PRENATAL COCAIN DYSREGULATES BDNF–TRKB AND $P75^{NTR}$ SIGNALING IN THE HIPPOCAMPUS AND PREFRONTAL CORTEX OF ADOLESCENT RATS

by

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Abstract

PRENATAL COCAINE DYSREGULATES BDNF-TRKB AND P75 SIGNALING IN THE HIPPOCAMPUS AND PREFRONTAL CORTEX OF ADOLESCENT RATS.

By

Andres Wood Stucky

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Brain-derived neurotrophic factor (BDNF) upregulates glutamatergic transmission and N-Methyl-D-Aspartate receptor (NMDAR) function through the activation of tropomyosin-related kinase receptor type B (TrkB). Conversely, NMDAR activation influences BDNF release. Because prenatal cocaine exposure can markedly alter glutamatergic transmission and NMDAR activation, we hypothesized that a dysregulation of the glutamatergic system following prenatal cocaine exposure could result in long-lasting alteration of TrkB signaling, thereby influencing the interaction between TrkB and glutamatergic NMDARs. In agreement with this hypothesis, we found that activated (i.e. tyrosine-phosphorylated) TrkB (pY-TrkB) levels in response to exogenous BDNF were increased in both the prefrontal cortex and hippocampus of 21-day-old rats that were exposed prenatally to cocaine. This cocaine-induced effect was corroborated by an elevated pY-TrkB-associated phospholipase, C-γ1, and adapter protein, Shc, as well as increases in downstream extracellular signal-regulated kinase 2 (ERK2) and PI3K signaling.

We report a significant decrease in the levels of BDNF released at the synapse of prenatal cocaine-exposed rats compared to control after NMDA and K+ stimulation and a marked increased affinity of receptor TrkB to its ligand BDNF. This suggests that

increased activation and signaling of TrkB in prenatal cocaine-exposed rats is the result of increased affinity of TrkB to BDNF, possibly as a functional compensation for decreased levels of activity-dependent BDNF released at the synapse. Moreover, we found a decreased activity of the p75 neurotrophin receptor($p75^{NTR}$) death-inducing pathways, as assessed by $p75^{NTR}$ recruitment of adaptor proteins TRADD, FADD, and TRAF2/6, and corroborated by decreased downstream Janus kinase 1 (JNK1) activation, as indicated by lower JNK1 phosphorylation (p-JNK1) levels.

Our data suggest that BDNF–TrkB and BDNF–/proBDNF–$p75^{NTR}$ activities are reduced following prenatal cocaine exposure due to a marked reduction in BDNF/Thrown Away proBDNF release. Given that neurotrophins and glutamate receptors interact to modulate the health and excitability of glutamatergic synapses, upregulation of BDNF–TrkB signaling and downregulation of BDNF-/proBDNF $p75^{NTR}$ pathways suggests a more efficient neurotrophin signaling in an attempt to reestablish synaptic homeostasis when supplies of BDNF are restored.

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Introduction

What is prenatal cocaine exposure? What are its effects?

Cocaine exposure during pregnancy causes abnormalities in the fetal brain development of exposed offspring (Thomphson et al. 2009; Campillo et al. 2004) and has been associated with postnatal cognitive and behavior function impairment of children exposed in utero (Singer et al. 2002;Morrow et al. 2006;McLaughlin et al. 2011). The underlying cellular mechanisms of these effects are not yet well understood, but they are thought to be mediated by long-term neurochemical and morphological adaptation in the striatum, cortex, and hippocampus of exposed animals (Bakshi et al. 2009; Stanwood and Levit 2007).A previous study from our lab has shown that prenatal cocaine exposure increases spine density in the prefrontal cortex and hippocampus of adolescent rats (Frankfurt et al. 2009). This is particularly significant during the critical period between gestation days 10 and 21 (Fujita et al.1993; Sanders et al.2005). In mice and rabbits Prenatal cocaine exposure can elicit deficits in glutamate transmission in the hippocampus and prefrontal cortex that could be associated with impairments in brain development, discriminative learning, and motor function (Romano et al. 1995; Romano and Harvey 1996; Stanwood et al. 2001; Brunzell et al. 2002; Bashkatova et al. 2005; Liu et al. 2010; Mameli et al. 2011). Altered glutamate transmission may play a critical role in mediating alpha-amino-3-hydroxy-5-methyl-4- isoxazole-propionic acid (AMPA) prenatal cocaine-induced learning and cognitive deficits.

Brain-derived neurotrophic factor (BDNF) upregulates glutamatergic transmission and N-Methyl-D-Aspartate receptor (NMDAR) function through the activation of

tropomyosin-related kinase type B (TrkB) receptor which will catalyze NMDA receptors phosphorylation and activity dependent BDNF release.

Because BDNF/TrkB signaling plays I important modulator of glutamatergic synaptic maintenance and plasticity, it is increasingly apparent that BDNF signaling is critical to modulating the long-term effects of prenatal cocaine exposure.

Molecular correlates of learning and memory are associated with drug addiction

A substantial body of evidence suggests that prenatal cocaine exposure causes dysregulation-of glutamate neurotransmission, cell excitability and long-term synaptic potentiation, or LTP (Bellone et al.2011; McKee and Meshul Thrown Away 2005; Pierce et al. 1996). At the molecular level, both developmental and cocaine-induced plasticity are mediated by regulating the excitability of active glutamatergic receptors and their traffic to the membrane. Early synaptic activity stimulates plasticity by controlling the synaptic function and the abundance of NMDA and AMPA of glutamate receptors (Collingridge and Bliss 1995; Malinow and Malenka, 2002). Data indicates that prenatal cocaine exposure causes an increase in NMDA receptor density in rat cortical membranes (Yablonsky-Alter et al. 2005) and reduces synaptic targeting of AMPA receptors containing GluR2/3 subunits in the frontal cortex (Bakshi et al. 2009) Recently, similar observations have been made regarding the shorter, postnatal effects of cocaine on the NMDAR/AMPAR functional ratio in the ventral tegmental area (Mameli et al 2011). Since sustained glutamatergic activity stimulates synaptic release of BDNF and in turn activity dependent BDNF release initiates growth and survival signals that go on to promote transcription-dependent synaptic accommodation, the formation of new synapses, and the establishment of existing ones (Turrigiano 2007), we wanted to see if

BDNF–TrkB signaling is altered by prenatal cocaine exposure.

Addiction-mediated synaptic plasticity involves BDNF signaling

BDNF binds with high specificity toTrkB and to the low-affinity neurotrophin receptor $p75^{NTR}$. Recently, BDNF and its complementary, TrkB, a member of the neurotrophin receptor tyrosine kinase family, have been identified as critical upstream regulators of LTP (Lu et al. 2008) and synaptic plasticity (Pang et al. 2004; Carvalho et al. 2008; Kuczewski et al. 2010; Bramham and Wells 2007). TrkB exists as 145KD, a fully functional protein, and as 95KDa in its truncated form, lacking the catalytic kinase domain. TrkB is widely expressed throughout the brain, mainly in the striatum (Riquelme et al. 2012) brainstem (Liu and Wong-Riley 2012), cortex, hippocampus (Braun et al. 2012; Callaghan and Kelly 2012), and spinal cord (Lin et al. 2011; Huie et al. 2012). The induction of either LTP or long-term depression (LTD) in the hippocampus requires TrkB activation by BDNF as well as the activation of NMDA receptors (Lu et al. 2008). BDNF signaling involves activation of both PI3K-AKT and protein kinase C (or the specific protein kinase Mζ Active TrkB also recruits the NMDAR-scaffolding protein PSD-95 from the Golgi and consequently brings AMPA-Rs to the plasma membrane (Yoshii and Constantine-Paton 2007, 2010; Yoshii et al. 2011). All these events converge to facilitate the generation of drug-induced sensitization. Additionally, BDNF-activated TrkB can also associate with NMDA receptors to increase its open probability (Turrigiano 2007) Together NMDA and TrkB activation converge to trigger late-phase LTP, which could bring about the long-term effects of cocaine (Messaoudi et al. 2002; Bramham and Wells 2007).

Binding of BDNF to TrkB causes receptor dimerization and activation triggered by the cross phosphorylation of its intracellular tyrosine kinase domain (Binder and Scharfman 2004). Phosphorylated tyrosine residue in the kinase domain later serves as an anchoring site for Src homology domain-containing adaptor proteins Shc, N-Sch and phospholipase Cγ1 (PLCγ1), which are the three major branches of TrkB signaling (Roux and Barker 2002). Adaptor protein N-Sch binds with Grab2/Sos and initiates cell survival through activation of downstream kinases Ras-Raf and extracellular signal-regulated kinase 2(ERK2). Sch association with insulin receptor substrate 1 activates phosphatidylinositiol 3kinase (PI3K), phosphatidyl dependent kinase and protein kinase B, which depending on the cellular environment help promote early gene expression and synaptic plasticity (Arévalo and Wu 2006). Finally PLCγ1 activation cleaves membrane bound inositol phosphate and provides a diacylglycerol docking site for protein kinase Cat the membrane which can then be activated by increasing intracellular Ca^{2+} via inositol phosphate 3. The upregulation of these pathways after cocaine exposure links BDNF–TrkB to drug-induced plasticity (Pu et al. 2006; Russo et al. 2009). Indeed, levels of BDNF are altered in the hippocampus and prefrontal cortex of rats after chronic cocaine administration (Sadri-Vakili et al. 2010). Furthermore, evidence suggests that BDNF-induced LTP underlies long-lasting changes that are critical to cocaine-seeking behaviors (Pu et al. 2006).

$p75^{NTR}$ can modulate TrkB signaling and influence neuronal death or survival outcomes

The low-affinity pan-neurotrophin receptor $p75^{NTR}$ is a member of the tumor

necrosis factor (TNFR) death receptor super family $p75^{NTR}$ is widely expressed in both neurons and glia cells, and is usually found in clusters of homotrimers, but can also dimerize and partner with filamin or with TrkB receptors to modulate signal outcomes (Nykjaer et al. 2005). Like TrkB, $p75^{NTR}$ has an extracellular cysteine rich domain which accounts for neurotrophin ligand specificity (Hsu et al. 1993). However, unlike the tyrosine kinases, $p75^{NTR}$ has no intrinsic catalytic activity. Instead it has a 60–70 amino acid intracellular death domain (Ashkenazi and Dixit 1998) by which it interacts with a series of death domain-containing proteins to modulate cell death or survival depending on its associated partners (Roux and Barker 2002). To date $p75^{NTR}$ has been shown to interact with at least 15 partners to modulate the nuances of its downstream signaling pathways, however $p75^{NTR}$ is best known for eliciting process retraction and cell programmed cell death.

A recent study has indicated that the uncleaved 32 kDa neurotrophin precursor proBDNF may also activate a cell signaling cascade distinct from that of mature BDNF or mBDNF (Lee et al. 2001) $p75^{NTR}$ not only responds to mature (cleaved) neurotrophins, but will also bind unprocessed proneurotrophins with a much higher affinity. Shortly after ligand binding, $p75^{NTR}$ death domain recruits TNFR-associated death domain (TRADD). TRADD can then function as a receptor-coupling platform for an assortment of death domain and tumor necrosis factor (TNF)-associated effector proteins (Hsu et al. 1996). TRADD association can activate two sequential pathways. TNF-associated effector protein (TRAF) recruitment to the receptor complex activates RIP1- MEK7 and the stress-induced kinase janus (JNK) which provoke dendrite retraction and discourages cell growth, while recruitment of Fasassociated death domain (FADD) induces

dimerization of RIP1 and RIP3 which will dissociate the complex and initiate Caspase activation and programmed cell death.

Until now the specific effects of prenatal cocaine on $p75^{NTR}$ signaling have not been studied. It has been shown that $p75^{NTR}$ expression is downregulated in the nucleus accumbens and caudate putamen after chronic intravenous cocaine self-administration (Smith et al. 2004). Here we report a decrease in activity of $p75^{NTR}$ death-inducing pathways as assessed by decreased $p75^{NTR}$ recruitment of adaptor proteins TRADD, FADD, and TNF receptor-associated factors 2 and 6 (TRAF2 and TRAF6). This effect was corroborated by decreased downstream JNK1 activation, as indicated by lower JNK1 phosphorylation (p-JNK1) levels. Since neurotrophin and glutamate receptors interact to modulate the health and excitability of synapses, upregulation of BDNF–TrkB signaling and downregulation of BDNF-/proBDNF $p75^{NTR}$ pathways suggest an effort to improve the efficiency of neurotrophin signaling in an attempt to regain synaptic homeostasis when supplies of BDNF are restored.

Effect of prenatal cocaine exposure on BDNF-TrkB

In this study we investigated how prenatal cocaine exposure affects both mediated TrkB and $p75^{NTR}$ signaling as well as TrkB-NMDAR interaction in the prefrontal cortex (PFCX) and hippocampus of 21-day-old rats. We show that prenatal cocaine exposure elevates BDNF–TrkB signaling in rats, indicated by higher tyrosine-phosphorylated TrkB levels and an increase in TrkB-associated PLC-γ1 and adaptor proteins Shc/N-Shc. We also show increased downstream ERK2 and Akt phosphorylation in addition to increased TrkB-NMDAR association in both the hippocampus and PFCX of prenatal cocaine-

exposed rats after stimulation with BDNF. Subsequently, we wanted to determine whether prenatal cocaine could affect the functional release of BDNF from synapses after simulation with NMDA/ Glycine and depolarization by K+. Notably

Experimental Results

Prenatal cocaine exposure enhances BDNF–TrkB signaling

We first tested whether prenatal exposure to cocaine alters TrkB activation in the hippocampus and PFCX of P21 rats. Prenatal cocaine exposure did not affect the expression of the 145-KDa (full-length) and 95-KDa (truncated) forms of TrkB. In response to exogenous 50 ng/ml of BDNF, the levels of activated (tyrosine-phosphorylated, pY) 145- and 95-KDa TrkB were increased in both hippocampus and PFCX. In the prenatal cocaine exposed group, the level of BDNF-induced pY-145-KDa TrkB was 122% and 96% higher in the hippocampus and PFCX, respectively, than in the saline-exposed group (Fig 1). In contrast, levels of BDNF-induced pY-95-KDa TrkB were similar in prenatal cocaine- and saline-exposed brains (Fig 1).

The finding of a higher BDNF-induced pY-145-KDa in prenatal cocaine exposed brains was substantiated by higher levels of BDNF-induced recruitment of phospholipase C-γ1 (173% and 86% in hippocampus and PFCX, respectively) and adaptor proteins Shc (70% and 50% in hippocampus and PFCX, respectively) and N-Shc (91% and 42% in hippocampus and PFCX, respectively) to specific pY-TrkB docking sites (Fig 2). A similar conclusion was also derived from the higher levels of downstream signaling pathways of TrkB. In accord, BDNF-induced PI3K and ERK2 activation was indicated by the higher pS^{473}Akt (55% and 73% in hippocampus and PFCX, respectively; Fig 3a) and pY/pTERK2 (59% and 59% in hippocampus and PFCX, respectively; Fig 3b) in prenatal cocaine-exposed brains when compared to saline-treated group. Given that BDNF–TrkB activation regulates long-term synaptic plasticity in cooperation with

NMDARs, we measured the levels of obligatory NMDAR subunit -NR1, co-immunoprecipitated with TrkB after BDNF receptor activation. Our results show that BDNF stimulation increased the level of TrkB–NMDAR association in brain regions from both prenatal cocaine- and saline-exposed P21 rats as indicated by a marked increase in NR1 in anti-TrkB immunoprecipitates. This BDNF-induced effect was increased by 56% and 52% in the hippocampus and PFCX, respectively by prenatal cocaine exposure (Fig 4). Collectively, these data indicate that prenatal cocaine exposure heightens the efficacy of BDNF–TrkB signaling and improves the proteins' interactions with the NMDAR complexes.

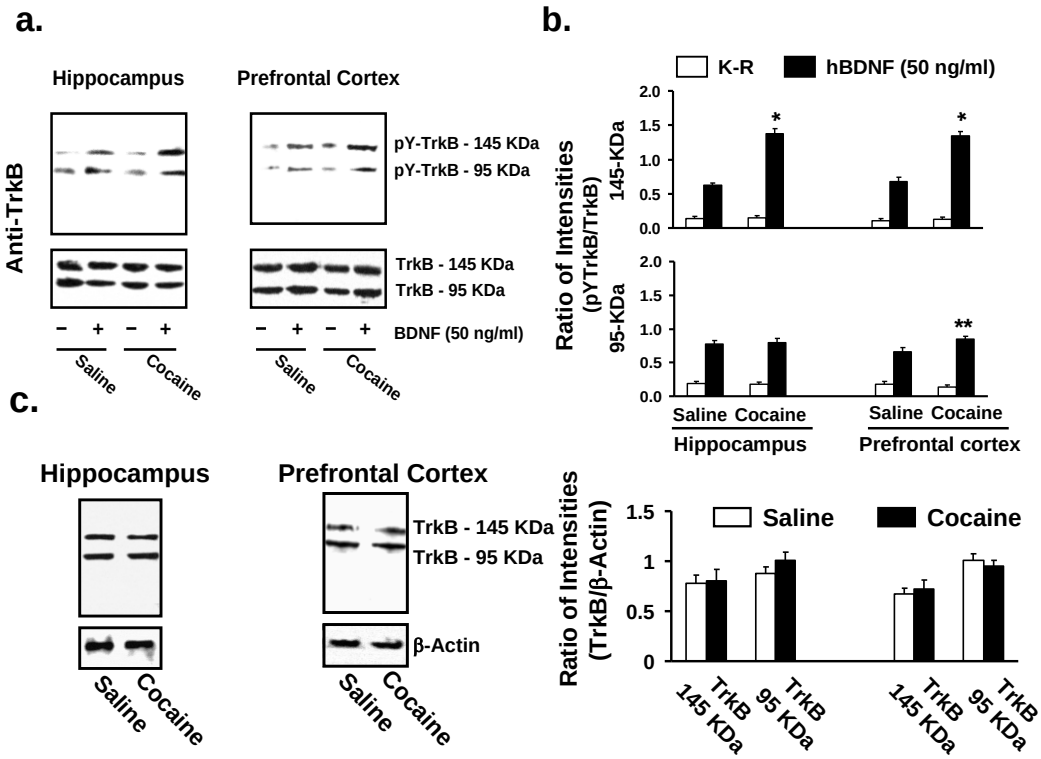


Fig.1 (*a*) The effect of prenatal cocaine exposure on TrkB activation was assessed in hippocampal and PFCX slices from P21 prenatal cocaine- and saline-exposed rats. Brain slices (100 μm x 100 μm x 3 mm) were incubated with 50 ng/ml recombinant human BDNF at 37°C for 30 min. In a preliminary experiment, 50 ng/ml of BDNF was shown to be the ED50 for activating TrkB. The level of activated (tyrosine-phosphorylated [pY]) TrkB in anti-TrkB immunoprecipitate was determined by Western blotting. Blots were stripped and re-probed with anti-TrkB to validate equal loading. Our results show that tyrosine phosphorylation of both the full-length (145-KDa) and truncated (95-KDa) was markedly increased in response to BDNF stimulation in prenatal saline- and cocaine-treated rats. (*b*) *Densitometric quantification of blots*. The data are expressed as the ratios of pY-TrkB optical intensity normalized by the optical intensity of total TrkB. n = 6. Data are means ± s.e.m. of the ratio of pY-TrkB to TrkB optical intensities. *$p < 0.01$, **$p < 0.05$ compared to respective protein in the saline-treated group. (*c*) The effect of prenatal cocaine exposure on TrkB expression was determined in hippocampal and PFCX slice lysate from P21 prenatal cocaine- and saline-exposed rats. Blots were stripped and re-probed with anti-β-actin. Densitometric quantification of blots revealed no discernible differences in both 145- and 95-KDa TrkB expression levels. n = 4. Data are mean ± s.e.m. of the ratio of 145- or 95-KDa TrkB to β-actin optical intensities.

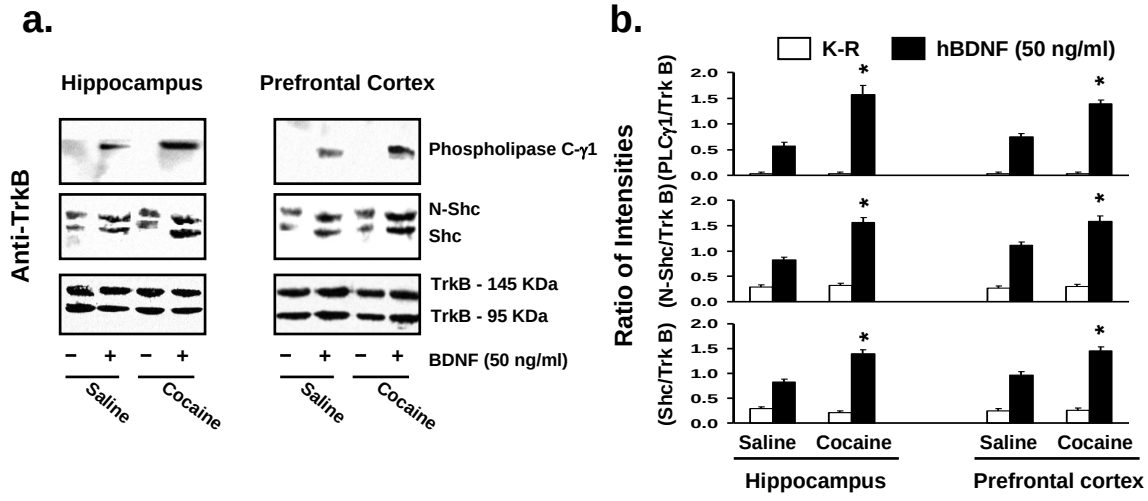


Fig. 2 (*a*) The effect of prenatal cocaine exposure on TrkB activation was evaluated by the recruitment of the downstream signaling molecules, phospholipase C-γ1 (PLC-γ1), and various adaptor proteins (Shc and N-Shc) to activated TrkB in hippocampal and PFCX slices from P21 prenatal saline- and cocaine-treated rats. The levels of PLC-γ1, N-Shc, and Shc in the anti-TrkB were determined by Western blotting. Blots were stripped and re-probed with anti-TrkB to validate equal loading. Our results show that BDNF-induced TrkB activation is accompanied by an increase in PLC-γ1, Shc, and N-Shc levels in the anti-TrkB immunoprecipitate in both the hippocampus and PFCXof P21 prenatal saline- and cocaine-treated rats. (*b*) *Densitometric quantification of blots*. The data are expressed as the ratios of PLC-γ1, N-Shc, and Shc optical intensity normalized by the optical intensity of total TrkB. n = 6. Data are means ± s.e.m. of the ratios of PLC-γ1, N-Shc, or Shc to TrkB optical intensities. *$p < 0.01$, compared to respective protein in the saline-treated group.

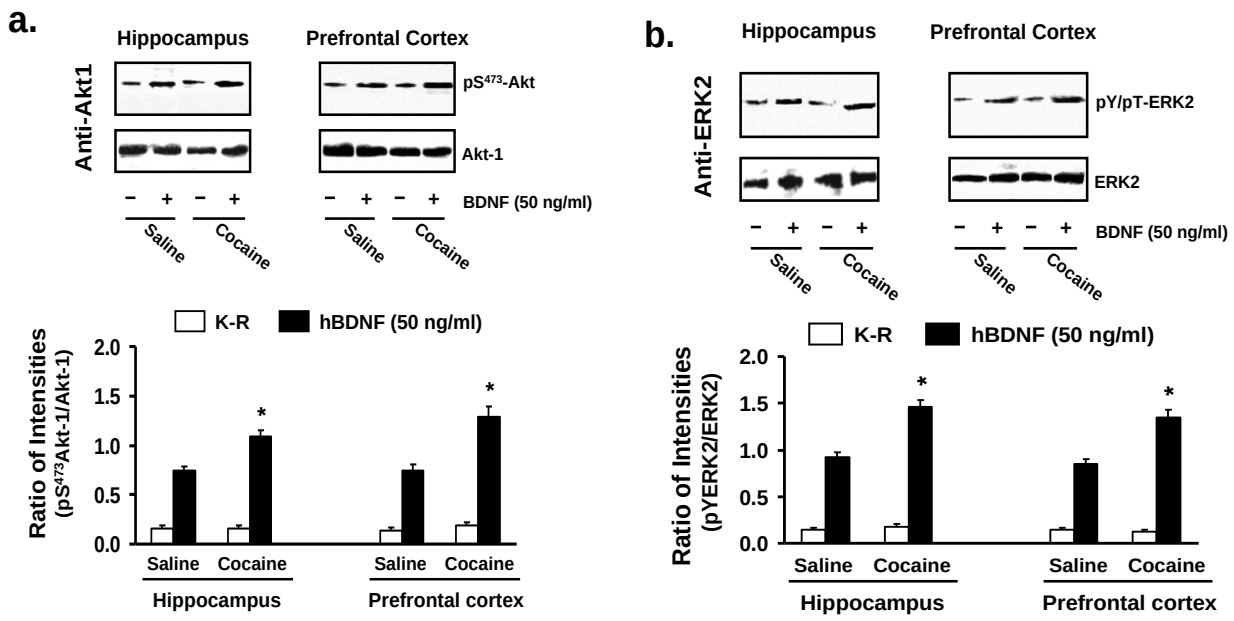


Fig. 3 (*a*) The effect of prenatal cocaine exposure on TrkB activation was evaluated by the activation of the PI3K signaling downstream of TrkB in hippocampal and PFCX slices from P21 prenatal saline- and cocaine-treated rats. The level of activated (pS^{473})Akt1 in the anti-Akt1 was determined by Western blotting. Blots were stripped and re-probed with anti-Akt to validate equal loading. Our results show that BDNF increased pS^{473}-Akt in both hippocampus and prefrontal cortices of P21 prenatal saline- and cocaine-treated rats. *Densitometric quantification of blots*. The data are expressed as the ratios of pS^{473}-Akt1/Akt1 optical intensities. n = 6. Data are means ± s.e.m. of the pS^{473}-Akt1/Akt1 optical intensities. (*b*) The effect of prenatal cocaine exposure on TrkB activation was evaluated by the activation of the ERK2 signaling downstream of TrkB in hippocampal and PFCX slices from P21 prenatal saline- and cocaine-treated rats. The level of activated (pY/pT)ERK2 in the anti-ERK2 was determined by Western blotting. Blots were stripped and re-probed with anti-Akt to validate equal loading. Our results show that BDNF increased pY/pT-ERK2 in both hippocampus and PFCX of P21 prenatal saline- and cocaine-treated rats. *Densitometric quantification of blots*. The data are expressed as the ratios of pY/pT-ERK2/ERK2 optical intensities. n = 6. Data are means ± s.e.m. of the ratio of pY/pT-ERK2 to ERK2 optical intensities. *$p < 0.01$, compared to respective protein in the saline-treated group.

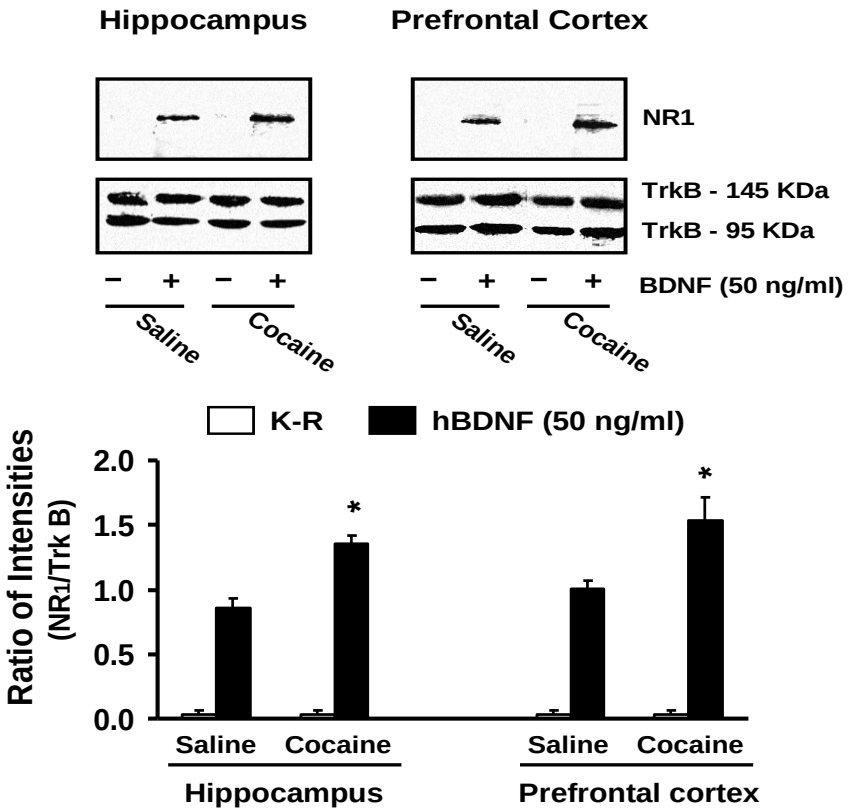


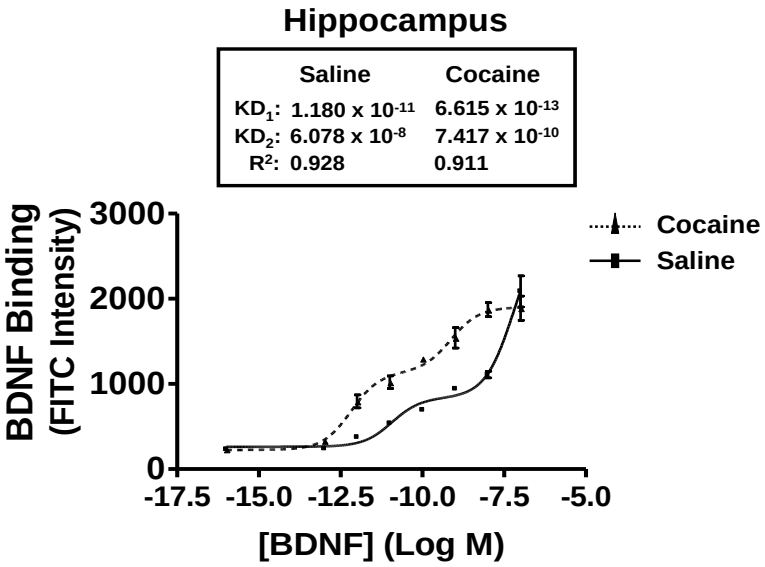
Fig.4 (*top*) The effect of prenatal cocaine exposure on TrkB–NMDAR interaction was assessed by the level of obligatory subunit of the NMDARs, NR1, to activated TrkB in hippocampal and PFCX slices from P21 prenatal saline- and cocaine-treated rats. Levels of NR1 in the anti-TrkB were determined by Western blotting. Blots were stripped and re-probed with anti-TrkB to validate equal loading. Our results show that BDNF promoted NMDAR–TrkB association, as evidenced by increased NR1 levels in the anti-TrkB immunoprecipitate in both hippocampus and PFCX of P21 prenatal saline- and cocaine-treated rats. (*bottom*) *Densitometric quantification of blots*. The data are expressed as the ratios of NR1 optical intensity normalized by the optical intensity of total TrkB. n = 6. Data are means ± s.e.m. of the NR1/TrkB optical intensities. *$p < 0.01$, compared to respective protein in the saline-treated group. This may eventually reshape the synaptic plasticity in prenatal cocaine-exposed brains.

Improved BDNF binding affinity for TrkB is responsible for prenatal cocaine-induced increases in BDNF–TrkB signaling

Next, we attempted to resolve the underlying mechanism responsible for the increased BDNF–TrkB signaling (Fig 1–3) and heightened BDNF–TrkB/NMDARs interaction. In light of the fact that prenatal cocaine exposure did not affect TrkB expression level, we tested whether prenatal cocaine exposure affects the affinity of BDNF for TrkB. Using biotinylated surface receptors as the tissue source, we performed a modified binding assay and measured the BDNF binding with fluorescence detection.

We found that the BDNF saturation curves in the brain areas from both prenatal cocaine- and saline-exposed P21 rats fit significantly better when the nonlinear regression curve-fit algorithm assumed the presence of two saturation sites rather than one presumable reflecting the presence of TrkB and $p75^{NTR}$ ($r^2 > 0.911$). Calculated *K*d values for the prenatal saline-exposed brains were 11.8 pM and 60.8 nM in the hippocampus and 3.9 pM and 21.9 nM in the PFCX. Prenatal cocaine exposure increased BDNF binding affinities at the high-affinity binding site by 17.8-and 15-fold in hippocampus and PFCX, respectively, to 0.66 pM and 0.26 pM. Moreover, prenatal cocaine exposure improved the BDNF affinities at the low-affinity binding sites by 80.1- and 12-fold in the hippocampus and PFCX, respectively, to 0.74 nM and 1.86 nM (Fig 5). The data strongly suggest that an improved BDNF binding affinity is responsible for more efficacious BDNF–TrkB signaling in prenatal cocaine-exposed brains.

a.


Hippocampus
Saline Cocaine
KD1: 1.180 x 10-11 6.615 x 10-13
KD2: 6.078 x 10-8 7.417 x 10-10
R2: 0.928 0.911
BDNF Binding (FITC Intensity)
3000
2000
1000
0
-17.5 -15.0 -12.5 -10.0 -7.5 -5.0
[BDNF] (Log M)
Cocaine
Saline

b.

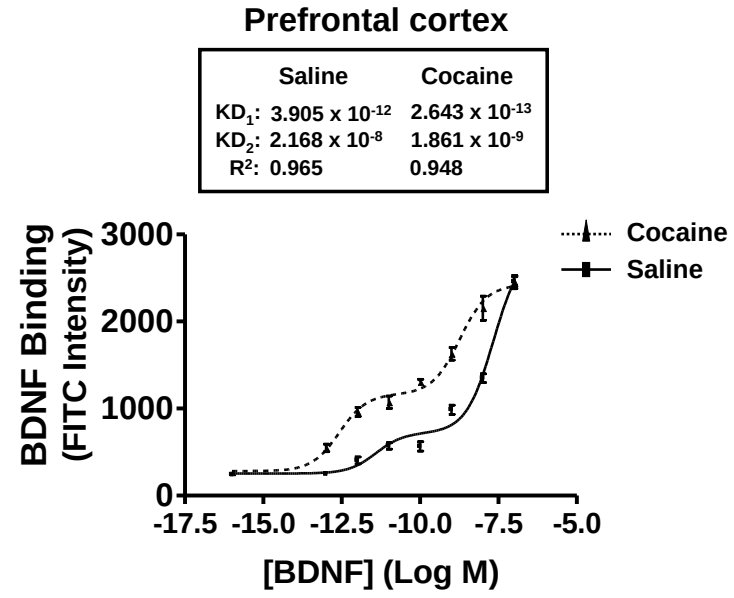

Prefrontal cortex
Saline Cocaine
KD1: 3.905 x 10-12 2.643 x 10-13
KD2: 2.168 x 10-8 1.861 x 10-9
R2: 0.965 0.948
BDNF Binding (FITC Intensity)
3000
2000
1000
0
-17.5 -15.0 -12.5 -10.0 -7.5 -5.0
[BDNF] (Log M)
Cocaine
Saline

Fig 5. The effect of prenatal cocaine exposure on the interaction of BDNF with TrkB was assessed by a ligand binding assay. Synaptic membranes were prepared from both hippocampi (*a*) and PFCX (*b*) of P21 prenatal cocaine- and saline-exposed rats. The membrane-bound proteins were first biotinylated using a biotinylation kit (Pierce). Following detergent solubilization and then dilution, the biotinylated surface proteins were coated onto streptavidin-coated plates (Reacti-BindTM NeutrAvidinTM High binding capacity coated 96-well plates). Plates were washed and incubated at 30°C with K-R, and BDNF (100 fM – 10 nM) was added for 1 hr. The plate was washed and then sequentially incubated with anti-BDNF followed by FITC-conjugated anti-rabbit IgG. Plates were washed and the residual FITC signals were determined by multimode plate reader, DTX880 (Beckman). Nonlinear regression data curve fit was performed using Prism. Data points are means and vertical bars are the s.e.m. derived from six independent rats in each treatment group.

Conformational change in TrkB as reflected by a shifted isoelectric point in prenatal cocaine-exposed brains

To determine whether conformational changes in TrkB in the prenatal cocaine-exposed brains contribute to BDNF's augmented binding affinity for TrkB, we individually isolated TrkB immunoprecipitation with immobilized anti-TrkB from the hippocampus and PFCX lysate of prenatal cocaine- and saline-treated P21 rats. Following elution, the TrkB-containing eluate was then passed through a 100-KDa molecular weight cut-off filter to remove 95-KDa, the truncated form of TrkB. The TrkB receptor's isoelectric point (pI) was determined by separation on isoelectric-focusing gels followed by Western blotting. The results summarized in Figure 6 indicate that prenatal cocaine exposure causes a shift in TrkB's calculated pI from 6.1 to 6.9. The data therefore suggest that conformational changes in TrkB contribute to the observed increase in BDNF affinity for TrkB.

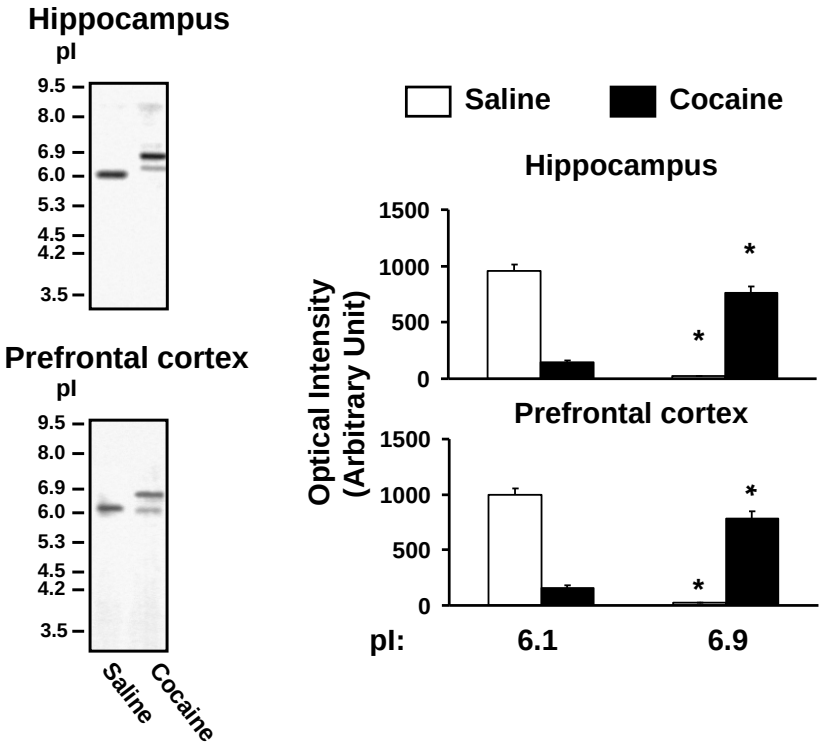


Fig. 6. The effect of prenatal cocaine exposure on 145-KDa TrkB conformation in PFCX (*top)* and hippocampal (*bottom*) slices prepared from P21 prenatal cocaine- and saline-treated rats. Synaptic membranes were prepared from approximately 10 mg PRCX and hippocampus. The membranes were then solubilized, treated with 1% SDS for 1 min, diluted, and then immunoprecipitated with anti-TrkB. The obtained immunoprecipitate was solubilized and passed through a 100-KDa cut-off filter. Proteins retained on the filter were separated on pH3-10 isoelectric focusing gels and then Western blotted with anti-TrkB. Blots were quantified by densitometric scanning. Data are means ± s.e.m. of the pI 6.1 and pI 6.9. $n = 5$. $p < 0.01$ compared to respective protein in the saline-treated group.

Prenatal cocaine decreases endogenous proBDNF and BDNF release

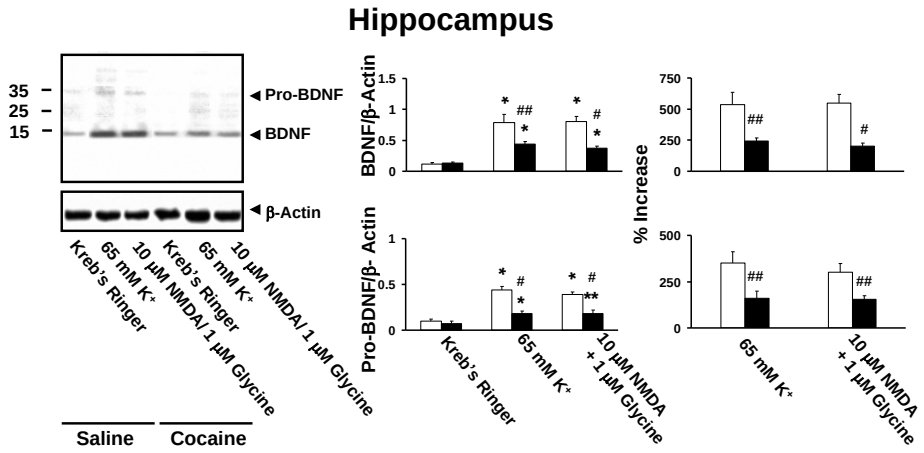
BDNF–TrkB signaling is an important regulator of long-term plasticity and function at glutamatergic synapses. In turn, BDNF release is triggered and regulated by glutamatergic synaptic activity. BDNF can be released from both presynaptic terminals and postsynaptic dendritic fields (Lessmann and Brigadski, 2009 Kuczewski et al. 2010). To further probe the underlying mechanism responsible for upregulated BDNF–TrkB signaling, we determined BDNF and probing expression levels, as well as the levels of BDNF/proBDNF released from both presynaptic terminals and postsynaptic dendritic fields using K^+-depolarization and NMDA/glycine. Endogenous BDNF release was evoked by 10 μM NMDA/1 μM glycine, presumably from postsynaptic dendritic field, or by 65 mM K^+-depolarization, presumably from the presynaptic axonal terminals in both the hippocampus and PFCX (Fig 7). Prenatal cocaine exposure did not have a discernible effect on proBDNF or BDNF expression nor on spontaneous BDNF and proBDNF efflux in either brain region. K^+-depolarization and NMDA/glycine markedly increased BDNF and proBDNF in the perfusate by approximately 6- and 4-fold, respectively, in the hippocampus and PFCX of the prenatal saline-exposed control rats. Conversely, BDNF and proBDNF release was reduced by 50–60% in the prenatal cocaine-exposed brains (Fig 7). Observed levels of the 32-kD proBDNF were significantly lower than the 14-kD mBDNF form, indicating that most of the proBDNF was converted to mBDNF within the time frame of the test.

To further ascertain the effect of prenatal cocaine exposure on the release of proBDNF, brain slices were first incubated with metaloprotease and tissue plasminogen activator (tPA)/serine protease inhibitors. MMP-9 and tPA are the primary proteases that

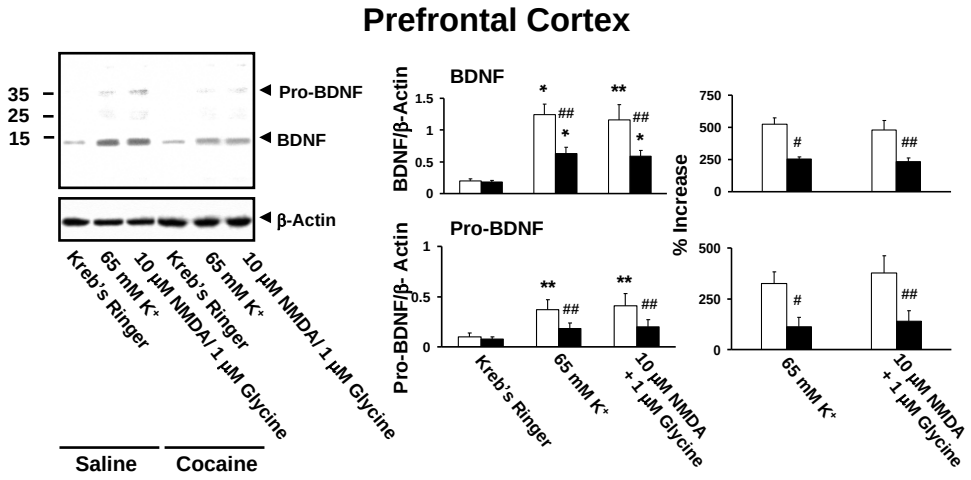
convert proBDNF to mBDNF. Similarly, we induced proBDNF release by K^+-depolarization and 10 μM NMDA/1 μM glycine. K^+-depolarization and NMDA/glycine markedly increased proBDNF in the perfusate by approximately 3.5- to 5.5-fold in the hippocampus and PFCX of prenatal saline-exposed P21 control rats. Compared to the saline-exposed controls, prenatal cocaine exposure decreased proBDNF release by 51–55% (Fig. 8).

tPA activates plasminogen, a serine protease that breaks down proBDNF to mBDNF. Although the ratio of proBDNF and BDNF released were comparable between prenatal cocaine- and saline-exposed rats, the tPA levels in both brain regions were measured by Western blotting to further confirm that the reduction in BDNF release found in the prenatal cocaine-exposed brains was not caused by an alteration in tPA levels. The data summarized in Figure 9 indicate that there were no discernible changes in tPA levels in the hippocampus and PFCX of prenatal cocaine- and saline-exposed rats.

a.


Hippocampus
35
25
15
Pro-BDNF
BDNF
β-Actin
Kreb's Ringer
65 mM K+
10 μM NMDA/ 1 μM Glycine
Kreb's Ringer
65 mM K+
10 μM NMDA/ 1 μM Glycine
Saline
Cocaine
BDNF/β-Actin
Pro-BDNF/β- Actin
% Increase
Kreb's Ringer
65 mM K+
10 μM NMDA + 1 μM Glycine
65 mM K+
10 μM NMDA + 1 μM Glycine

b.


Prefrontal Cortex
35
25
15
Pro-BDNF
BDNF
β-Actin
Kreb's Ringer
65 mM K+
10 μM NMDA/ 1 μM Glycine
Kreb's Ringer
65 mM K+
10 μM NMDA/ 1 μM Glycine
Saline
Cocaine
BDNF
BDNF/β-Actin
Pro-BDNF
Pro-BDNF/β- Actin
% Increase
Kreb's Ringer
65 mM K+
10 μM NMDA + 1 μM Glycine
65 mM K+
10 μM NMDA + 1 μM Glycine

c.

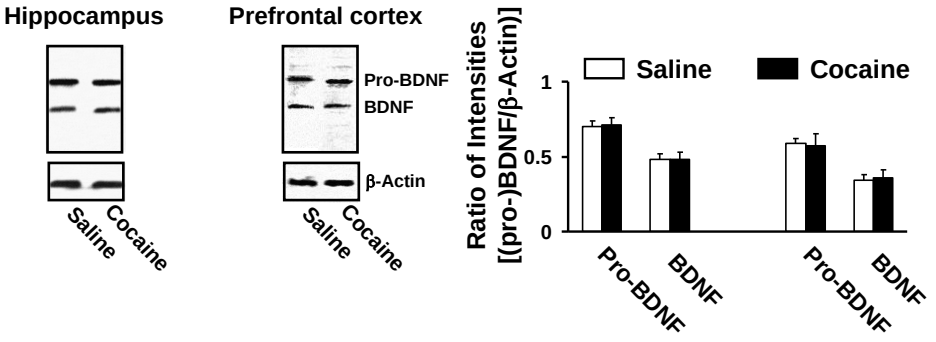

Hippocampus
Prefrontal cortex
Pro-BDNF
BDNF
β-Actin
Saline
Cocaine
Saline
Cocaine
Ratio of Intensities [(pro-)BDNF/β-Actin)]
Saline
Cocaine
Pro-BDNF
BDNF
Pro-BDNF
BDNF

Fig 7. The effect of prenatal cocaine exposure on BDNF release was determined in hippocampal (*a*) and PFCX (*b*) slices prepared from P21 prenatal cocaine- and saline-treated rats. Brain slices (100 μm x 100 μm x 3 mm) were superfused with 0.2 ml/min 0.3 mM Mg^{2+}-containing Kreb's-Ringer (LMKR) and 10 μM NMDA/1 μM glycine in LMKR for 30 min, or 65 mM K^{+}-depolarization for 10 min followed by 20 min of LMKR at 37°C. BDNF and proBDNF in the perfusate were then immunoprecipitated with immobilized anti-BDNF and determined by Western blotting. The brain slices were collected, homogenized, and solubilized, and the level of actin in the brain slices was determined by Western blotting. The blots were quantified by densitometric scanning. Data are means ± s.e.m. of the ratios of BDNF or proBDNF optical intensity to the optical intensity of actin. n = 6. **$p < 0.05$, *$p < 0.01$ compared to LMKR-treated in the same group. ##$p < 0.05$, #$p < 0.01$ compared to respective protein in the saline-treated group. (*c*) The effect of prenatal cocaine exposure on proBDNF/BDNF expression was determined in hippocampal and PFCX slice lysate from P21 prenatal cocaine- and saline-exposed rats. Blots were stripped and re-probed with anti-β-actin. Densitometric quantification of blots revealed no discernible differences in proBDNF and BDNF expression levels. n = 4. Data are mean ± s.e.m. of the ratio of proBNDF or BDNF to β-actin optical intensities.

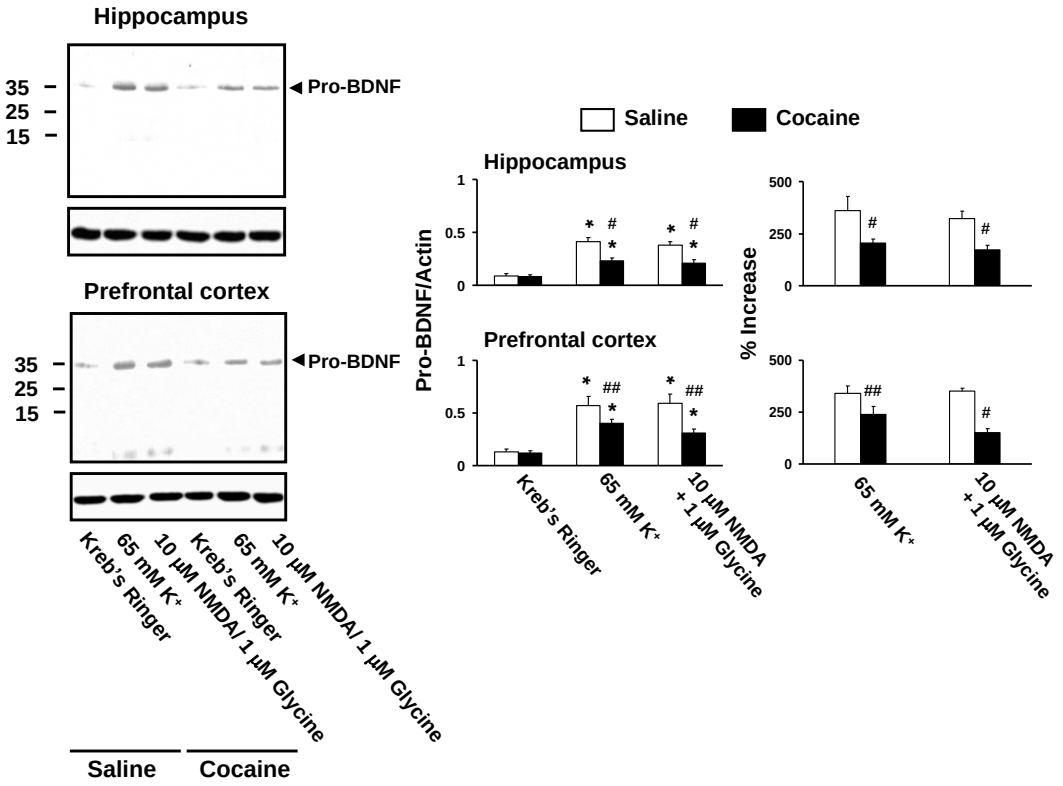


Fig 8. The effect of prenatal cocaine exposure on BDNF release was determined in hippocampal (*a*) and PFCX (*b*) slices prepared from P21 prenatal cocaine- and saline-treated rats. Brain slices (100 μm x 100 μm x 3 mm) were superfused with 0.2 ml/min LMKR for 30 min (spontaneous efflux) or with 10 μM NMDA/1 μM glycine in LMKR or 65 mM K^+-depolarization for 10 min followed by 20 min of LMKR at 37°C. BDNF and proBDNF in the perfusate were then immunoprecipitated with immobilized anti-BDNF and determined by Western blotting. The brain slices were collected, homogenized and solubilized and the level of β-actin in the brain slices was determined by Western blotting. The blots were quantified by densitometric scanning. Data are means ± s.e.m. of the ratios of BDNF or proBDNF optical intensity to the optical intensity of actin. n = 6. **$p < 0.05$, *$p < 0.01$ compared to LMKR-treated in the same group. ##$p < 0.05$, #$p < 0.01$ compared to respective protein in the saline-treated group. (*c*) The effect of prenatal cocaine exposure on proBDNF/BDNF expression was determined in hippocampal and PFCX slice lysate from P21 prenatal cocaine- and saline-exposed rats. Blots were stripped and re-probed with anti-β-actin. Densitometric quantification of blots revealed no discernible differences in both proBDNF and BDNF expression levels. n = 4. Data are mean ± s.e.m. of the ratio of proBDNF or BDNF to β-actin optical intensities.

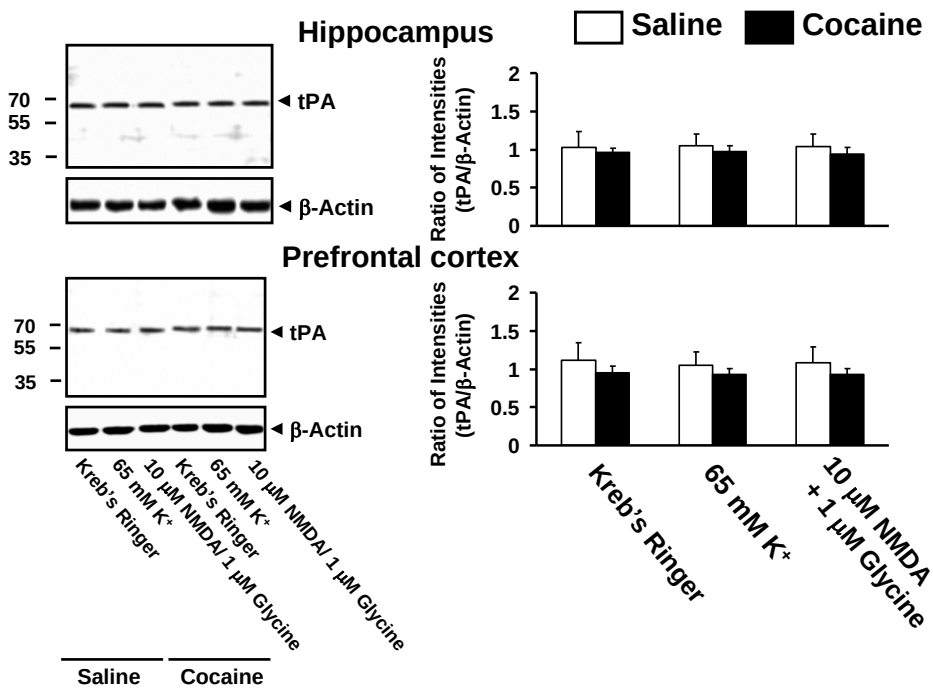


Fig 9. The effect of prenatal cocaine exposure on proBDNF release was determined in hippocampal (*top*) and PFCX (*bottom*) slices prepared from P21 prenatal cocaine- and saline-treated rats. Brain slices (100 μm x 100 μm x 3 mm) were superfused with 0.2 ml/min LMKR for 30 min (spontaneous efflux) or with 10 μM NMDA/1 μM glycine in LMKR or 65 mM K^+-depolarization for 10 min followed by 20 min of LMKR at 37°C. BDNF and proBDNF in the perfusate were then immunoprecipitated with immobilized anti-BDNF and the level of proBDNF was determined by Western blotting with specific anti-proBDNF. The brain slices were collected, homogenized, and solubilized and the level of actin in the brain slices was determined by Western blotting. The blots were quantified by densitometric scanning. Data are means ± s.e.m. of the ratios of proBDNF optical intensity to the optical intensity of actin. n = 5. **$p < 0.05$, *$p < 0.01$ compared to LMKR-treated in the same group. ##$p < 0.05$, #$p < 0.01$ compared to respective protein in the saline-treated group.

Prenatal cocaine exposure reduces BDNF–/proBDNF–p75^{NTR} signaling

In addition to binding to TrkB with a high affinity, thereby transmitting pro-survival signaling and improving synaptic plasticity, BDNF also binds to p75^{NTR} with a lower affinity, thereby promoting death signaling and reducing synaptic efficiency. To this end, we tested the effect of prenatal cocaine exposure on BDNF-/proBDNF-induced p75^{NTR} signaling in the hippocampus and PFCX of P21 rats.

Prenatal cocaine exposure did not affect p75^{NTR} levels, as indicated by the similar levels of p75^{NTR} immunoprecipitated by anti- p75^{NTR} in both the experimental and control groups (Fig 10). A preliminary experiment applying exogenous of 200 ng/ml BDNF (an estimated ED50) found increased recruitment of TRAF2 and TRAF6 in both the hippocampus and PFCX, indicating p75^{NTR} activation levels. Compared to the control, the level of BDNF-induced TRAF2 and TRAF6 recruitment was reduced by 44% and 60% in the hippocampus and PFCX of the cocaine-exposed rats (Fig 10).

This finding was substantiated by a 45% reduction in BDNF-induced recruitment of FADD and TRADD in both the hippocampus and PFCX (Fig 11 and Fig 12). We derived a similar conclusion from the reduced levels of downstream signaling pathways of p75^{NTR}. In accord, BDNF-induced stress kinase JNK1 activation was indicated by a pJNK1 decrease of 43% and 45% in the hippocampus and PFCX, respectively—although with comparable basal pJNK1 (Fig 13). Prenatal cocaine exposure did not affect the expression of JNK1.

In addition to binding to BDNF with low affinity, p75^{NTR} can also be stimulated by pro-neurotrophins such as proBDNF leading to synaptic retraction and long-term

depression (Yang et al. 2009; Woo et al. 2005). Therefore, we tested whether proBDNF–$p75^{NTR}$ signaling is also reduced in prenatal cocaine-exposed brains. When we added 50 ng/ml recombinant mutated human proBDNF (which resists protease digestion to BDNF), TRAF2 and FADD recruitment to $p75^{NTR}$ was also reduced by 42–47% and 47–57%, respectively (Fig 13 and Fig 14).

Collectively, these data indicate that prenatal cocaine exposure attenuates the effectiveness of $p75^{NTR}$ signaling in response to either BDNF or proBDNF. The reduced BDNF/proBDNF – $p75^{NTR}$, together with enhanced BDNF–TrkB signaling, suggests a compensatory increase in survival drives and synaptic efficiency to offset the impact of defective glutamatergic synapses (Bakshi et al. 2009; Lu et al. 2009, Luscher et al. 2009).

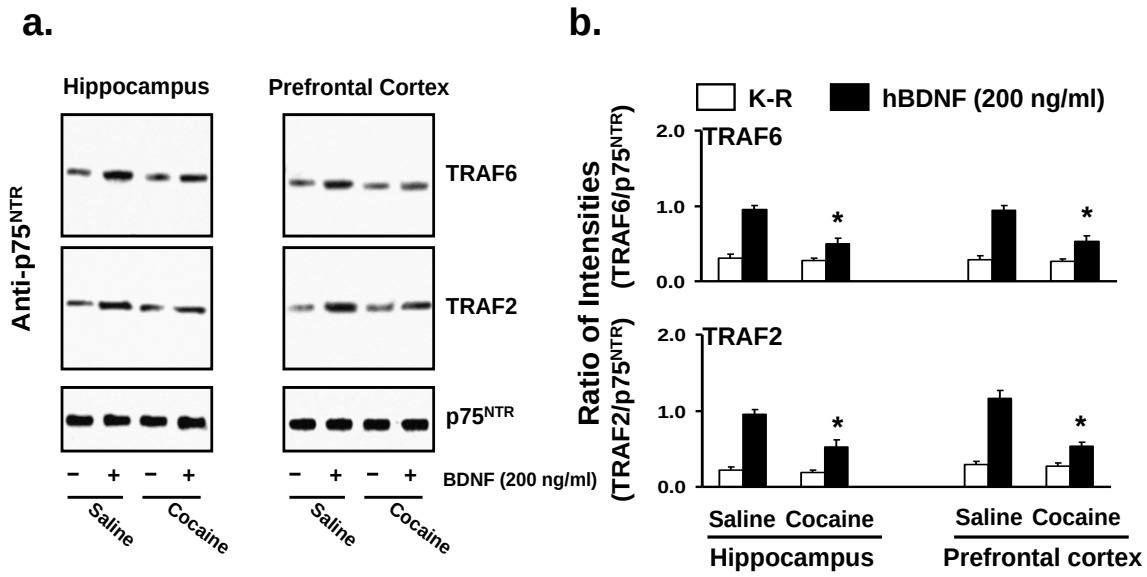


Fig 10. (*a*) The effect of prenatal cocaine exposure on $p75^{NTR}$ activation was assessed in hippocampal and PFCX slices from P21 prenatal cocaine- and saline-exposed rats. Brain slices (100 μm x 100 μm x 3 mm) were incubated with 200 ng/ml recombinant human BDNF at 37°C for 30 min. In a preliminary experiment, 200 ng/ml of BDNF was shown to be the ED50 in activating $p75^{NTR}$. The levels of TRAF6 and TRAF2 recruited to $p75^{NTR}$ were determined in anti- $p75^{NTR}$ immunoprecipitate by Western blotting. Blots were stripped and re-probed with anti- $p75^{NTR}$ to validate equal expression and loading. Our results show that BDNF-induced TRAF2 and TRAF6 recruitment was substantially lower in both brain areas from prenatal cocaine exposed rats as indicated by a lower TRAF2 and TRAF6 in the $p75^{NTR}$ immunoprecipitates. There were no detectable differences in $p75^{NTR}$ levels. (*b*) Densitometric quantification of blots. The data are expressed as the ratios of TRAF2 or TRAF6 optical intensity normalized by the optical intensity of total $p75^{NTR}$. n = 4. Data are means ± s.e.m. of the ratio of pY-TrkB to TrkB optical intensities. *$p < 0.01$ compared to respective protein in the saline-treated group.

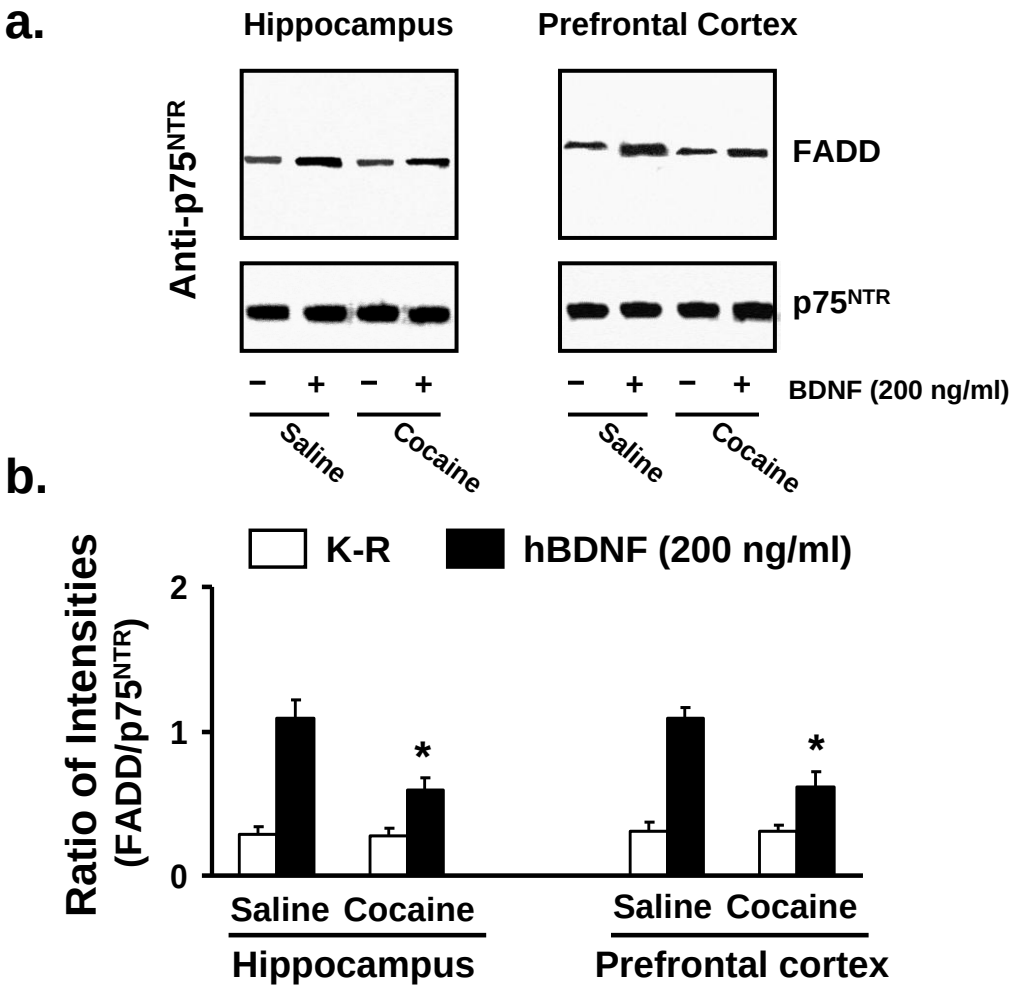


Fig 11. (*a*) The effect of prenatal cocaine exposure on $p75^{NTR}$ activation was assessed in hippocampal and PFCX slices from P21 prenatal cocaine- and saline-exposed rats. Brain slices (100 μm x 100 μm x 3 mm) were incubated with 200 ng/ml recombinant human BDNF at 37°C for 30 min. The level of FADD recruited to $p75^{NTR}$ was determined in anti- $p75^{NTR}$ immunoprecipitate by Western blotting. Blots were stripped and re-probed with anti- $p75^{NTR}$ to validate equal loading. Our results show that BDNF-induced FADD recruitment was substantially lower in both brain areas from prenatal cocaine exposed rats as indicated by a lower FADD and TRAF6 in the $p75^{NTR}$ immunoprecipitates. There were no detectable differences in $p75^{NTR}$ levels. (*b*) *Densitometric quantification of blots*. The data are expressed as the ratios of FADD optical intensity normalized by the optical intensity of total $p75^{NTR}$. n = 4. Data are means ± s.e.m. of the ratio of pY-TrkB to TrkB optical intensities. *$p < 0.01$ compared to respective protein in the saline-treated group.

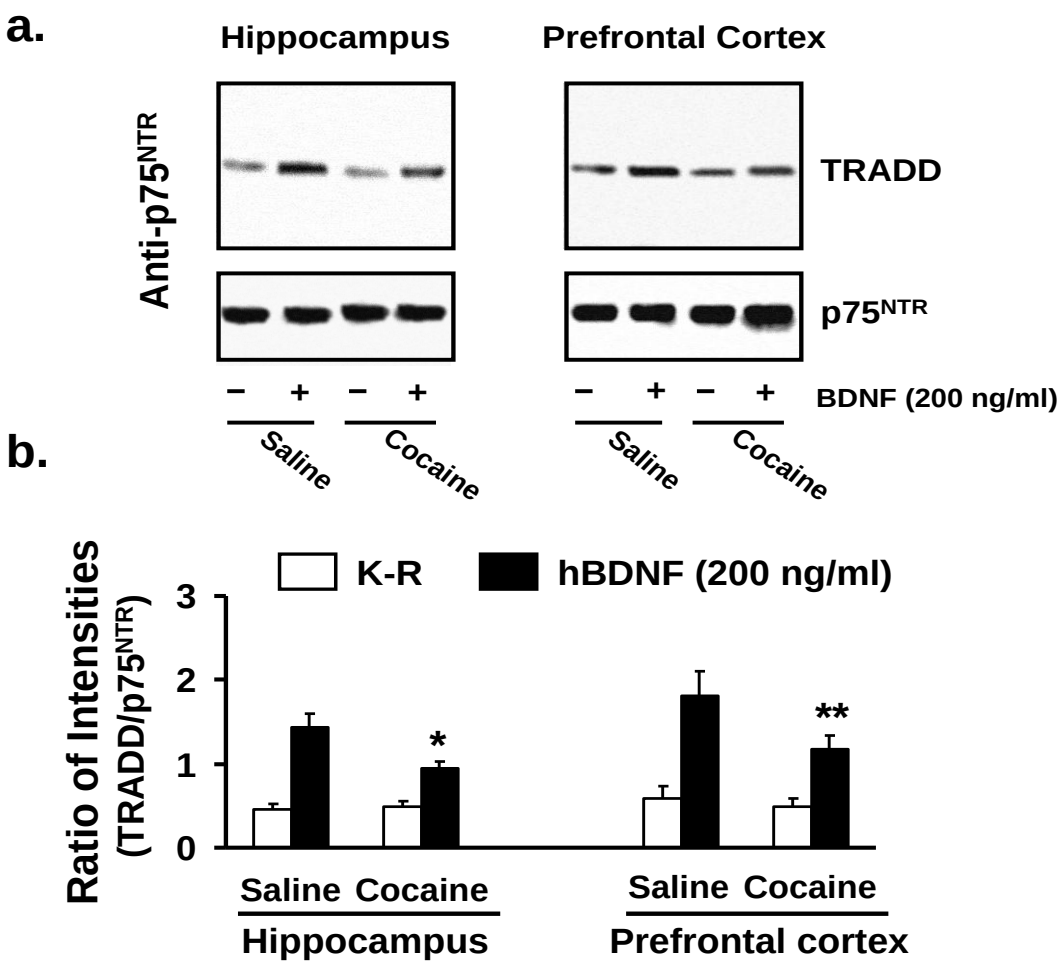


Fig 12. (*a*) The effect of prenatal cocaine exposure on $p75^{NTR}$ activation was assessed in hippocampal and PFCX slices from P21 prenatal cocaine- and saline-exposed rats. Brain slices (100 μm x 100 μm x 3 mm) were incubated with 200 ng/ml recombinant human BDNF at 37°C for 30 min. The level of TRADD recruited to $p75^{NTR}$ was determined in anti- $p75^{NTR}$ immunoprecipitate by Western blotting. Blots were stripped and re-probed with anti- $p75^{NTR}$ to validate equal loading. Our results show that BDNF-induced TRADD recruitment was substantially lower in both brain areas from prenatal cocaine exposed rats as indicated by a lower FADD and TRAF6 in the $p75^{NTR}$ immunoprecipitates. There were no detectable differences in $p75^{NTR}$ levels. (*b*) *Densitometric quantification of blots*. The data are expressed as the ratios of TRADD optical intensity normalized by the optical intensity of total $p75^{NTR}$. n = 4. Data are means ± s.e.m. of the ratio of TRADD to $p75^{NTR}$ optical intensities. *$p < 0.01$, **$p < 0.05$ compared to respective protein in the saline-treated group

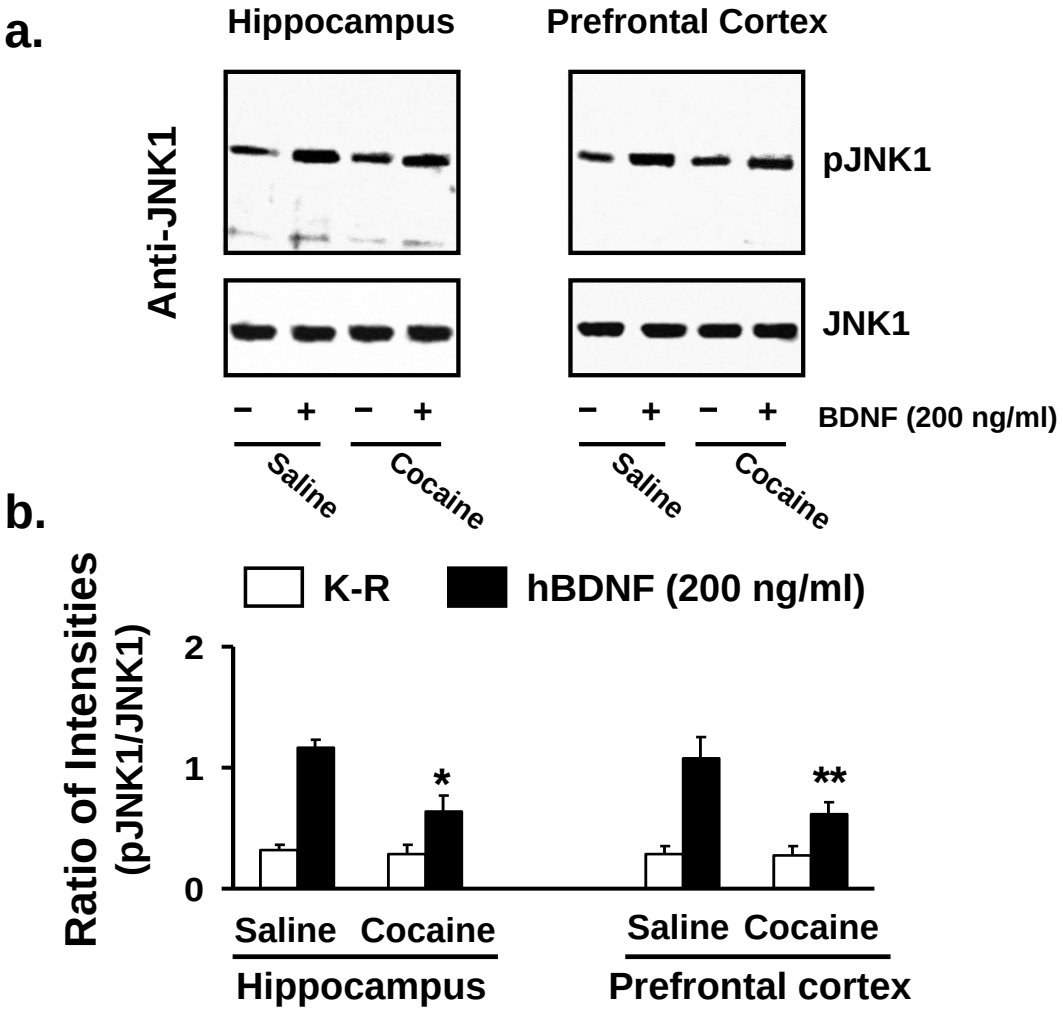


Fig 13. (*a*) The effect of prenatal cocaine exposure on $p75^{NTR}$ signaling was assessed in hippocampal and prefrontal cortical slices from P21 prenatal cocaine- and saline-exposed rats. Brain slices (100 μm x 100 μm x 3 mm) were incubated with 200 ng/ml recombinant human BDNF at 37°C for 30 min. In a preliminary experiment, 200 ng/ml of BDNF was shown to be the ED50 in activating $p75^{NTR}$. The activated level of stress kinase, JNK1 is indicated by the level of phosphorylated JNK1 (pJNK1) in anti-JNK1 immunoprecipitate by Western blotting. Blots were stripped and re-probed with anti-JNK1 to validate equal expression and loading. Our results show that BDNF-induced TRAF2 and TRAF6 recruitment was substantially lower in both brain areas from prenatal cocaine exposed rats as indicated by a lower pJNK1 in the pJNK1 immunoprecipitates. There were no detectable differences in JNK1 levels. (*b*) *Densitometric quantification of blots*. The data are expressed as the ratios of pJNK1 optical intensity normalized by the optical intensity of total JNK1. n = 4. Data are means ± s.e.m. of the ratio of pJNK1 to JNK1 optical intensities. *$p < 0.01$; **$p < 0.05$ compared to respective protein in the saline-treated group.

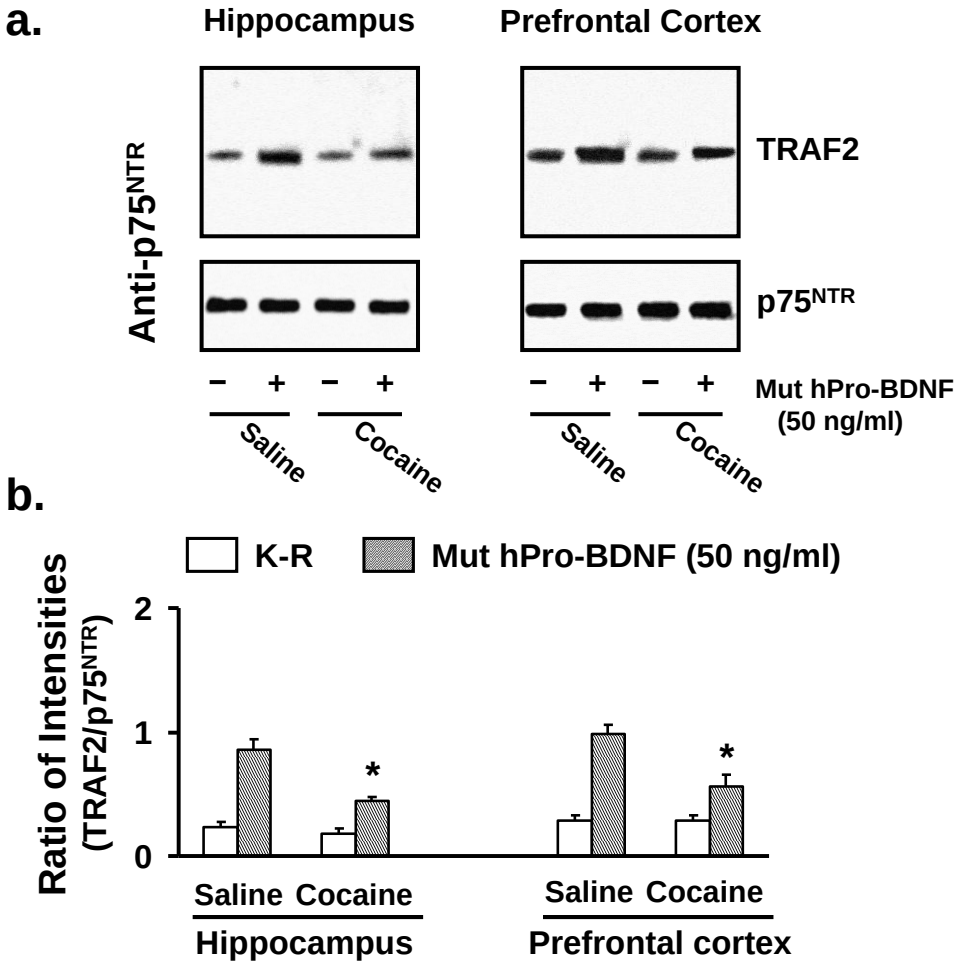


Fig 14. (*a*) The effect of prenatal cocaine exposure on $p75^{NTR}$ activation was assessed in hippocampal and PFCX slices from P21 prenatal cocaine- and saline-exposed rats. Brain slices (100 μm x 100 μm x 3 mm) were incubated with 50 ng/ml mutated recombinant human proBDNF at 37°C for 30 min. In a preliminary experiment, 50 ng/ml of proBDNF was shown to be the ED50 in activating $p75^{NTR}$. The level of TRAF2 recruited to $p75^{NTR}$ was determined in anti- $p75^{NTR}$ immunoprecipitate by Western blotting. Blots were stripped and re-probed with anti- $p75^{NTR}$ to validate equal loading. Our results show that BDNF-induced TRAF2 recruitment was markedly lower in both brain areas from prenatal cocaine exposed rats as indicated by a lower TRAF2 in the $p75^{NTR}$ immunoprecipitates. There were no detectable differences in $p75^{NTR}$ levels. (*b*) *Densitometric quantification of blots*. The data are expressed as the ratios of TRAF2 optical intensity normalized by the optical intensity of total $p75^{NTR}$. n = 4. Data are means ± s.e.m. of the ratio of TRAF2 to $p75^{NTR}$ optical intensities. *$p < 0.01$ compared to respective protein in the saline-treated group.

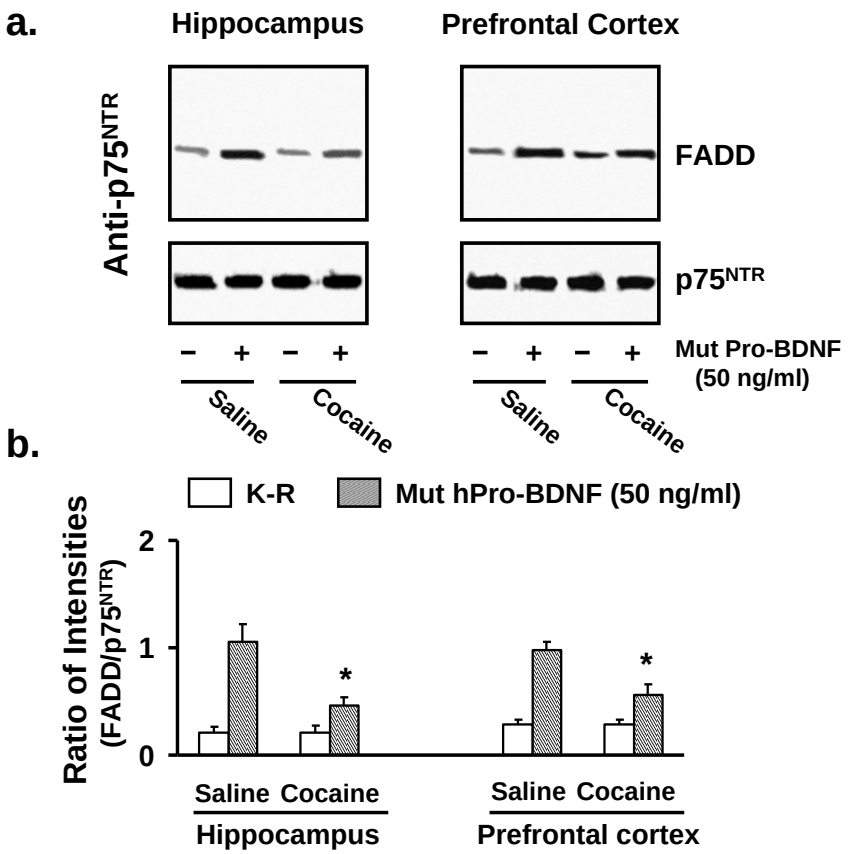

a.
Hippocampus
Prefrontal Cortex
Anti-p75NTR
FADD
p75NTR
– + – +
– + – +
Mut Pro-BDNF
(50 ng/ml)
Saline
Cocaine
Saline
Cocaine
b.
K-R
Mut hPro-BDNF (50 ng/ml)
Ratio of Intensities
(FADD/p75NTR)
2
1
0
*
*
Saline Cocaine
Hippocampus
Saline Cocaine
Prefrontal cortex

Fig 15. (*a*) The effect of prenatal cocaine exposure on $p75^{NTR}$ activation was assessed in hippocampal and PFCX slices from P21 prenatal cocaine- and saline-exposed rats. Brain slices (100 μm x 100 μm x 3 mm) were incubated with 50 ng/ml mutated recombinant human proBDNF at 37°C for 30 min. The level of FADD recruited to $p75^{NTR}$ was determined in anti-$p75^{NTR}$ immunoprecipitate by Western blotting. Blots were stripped and re-probed with anti- $p75^{NTR}$ to validate equal loading. Our results show that BDNF-induced FADD recruitment was significantly lower in both brain areas from prenatal cocaine exposed rats as indicated by a lower FADD level in the $p75^{NTR}$ immunoprecipitate. There were no detectable differences in $p75^{NTR}$ levels. (*b*) *Densitometric quantification of blots*. The data are expressed as the ratios of FADD optical intensity normalized by the optical intensity of total $p75^{NTR.}$ n = 4. Data are means ± s.e.m. of the ratio of FADD to TrkB optical intensities. *$p < 0.01$ compared to respective protein in the saline-treated group.

Methods

Animal treatment

Pregnant female rats were housed individually with no disturbance other than a daily injection of cocaine or saline. From gestation days 8–21, pregnant dams received daily intra-peritoneal injections of either 30 mg/kg cocaine HCl, in 0.9% saline, or 2 ml/kg saline. Animals were injected daily between 10–11 a.m. Following each injection, pregnant rats were observed for 1 h and behavioral abnormalities were recorded. There were apparent increases in locomotor activity in cocaine-treated rats.

Progeny was cross-fostered group housed with surrogate mothers until they were sacrificed at 21 days of age (P21). Food and water were freely available. They were subjected to the minimum handling associated with routine animal husbandry. Both sexes from multiple litters were employed in these experiments. Pups were sacrificed by rapid decapitation, brains were removed immediately, and frontal cortices were dissected on ice. All animal procedures complied with the National Institutes of Health Guide for Care and Use of Laboratory Animals. The experimental protocol was approved by the City College of New York Animal Care and Use Committee.

Materials and chemicals

Recombinant human BDNF (rhBDNF), MuthproBDNF Leupeptin, aprotinin, phenylmethylsulfonyl fluoride (PMSF), pepstatin A, soybean trypsin inhibitor, NaF, sodium vanadate, β-glycerophosphate, 2-mercaptoethanol, NMDA, glycine, Tween-20, NP-40, and Histpaque-1077 were from Sigma-Aldrich (Saint Louis, MO). Anti-phosphotyrosine (SC-508), TrkB (SC-119) -ERK2 (SC-154, SC-81457), -pY-ERK (SC-

7383), -pS^{473}Akt (SC- 7985-R), -Akt1 (SC-65487), -Akt2 (SC-81436), -Akt1/2/3 (SC-8312), -phospholipase C-γ1 (SC-7290), -Shc (SC-967), -N-Shc (SC-28833) -NR1 (SC-9058), -actin (SC-7210), -β-actin (SC-47778), NTR P75(SC-8317), -FADD (SC-271520), -TRADD (SC-46653), -TRAF6 (SC-8408), -TRAF2 (SC-136999), -JNK1 (SC- 571) P-JNK (SC-6254) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Seize-X immunoprecipitation kit, antigen elution buffer, BindTM NeutrAvidinTM, high binding capacity coated 96-well plates, and West pico chemoluminescent reagents were from Pierce Endogen, Inc. (Rockford, IL). Bradford reagent, SDS-PAGE reagents, and pre-stained molecular weight markers were from Bio-Rad Laboratories (Hercules, CA). Protease inhibitors (EDTA-free) and protein phosphatase inhibitor tablets were from Roche (Mannheim, Germany).

BDNF was reconstituted according to the manufacturer's instruction. To avoid freezing damage, 10% glycerol was added to achieve 10 ng/μl BDNF and stored in -80°C until use. All other test agents were made fresh according to the manufacturer's recommendation. The DMSO concentration in the incubation medium was ≤ 1% when used.

Brain slice preparation

Hippocampal and PFCX tissue was sliced using a chilled McIlwain tissue chopper (100 μm x 100 μm x 3 mm). Brain slices of approximately 10 mg were suspended in 1 ml of ice-cold oxygenated low Mg^{2+} Kreb's-Ringer (LMKR), containing 25 mM HEPES, pH7.4, 118 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM KH_2PO_4, 0.3 mM $MgSO_4$, 25 mM $NaHCO_3$, 10 mM glucose, 100 μM ascorbic acid, 50 μg/ml leupeptin, 0.2 mM

PMSF, 25 μg/ml pepstatin A, and 0.01 U/ml soybean trypsin inhibitor, and centrifuged briefly. Brain slices were washed twice more and suspended in 1 ml of LMKR.

*Ex vivo tissue treatment for BDNF–TrkB signaling, TrkB-NMDAR association assessments, and proBDNF/BDNF–p75^{NTR}signaling*To determine effects of prenatal cocaine exposure on TrkB and p75^{NTR} signaling in brain tissue, brain slices were incubated for 30 min at 37°C in 0.5 ml oxygenized LMKR with or without 50 ng/ml rhBDNF (for BDNF–TrkB signaling), or 200 ng/ml BDNF, or 50 ng/ml mutant human cleavage-resistant proBDNF (mut proBDNF; for BDNF/proBDNF – p75^{NTR} signaling)..A preliminary experimental series that measured the dose–response relationship of BDNF to the activation of TrkB and p75^{NTR} found the respective ED50 for stimulating TrkB and p75^{NTR} to be 50 and 200 ng/ml of BDNF. Likewise, the ED50 for activating p75^{NTR} was 50 ng/ml mut hproBDNF

During incubation, the incubation mixture was aerated with 95% O_2/5% CO_2 for 1 min every 10 min. Ligand stimulation was terminated by adding 1 ml of ice-cold Ca^{2+}-free LMKR containing 0.5 mM EGTA/0.1 mM EDTA and phosphatase inhibitors, andhcentrifugated briefly. Supernatant was discarded and tissue slices were homogenized in 0.25 ml ice-cold immunoprecipitation buffer (25 mM HEPES, pH 7.5, 200 mM NaCl, 1 mM EDTA, 50 μg/ml leupeptin, 10 μg/ml aprotinin, 2 μg/ml soybean trypsin inhibitor, 0.04 mM PMSF, 5 mM NaF, 1 mM sodium vanadate, 0.5 mM β-glycerophosphate, and 0.02% 2-mercaptoethanol containing 0.5% digitonin, 0.2% sodium cholate, and 0.5% NP-40. The resulting homogenates were centrifuged at 1000g for 5 min (4°C), the supernatant (post-mitochondrial fraction) was sonicated for 10 s, and protein

concentrations were measured by the Bradford method (Bio-Rad). We solubilized 200 μg of lysate was in 0.5% digitonin/0.2% sodium cholate/0.5% NP-40 for 60 min at 4°C via end-to-end rotation. Resultant lysates were cleared by centrifugation at 50,000g for 5 min and diluted with 0.75 ml of immunoprecipitation buffer.

Assessment of TrkB activation; phospholipase C-γ1, shc, and N-shc recruitment; TrkB–NMDAR interaction; and ERK and PI3K activation by co-immunoprecipitation

TrkB signaling complexes ERK2 and Akt were immunoprecipitated separately in 200 μg of tissue lysate for a 2-h incubation (4°C) with 1 μg of immobilized anti-TrkB (for assessment of pY-TrkB; PLC-γ1, shc, and N-shc recruitment; and TrkB–NMDAR interaction), anti-ERK2 (pYERK2), and mouse anti-Akt1/2 (pS^{473}-Akt) followed by addition of 30 μl of protein A/G-conjugated agarose beads. Incubation continued at 4°C for 16 hr. The resultant immunocomplexes were pelleted by centrifugation (4°C), washed three times with 1 ml of ice-cold PBS (pH 7.2), and centrifuged. The resultant immunocomplexes were solubilized by boiling for 5 min in 100μl of SDS-PAGE sample preparation buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, and 0.1% bromophenol blue). The contents of the pY-TrkB, PLC-γ1, shc, N-shc, and NMDAR subunits, in anti-TrkB immunoprecipitate, pY/pT-ERK2 in 50% anti-ERK2 immunoprecipitate, and pS^{473}Akt in 50% anti-Akt immunoprecipitate were determined by Western blotting. The blots were stripped and re-probed with anti-TrkB, -ERK2, or -Akt1/2/3 to illustrate even immunoprecipitation efficiency and loading.

Assessment of $p75^{NTR}$ activation; TRAF2, TRAF6, TRADD, and FADD recruitment;

and JNK1 activation by co-immunoprecipitation

$p75^{NTR}$ signaling complexes and JNK1 were immunoprecipitated separately in 200 μg of tissue lysate for a 2-hr incubation (4°C) with 1 μg of immobilized anti-$p75^{NTR}$ (for assessment of TRAF2, TRAF6, TRADD and FADD and N-shc recruitment in response to 200 ng/ml BDNF or TRAF2/FADD in response to 50 ng/ml mut hproBDNF) and anti-JNK1 (pJNK1), followed by addition of 30 μl of protein A/G-conjugated agarose beads. Incubation continued at 4°C for 16 h. The resultant immunocomplexes were pelleted by centrifugation (4°C), washed three times with 1 ml of ice-cold PBS, pH 7.2, and centrifuged. The resultant immunocomplexes were solubilized by boiling for 5 min in 100_l of SDS-PAGE sample preparation buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, and 0.1% bromophenol blue). The contents of the TRAF2, TRAF6, TRADD, and FADD in 50% anti-$p75^{NTR}$ immunoprecipitate, pJNK1 in 50% anti-JNK1 immunoprecipitate were determined by Western blotting. The blots were stripped and re-probed with anti-$p75^{NTR}$ to illustrate even immunoprecipitation efficiency and loading.

Western blot analysis

Solubilized immunoprecipitates, size-fractionated by either 7.5 or 10% SDS-PAGE, were electrophoretically transferred to nitrocellulose membranes. The membranes were washed with PBS and blocked overnight (4°C) with 10% milk in 0.1% Tween 20-containing PBS (PBST). Following three 5min 0.1% PBST washes, membranes were incubated at 25°C for 2 h with 1:500 to 1:1,000 dilutions of selected antibodies. After three 2-min 0.1% PBST washes, membranes were incubated for 1 h with anti-

species IgG-HRP (1:5000 dilution) and washed three times with 0.1% PBST for 2 min each. The signals were detected using a chemiluminescent method and visualized by exposure to x-ray film. Specific bands were quantified by densitometric scanning (GS-800 calibrated densitometer, Bio-Rad).

BDNF binding

We used hippocampal and PFCX synaptosomes of prenatal cocaine- and saline-exposed P21 rats. Membrane-bound proteins in the synaptosomes were first biotinylated using a biotinylation kit. The biotinylated surface proteins containing synaptosomes were sonicated in 500 μl LMKR for 10 s on ice and solubilized by 0.5% digitonin/0.2% sodium cholate/0.5% NP-40 and diluted 1:5. Protein concentrations were measured using the Bradford method. The biotinylated protein solution was then diluted with LMKR to 50 μg/100 μl. To coat the plate with biotinylated proteins, streptavidin-coated plates (Reacti-Bind NeutrAvidin high binding capacity 96-well plates) were washed three times with 200 μl of ice-cold LMKR and then incubated at 30°C with 50 μg/well biotinylated receptor solution for 1 h in the presence of 5% blocking reagent (Thermo). Following two LMKR washes, BDNF was added (100 fM to 10 nM), and incubation was performed at 30°C for 1 h. Plates were washed with ice-cold LMKR and incubated at 30°C for 1 h with anti-BDNF (0.5 μg/well) in PBST, followed by FITC-conjugated anti-rabbit IgG (0.5 μg/well) in PBST for 1 h. Plates were washed twice with 200 μl of ice-cold PBST, and the FITC signals were determined by a multimode plate reader, DTX880 (Beckman). Negligible FITC signal was noted when vehicle other than BDNF was added.

Isoelectric point assessment

TrkB derived from the hippocampus and PFCX synaptosomes of prenatal saline- or cocaine-exposed P21 rats was sonicated for 10 s on ice, solubilized using 0.5% digitonin/0.2% sodium cholate/0.5% NP-40 at 4°C with end-over-end rotation for 1 hr. Following centrifugation to remove insoluble debris, the obtained lysate was then treated with 1% SDS for 1 min to dissociate the TrkB-associated proteins, diluted 10-fold with immunoprecipitation buffer, and imunopurified with immobilized anti-TrkB. The resultant TrkB (145- and 95-KDa) was then eluted using 200 µl antigen-elution buffer (Thermo), neutralized immediately with 100 mM Tris HCl (pH9.0), diluted to 500 µl with 50 mM Tris HCl, pH7.5, and then passed through a 100 kD cut-off filter to remove 95-KDa TrkB isoform. Once purified, the 145-KDa TrkB was suspended in the 100 µl isoelectric focusing sample buffer. Samples (50 µl) were loaded onto pH3-10 isoelectric focusing gels (Biorad) and the proteins were fractionated (100 V for 1 hr, 200 V for 1 hr, and 500 V for 30 min). The separated proteins were then electrophoretically transferred to nitrocellulose membranes. TrkB was identified by Western blotting with anti-TrkB.

BDNF and proBDNF release.

BDNF and proBDNF release was determined in hippocampal and PFCX slices (100 µl x 100 µl x 3 mm) from prenatal cocaine- and saline-exposed P21 rats. Brain slices of approximately 10 mg of were suspended in 250 µl oxygenized LMKR and loaded onto a superfusion chamber and superfused at 0.2 ml/min with oxygenated LMKR at 37°C for 30 min. Spontaneous (basal) BDNF/proBDNF efflux was defined by the levels of BDNF/proBDNF in LMKR perfusate at the 30-min mark. Depolarization- and

NMDA(10 μM)/glycine(1 μM)-induced (stimulated) BDNF/proBDNF release was evoked by superfusing brain slices with 65 mM K^+ (replacement of NaCl with KCl) or LMKR-containing NMDA(10 μM/glycine(1 μM) for 10 min. This was followed by superfusion of oxygenated LMKR at 37°C for 20 min. Slices were also collected at the conclusion of superfusion. For the proBDNF release with minimal Proteolytic conversion to BDNF, brain slices were superfused with LMKR-containing MMP-9 inhibitor I (Calbiochem) and tPA inhibitor (American Diagnostica).

To assess levels of BDNF and proBDNF in the perfusate (6 ml), protease inhibitors and detergents (digitonin/sodium cholate/NP-40) were first added to the perfusate to achieve final concentrations of 0.05% digitonin/0.02% sodium cholate/0.05% NP-40). BDNF and proBDNF were then immunoprecipitated with anti-BDNF (1 μg/ml) followed by protein A/G-conjugated agarose beads. The levels of BDNF and proBDNF were measured using Western blotting with anti-BDNF and anti-proBDNF.

To assess the percentage of brain tissue from which BDNF/proBDNF was released, tissue slices were homogenized and diluted 10-fold in immunoprecipitation buffer. Following centrifugation at 1000g for 10 min, the resultant supernatant (post-mitochondrial fraction) was used to measure the protein content by the Bradford method and solubilized by boiling in the sample preparation buffer for 5 min. The level of the β-actin was then measured by Western blotting to serve as the control of tissue quantity.

Statistical analyses

All data are presented as mean ± standard error from the mean ± s.e.m. Treatment effects were evaluated by ANOVA. Specifically, the treatment effects of TrkB or $p75^{NTR}$

activation-related biochemical indices in animal experiments were evaluated using Newman-Keul's test for multiple comparisons or by two-tailed Student's t test, as appropriate. The threshold for significance was $p < 0.05$. Between-group comparisons were also conducted for all parameters.

Discussion

Our results indicate that exposure to cocaine during the gestational period produces synaptic plasticity changes by promoting BDNF–TrkB signaling, facilitating TrkB-NMDAR interaction, and reducing $p75^{NTR}$ signaling and its associated stress kinase JNK1 activity in the PFCX and hippocampus of P21 rats. This prenatal cocaine-induced effect on neurotrophin signaling is accompanied by decreased levels of activity-dependent BDNF and proBDNF released from both presynaptic neuronal terminals and postsynaptic dendritic field as indicated, respectively, by a lower K^+-depolarization- and NMDA/glycine-induced BDNF/proBDNF in perfusate. Noticeably, prenatal cocaine exposure also altered TrkB conformation, as evidenced by a shift in the isoelectric point of the full-length receptor.

Studies in both humans and animal models indicate that prenatal cocaine exposure negatively affects attention, motor, and language skills, as well as associative and discrimination learning, all of which involve excitatory synapses (Mayes et al. 1995; Romano and Harvey, 1996; Delaney-Black et al. 1996; Bandstra et al. 2002). These findings suggest that exposure to cocaine during early development can modify synaptic plasticity at the excitatory synapses, resulting in enduring changes in brain function. In support of this possibility, our earlier data indicate that cocaine exposure in utero attenuates AMPAR-mediated LTD without affecting basal transmission (Bakshi et al. 2009). This reduction in prenatal cocaine-exposed brains is accompanied by reduced synaptic expression of the AMPARs with apparent retention of GluR2/3 in cytosol resulting from disrupted GluR2/3–GRIP interaction (Bakshi et al. 2009) and enhanced

GRASP-1–GRIP association (Bakshi et al. 2011). Prenatal cocaine exposure has also been shown to affect numerous neurotransmitter systems, including dopamine D1 receptor–Gs/olf coupling (H. Wang et al. 1995; Friedman et al. 1996; Jones et al. 2000; Zhen et al. 2001), GABAergic neurons (X. Wang et al. 1995, Lu et al. 2009), and the noradrenergic system (Booze et al. 2006). Despite causing the dramatic synaptic dysfunction outlined above, prenatal cocaine exposure actually increases the number of synapses on the synaptic spines of prelimibic rat cortices (Morrow et al. 2007).

The relationship of prenatal cocaine-induced neuronal injuries to cognition and functional changes is undoubtedly complex, and the lesions themselves may only account for a relatively small portion of cognition variance. Thus, other as-yet-undefined contributions to neural damage, or functional resilience to that damage, must be at play. These factors may be initiated by, related to, or independent of the changes in numbers of cells or membrane receptors per se, since there was no discernible difference in these parameters in the prenatal cocaine-exposed brains. Despite the possibility of regional variations in BDNF and TrkB expression, as well as BDNF–TrkB and $p75^{NTR}$ signaling, we chose to examine the prefrontal cortex and hippocampus because they play an important role in cognition, emotion, and attention, all of which involve BDNF-mediated TrkB signaling counterbalanced by $p75^{NTR}$ activity.

BDNF–TrkB signaling regulates a complex range of neuronal properties, including cell survival, process outgrowth, neurotransmission, firing properties, and some forms of activity-dependent synaptic plasticity (Lee et al. 2001). During brain development, BDNF–TrkB signaling is essential for the establishment of proper synaptic connectivity and brain function in the postnatal period (Gao et al. 2009). Extensive data

demonstrate that BDNF affects neurotransmission through both pre- and postsynaptic mechanisms, leading to cognitive effects (Kohara et al. 2001; Kovalchuk et al. 2002). BDNF-induced TrkB activation leads to enhanced glutamatergic neurotransmission (Levine et al. 1995), rapid NMDAR subunit phosphorylation (Suen et al. 1997), and LTP (Figurov et al. 1996). In spatial memory tasks with rodents, activated TrkB in the hippocampus was selectively and transiently increased after maze training (Mizuno et al. 2003). The role of TrkB activation in learning and memory is substantiated by data showing that intracerebroventricular administration of antisense BDNF oligonucleotides to maze-trained rats dampens their previously acquired spatial memory (Mizuno et al. 2000). Moreover, addition of exogenous BDNF rescues LTP defects in BDNF-deficient mutant mice (Patterson et al. 1996) and anti-BDNF antibodies impair memory in water maze and passive avoidance tests (Mu et al. 1999; Alonso et al. 2002). Conditioned TrkB-CRE knockout in the forebrain during postnatal development severely impaired complex learning and short-term plasticity (Minichiello et al. 1999). Moreover, TrkB activation (tyrosine-phosphorylation) is closely correlated with synaptic plasticity elicited in the fear-learning and extinction paradigms (Musumeci et al. 2009). BDNF–TrkB signaling is also required for cocaine-induced behavioral sensitization and conditioned place preference (Crooks et al. 2010). Collectively, these findings indicate that activation of the BDNF–TrkB system regulates NMDAR-mediated, LTP-associated learning, memory, and cognition. Hence, increased BDNF-induced TrkB tyrosine phosphorylation, together with elevated catalytically capable full-length TrkB in the PFCX and hippocampus could represent a neuroprotective plastic response whereby hyperactivity of BDNF–TrkB signaling is an attempt to offset deteriorating synaptic function in the

prenatal cocaine-exposed brain. Consistent with this adaptive hypothesis is our data showing increased association of NR1 with TrkB following incubation with exogenous BDNF.

The increase in BDNF-stimulated TrkB activation is further supported by our data showing commensurate increases in BDNF-induced ERK2 and Akt phosphorylation. TrkB activates downstream intracellular Ras/ERK and PI-3K signaling pathways predominantly through the binding of adaptor proteins such as shc and N-Shc to phosphotyrosine residues on BDNF-activated TrkB (Skaper 2008). Shc has been shown to be tyrosine-phosphorylated following recruitment/association with activated TrkB, presumably through the associated tyrosine kinases (Tong et al. 2004). Tong et al. (2008) postulated that Shc's reduced tyrosine phosphorylation is responsible for the reduction in BDNF-induced ERK and PI-3K signaling. In accord with the notion that prenatal cocaine exposure enhances BDNF–TrkB signaling, the levels of Shc and N-Shc associated with activated TrkB following BDNF stimulation were significantly higher in brain tissues from prenatal cocaine-exposed P21 rats.

Previous studies have shown that even small changes in BDNF–TrkB signaling levels during critical developmental stages can greatly influence brain architecture and function at later stages in life (Gao et al. 2009; Park and Poo 2012)By altering the magnitude of BDNF–TrkB signaling and TrkB-NMDAR interaction, prenatal cocaine exposure may lead to persistent, if not permanent changes to the cortical and sub-cortical structures and functions, including dendritic architecture and their synaptic connectivity functions (Jones et al. 2000; Churchill et al. 2002; Frankfurt et al. 2009), as well as neurotransmission, especially within the glutamatergic system (Levine et al. 1995; Suen

et al. 1997; Gottschalk et al. 1998; Bakshi et al. 2009; Lu et al. 2009).

To gain insight into the mechanism responsible for increased BDNF–TrkB signaling, we considered the possibility of an altered BDNF affinity for TrkB. Similar to our earlier report (H. Wang et al. 2011), BDNF was shown to bind with two binding affinities in both hippocampus and PFCX. Presumably, the high-affinity pM binding site is on TrkB, whereas the low-affinity nM binding site is on $p75^{NTR}$ (Lu et al. 2005). BDNF binding affinities in the PFCX are three times higher than in the hippocampus. This may imply that subtle differences in BDNF interaction with its targets TrkB and $p75^{NTR}$ do exist in different brain regions, despite their identical functionality and associated signaling pathways. Similar to the effect of repetitive transcranial magnetic stimulation in adult rats and humans (H. Wang et al. 2011), prenatal cocaine exposure markedly increases BDNF binding affinity to both high- and low-affinity binding sites. Together, these results support the idea that increased BDNF binding affinity to TrkB is at least in part responsible for heightened BDNF–TrkB signaling in the brains of prenatal cocaine-exposed P21 rats.

To assess this altered TrkB hypothesis, we specifically assessed the possible change in TrkB conformation. An altered isoelectric point (*pI*) has long being utilized to indicate a change in the conformation of a protein (Ui 1973). Consistent with the conformational change hypothesis, the majority of the full-length TrkBs purified from prenatal cocaine-exposed brains under non-stimulated condition exhibited a higher *pI*. While the underlying mechanism responsible for this *pI* change in TrkB is currently unknown, the observed shift in TrkB's net charge is consistent with the finding of higher BDNF affinity for TrkB and greater BDNF–TrkB signaling in the brains of prenatal

cocaine-exposed P21 rats.

To delineate the cause of this heightened BDNF binding affinity, we assessed the level of activity-dependent BDNF and proBDNF release, since there was no discernible change in proBDNF and BDNF expression by prenatal cocaine exposure. In our previous study, we demonstrated that K^+-depolarization and NMDA/glycine increase BDNF–TrkB signaling by promoting BDNF release, presumably from respective presynaptic neuronal terminals and postsynaptic dendritic fields (H. Wang et al. 2011). In accord, here we show that K^+-depolarization and NMDA/glycine elicit release of both proBNF and mBDNF. More importantly, prenatal cocaine exposure profoundly reduced the levels of proBDNF and mBDNF released in response to K^+-depolarization and NMDA/glycine stimulation. In contrast, prenatal cocaine exposure did not alter the level of spontaneous BDNF efflux. The reduction of activity-dependent BDNF released in prenatal cocaine-exposed brains may thus lead to compensatory upregulation of BDNF–TRkB signaling to restore adequate synaptic activities.

BDNF is initially synthesized as a precursor protein (proBDNF) in the endoplasmic reticulum. Following cleavage of the signal peptide, the resultant proBDNF is then transported to the Golgi for sorting into either constitutive or regulated secretory vesicles for spontaneous and activity-regulated release. proBDNF may be converted into mBDNF intracellularly in the trans-Golgi by various subtilisin-kexin of the endoprotease family such as furin, or in the immature secretory granules by proprotein convertases (Mowla et al. 1999). Recent studies have further demonstrated that proBDNF does secrete and convert to mBDNF in vitro via tissue plasminogen activator (tPA)-dependent activation of plasminogen and by matrix metalloproteases such as MMP-9, thereby

suggesting that proBDNF may be biologically active (Pang et al. 2004), although the efficiency of intracellular cleavage remains controversial (Matsumoto et al. 2008) and likely vary among neuronal cell types. In accord with the notion that BDNF–TrkB signaling regulates NMDAR activity, BDNF and tPA have both been implicated in late-phase LTP and long-term memory since mBDNF but not uncleavable proBDNF, rescues L-LTP in tPA and in plasminogen knock-out mice (Pang et al. 2004). These studies therefore suggest that the conversion of proBDNF to BDNF promotes L-LTP expression in the hippocampus, and perhaps other brain regions. The fact that prenatal cocaine exposure dampens the activity-dependent release of both BDNF and proBDNF without evidence of altered tPA level suggests that prenatal cocaine exposure may result in defects in depolarization–exocytosis coupling and/or release of neurotransmitters and NMDAR function. Indeed, previous studies have shown that prenatal cocaine exposure reduces dopamine release and NMDAR activity (H,Wang et al. 1995; Yablonsky-Alter et al. 2005). Altogether, these data provide a mechanistic insight for the upregulated BDNF–TrkB signaling and enhanced TrkB-NMDAR coupling observed in prenatal cocaine-exposed brains. It is well established that the pro-survival and synaptic-promoting activity of BDNF–TrkB signaling is opposed by the pro-apoptosis and synaptic downgrading activity of $p75^{NTR}$ receptors. We therefore investigated the effect of prenatal cocaine exposure on $p75^{NTR}$-mediated signaling. This is particularly important in light of the finding that activity-dependent BDNF and proBDNF release is reduced in prenatal cocaine-exposed brains.

$p75^{NTR}$ receptors can be activated by BDNF, although a markedly higher BDNF level is required to trigger $p75^{NTR}$ signaling. ProBDNF also binds and activates the

p75^{NTR} receptor (Teng et al. 2005). Using the uncleavable proBDNF, an earlier study revealed that through activation of p75^{NTR}, proBDNF significantly enhances long-term depression (LTD) in the hippocampus, an effect not observed in 3–4-week-old p75^{NTR}-deficient mice (Woo et al. 2005). In addition, proBDNF can induce neuronal apoptosis via activation of p75^{NTR}/sortilin complex (Teng et al. 2005). Such a pro-apoptosis action of p75^{NTR} can be also triggered by BDNF, although high non-physiological concentrations are required to induce even modest levels of cell death. In the developing brain, BDNF activates p75^{NTR} to promote developmental pruning that eliminates less-active competing axons (Singh et al. 2008). These results suggest that activation of TrkB and p75^{NTR} elicit opposing synaptic effects and cell fate. Interestingly, complete deletion of the *Bdnf* gene, which abolishes both proBDNF and mBDNF, did not affect LTD (Matsumoto et al. 2008), suggesting that complete elimination of proBDNF and mBDNF may obscure the expression of opposing synaptic activities regulated by TrkB and p75^{NTR} activation. The latter finding may be relevant in the context of prenatal cocaine exposure in light of the reduced activity-dependent proBDNF and mBDNF.

Although lacking kinase domain, p75^{NTR} can cooperate with many different protein partners, such as TRAF2, TRAF6, TRADD and FADD to form multimeric receptor complexes to activate various intracellular signaling cascades such as stress-activated kinase, JNK, and produce a number of cellular responses, including apoptosis, neurite outgrowth and myelination (Gentry et al. 2004; Nykjaer et al. 2005). Hence, the levels of BDNF/proBDNF-induced, p75^{NTR}-associated TRAF2, TRAF6, TRADD and FADD, along with p75^{NTR} downstream JNK activation, can be used to gauge the efficiency of p75^{NTR} signaling. In this regard, our findings of significant reductions in

BDNF- and proBDNF induced TRAF2, TRAF6, TRADD, and FADD association with $p75^{NTR}$ in the prenatal cocaine-exposed brains support the notion that prenatal cocaine exposure dampens $p75^{NTR}$ function. This cocaine-mediated effect in utero is substantiated by data showing that BDNF-induced JNK1 activation is also lower in the prenatal cocaine-exposed brain. Thus, in contrast to an upregulation of TrkB alongside a markedly reduced depolarization- and NMDA/glycine-induced BDNF/proBDNF release, $p75^{NTR}$ signaling is downregulated following prenatal cocaine exposure and postnatal (1–3 weeks) abstinence. Collectively, even in the presence of reduced BDNF release, reduced $p75^{NTR}$ signaling enables a more effective BDNF–TrkB signaling and thereby facilitates the recovery of cocaine-induced synaptic damages incurred in utero. Our data, however, do not indicate the effectiveness of such an attempt on reversing prenatal cocaine-induced brain damage.

Data indicate that BDNF–TrkB signaling is associated with psychological distress, and may also account for at least some of the association between psychological distress and incidental cognitive impairment. In fact, people with psychiatric illnesses such as depression and schizophrenia (Wilson et al. 2006; Thomas and O'Brian 2008), or those who experience increased psychological distress are approximately twice as likely to develop dementia or cognitive decline in old age (Wilson et al. 2006, 2007). Nevertheless, whether abnormal BDNF–TrkB expression or functioning contributes to both mood and cognition disturbance independently or in synergy is currently not clear, although the potential relationships have been noted (Martinowich et al. 2007; Groves 2007). Reduced BDNF serum levels have been reported in people with depression and depression-prone personality traits, and were shown to increase with antidepressant

treatment (Lang et al. 2004; Sen et al. 2008). The functional Val^{66}Met BDNF polymorphism, which reduces activity-dependent release and affects BDNF distribution, has been reported to predispose to depression, anxiety, and cognitive impairments (Egan et al. 2003; Hariri et al. 2003). Brain BDNF levels are lower in rodent models of depression (Smith et al. 1995). Conditional BDNF knockout mice exhibit depression-like behaviors (Monteggia et al. 2007). Antidepressant treatment increases BDNF expression in rodents (Nibuya et al. 1995), and central administration of BDNF may ameliorate depressive behaviors (Shirayama et al. 2002). Likewise, a strong correlation between upregulated BDNF–TrkB signaling and anxiety traits has been revealed (H. Wang et al. 2011). Thus, reduced activity-dependent proBDNF and BDNF release, together with a heightened BDNF–TrkB signaling that is un-opposed by a less effective p75^{NTR} in prenatal cocaine-exposed brains, may suggest that the offspring of the pregnant cocaine users are more prone to have depression, anxiety, and some forms of cognitive impairments. In support of this depression/anxiety-prone hypothesis, prenatal exposure to relatively high dose of cocaine (40 mg/kg/day) and/or nicotine (5 mg/kg/day) was found to promote the development of depression and anxiety in aging rats (Sobrian et al. 2003). In contrast, a longitudinal human study of the prenatal cocaine-exposed adolescent revealed that prenatal cocaine exposure impairs incidental face memory but without latent effect on inhibitory control, working memory, and receptive language (Betancourt et al. 2011).

There are potential translational implications of this work. First, this work demonstrates abnormalities in BDNF–TrkB and proBDNF/BDNF-p75^{NTR} signaling in the prenatal cocaine-exposed brains and thus may indicate that BDNF (or other

neurotrophin)-based therapies may not be effective in modifying synaptic and brain function at earlier stages because the system is already hyperactivated. It may even be possible that this hyperactivity promotes some of the observed brain function abnormalities. One effect of activated TrkB interaction with non-receptor Src family tyrosine kinase, fyn is phosphorylation of tau at Tyrosine18, which has been identified in paired helical filament preparations and neurofibrillary tangles of Alzheimer's disease and Down's syndrome patients (Goedert et al. 1991; Williamson et al. 2002; Lee et al. 2004). Fyn also phosphorylates GSK3β, which phosphorylates multiple Ser and Thr residues of tau, leading to neurofibrillary lesions and various signaling complications (Lesort et al. 1999; Jope et al. 2004). Regardless of whether upregulated BDNF–TrkB, in combination with downregulated $p75^{NTR}$ signaling, exacerbates synaptic damages in developing brains or is an adaptive response intended to offset the existing prenatal cocaine-elicited damages, our data presented here indicate that hyperactivated BDNF–TrkB signaling and dampened $p75^{NTR}$ are associated with brain changes elicited by prenatal cocaine exposure.

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