Effects of Cocaine and Estrogen on BDNF-Trk B Signaling in Hippocampi of Ovariectomized Rats.

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Abstract:
Cocaine addiction has been associated with changes in synaptic plasticity, mediated in part by neurotrophin cascades. Given sex differences in cocaine addiction, we wanted to assess whether estradiol alters signaling of the brain-derived neurotrophic factor (BDNF), through interaction with its cognate receptor, tyrosine kinase B (Trk B), after repeated cocaine treatment. Adult ovariectomized (OVX) Sprague-Dawley rats were injected intraperitoneally with saline or 30mg/kg cocaine daily and 8ug/kg estradiol benzoate (EB) or oil subcutaneously every other day for 9 days. Hippocampal neurotrophin receptor signaling was initiated by BDNF stimulation and measured by the level of Trk B phosphorylation, as well as the magnitude of down-stream ERK and PI_3K activation. Expression levels of Trk B, BDNF, AKT, and ERK were also determined. While no differences in protein expression were found, BDNF-induced Trk B activation was increased following repeated cocaine administration. Furthermore, the effect of cocaine was reduced in groups receiving co-administration of EB. Neither cocaine nor EB affected phosphorylation levels of AKT or ERK. Further research is necessary to determine the nature of the interaction between cocaine and estrogen on BDNF-induced Trk B activation.

Keywords: Cocaine, TrkB signaling, BDNF, Estrogen

I. Introduction

The mechanisms underlying sex-related differences in cocaine addiction are unknown. Studies show that chronic cocaine exposure leads to lasting changes in synaptic plasticity [1, 2]. Brain-derived neurotrophic factor (BDNF), receptor tyrosine kinase B (Trk B) and its signaling cascades mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK), and phosphoinositol 3-kinase (PI3K) modulate activity-dependent neuronal plasticity in the adult brain [3]. . BDNF synaptic secretion is associated with increased glutamatergic activity, long-term potentiation, dendritic protein synthesis, and dendritic spine formation. It has been shown that cocaine exposure affects BDNF-Trk B signaling [4]. Increased BDNF levels in the ventral tegmental area (VTA) or the nucleus accumbens (NAc) lead to behaviors associated with addiction[5] [6]. There is also growing evidence that estrogen can affect BDNF signaling[7]. The BDNF gene contains a sequence homologous to the estrogen-response-element and estrogen ligand-receptor complexes can bind to this sequence [8], thus providing a mechanism for the complexes act as nuclear transcription factors and directly influence BDNF expression. Estrogen replacement in ovariectomized (OVX) rats upregulates BDNF expression in several brain regions, including the hippocampus [8, 9]. OVX rats given estradiol also show increased TrkB[10, 11] and increased TrkB mRNA levels in the hippocampus [12]. Therefore, it is conceivable that some of the cocaine-induced gender-related changes in brain function may be due to an interaction between cocaine and estrogen on BDNF signaling.

The addictive properties of cocaine appear to be due to the ability of the drug to alter synaptic plasticity [1, 2, 13-15]. These neural adaptations appear to involve similar cellular mechanisms to those underlying learning and memory [13, 16, 17]; hence, it has been proposed that the hippocampus could play a critical role in addictive behaviors. The hippocampus has extensive interconnections with reward-related and learning and memory pathways, including dopaminergic input from the VTA, as well as glutamatergic output to the NAc shell, for review see [18]. Coincidentally, synaptic function and plasticity in this region may be influenced by both estrogen and BDNF signaling [19-23]. In adult cycling female rats large fluctuations occur in the levels of dendritic spines on pyramidal cells of the CA1 region of the hippocampus [24, 25]. In the hippocampus, spine density is greatest on proestrous and low in OVX animals [26]. Hippocampal LTP is facilitated in OVX rats when treated with estrogen as compared to the untreated OVX animals [27]. LTP is also maximal in cycling females when estrogen

concentrations are highest in the cycle [28]. Neurotrophin signaling increases the phosphorylation of NR1 and NR2 subunits of NMDA receptors and the expression of GluR1 and GluR2/3 levels of AMPA receptors [29], and thus enhances LTP in the hippocampus [30]. Therefore, changes in hippocampal synaptic plasticity may be relevant to the sex differences associated with the addictive properties of the drug. The objective of the present study was to examine the effects of estrogen on BDNF-Trk B signaling in the hippocampi of OVX rats that were treated repeatedly with cocaine.

II. Methods

OVX Sprague-Dawley rats (200g) were purchased from Taconic Farms (Germantown, NY), housed three per cage and maintained at constant temperature and humidity under a 12-h light/dark cycle with free access to food and water. All animal procedures were carried out in compliance with the Guide for the Care and Use of Laboratory Animals of the National Institute of Health (NIH) and were approved by the City College of New York Animal Care and Use Committee. One week after acclimatization, animals were assigned to one of four groups: saline/oil, saline/estrogen, cocaine/oil, cocaine/estrogen. Animals received daily injections (i.p. 0.2ml) for nine consecutive days of either saline or cocaine (30 mg/kg in 0.9% saline). β-Estradiol benzoate (EB), 8µg/kg, or corn oil was injected s.c. every other day during the same time period. One hour after the last cocaine injection, animals were sacrificed by rapid decapitation, brains removed, and the hippocampus dissected on ice. The tissue was immediately frozen in liquid nitrogen, and stored at -80°C until assay.

To determine whether changes in hippocampal BDNF-Trk B signaling in cocaine and/or EB treated rats is mediated by alterations in the expression of signaling molecules, we evaluated the levels of BDNF, Trk B, as well as the downstream signaling proteins AKT and ERK. For western blot analysis, approximately 20 mg of frozen hippocampus was thawed and homogenized in lysis buffer (20mM Tris HCL-pH 7.4, 20mM NaCl, 1mM EDTA, 1mM EGTA, 20mM NaF, 25mM β-glycerophosphate, 5mM Sodium pyrophoaphate, 1% Triton X-100) by sonicating for 20 sec. in ice. The hippocampal homogenate was then centrifuged at 1000g for 5 min. The supernatant, post-mitochondrial fraction, was used to determine protein content by the Bradford method (Bio-Rad). The tissue homogenate was then solubilized by boiling in sample preparation buffer for 5 min. Fifty ug of the solubilized hippocampal proteins were size-fractionated on 10 or 12% SDS–PAGE according to Laemmli (1970). The separated proteins were transferred electrophoretically to nitrocellulose membranes. Following brief washes, the membranes was incubated overnight at 4°C in phosphate-buffered saline, pH 7.2 (TBS) containing 0.1% Tween 20, 10% fat-free milk to block non-specific binding of antibody, washed 4 times with 0.1% TBS (10-min each), and then incubated with the appropriate antibody. Following three washes with 0.1% TBS, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 1 hr at room temperature. After washing with 0.1% TBS three times, the immunocomplexes were detected by a chemiluminescent method and visualized by exposure to film. Membranes were stripped and reprobed with anti-β-actin or β-tubulin antibody to control for loading. Films were scanned and specific protein bands were quantified using Image J software (NIH). Statistical differences between the treatments, cocaine administration and hormone replacement, were assessed by 2-way ANOVA (drug treatment, hormone) followed by the Bonferroni test for post hoc comparisons ($p < 0.05$ considered significant).

We assessed Trk B activation by measuring the level of pY-Trk B in response to exogenous BDNF. The effect of repeated cocaine and/or EB on Trk B signaling was compared in hippocampal slices from OVX rats that were treated with vehicle, cocaine and/or EB. This was accomplished by cutting the hippocampi into 100 µm x 100 µm x 3 mm slices using a chilled McIlwain tissue chopper and suspended in ice-cold oxygenated Krebs'-Ringer (K-R) solution: 25 mM HEPES, pH 7.4; 118 mM NaCl, 4.8 mM KCl, 25 mM $NaHCO_3$, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 10 mM glucose, 100 µM ascorbic acid, 50 µg/ml leupeptin, 10 µg/ml aprotinin, 2 µg/ml soybean trypsin inhibitor, 0.04 mM PMSF. The slices were washed, resuspended in 0.5 ml of K-R, and divided equally into two tubes, which were incubated with 50 µg/ml of BDNF or K-R (control) for 30 minutes at 37◦C. Every 15 min the tubes were oxygenated (95% O_2/5% CO_2) for 1 min, and the reaction was terminated by adding 750 µl of ice-cold K-R and centrifuged immediately. Following removal of the supernatant, the pellet was resuspended in 200µl of immunoprecipitation (IP) buffer (25 mM Tris-HCl, pH 7.5, 200 mM NaCl, 2 mM EDTA, 0.5 mM EGTA, 50 µg/ml leupeptin, 0.2 mM PMSF, 25 µg/ml pepstatin A, 0.01 U/ml soybean trypsin inhibitor, 5 mM NaF, 1 mM sodium vanadate, 0.5 mM β-glycerophosphate and 0.1% 2-mercaptoethanol) by 10 sec sonication, and the protein content was determined by the Bradford method (Bio-Rad). Tissue homogenates were solubilized with 0.5% digitonin,

0.2% Na cholate and 0.5% Nonidet-P40 for 1 hr at 4°C with constant end-over-end rotation. Following centrifugation to remove insoluble debris, 200 µg hippocampal homogenate was used to assess components of BDNF-Trk B signaling cascade by immunoprecipitation with selected antibodies.

Trk B and its downstream proteins ERK_2 and Akt_1/Akt_2 were immunoprecipitated respectively with 1 µg of antibody for TrkB (sc-8316), ERK_2 (sc-154) AKT_1 (sc-5298)/AKT_2 (sc-5270) (all from Santa Cruz Biotechnology, Santa Cruz, CA) in IP buffer (total incubation volume: 1 ml). The tissue lysate/antibody mixture was first incubated for 30 min at 4°C with constant end-over-end rotation, combined with a 40 µl/tube of Agarose-conjugated protein A/G (Santa Cruz Biotechnology) and further incubated overnight at 4°C while shaking. The following day, the antigen-antibody-protein A/G-agarose complexes were retrieved by centrifugation and washed twice with 1 ml of PBS. The resultant immunoprecipitates were resuspended and solubilized by boiling for 5 min in 150 µl of sample preparation buffer. (62.5 mM Tris-HCl, pH6.8; 10% glycerol, 2% SDS; 5% 2-mercaptoethanol, 0.1% bromophenol blue). The activated TrkB (pY-TrkB), ERK2 (pY/pT-ERK2), and Akt (pS^{473}-Akt) levels in 50% of the anti-TrkB, -ERK2 and -Akt immunoprecipitates were determined by western blotting (as described above) using specific antibodies to activated Trk (sc-8058), p-ERK (sc-7383), and p-AKT 1/2/3 (Serine 473) (sc-33437) (Santa Cruz Biotechnology, Santa Cruz, CA). The membranes were then stripped and reprobed with the appropriate antibody to assess the total amount of protein for each group. These films were scanned, protein bands quantified using Image J, and analyzed using the same statistical paradigms as described for the western blotting section.

III. Results and Discussion

Western blot analysis revealed no significant differences in expression of BDNF, Trk B, AKT or ERK among the different treatment groups (data not shown). Recent studies also reported no changes in BDNF expression after cocaine treatment [31] Others have reported changes in BDNF mRNA levels in the hippocampus and amygdala following repeated cocaine exposure only after withdrawal of the drug [32, 33], while others only detect changes BDNF mRNA, after repeated cocaine exposure, specifically in the shell of the NAc [33]. Thus, the lack of changes in protein expression reported here may be due to the administration paradigm and/or region-specific effects.

A significant increase in tyrosine-phosphorylated (pY) Trk B, in response to 30-min incubation with 50µg/ml BDNF, was observed. In comparison to hippocampi from saline-treated rats, BDNF-induced pY-Trk B was increased by 42% in the repeated cocaine-administered group (Mean ± SEM: saline/oil vehicle: 0.9569±.05 and saline/oil BDNF-stimulated: 0 .9828±.07; cocaine/oil vehicle: 0.9608±.04/ and cocaine/oil BDNF stimulated: 1.6590±.08, P=0.001) See figures 1A and 1B. This cocaine-induced increase in pY-Trk B was reduced to 29.7% in rats that received simultaneous treatment with EB (cocaine/estrogen group vehicle: 1.0557±.04; cocaine/estrogen BDNF stimulated: 1.3607±.06), and this interaction between drug and EB was significant P=0.005 (Figures 1A and 1B). EB administration to OVX rats alone only modestly increased pY-Trk B by 26.1% (saline/estrogen vehicle: 0.9615±.04; saline/estrogen BDNF: 1.2944±.06) in the hippocampus. These results are consistent with the idea that the presence of EB can alter cocaine-induced synaptic plasticity, particularly along the BDNF signaling cascade.

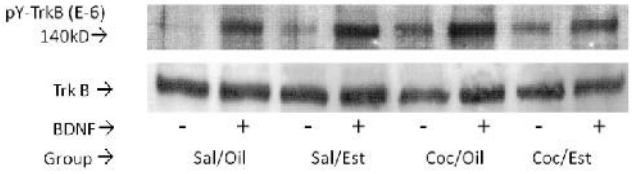

Fig 1. A Western blots of hippocampal lysates using p-Trk antibody (upper panel) or anti-TrkB antibody (lower panel). All four groups showed increased pY-TrkB associated with BDNF stimulation, as compared to vehicle. However, the cocaine-treated group had the greatest increase in pY-TrkB (42%) as compared to the saline/oil group (P=0.001). The group receiving both cocaine and EB had a less pronounced increase in this phosphorylated protein (29.7%), when compared to vehicle.

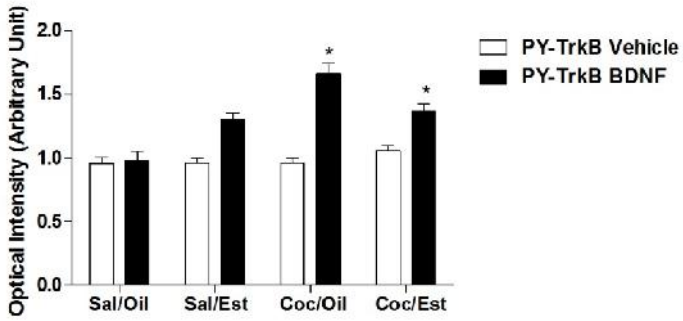

Fig 1.B. Cocaine alters BDNF-induced pY-Trk B levels in hippocampi of OVX rats. The four groups depicted are saline/oil,saline/estrogen, cocaine/oil, and cocaine/estrogen. Levels of pY-TrkB are expressed as Mean ± SEM obtained from 9 animals for each group. *p <0.05.

Since ERK and PI3K signaling pathways are downstream of Trk B, we compared the levels of pY-ERK2 and pS^{473}-Akt after BDNF stimulation. Repeated cocaine administration showed a tendency to increase phosphorylated AKT after BDNF stimulation (saline/oil vehicle: 0.7594±.08, after BDNF stimulation: 1.2609±.16; cocaine/oil vehicle: 1.0434±.13, with BDNF stimulation: 1.6924±.18); however, this apparent increase was not significant P=0.4 (figure 2). Cocaine treatment did not affect BDNF-induced levels of phosphorylated ERK2 either (saline/oil vehicle: 0.8450±0.2, with BDNF: 1.0433± 0.2; cocaine/oil vehicle: 0.8542±0.2, with BDNF: 1.0941±0.3, P>0.05) (figure 3). Previous studies have shown increased phosphorylation of ERK (p-ERK), but not AKT (p-AKT), in the mPFC of rats after acute cocaine treatment [34]. Others found that extended access to cocaine self-administration (SA) increased p-ERK in the mPFC and NAc, immediately after the final SA dose [35]. Yet recently, it was reported that early cocaine withdrawal was associated with a significant decrease in p-ERK, but not p-AKT in the rat mPFC after an intraprefrontal cortical infusion of BDNF [36]. This research suggests that cocaine may have different effects on the same signaling pathway depending on the route of drug administration, the time post-drug, and the brain region studied. This may explain why we did not find any significant changes in the phosphorylation of AKT or ERK, in repeatedly cocaine-treated rats, after an in-vitro BDNF stimulation of hippocampal tissue.

Similarly, we found that EB treatment alone did not alter AKT phosphorylation (estrogen/saline group vehicle: 0.9942±0.1, with BDNF: 1.6973±0.2) (Figure 2). Although, as in the cocaine treated animals, pAKT levels appeared to be higher in the estrogen-treated groups after exposure to BDNF when compared to control rats (P=0.4) (figure 2). Phosphorylated ERK was not affected by estrogen replacement either (estrogen/saline vehicle: 1.009±0.3; with BDNF: 1.549±0.2, P=0.3) (Figure 3), a finding consistent with research reporting that estrogen activates AKT and ERK1/2 in a time-dependent manner. AKT and ERK1/2 activation was evident 5-10 minutes after EB exposure, reaching a maximal at 30 min [37]. Our groups received the last EB injection 24 hours prior to decapitation. Further research is necessary to determine the acute effects of cocaine and EB on BDNF stimulation-induced TrkB, AKT, and ERK phosphorylations.

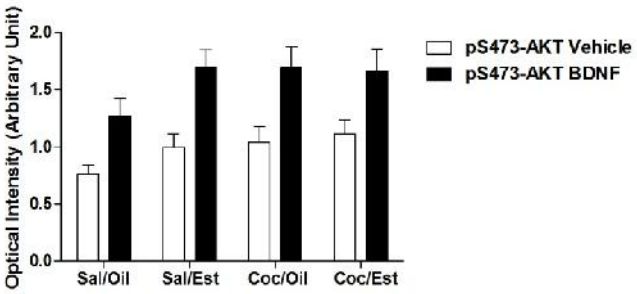

Fig 2. Signaling pathways downstream of Trk B were analyzed. Levels of pAKT, following BDNF stimulation of Trk B, were assessed. There is an apparent increase in pAKT levels in all three experimental groups as compared to the sal/oil control group. However, these changes do not reach significant levels for any of the groups. Data are expressed as Mean ± SEM obtained from 8 animals for each group (p>0.05).

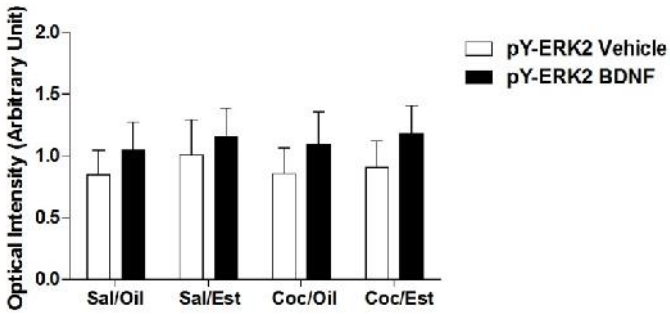

Fig 3. ERK signaling was assessed as indicated by levels of pY-ERK2. Following exogenous BDNF stimulation of the Trk B receptor, pY-ERK levels did not change significantly for any of the groups when compared to control: sal/oil group. Data are expressed as Mean ± SEM obtained from 8 animals for each group (p>0.05).

IV. Conclusion

Our results show that repeated cocaine treatment may increase neurotrophin activity by facilitating BDNF-induced Trk B activation; this suggests a possible increase in activity-dependent cellular adaptations in the hippocampus during prolonged cocaine exposure. Our data also indicate that EB attenuates cocaine-induced Trk B activation, which is consistent with a gender-sensitivity in the effects of cocaine. Although the nature of this interaction cannot be inferred from the present data there are several possibilities. If the interaction is on the same cell then EB may be acting at the genomic level to alter the sensitivity of BDNF signaling to cocaine at multiple levels. Alternatively the effects of cocaine and estrogen may be on different cells that converge at some point. While further investigations

are necessary to fully understand the interactions between cocaine and estrogen, the present results indicate that plastic changes in TrkB signaling may provide a substrate contributing to the sex differences seen in cocaine addiction, and suggest potential gender-based therapeutic strategies for treatments of cocaine addiction and related brain dysfunction.

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VI. References

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