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Dopamine agonist signalling in the hypothalamus of female rats is independent of calcium-dependent kinases

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Abstract

We have previously has demonstrated that dopamine agonist, SKF38396 (SKF), can substitute for progesterone (P) in the facilitation of female reproductive behavior in oestradiol benzoate (EB)-primed female rats and mice. We also reported that both P- and SKF-initiated signalling was mediated by cAMP-dependent PKA signal transduction cascade. As the rapid effects of P are also mediated by calcium-dependent kinases, calcium and calmodulin-dependent kinase (CaMKII) and protein kinase (PKC), we sought to determine whether SKF-initiated signalling also recruited calcium as a second messenger. We measured the changes in the activation of CaMKII and PKC in the ventromedial nucleus (VMN) of the hypothalamus and preoptic area (POA) of the rat brain, the two regions implicated in the regulation of female reproductive behavior in rodents. We measured the basal activities representing the activation of the kinases by *in vivo* treatments as well as the total kinase activities assayed in the presence of exogenous cofactors *in vitro*. We report that in contrast to P-initiated signalling, there was no recruitment of calcium by SKF in the hypothalamus as shown by the absence of changes in CaMKII activities in the VMN and POA. Furthermore, SKF-treatment resulted in a rapid increase in calcium-independent basal PKC activity in the VMN but not the POA. These rapid changes were not due to changes in PKC protein levels or phosphorylation status. These data indicate that P and SKF-recruit distinct signalling molecules within the same regions of the brain to activate region-specific signal transduction pathways.

Introduction

We have previously demonstrated a cross talk between signalling pathways activated by progesterone (P) and dopamine agonist, SKF38393 (SKF) in the facilitation of female receptive behavior in rats and mice (1, 2). The signal transduction cascade events involved in this cross talk included the activation of cAMP-mediated protein kinase A (PKA), leading to the phosphorylation of the neuronal phosphoprotein, dopamine and cAMP-regulated phosphoprotein-32 (DARPP-32), an obligatory step in both P- and dopamine (DA)-facilitated female receptive behavior (3). Interestingly, this study also revealed that while both P and SKF stimulated PKA activation, the signal transduction pathways initiated by both, though inter-dependent, are independent of each other. This was evident in the ability of D₁ antagonist to inhibit SKF-stimulated-, but not P-initiated-, increases in cAMP levels and PKA activities, suggesting that SKF and P activate distinct upstream signalling kinase pathways (3).

We have recently reported that in addition to PKA, P rapidly activates protein kinase C (PKC; 4) and calcium and calmodulin kinase II (CaMKII) in the ventromedial nucleus (VMN) of the hypothalamus and preoptic area (POA) of female rats (5). The activation of cytosolic CaMKII by P implicates the mobilization of calcium stores since CaMKII is a unique sensor of intracellular calcium levels. In addition, calcium is known to play an important role in the transduction of rapid neuronal signalling events such as neuroplasticity (6–8). Recent studies have demonstrated the recruitment of calcium (Ca^{+2}) binding proteins, such as Calcyon and neuronal Ca^{+2} sensor 1 in DA-mediated signal transduction *in vivo* (7).

While P regulation of calcium-mediated signalling has been established, not much is known about the similarities or differences in SKF-initiated, Ca^{+2} –mediated signalling in the VMN and POA. We therefore hypothesized that calcium regulation by SKF, mediated by PKC and CaMKII, could be distinct from that of P, and could potentially differentiate the signal transduction mechanisms between the two. The present experiments were performed to determine whether SKF-initiated signalling involved Ca^{+2} recruitment in the VMN and POA. We therefore examined whether CaMKII and PKC activities were also regulated by SKF in the VMN and POA of female rats under behaviorally relevant conditions. Our current findings demonstrate that there was no recruitment of calcium in the VMN of the hypothalamus as demonstrated by the absence of changes in the activity of CaMKII by SKF treatment. While SKF treatment activated PKC, this activity was independent of Ca^{+2} in this region. There were no changes in SKF-mediated induction of either CaMKII or PKC activities in the POA. These data suggest that SKF activation of the signal transduction cascade in the VMN and POA regions in the female rat is not dependent on CaMKII and PKC activities, clearly differentiating the signalling mechanisms from those initiated by P.

Materials and Methods

Oestradiol benzoate (EB), progesterone, calmodulin, phosphatidyl-serine, SKF38393 were purchased from Sigma chemicals (St Louis, MO). Diacyl glycerol (DAG) was purchased from Cayman chemicals (Ann Arbor, MI). The antibodies and their source are as follows: α PKC (rabbit polyclonal) from Upstate Biotech Inc (Lake Placid, NY), phospho- α PKC (rabbit polyclonal) from Cell Signalling (Millipore; Danvers, MA) and GAPDH antibody (rabbit polyclonal) from Santa Cruz Biotech Inc (San Francisco, CA). All other chemicals were of reagent grade and were purchased from either Sigma (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

Animal Protocol and Treatments

Sprague-Dawley ovariectomized rats (18–220g) were purchased from the supplier (Charles River Laboratories, MA). The animals were maintained on a 12:12 h reversed light cycle with lights off at 12:00 h and provided food soy-free diet (Lab Diet 5001; PMI International, Richmond, IN) and water, *ad libitum*. Animal studies were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine and were in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Stereotaxic Surgeries

Stainless steel guide cannulae (23gauge) were stereotaxically implanted into the 3rd cerebral ventricle of ovariectomized rats using a Lab Standard stereotaxic instrument (Stoelting, Wood Dale, IL) as previously described (1). Animals were allowed to recover from surgery for 1 week prior to their use in the experiments. 2 μ g of EB was administered subcutaneously (sc) followed by intracerebroventricular (icv) infusion of 100ng/ μ l of SKF 48 hr later. 30 min post-SKF administration, the animals were killed, the brains isolated, and the VMN of

the hypothalamus, POA and cerebral cortex (CTX) dissected as described below. Other treatment groups included Vehicle (oil) + saline, EB + saline, Vehicle (oil) + SKF and EB + P (positive internal control).

Tissue isolation and Processing

Fresh brain dissection was carried out at 4°C. Using a McIlwain tissue chopper, the POA slab was cut beginning 2mm rostral to the optic chiasm to 0.5mm caudal to the chiasm. A second cut (the medial basal hypothalamus) was made 4mm caudal to the caudal edge of the first slab. The slabs were placed on a cold microscope stage and brain areas of interest were viewed under a dissecting microscope with transillumination system (Zeiss Stereo Discovery V8). Bilateral punches were made using the Palkovits punch method from the slabs to dissect the VMN and POA using a 1mm internal diameter stainless steel punch. The cerebral cortex included the frontal cortex without the white matter from the slabs. The punches were immediately frozen on dry ice/isopropanol and stored at -80° C until processed.

Tissue samples were homogenized in buffer containing 10 mM Tris-HCl (pH 7.4), 1 mM EGTA, 1 mM EDTA, 0.5 mM DTT, 1mM thioglycerol, cocktail of protease inhibitors and phosphatase inhibitors (1:100) in a hand-held glass homogenizer on ice. The samples were centrifuged at 1000 × g for 10min, the pellet consisting of nuclear, mitochondrial and cellular debris discarded, and the supernatant solution centrifuged at 20,000 × g for 20min. The protein was quantified using a BCA assay (Pierce Inc. Rockford, IL). The supernatant was stored at -80°C in aliquots for the kinase assays and western analysis.

Kinase Assays

CaMKII activity was measured using a synthetic peptide substrate, bearing the auto-phosphorylation sequence from the CaMKII sequence (autocamtide-3; KKALHRQETVDAL). The assay was performed according to the method of Moore *et al* (9) with minor modifications as described. 5µl of protein samples (~1–5 µg) was added to the reaction mixture (final volume 25µl) containing 50 mM HEPES, pH 7.4, 1mM DTT, 20 µM substrate peptide, 20 µM ATP, 5 mM MgCl₂, 2µCi [γ -³²P]-ATP (3000Ci/mmol) and incubated at 30°C for 2 min. The assay was performed in the presence of 2mM EGTA in the reaction mixture to determine the basal activity and in the presence of 1mM CaCl₂ and 3 µM calmodulin to determine the total activity.

The PKC assays were performed using a modified procedure of Klann *et al* (10), using a synthetic peptide (AAAKIQASFRGHMAR) derived from neurogranin (aa 28–43, NG) as the substrate. The reaction mixture (final volume, 25µl) contained 50mM HEPES (pH 7.4), 1mM DTT, 2mM sodium pyrophosphate, 100 µM ATP, 2µCi of [γ -³²P] ATP and 10µM NG. Assays for PKC activity were carried out with 1mM EGTA and 1 mM EDTA in the reaction mixture for basal activity or in the presence of 4 mM CaCl₂ and lipid cofactors for total activity [final concentrations: 320 µg/ml phosphatidyl serine (PS) and 30µg/ml sn-1, 2-diocanoylglycerol (DAG)]. The reaction was started by the addition of 5µl of protein extract (~1–5µg) to aliquots (20µl) of the reaction buffer either containing NG or not, incubated at 30°C for 2 min.

After the incubation, all the reactions from the assays were similarly processed. The control reactions for background determination were run in parallel by omitting substrate peptide. 17 µl of the reaction mixture was spotted on P-81 phospho-cellulose filters and the reaction stopped by 75mM phosphoric acid. The filters were washed twice (10 min each) in fresh phosphoric acid solution, rinsed in methanol, and air-dried. The radioactivity on the phosphorylated peptide substrate was quantified by the Cerenkov method. Autocamtide-

phosphorylation was determined to be linear with respect to time and protein concentration under these conditions. For each experimental determination, the values for control reactions lacking the substrate peptide were subtracted from those containing the substrate peptide. The counts were normalized using protein concentrations determined by the BCA assay. The assay was performed for the samples from each animal independently. Data are expressed as either specific activity (pmols of phosphate transferred/min/mg of protein) for each of the treatment groups (average of 6 per group) or as a percent of Vehicle control group (set to 100%). All activities were converted to a percent of their respective control so that the basal and the total activities could be presented in the same graph.

Western Immunoblots for PKC

The tissue samples from VMN, POA and CTX were subjected to Western immunoblotting. Equal amounts of protein (5µg) from each sample were electrophoresed on SDS-PAGE [10% polyacrylamide](11) followed by transfer to a nitrocellulose membrane for Western blotting (12). Magic Marker XP Western standards (Invitrogen, Carlsbad, CA) were included in the gels for molecular weight determination. The membranes were blocked and sequentially probed with commercially available antibody to phosphorylated conventional PKC (c-PKC; homologous to Threonine514 of γ PKC), followed by a cPKC-antibody that recognized all the conventional and other isoforms, at a 1:2000 dilution. The antibody to GAPDH (as a normalizing control) was used at 1:1000 dilutions in conjunction with both the PKC-antibodies. The antibody binding was revealed by incubation with donkey anti-rabbit horseradish peroxidase-conjugated IgG (1: 10,000) followed by detection with the enhanced chemiluminescence reagent ECL (Amersham Pharmacia Biotech, Piscataway, NJ). The PKC and GAPDH bands were quantified by densitometry using Phosphor Imager: SF (Molecular Dynamics, Sunnyvale, CA). The density of the immunoreactive protein bands was normalized to the GAPDH. Linear range of signals for densitometry was obtained by exposing the chemiluminescent membranes to x-ray film for varying lengths of time. The protein samples were separately run with the controls, V and EB in one gel and SKF and EB +SKF on another. The protein extracts were also examined for presence of the neuronal membrane and cytosol components by probing for the CNPase protein (2', 3'-cyclic nucleotide 3'-phosphodiesterase) and the GAD67 protein (L-glutamic Acid Decarboxylase) respectively. These markers confirmed the presence of neuronal plasma membrane fraction in the protein extracts.

Statistical analysis

Statistical analysis was carried out using Prism (Graph Pad, San Diego). For each significant analysis of variance (ANOVA), post-hoc comparisons were made using Dunnett's method for comparison all groups versus the control group or the Newman-Keuls for multiple comparisons.

Results

The kinase assays performed in the presence of cation-chelators, EGTA and EDTA, determined the changes in basal activities as a result of various *in vivo* treatments. The total activities refer to a further stimulation of the kinases present in the tissue homogenate and measured, *in vitro*, in the presence of exogenous cofactors (Ca^{+2} , phosphatidyl serine (PS) and diacylglycerol (DAG) for PKC, and, Ca^{+2} and calmodulin for CaMKII). The basal and total activities represent the changes in kinase activities with respect to the vehicle (V) treatment. A comparison between the basal and total activities also provided information on the activated fraction of the kinase pool activated by SKF at the time point measured.

Dopamine agonist SKF38393 had no effect on CaMKII activation in the VMN and POA

CaMKII is uniquely sensitive to changes in intracellular calcium due to its absolute dependence on Ca^{+2} for its initial activation (13). To determine whether SKF initiated signalling is dependent upon Ca^{+2} , the basal and total CaMKII activities were assayed in the VMN and POA tissue homogenates from the vehicle (V)-, EB-, SKF- and EB+SKF-treated animals. We also used tissue homogenates from EB+P treated animals as positive controls. While EB+P treatment significantly enhanced both basal and total CaMKII activities, SKF treatment either singly or in combination with EB did not affect the basal or total CaMKII activities in the VMN (Fig.1A). Interestingly, in the POA, EB+P treatment caused a significant change in both basal and total CaMKII activities. While the basal activity was enhanced, the total activity was suppressed to levels below that of V. SKF treatment by itself had no significant effect on the basal CaMKII activity. However, EB-priming repressed this activity significantly, similar to that observed for EB+P treatment. The total CaMKII activity was severely attenuated by the SKF-treatment both in the presence and absence of EB-priming (* $P < 0.01$; Fig.1B). Neither P nor SKF± EB had any effect on the basal and total activities in the CTX (Fig.1C). The CaMKII specific activities for the vehicle controls set to 100% of these regions. In the VMN the specific activities of the basal and total were 12.2pmols phosphate transferred/min/mg protein and 151.2pmols phosphate transferred/min/mg protein respectively. Those for the POA were 2.8 pmols ATP transferred/ min/mg protein and 76.7 pmols ATP transferred/min/mg protein for basal and total activities respectively. In the CTX, the specific activities of basal and total CaMKII were equal to 5.96 pmols and 10.31pmols respectively.

Dopamine agonist SKF38393 had significant effect on PKC basal activity in the VMN of the Hypothalamus

The PKC family of Ser/Thr kinases has a complex cofactor requirement (14). The conventional isoforms (cPKC; α , β I, β II and γ) require both Ca^{+2} and PS/DAG for its activation, whereas the novel forms (nPKC; η and ϵ) require only PS/DAG while the atypical forms (aPKC; δ , ζ and ι) require neither calcium nor PS/DAG for its activation (15–17). The tissue homogenates were therefore assayed for both the basal and the total PKC activities *in vitro*. The PKC basal and total activities the VMN are presented in Fig. 2A. EB- treatment by itself had no significant effect on either basal or total PKC activity compared to the vehicle control (V). SKF-treatment caused a statistically significant increase in basal activity (* $P < 0.01$), while not significantly affecting the total activity. Interestingly, EB- priming did not significantly affect the basal PKC activity while significantly increasing (* $P < 0.01$) total PKC activity, indicating a latent kinase activity that is capable of further enhancement by additional cofactor-levels *in vivo*. The specific activities of basal and total PKC of the vehicle control (V) set to 100% was equal to 17.1 ± 0.7 and 203.7 ± 74 pmol of phosphate transferred/min/mg protein respectively.

In order to determine if the changes in PKC activities in SKF or EB+SKF treated VMN tissue homogenates were due to changes in protein levels, we examined the total and phosphorylated c-PKC protein levels (α , β I, β II and γ) in the tissue homogenates assayed for PKC activity. Normalization of the protein content of the 80kDa band corresponding to total cPKC, using GAPDH immuno-reactive band (37kDa), indicated no significant changes in the protein levels due to EB±SKF treatments (Fig. 2A). The actual values are tabulated and shown in Table 1. Quantification of the phosphorylated-cPKC band (phosphorylated @ Thr 514) also demonstrated no significant changes in response to EB ± SKF compared to the V treatment, suggesting that the phosphorylation status and protein levels of cPKC at Thr514 was not affected by SKF treatment. The ratio of phospho- to total cPKC for each of the treatment groups, are shown in Table 2. The data in the table presented is representative of cPKC protein, the most abundant PKC isoforms. Quantification of the other isoforms

showed no apparent change in either total or phosphophorylated PKC proteins of the nPKC and aPKC isoforms.

Dopamine agonist, SKF38393 inhibited PKC response in the POA

An examination of the basal PKC activity in the POA revealed no significant effect ($P>0.05$) of EB-priming compared to the vehicle control (Fig. 2B). In contrast, SKF-treatment caused a significant decrease ($*P<0.01$) in PKC basal activity, which was not alleviated by EB-priming ($*P<0.01$). There was no augmentation of total PKC inducible activity in the presence of exogenous cofactors, demonstrating a complete lack of PKC activation in this region of the brain. The specific activity of the basal and total PKC activities were 17.6 ± 7 and 289.9 ± 76 pmols of ATP transferred/min/mg protein in the V treatment, respectively, set to 100%.

Quantification and normalization of the 80kDa c-PKC protein levels in the POA samples used in the kinase activity assays revealed no significant change in the total protein levels due to EB or SKF treatments independently or in combination (Table 1). An examination of the phosphorylated c-PKC (corresponding to Thr 514 of the γ -isoform) also revealed no significant changes due to SKF $\pm$ EB compared to the vehicle (Table 2).

SKF-regulation had no effect on the basal PKC activity in the frontal Cortex

The basal activities in the homogenates from the prefrontal cortex showed no significant changes in response to either SKF or EB+SKF treatments (Fig. 2C). The specific PKC activity due to the vehicle treatment set to 100% was equal to 23.6 ± 7 pmols of phosphate / min/mg protein. There was no change in total PKC activity assayed in the presence of exogenous cofactors calcium and PS & DAG ($P>0.05$), due to the vehicle or SKF $\pm$ EB. The 100% of vehicle treatment was equal to of 289.4 ± 72 pmol of ATP transferred per min per mg protein.

The western blot analysis, shown in Fig. 2C and Table 1, demonstrated no significant differences in either the total protein or its phosphorylation at Thr 514 residue, due to SKF $\pm$ EB treatments.

Discussion

The study extends our current understanding of the signal transduction mechanisms mediated by DA-agonist in the VMN and POA, the two regions implicated in female reproductive behavior. The results demonstrate that recruitment of calcium and calcium-dependent kinases is not a necessary step in the signal transduction pathway initiated by D_1 agonist, SKF, in the VMN and POA. These findings are in stark contrast to our earlier observations demonstrating P-initiated activation of calcium-dependent kinases, CaMKII and PKC, (4–5) and their regulation by steroid hormones (18–20). Taken together, these studies suggest that the SKF- and P-initiated signalling pathways are distinct and tissue-specific. While PKA-mediated signalling is a common pathway recruited by both compounds, calcium recruitment and mobilization appears to be specific to P and not to SKF. The differential regulation of the signalling pathways suggests the possibility of modulation of distinct downstream targets stimulated by P and SKF.

Icv administration of SKF, with and without EB priming, had no effect on the basal and total activities of CaMKII in the VMN or POA. In contrast, P treatment caused significant changes in both basal and total activities of CaMKII in the same tissues, indicating differential regulation by SKF and P. Interestingly, while SKF treatment had no effect on basal CaMKII activity in the POA, it caused a reduction in the total CaMKII activity. EB treatment resulted in a further reduction of the total CaMKII activity. It is possible that EB

treatment converts CaMKII to an autoactive kinase whose activity becomes independent of calcium and calmodulin (21). Studies are in progress to examine this interesting regulatory aspect by SKF in the POA.

SKF-treatment resulted in a rapid activation of basal PKC, but not the total PKC, in the VMN. While EB-priming had little or no effect on SKF-mediated activation of basal PKC activity, the activity levels of total PKC was increased four-fold, suggesting that the observed effects of SKF on the basal activity were independent of co-factor requirement and mediated by conventional forms of PKC. In contrast to VMN, SKF had no effect on the activation of PKC in the POA, with or without EB-priming, suggesting a region-specific regulation by SKF. It is plausible that EB-stimulated changes are mediated by calcium independent isoforms of PKC, as has been reported to be regulated by EB (22–26). Alternatively, in the absence of calcium recruitment, the SKF-stimulated PKC-dependent kinase signals could be transduced through pathways such as the activation of the membrane bound phospholipase C (PLC), as has been observed in the D₁ receptor interaction in the striatum (27), or the selective activation of PKC via phosphatidylinositol-3 kinase pathway (28).

Calcium recruitment may be one of the mechanisms by which differential signalling pathways stimulated by SKF and P independently regulate reproductive behavior. SKF-stimulated pathways do not utilize calcium-dependent kinases for their signal transduction, while P activates both CaMKII and PKC-mediated signalling mechanisms. One explanation could be that calcium-dependent mechanisms could have detrimental effect on the SKF-initiated behaviors. Previous reports from our laboratory have shown that SKF stimulates D₁ receptors in the hypothalamus, leading to sequential activation of cAMP-dependent PKA, phosphorylation of DARPP-32 on Thr³⁴ and facilitation of female receptive behavior (3). Thus, regulation of DARPP-32 by SKF is critical for the expression of behavior. Calcium has been demonstrated to induce a dramatic decrease in the phospho-DARPP-32 levels in the neostriatal tissues of mice (29). CaMK-dependent protein phosphatase calcineurin has been demonstrated to dephosphorylate DARPP-32 with high efficiency both in vitro and in vivo (29–30). Thus, calcium-dependent processes that increase intracellular calcium could contribute to the inactivation of DARPP-32 and decreased SKF-facilitation of female receptive behavior. However, such a scenario will not explain published reports by us (3–5) and others (18–20) demonstrating increases in (a) activities of CaMKII and PKC by P, (b) phosphorylation of DARPP-32 by P and (c) involvement of PKC and CaMKII in P-facilitation of female reproductive behavior.

Alternatively, P and SKF could be affecting multiple signalling molecules within the same regions of the brain, to activate distinct signalling pathways. The pathways perhaps modulate different downstream effectors required for reproductive behavior. Recent studies have shown that multiple effectors, i.e., ion channels, transcription factors, coregulators, are regulated through multiple signalling mechanisms, including membrane-initiated steroid and DA signalling (31–36). Future studies should help us understand the cross talk between the rapid signalling events and their integration with nuclear steroid hormone receptors to bring about changes in gene expression and reproductive behavior.

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A

POA

B

C

Fig. 1.

Dopamine receptor (D_1R) agonist, SKF38393, (SKF)- and progesterone (P)-regulation of CaMKII basal and total activities in the (A) ventromedial nucleus of the hypothalamus (VMN), (B) preoptic area (POA) and (C) frontal cortex (CTX), of ovariectomized, oestrogen benzoate (EB)-primed female rats with chronic indwelling cannula in the third cerebral ventricle. In vivo treatment of animals, tissue isolation, preparation of tissue homogenates and kinase assays were performed as described in Materials and Methods. The changes in the basal and total activities of CaMKII due to the vehicle (V)-, EB-, SKF-, EB+SKF- and EB+P- treatments are expressed as percentage of V (V= 100%) and are Mean $\pm$ SEM of 6 independent determinations, each assayed in triplicate. (N=6 for each treatment group). *, #, Significantly different from V (* $P < 0.01$; # $P < 0.05$). No significant difference was observed between V and EB and V and SKF $\pm$ EB in the frontal cortex, $P > 0.05$.

VMN

A

POA

B

CTX

Fig. 2.

SKF-modulated changes in basal and total activities of PKC (Top panel) and representative autoradiogram of western immunoblots for PKC protein (Bottom panel) in tissue homogenates from (A) VMN, (B) POA and (C) CTX. Kinase assays on the tissue homogenates from vehicle (V), oestradiol benzoate (EB)- and SKF- treated animals were performed as described in materials and methods. The counts were normalized using protein concentrations in each sample. The assay was performed on samples from each animal independently. Data are expressed as percentage of vehicle control (: V=100%) and represent Mean $\pm$ SEM of 6 homogenate samples, each assayed in triplicate. *, #, Significantly different from V (* P<0.01; # P< 0.05).. The bottom panel of each figure shows the representative autoradiograph of the western immunoblot for PKC protein, the 80kDa band corresponding to conventional PKC isoforms and the 37kDa band corresponding to GAPDH. The GAPDH band at 37kDa was used as a protein loading control for the respective sample.

Table 1
Densitometric analyses of total c-PKC proteins

The intensities of c-PKC (80kDa) and GAPDH (37kDa) bands identified by western immunoblotting (Fig. 2) were quantified using a densitometer. The ratio of c-PKC to GAPDH in each lane was calculated for normalization for protein loading. This ratio for the vehicle control (V) was equated to 1, and compared to those of oestrogen benzoate (EB) and /or dopamine agonist SKF39383 (SKF) treatments. ANOVA followed by Dunnett's post-hoc comparisons showed no significant differences ($P > 0.05$) between the vehicle and treatment groups in each of the regions examined. The values are represented as the mean values of the ratio $\pm$ SEM (n=6 per treatment group) for the three brain regions examined.

Treatment	VMN	POA	CTX
V	1	1	1
EB	1.0 ± 0.4	1 ± 0.1	0.99 ± 0.02
SKF	0.7 ± 0.2	0.8 ± 0.1	1.13 ± 0.04
EB+SKF	0.9 ± 0.3	1.0 ± 0.1	1.2 ± 0.1

Table 2
Ratio of the phosphorylated PKC to total PKC

Densitometric intensities of the phosphorylated PKC (@ Thr514) and total PKC bands identified in the western immunoblots were quantified. The ratio of the intensities of phospho- to total band for each treatment group was calculated. This ratio for vehicle control (V) was set to 1 and compared to those from the treatment groups (EB & SKF). ANOVA followed by post-hoc comparisons demonstrated no significant differences in the ratios between the V and treatment groups in each of the brain regions examined ($P > 0.05$). The values are given as the mean values of the ratio $\pm$ SEM (n=6 per treatment group) for the brain regions examined.

Treatment	VMN	POA	CTX
V	1	1	1
EB	1.0 $\pm$ 0.5	1.1 $\pm$ 0.2	3.8 $\pm$ 1.4
SKF	0.7 $\pm$ 0.2	0.5 $\pm$ 0.1	1.0 $\pm$ 0.4
EB+SKF	0.9 $\pm$ 0.3	0.5 $\pm$ 0.1	1.0 $\pm$ 0.4