [24] [25] and adaptations to pathological threats, such as developing mood disorders [26]. Consistently, PKD phosphorylation increased upon glutamate receptor stimulation [27], which is putatively activated during long-term potentiation [28]. One key protein that has received much attention because of its role in such plasticity is GAP-43 [29], whose expression in the adult hippocampus is coincident with dendritic outgrowth [30] and is a substrate for PKC [31]. There is, however, no evidence for whether GAP-43 is also a substrate for PKD.

Because both BDNF [32] [33] [34] and GAP-43 [35] are potent mediators of plasticity, there is much evidence that these two proteins are both up- or down-regulated, although not necessarily at the same time and place in the hippocampus [36] [37]. Rather, the extent of their co-expression depends on the type of conditions that may cause the hippocampus to adapt to new challenges, such as with physical exercise [38], learning [33], aging [39] and withstanding toxic [40], pathological [41] [42] or traumatic [43] insult. On the other hand, neither stress [44] nor antidepressant-induced reversal of stress [45] changed hippocampal BDNF and GAP-43 levels, compared to those of untreated controls.

However, neuronal or animal age is also an important factor in determining the levels of BDNF and GAP-43 upon stimulation. Seizures induced into the brains of young animals resulted in an increase in BDNF, although GAP-43 le-

vels did not change, relative to that of controls [46]. More recently, low-frequency stimulation to cultured hippocampal neurons resulted in increased expression of a wide battery of synaptic proteins involved in structural changes that accompany synaptic plasticity, among them of which were BDNF and GAP-43 [47]. And application of norepinephrine to hippocampal astrocytes resulted in increased BDNF release into the culture medium [48].

Thus, given the relatively sparse literature on PKD, it is hypothesized that norepinephrine induces BDNF expression via PKD activation. We therefore extend our earlier findings done with the PI-3K and MAPK cascades [8] to PKD-mediated BDNF expression and CREB activation via the application of norepinephrine to cultured hippocampal neurons.

2. Materials and Methods

2.1. Drugs and Chemicals

Norepinephrine and bisindoylmaleimide were purchased from Sigma Chemical Co. (St. Louis, MO). Dulbecco's modified eagle medium (DMEM) was purchased from ATCC (Manassas, VA). Bisindoylmaleimide (GF109203X (GF)) was dissolved in DMSO; at no time did the concentration of DMSO exceed 0.5% of the total volume of each tissue culture well.

2.2. Antibodies

Anti-BDNF was purchased from Santa Cruz Biotech (Santa Cruz, CA). Anti-phospho-PKCμ (PKD), anti-phospho-PKCμ_{S916}, anti-phospho-PKC$\mu_{S744/748}$, anti-phospho-PKCa/β, anti-phospho-PKCδ_{S643}, anti-phospho-PKCδ_{T505}, anti-phospho-PKCθ_{T538}, anti-phospho-PKC$\zeta\lambda$, anti-pan PKC, anti-phospho-GAP-43, anti-GAP-43, anti-phospho-CREB and anti-CREB were purchased from Cell Signaling Technology (Danvers, MA). Anti-GAPDH was purchased from Advanced Immunochemical Inc. (Long Beach, CA). Anti-rabbit IgG and anti-mouse IgG, were purchased from Amersham-Pharmacia Biotech (Piscataway, NJ).

2.3. Animals: Ethics Statement

All efforts were made to minimize the number of rats used. We have abided by the use of the ethical treatment of laboratory animals as specified in the National research Council's Guide for the Care and the Use of Laboratory Animals (1996). The Institutional Animal Care and Use Committee (IACUC) and the Institutional Review Board-Human Subjects at California State University, Los Angeles, approved this project. The IACUC Protocol number is AW 10-1.

2.4. Hippocampal Dissection at Embryonic Day 18 (E18)

Dissection of embryonic hippocampi at E18 has been described in great detail elsewhere [8] [49]. Briefly, four pregnant female Sprague-Dawley rats (120 - 150 g, two months of age, Charles River, Wilmington, MA) were sacrificed by deca-

pitation on their 18th day of gestation. The embryos (14 per mother rat) were then quickly placed on ice and their hippocampi excised into Ca-Mg-free medium [50] and rinsed therein. Then, 0.125% trypsin was added, and the hippocampi placed in a 37˚C-water bath and gently shaken every several minutes for 10 min, followed by adding 10% fetal bovine serum/DMEM to quench the reaction. All medium was then drained and replaced with 5 ml DMEM in which the hippocampi were triturated through a siliconized glass Pasteur pipet to liberate individual cells. Neurons were then counted on a glass hemacytometer and then plated in tissue culture plates that were pre-coated with poly-*l*-lysine at a density of 50,000 cells/cm^2. Neurons were then allowed to acclimate to their new environment for three days in an incubator at 37˚, 5% CO_2.

2.5. Application of Norepinephrine and Bisindoylmaleimide (GF) to Hippocampal Neurons

On the day of drug treatment, GF (5 μM [51]) and/or norepinphrine (100 nM [8]) was added first to randomly selected wells in the tissue culture plates in the following order: GF + norepinephrine, norepinephrine alone, GF alone, vehicle (controls, DMSO). Returned plates to the incubator for 2 hr, at the end of which, cells were lysed and harvested in lysis buffer. Cells were then boiled for 5 min and then triturated through a 26-G needle/syringe to sheer genomic DNA. The volumes were measured, equal volumes of Laemmli buffer [52] added, and then the cells were stored at −70˚C till SDS-PAGE/Western blotting. Protein concentrations were determined using the method of Lowry [53].

2.6. SDS-PAGE/Western Blotting

SDS-PAGE/Western blotting has been described in great detail elsewhere [49]. Briefly, equal volumes of cell lysates were added to 10% polyacrylamide gels, electrophoresed at 100 V for 2 hr, electrotransferred to nitrocellulose membranes (Biorad, Hercules, CA) at 100 V for 2 hr, and then Western blotting performed according to the manufacturer's specific instructions for each respective antibody. Following probing with the secondary antibody, to visualize protein immunoreactivity, nitrocellulose membranes were incubated in enhanced chemiluminescence reagents (Amersham Pharmacia-Biotech, Piscataway, NJ) and apposed to hyperfilm (Amersham Pharmacia-Biotech, Piscataway, NJ). Membrane blots first exposed to anti-BDNF were then stripped (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM tris-HCl, pH 6.7, 55˚C, for 10 min) and then re-probed with anti-GAPDH; all membrane blots first probed with the phospho-form of an antibody were then stripped and then re-probed with the total (pan) form of the respective antibody.

Optical densities of lightly exposed bands were then quantified using computer-assisted densitometry (MCID, St. Catherine's, Ontario, Canada). Optical densities of BDNF and the phospho-forms were then arithmetically divided by those

of GAPDH and the pan forms, respectively. The linear portion of a standard curve was used to calibrate the range of a gray scale where all optical densities were taken.

2.7. Statistical Analyses

Western blot data of BDNF, phospho-CREB and phospho-GAP-43 were based on four treatment groups: controls, norepinephrine-treated, GF-treated, and norepinephrine-+-GF-treated. Omnibus F values were calculated using a one-way ANOVA, followed by Fisher's post-hoc LSD to evaluate statistically significant differences of pair-wise comparisons.

3. Results

3.1. PKCδ_{S643} and PKCμ (PKD) Are the only PKCs That Responded to Bisindoylmaleimide

Western blotting screening of the eight phospho-PKC isozymes resulted in, for the most part, no significant differences among the four treatments in E18 hippocampal neurons. In most cases, GF did not suppress the activity of any of the phospho-PKC isoforms, relative to that of controls; the only exception was that of phospho-PKCδ_{S643} ($F_{(3,8)} = 4.70$, $p = 0.0355$; Figure 1), whose activity was significantly less than that of controls as a result of GF. Likewise, application of

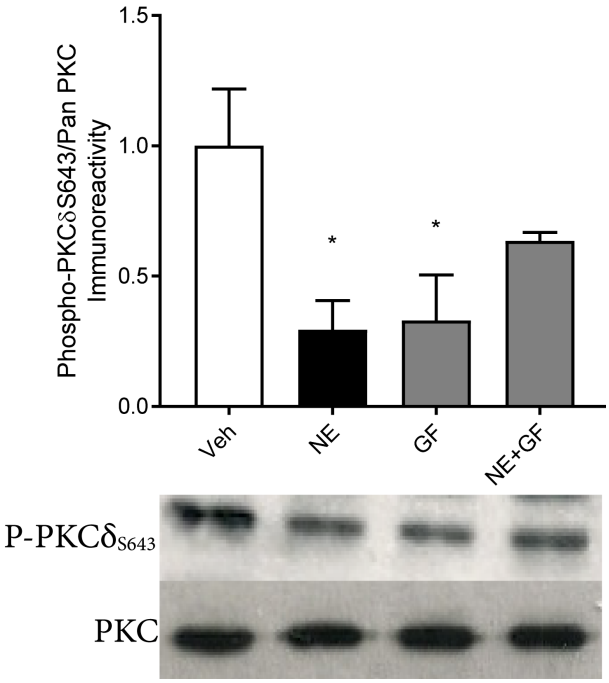

Figure 1. Both norepinephrine and GF decrease phospho-PKCS643 activity. Both norepinephrine ($p = 0.011$) and GF ($p = 0.014$) significantly decreased P-PKCδ_{S643} activity, relative to that of vehicle controls. The combination of the two (NE + GF) increased phosphorylation of PKCδ_{S643} to a level where it was statistically comparable to that of controls. Veh, vehicle control; NE, norepinephrine (100 nM); GF, bisindoylmaleimide (5 μM); *Significantly different from vehicle controls.

norepinephrine did not result in increased activity with most PKC isoforms and in some cases (e.g., phospho-PKCδ_{S643} (Figure 1)), actually resulted in less activity, compared to those of controls. Only phospho-PKCμ ((PKD) $F_{(3,8)} = 4.354$, $p = 0.0427$; Figure 2(a))) and phospho-PKCθ_{T538} ($F_{(3,8)} = 4.647$, $p = 0.0366$, Figure 2(b)) increased in response to norepinephrine, compared to those of controls. Application of norepinephrine, whether GF was present or not, resulted in a significant increase in phospho-PKD activity, compared to those of vehicle-treated controls (Figure 2(a)).

3.2. Norepinephrine Activates GAP-43

Application of norepinephrine to E18 hippocampal neurons resulted in a significant increase in phospho-GAP-43, compared to that of vehicle-treated controls, whereas GF significantly decreased phospho-GAP-43 levels ($F_{(3,32)} = 91.02$, $p < 0.001$; Figure 3).

3.3. Norepinephrine Induces BDNF

Application of norepinephrine to E18 hippocampal neurons resulted in a significant increase in BDNF, compared to those of vehicle-treated controls, whereas GF significantly decreased BDNF levels, relative to those receiving norepinephrine ($F_{(3,32)} = 38.22$, $p < 0.001$; Figure 4). However, although GF decreased

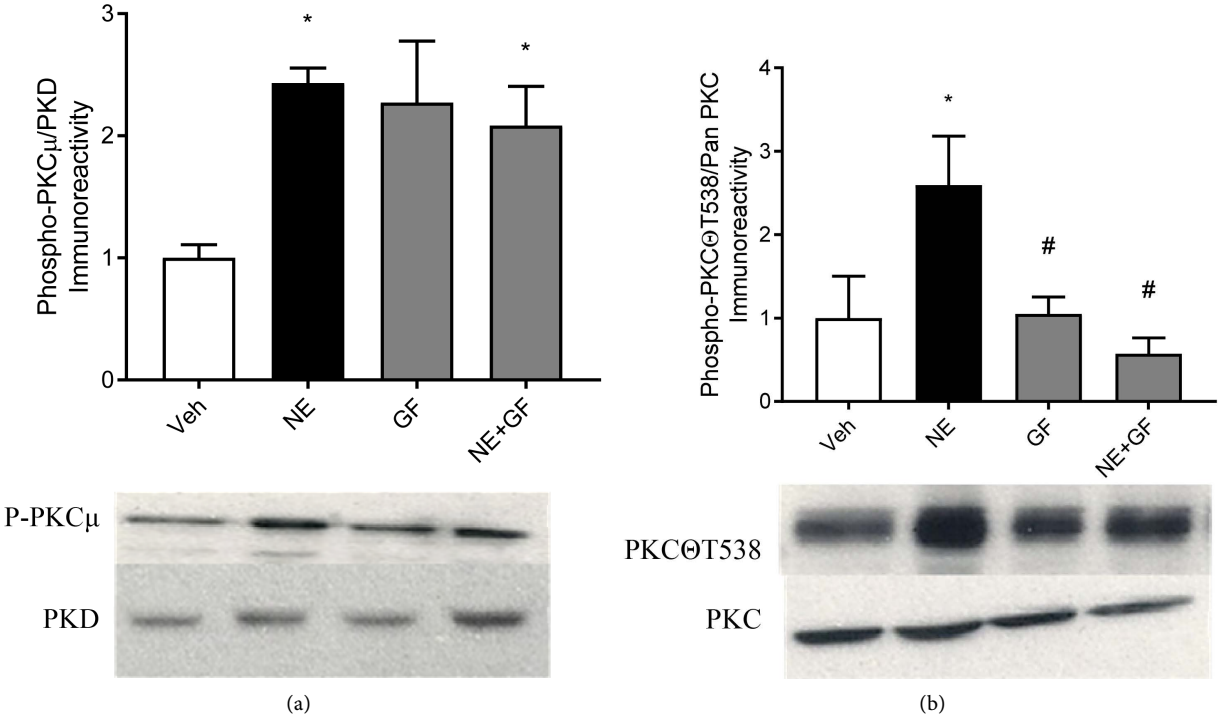

Figure 2. Norepinephrine activates PKC isoforms. (a) Norepinephrine alone ($p = 0.0114$), GF alone ($p = 0.020$) and the two combined ($p = 0.0388$) significantly increased phosphorylation of PKD above that of controls; (b) Norepinephrine alone activates PKCθ_{T538} relative to that of vehicle controls ($p = 0.0253$). Norpinephrine activation of PKCθ_{T538} is also significantly higher than that of NE + GF ($p = 0.0084$) and GF alone ($p = 0.0288$). Veh, vehicle control; NE, norepinephrine (100 nM); GF, bisindoylmaleimide (5 μM); *Significantly different from vehicle controls; #Significantly different from NE.

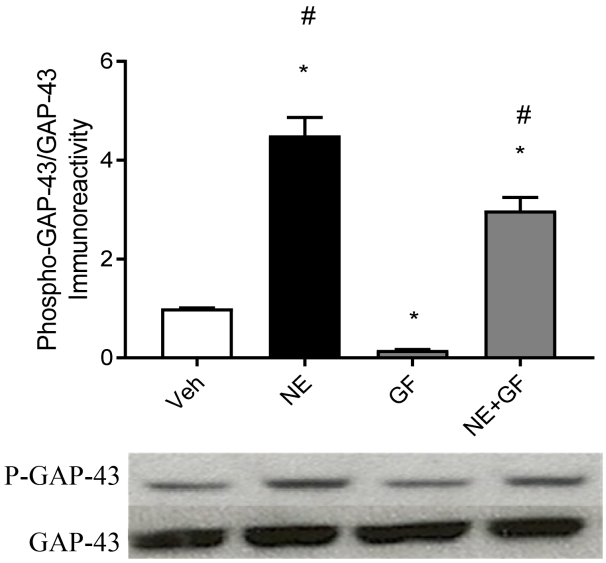

Figure 3. Norepineprhine increases, while GF decreases GAP-43 phosphorylation. Norepinephrine significantly increased phosphorylation of GAP-43 above that of vehicle controls ($p < 0.001$) and when combined with GF (NE + GF), was decreased, but still significantly higher than that of vehicle controls ($p < 0.001$). GF, on the other hand, resulted in significantly lower GAP-43 phosphorylation levels, compared to that of controls ($p < 0.001$). Veh, vehicle control; NE, norepinephrine (100 nM); GF, bisindoylmaleimide (5 μM); *Significantly different from vehicle controls; #Significantly different from GF at $p < 0.001$.

BDNF levels, compared to that of norepinephrine, BDNF levels still remained significantly higher than those of controls ($p < 0.001$, **Figure 4**).

3.4. Norepinephrine Activates CREB

Application of norepinephrine to E18 hippocampal neurons resulted in a significant increase in phosphorylated CREB, compared to that of vehicle-treated controls, whereas GF significantly decreased phospho-CREB levels, relative to that of controls ($F_{(3, 32)} = 136.76$, $p < 0.001$; **Figure 5**).

4. Discussion

Our previous studies conducted in cultured hippocampal neurons showed that application of norepinephrine resulted in increased BDNF expression, CREB phosphorylation and activation of two key cell survival-promoting cascades [8], which were also up-regulated in response to nutrient deprivation stress from the cultured medium [54] [55]. In the current study, we explored the effects of norepinephrine on PKD, a key regulator in plasticity [15] [16], and its effects on BDNF, CREB and GAP-43, which PKC is known to phosphorylate [31]. As before, we found that norepinephrine up-regulated BDNF and activated CREB [8]. However, this could also indicate that PKA [56], MAPK [57] [58] or PI-3K/Akt [7] [54] [57] [59] [60] are also phosphorylating CREB through pathway cross-talk [8] [55] [58]. And although this narrows down the list of possibilities and

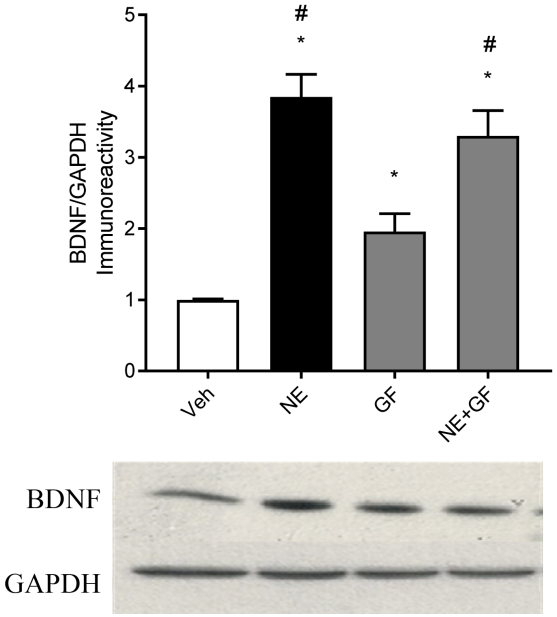

Figure 4. Norepinephrine increases BDNF expression. Norepinephrine significantly increased expression of BDNF above that of vehicle controls ($p < 0.001$), but when combined with GF (NE + GF), was decreased only very slightly and was statistically higher than that of vehicle controls ($p < 0.001$) and GF alone ($p = 0.001$). GF alone, on the other hand, resulted in decreased BDNF expression, compared to that of norepinephrine ($p < 0.001$), but significantly higher than that of vehicle controls ($p < 0.001$). Veh, vehicle control; NE, norepinephrine (100 nM); GF, bisindoylmaleimide (5 μM); *Significantly different from vehicle controls. #Significantly different from GF at $p < 0.001$.

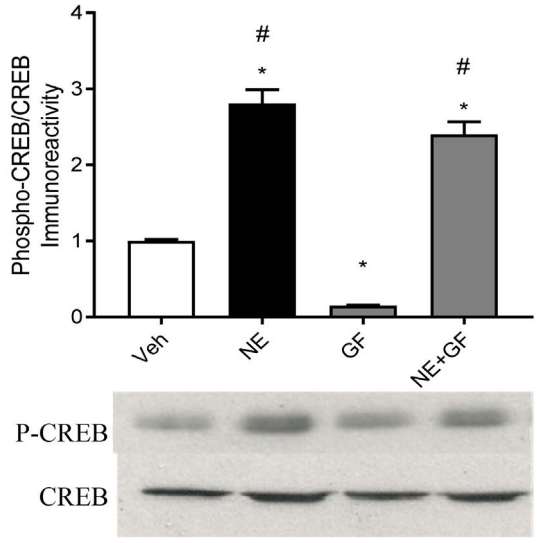

Figure 5. Norepinephrine increases, while GF decreases CREB phosphorylation. Norepinephrine alone ($p < 0.001$) or when combined with GF ($p = 0.026$) significantly increased phosphorylation of CREB above that of vehicle controls. GF, on the other hand, resulted in decreased CREB phosphorylation, compared to that of vehicle controls ($p < 0.001$). Norepinephrine significantly reversed the suppressing effects of GF, being significantly higher than that of GF alone ($p < 0.001$). Veh, vehicle control; NE, norepinephrine (100 nM); GF, bisindoylmaleimide (5 μM); *Significantly different from vehicle controls. #Significantly different from GF at $p < 0.001$.

implicates PKD as also responsible for phosphorylating CREB [61]. Other pathways/enzymes, such as CaMKII, which we did not evaluate, can still phosphorylate the transcription factor. Just as prior use of the PKA inhibitor, Rp-cAMPs, verified PKA involvement [55], our current use of the specific PKC/PKD inhibitor, bisindoylmaleimide, implicates PKC/PKD in the current study. Moreover, our results of increased phosphorylation of GAP-43 as a result of norepinephrine as a stimulator indicate that neurite outgrowth may be partially G-protein-coupled receptor (GPCR)-dependent (**Figure 3**); likewise, GF inhibition of both GAP-43 (**Figure 3**) and PKCδ_{S643} (**Figure 1**) phosphorylation lends support to the hypothesis that PKCδ_{S643} is a mediator of neurite outgrowth. At the same time, norepinephrine stimulated GPCR-mediated increase in PKD activity, whether GF is present or not. Ultimately, norepinephrine via PKCδ_{S643}/PKD/PKCθ_{T538} led to increases in CREB activation and BDNF expression. Although CREB is a molecular [62] [63] [64] [65] [66] and behavioral [67] substrate for so many converging and cross-talking pathways, our findings that GF alone suppressed CREB phosphorylation (**Figure 5**) to a much greater statistical extent than it did in suppressing BDNF expression (**Figure 4**) could seem to defy explanation. However, it is possible that other protein kinase c isoforms that we did not evaluate (*e.g.*, PKCε) were also sensitive to GF and consequently participated in suppressing CREB phosphorylation.

Herein, GF had no inhibitory effect on most of the other PKC isozymes, except for phospho-PKCδ_{S643}. Developmentally, this isozyme reaches its peak expression at 2 - 3 days in culture [13], which corresponds to the incubation period used in the current study. PKCα and PKCβ show maximal expression only after 10 days in culture, while PKCε (which was not evaluated in the current study) and PKC$\lambda\zeta$ are maximal after only 1.5 - 2 days in culture [13]. Therefore, one possible limitation of the current study would be that the effects of norepinephrine and/or GF were not evaluated when some of the isoforms (*e.g.*, PKCα, β, ε, $\lambda\zeta$, μ) were at its peak expression in culture. This is the post plausible explanation for the negative findings regarding some of these isoforms. Another possible limitation of the study is that antidepressant drugs were not evaluated for any effects on the various PKC isoforms. Neurotransmitter-specific antidepressants, such as serotonin- or norepinephrine-selective re-uptake inhibitors, as previously done [8], would make this study more clinically applicable. Because PKC inhibition by GF has been shown to interfere with BDNF action [68] and excitatory neurotransmission [69], future studies should strive to determine which of the many PKC isozymes are most heavily involved in psychiatric, neurodevelopmental and neurodegenerative disorders.

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