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Cocaine exposure *in vitro* induces apoptosis in fetal locus coeruleus neurons through TNF-α-mediated induction of Bax and phosphorylated c-Jun NH_2-terminal kinase

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Abstract

Cocaine exposure results in aberrant outgrowth and decreased survival for locus coeruleus (LC), a noradrenergic population of neurons that putatively regulates attentional function; however, the underlying mechanisms for these events are not known. We previously showed that cocaine exposure *in vitro* activates pro-apoptotic Bax, caspase-9, and caspase-3 in LC neurons dissected from embryonic day 14 rats, implicating that apoptosis may be orchestrated via signal transduction events. In the current study *in vitro*, we examined upstream events to determine the role of the pro-inflammatory cytokine, tumor necrosis factor alpha (TNF-α), on LC signal transduction, because cocaine exposure to LC neurons triggered TNF-α expression at 30 min as measured by ELISA. Exposure of LC neurons to *recombinant*-TNF-α resulted in decreased metabolic activity, an indicator of reduced neuron viability [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay], and increased apoptosis (terminal deoxynucleotidyl transferase-mediated DNA nick end labeling assay). Pro-apoptotic caspase-3 was induced by cocaine starting at 30 min. *Recombinant*-TNF-α induced caspase-3 activity earlier than cocaine (15 and 20 min). The caspase-3 levels were significantly reduced when cocaine and TNF-α were combined with neutralizing-TNF-α (nTNF-α), respectively. Further, cocaine alone elevated phospho-p38-mitogen-activated protein kinases that persisted when combined with nTNF-α. However, both cocaine and TNF-α independently increased phospho-c-Jun NH_2-terminal kinase and Bax levels at concurrent time periods (30 min and 1 h), and this elevation was attenuated in the presence of nTNF-α. These simultaneous molecular events triggered by cocaine and TNF-α implicate a potential apoptotic signal transduction pathway via induction of phospho-c-Jun NH_2-terminal kinase and Bax that may lead to caspase-3 activation and apoptosis in cocaine-exposed fetal LC neurons.

Keywords: apoptosis, cocaine, drug abuse, noradrenergic, rat, tumor necrosis factor-α.

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Attentional dysfunction is one of the prominent behavioral abnormalities associated with prenatal cocaine exposure (Richardson *et al.* 1996; Heffelfinger *et al.* 2002; Noland *et al.* 2005) and may result from deficits in noradrenergic locus coeruleus (LC) neurons, which innervate the prefrontal cortex (Bouret and Sara 2004). Previous studies *in vitro* and *in vivo* have shown that during early gestation, noradrenergic LC is a primary target for cocaine, influencing outgrowth and survival (Mactutus 1999; Snow *et al.* 2001, 2004; Foltz *et al.* 2004; Dey *et al.* 2006, 2007). The deleterious effects of cocaine on development are dependent upon the gestational period of exposure [Lidow and Song 2001 (primates); Stanwood *et al.* 2001 (rabbits); Snow *et al.* 2004; Dey *et al.* 2006 (rats)]. The latter studies showed that cocaine-induced alterations in development occur when cocaine is delivered during the time frame that includes peak LC neurogenesis, i.e. including embryonic days 11–13 (E11–13) in the rat, which corresponds to the fifth to sixth week in humans (Lauder and Bloom 1974;

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Abbreviations used: JNK, c-Jun NH_2-terminal kinase; LC, locus coeruleus; MAPK, mitogen-activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; nTNF-α, neutralizing-TNF-α; PARP, poly (ADP-ribose) polymerase; PBS, phosphate-buffered saline; PJNK, phosphorylated JNK; rTNF-α, recombinant TNF-α; TNF-α, tumor necrosis factor alpha; TUNEL, terminal deoxynucleotidyl transferase-mediated DNA nick end labeling.

Burgunder and Young 1990; Bayer *et al.* 1993). In the present study, we targeted this early gestational period (E14) to examine inhibitory effects of cocaine on LC survival. Recent studies indicate that cocaine-induced activation of Bax, caspase-9, and caspase-3, and cleavage of caspase-3 target proteins (Dey *et al.* 2007) can play a critical role in signaling pathways leading to apoptosis in embryonic LC neurons.

Neuronal death plays an important role in normal CNS development by precisely orchestrating synaptic connectivity. However, unregulated neuronal death may abrogate synaptic organization, leading to developmental and behavioral abnormalities in offspring. In this study *in vitro*, we sought to determine the potential initiators of the apoptotic signaling pathway triggered by cocaine exposure in fetal LC neurons. Although the CNS is often considered protected from the infiltration of cytokines and immunocompetent cells due to the blood–brain barrier, receptors for tumor necrosis factor alpha (TNF-α) and interleukin-1, as well as TNF-α protein and mRNA have been detected in neurons innervating various brain regions (Takao *et al.* 1990; Kinouchi *et al.* 1991; Breder *et al.* 1993; Ignatowski *et al.* 1996). In addition, accumulation and/or release of TNF-α by neurons has been reported (Ignatowski *et al.* 1997), affecting the release of neurotransmitters and the growth and degeneration of neurons (Mogi *et al.* 1994; Ignatowski *et al.* 1997). Importantly, TNF-α-mediated apoptosis involves activation of caspases (Chang and Yang 2000; Haeberlein 2004) and members of mitogen-activated protein kinases (MAPKs), the p38-MAPK and the c-Jun NH_2-terminal kinase (JNK-MAPK), through the TNF death domain (Park *et al.* 2002). TNF death receptor initiates phosphorylation of JNK in retinal ganglion cells following optic nerve injury (Tezel *et al.* 2004), which also plays an important role in apoptosis by inducing pro-apoptotic Bax expression, resulting in mitochondrial cytochrome *c* release (Tournier *et al.* 2000; Tsuruta *et al.* 2004). Studies also implicate p38-MAPK in cocaine-induced myocardial apoptosis (Zhang *et al.* 1999).

Because of the importance of TNF-α in initiating cell death pathways, in the present study we sought: (i) to determine whether cocaine-mediated, decreased LC survival could be due to TNF-α and (ii) to begin to define the molecular components of the TNF-α-induced apoptotic pathway in LC neurons. We exposed LC neurons to exogenous recombinant TNF-α (rTNF-α), verified its effects by combining it with neutralizing anti-TNF-α, and defined the signaling events triggered in LC neurons dissected from E14 embryos that were exposed to cocaine. The results of these studies showed that TNF-α is sufficient to induce a cell death cascade in LC neurons involving caspases, Bax and bcl-2, and may identify potential pharmacological targets for therapeutic interventions for cocaine ab(use) during pregnancy.

Materials and methods

Animals

Nulliparous Long Evans timed-pregnant rats were maintained according to NIH guidelines in The Association for Assessment and Accreditation of Laboratory Animal Care accredited facilities. Food (Teklad Global 18% Protein Rodent Diet No. 2018; Harlan Teklad, Madison, WI, USA) and water were available *ad libitum*. The animal facility was maintained at 21°C ± 2 and 50% ± 10, relative humidity and had a 12 h light : 12 h dark cycle with lights on at 07:00 hours (EST). The protocol for the use of rats in this project was approved by the Institutional Animal Care and Use Committee of the University of Kentucky (00656M2003). The animals were allowed to acclimate for 2 days after shipment. All procedures were conducted under a protocol approved by The University of Kentucky Animal Care and Use Committee and regulated in Pain Category I (no pain).

Tissue culture

Substratum preparation

Tissue culture flasks were coated with poly-L-lysine (100 μg/mL; Sigma, St Louis, MO, USA) in sterile calcium-magnesium free phosphate-buffered saline (PBS; pH 7.2) and stored at 37°C overnight in a room air-humidified, HEPA-filtered incubator. The flasks were rinsed twice with sterile double distilled water, then covered with 500 μL of culture media and incubated at 37°C in a 5% CO_2-air humidified incubator until required for use.

Tissue dissection

Timed-pregnant rats bearing E14 pups were killed by CO_2 inhalation according to The Association for Assessment and Accreditation of Laboratory Animal Care regulations. The uterine horns were removed under sterile conditions and placed in a Petri dish containing cold Hank's Balanced Salt Solution (pH 7.3) on ice. The embryos were removed and placed in cold L-15 medium containing 6 mg/mL glucose and 15 μg/mL gentamycin and further dissection was performed in the same medium. The brains were removed and areas containing LC (rhombencephalon) (Konig *et al.* 1989) neurons were dissected. The tissue dissected was a mixed population of LC neurons as well as non-neuronal cells (e.g. glia). However, the effect of cocaine was specifically studied on LC neurons. The tissues were completely dissociated by gentle trituration, using glass Pasteur pipettes with increasingly smaller-bored fire polished tips. Cells were plated at varying densities on previously prepared tissue culture flasks (see above) and chamber slides.

Media

Locus coeruleus neurons were cultured in minimum essential medium (Earle's; Invitrogen, Carlsbad, CA, USA), supplemented with 6 mg/mL glucose, 2 mmol/L glutamine, 5% horse serum (Hyclone, Logan, UT, USA), 5% fetal bovine serum (Hyclone), and 15 μg/mL gentamycin (Gibco Life Technologies Inc., Carlsbad, CA, USA). All cultures were grown using the same batch of serum to avoid variability among cultures. Four days after plating, the original media was discarded and culture dishes were either replenished with fresh media (controls) or media containing cocaine hydrochloride (500 ng/mL), or rTNF-α (10 ng/mL), or neutralizing anti-TNF-α (1 μg/mL) in combination with cocaine or rTNF-α.

Cocaine

Cocaine hydrochloride ($C_{17}H_{21}NO_4$) was obtained from the NIDA Drug Supply Program (RTI International, Research Triangle Park, NC, USA). Cocaine undergoes rapid degradation and is unstable at neutral or alkaline pH (Baselt 1983; Isenschmid *et al.* 1989; Bouis *et al.* 1990; Vorhees *et al.* 1995). Cocaine was dissolved in sodium citrate buffer (pH 5.0) at a concentration of 10 μg/mL to prevent degradation into its primary and secondary metabolites, benzoylecgonine and ecgonine methyl ester (Vorhees *et al.* 1995). Further dilutions were made using the growth media such that the final concentration added to the culture dishes was pharmacologically relevant to the dose of 500 ng/mL (1.5 μmol/L) (Evans *et al.* 1996; Mactutus 1999; Bunney *et al.* 2000).

ELISA for detection of activated TNF-α

Locus coeruleus neurons dissected from E14 embryos were left untreated or exposed to cocaine (500 ng/mL) for 30 min and 1, 4, and 24 h. Levels of TNF-α secreted in the culture supernatants of untreated and treated embryonic LC neurons were detected using the DuoSet ELISA development system (R&D Systems Inc., Minneapolis, MN, USA). Microtiter ELISA plates (DYNATECH LABS, Chantilly, VA, USA) were coated with mouse anti-rat TNF-α antibody overnight at 23°C, then washed with wash buffer, and blocked for 1 h with blocking buffer (reagent diluent, 1% BSA in PBS, pH 7.2–7.4). Next, 100 μL of standards (recombinant rat-TNF-α) and test supernatants were added in triplicates and incubated for 2 h at 23°C. Unbound substances were washed and incubated with 100 μL of biotinylated goat anti-rat TNF-α secondary antibody for 2 h at 23°C. Subsequently, plates were washed and the complex was conjugated with 100 μL of streptavidin-horseradish peroxidase for 20 min at 23°C. Sure Blue Reserve, 3,3′,5,5′ tetramethyl benzidine Peroxidase Substrate solution (KPL, Gaithersburg, MD, USA) was added and incubated for 20 min for the color reaction, which was stopped using a 2 N H_2SO_4 solution, and the optical density was measured using a microplate ELISA reader set at a wavelength of 450 nm. Mean values were obtained from three replicates ($n = 3$).

Cell survival assay

Survival of LC neurons following exposure to exogenous recombinant rat TNF-α (rTNF-α; R&D Systems Inc.) was determined by measuring the metabolic activity of LC neurons with a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Roche Applied Science, USA). Cells were cultured at a density of (1×10^5) in triplicates in a 96 well plate. Cells were treated with increasing concentrations of rTNF-α (0.1, 1, 5, 10, and 15 ng/mL) and incubated for 48 h. The resultant purple formazan product indicative of metabolic activity of the cells was solubilized and quantified using a multiwell ELISA spectrophotometer at an absorbance of 570 nm with reference wave length >650 nm. Mean values were obtained from three replicates ($n = 3$).

Quantitation of apoptosis

Neuronal apoptosis was analyzed by terminal deoxynucleotidyl transferase-mediated DNA nick end labeling (TUNEL), using a fluorescein *in situ* cell death detection kit (Roche Applied Science, USA). Cells were cultured for 4 days and then left untreated (controls) or exposed to exogenous recombinant rat TNF-α alone (rTNF-α 10 ng/mL; R&D Systems Inc.) or rTNF-α in combination with neutralizing anti-TNF-α (nTNF-α 1 μg/mL; R&D Systems Inc.), or cocaine alone (500 ng/mL), or nTNF-α in combination with cocaine. Cell death was measured after 24 and 48 h of treatment. Cell death was confirmed using fluorescent DNA-binding Hoechst dye 33258 (1 μg/mL in 1× calcium-magnesium free-PBS; Invitrogen) for 10 min to identify condensed or fragmented nuclei. Neuronal cells were identified by immunocytochemistry using the neuronal marker, TUJ1 (anti-βIII tubulin antibody, Covance, Berkely, CA, USA). The fixed cells after treatment were triple stained by immunocytochemistry using first, TUJ1 (neuronal marker), then by TUNEL and Hoechst staining, respectively. The stained specimens were visualized using a fluorescence microscope and Axiovision software v. 4.1 (Carl Zeiss Vision GmbH-v 4.1, Thornwood, NY, USA) and images were merged using multidimensional acquisition. The number of apoptotic cells was counted from 10 randomly selected fields and the percentage of apoptotic cells was calculated as (number of apoptotic cells/number of total cells × 100). Mean values were calculated from three replicates ($n = 3$).

Caspase-3 activity assay

Caspase-3 activity was measured using the caspase-3 substrate (Ac-DEVD-7-amino-4-methylcoumarin; Bachem Biosciences, King of Prussia, PA, USA). Briefly, LC neurons from E14 embryos were cultured for 4 days and treated with cocaine alone (500 ng/mL) or exogenous *recombinant* rat TNF-α alone (rTNF-α 10 ng/mL) to detect caspase-3 activity. To investigate the role of TNF-α in cocaine-induced activation of caspase-3, cocaine and rTNF-α treatments on LC neurons were combined with anti-TNF-α *neutralizing* antibody (nTNF-α 1 μg/mL, R&D Systems Inc.) and harvested at 30 min and 1 h after treatments. Additionally, the effects of rTNF-α on caspase-3 activity were studied at earlier time points as well (10, 15, and 20 min). Following treatment, cells were harvested in lysis buffer [25 mmol/L HEPES, pH 7.5 (Sigma), 5 mmol/L EDTA (Sigma), 1 mmol/L EGTA (Sigma), 5 mmol/L $MgCl_2$ (Fisher Scientific, Pittsburgh, PA, USA), 10 mmol/L sucrose (Fisher Scientific), 5 mmol/L dithiothreitol (Sigma), 1% 3-[-(3-chloramidopropyl) dimethylammonio]-1-propanesulfonic acid (Sigma), protease inhibitor cocktail (10 μL/mL; Sigma), and 1 mmol/L phenylmethylsulphonyl fluoride (Sigma)]. Crude cell lysates were freeze/thawed three times and centrifuged at 12 000 *g* for 60 min. The supernatants (cell lysates) were normalized for protein content and incubated in a buffer containing 25 mmol/L HEPES, pH 7.5, 10% sucrose, 0.1% 3-[-(3-chloramidopropyl) dimethylammonio]-1-propanesulfonic acid, and 1 mmol/L dithiothreitol supplemented with 50 μmol/L of caspase-3 substrate at 37°C in 96-well plates for 1 h. The enzyme-catalyzed release of the fluorogenic 7-amino-4-methylcoumarin moiety was quantified in a Wallac Victor III fluorimeter (Perkin Elmer, Waltham, MA, USA) using 360 nm excitation and 460 nm emission wavelengths. caspase-3 activity was expressed as units per microgram of total protein. Changes in cocaine and rTNF-α-induced caspase-3 activity was compared with the control cells (no treatment). Changes in levels of caspase-3 activity observed after combining nTNF-α with cocaine or rTNF-α were compared with their respective changes in caspase-3 activity when treated with cocaine and rTNF-α alone. Mean values were obtained from three replicates ($n = 3$). Total cell lysates were quantified using the Bicinchoninic Acid assay kit (Pierce, Rockford, IL, USA).

Western blot analysis

Embryonic LC neurons were left untreated or treated with (i) cocaine (500 ng/mL) alone, or (ii) cocaine in combination with nTNF-α (1 μg/mL), or (iii) rTNF-α alone, or (iv) rTNF-α (10 ng/mL) in combination with nTNF-α (1 μg/mL). Cell lysates were extracted at 30 min and 1 h after treatment using the lysis buffer described above. Cell lysates normalized for protein content were electrophoresed in 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions in running buffer (25 mmol/L Tris, 192 mmol/L glycine, and 0.1% sodium dodecyl sulfate, pH 8.3) and electrotransferred to polyvinylidene fluoride membrane in Towbin-transfer buffer (25 mmol/L Tris, 192 mmol/L glycine, and 20% MeOH, pH 8.3) at 100 constant volts for 2 h. Blots were blocked with 5% milk solution in wash buffer (20 mmol/L Tris and 500 mmol/L NaCl, pH 7.5) for 2 h and incubated overnight in 5% milk containing anti-JNK-polyclonal antibody, or anti-phospho-JNK monoclonal antibody, or anti-p38 polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), or anti-phospho-p38 monoclonal antibody (Cell Signaling Technology Inc. USA), or anti-Bax monoclonal antibody (Santa Cruz Biotechnology Inc), or anti-Bcl-2 polyclonal antibody (Santa Cruz Biotechnology Inc.). All antibodies were used at a dilution of 1 : 1000. The blots were washed and the bound antigen–antibody was detected by incubation for 1 h in the appropriate horseradish peroxidase-conjugated secondary antibody (Jackson Immunoresearch Laboratories, Bar Harbor, ME, USA). Mouse anti-actin monoclonal antibody (Chemicon International, Temecula, CA, USA) was used as an internal loading control at a dilution of 1 : 5000. Immunoreactive proteins were visualized using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotechnology, Buckinghamshire, UK). Results were quantified using the KODAK Imaging system and KODAK 1D Image Analysis Software (New Haven, CT, USA). The proteins of interest were normalized to actin and expressed as percent control of the protein levels within each group. Mean values were calculated from three replicates ($n = 3$).

Data analysis

Data were represented as mean ±SEM from replicate samples obtained from at least three separate experiments. Overall differences between experimental groups were analyzed using ANOVA (JMP IN statistical software, release version 5.1; SAS Institute Inc., Belmont, CA, USA). Specific ANOVAs used were: (i) TNF-α expression (2 × 4): (treatment – cocaine vs. control) × (time – 30 min, 1 h, 4 h and 24 h); (ii) neuron viability (2 × 4): (treatment – TNF-α vs. control) × (TNF-α dose 1, 5, 10, and 15 ng/mL); (iii) cell death (2 × 2): (treatment – TNF-α vs. control) × (time – 24 h and 48 h); (iv) caspase-3 activity: (2 × 2) (treatment – cocaine vs. control) × (time – 30 min and 1 h) or (2 × 5) (treatment – TNF-α vs. control) × (time – 10, 15, 20, 30 min and 1 h); (v) protein expression after cocaine exposure (2 × 2): (treatment – cocaine vs. control) × (time – 30 min and 1 h) or [treatment – cocaine vs. (cocaine + nTNF-α)] × (time – 30 min and 1 h); and (vi) protein expression after TNF-α exposure (2 × 2): (treatment – TNF-α vs. control) × (time – 30 min and 1 h) or [(treatment – TNF-α vs. (TNF-α + nTNF-α)] × (time – 30 min and 1 h). If significant differences were found between experimental groups, paired group differences of time and dose effects were analyzed *post hoc* using Dunnett's test (d). An α-level of $p < 0.05$ was considered significant for all statistical tests employed.

Results

Cocaine exposure *in vitro* induces TNF-α expression in fetal LC neurons as measured by ELISA

Cocaine induces the activity of the apoptotic indicator, caspase-3 (Dey *et al.* 2007), and apoptosis, documented in several neural systems, is induced via TNF-α (Barker *et al.* 2001; Lee *et al.* 2001). Thus, we determined whether cocaine exposure was sufficient to induce the expression of TNF-α in LC neurons, which could in turn mediate an apoptotic cascade. The expression of TNF-α was assayed by ELISA in cocaine-treated fetal LC neuron cultures and controls (untreated). As hypothesized, cocaine exposure resulted in a significant induction of TNF-α in LC neurons compared with controls ($F = 4.61$; $p < 0.008$), with a peak expression at 30 min following cocaine treatment (d: $p < 0.012$; Fig. 1).

TNF-α decreases fetal LC neuron viability (metabolic activity), as measured by the MTT assay

Given that cocaine exposure to fetal LC neurons induced TNF-α expression, we sought to determine whether TNF-α

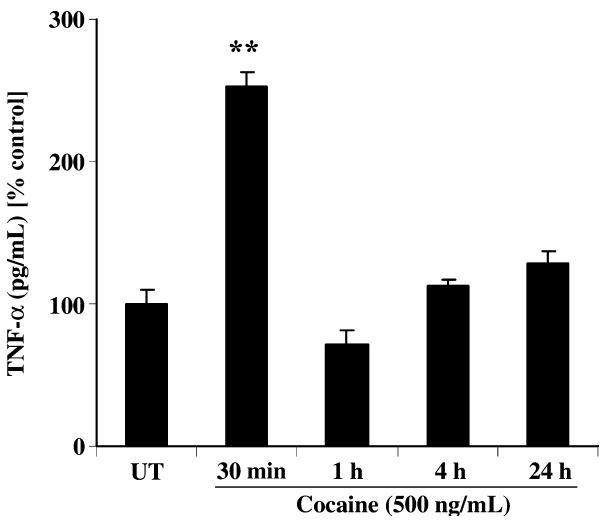


Fig. 1 Cocaine exposure *in vitro* induces tumor necrosis factor alpha (TNF-α) expression in fetal locus coeruleus neurons. Embryonic day 14 locus coeruleus neurons were treated with cocaine (500 ng/mL). TNF-α in the supernatants was measured after 30 min and 1, 4, and 24 h using ELISA. TNF-α expression was significantly induced following 30 min of cocaine exposure (~ 2.5-fold) compared with controls (ANOVA: $^*p < 0.008$; $n = 3$).

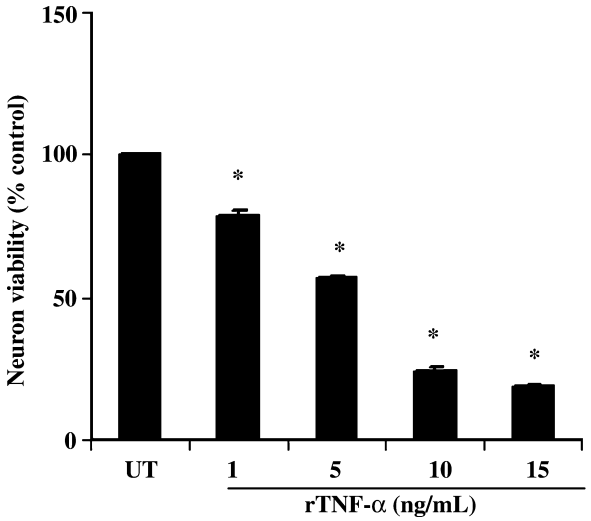


Fig. 2 Tumor necrosis factor alpha (TNF-α) decreases fetal locus coeruleus (LC) neuron survival. Survival of LC neurons *in vitro* was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, a colorimetric measure of metabolic activity, which serves as an indicator of neuron survival. LC neurons treated with 1, 5, 10 and 15 ng/mL of recombinant TNF-α (rTNF-α) demonstrated a linear dose-dependent decrease in survival with increasing doses of rTNF-α (ANOVA: $^*p < 0.0001$; $r^2 = 0.983$; $n = 3$), compared with controls. A floor effect was observed between 10 and 15 ng/mL (ANOVA: $p < 0.061$, $r^2 = 0.201$).

alone was sufficient to initiate LC neuron cell loss. The MTT assay measures changes in metabolic activity, indicative of changes in survival (neuron viability). The MTT assay was used in LC neurons exposed to recombinant rat TNF-α (rTNF-α) *in vitro* at increasing concentrations of 1, 5, 10 and 15 ng/mL TNF-α. The results of this assay showed a linear dose-dependent decrease in metabolic activity of LC neurons compared with controls, indicative of overall decreased neuron viability ($F = 590.4$; $p < 0.0001$; $r^2 = 0.983$). There was a significant decrease in neuronal viability at each concentration studied, with a floor effect between 10 and 15 ng/mL ($F = 4.04$; $p < 0.061$; d: $p < 0.061$; $r^2 = 0.201$; Fig. 2). Therefore, all subsequent experiments used a concentration of 10 ng/mL TNF-α.

Cocaine and TNF-α induce apoptosis in fetal LC neurons, assayed using TUNEL analysis and Hoechst staining

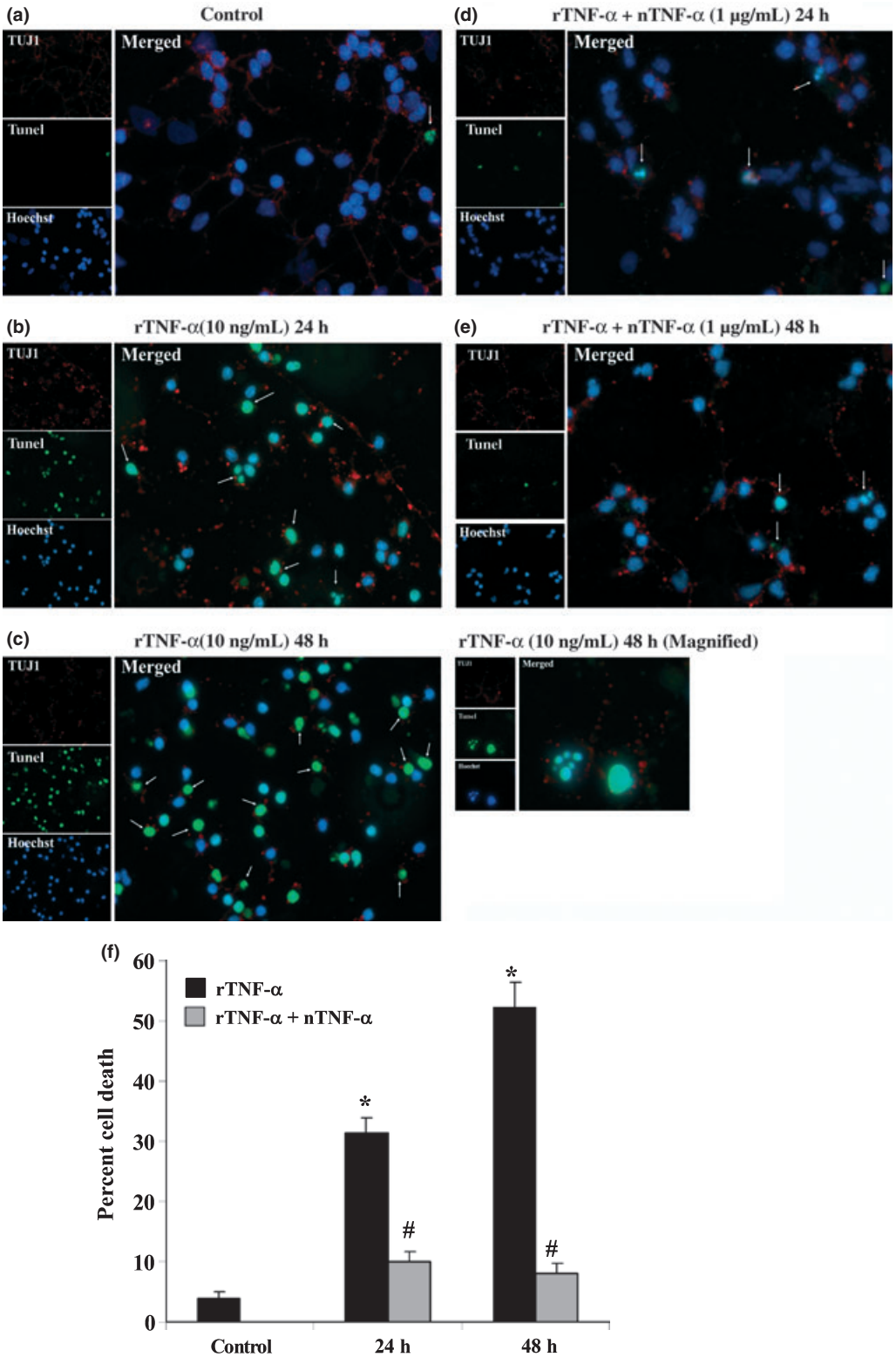
To determine whether decreased LC survival in response to rTNF-α (10 ng/mL) and cocaine was mediated through apoptosis, we performed TUNEL analysis to study DNA fragmentation, which is a hallmark of apoptosis (Nassogne *et al.* 1998; He *et al.* 2000b). TUNEL analysis indicated positive cell death profiles as evidenced by DNA fragmentation at 24 and 48 h after treatment with rTNF-α in LC neurons (Fig. 3b and c), compared with controls (Fig. 3a), and cocaine-treated LC neurons (Fig. 3h and i), in comparison with controls (Fig. 3g). Although DNA fragmentation is a well-accepted indicator of apoptosis, our results were further confirmed by staining with the DNA-binding fluorescent Hoechst dye, which showed neuronal chromatin condensation accompanied by DNA fragmentation in rTNF-α and cocaine-treated LC neurons (Fig. 3a, b, c, g, h, and i, respectively). That the effects were specific to neurons was confirmed by co-staining with the neuronal marker TUJ1 (anti-βIII tubulin antibody; Covance) (Fig. 3a, b, c, g, h, and i, respectively). Quantitative analysis showed a significant cell death effect of rTNF-α ($F = 74.31$; $p < 0.0001$; Fig. 3f) and cocaine ($F = 58.63$; $p < 0.0002$; Fig. 3l) on LC neurons. Apoptotic cells were expressed as percent cell death (number of apoptotic cells/number of total cells × 100). In contrast, neutralizing TNF-α (nTNF-α) when combined with rTNF-α significantly reduced cell death in LC neurons at 24 and 48 h (F's > 50.00; p's < 0.001, compared with respective rTNF-α treatments; Fig. 3d, e, and f), and showed a significant decrease in the percent of cell death compared with controls at both time points studied ($F = 3.14$; $p < 0.062$; Fig. 3f). Further, nTNF-α when combined with cocaine significantly reduced cell death in LC neurons at 24 and 48 h (F's > 44.00; p's < 0.0001, compared with

Fig. 3 Cocaine and tumor necrosis factor alpha (TNF-α) induces apoptosis in fetal locus coeruleus (LC) neurons. LC (embryonic day 14) neurons were treated with recombinant TNF-α (rTNF-α; 10 ng/mL) and cocaine (500 ng/mL) and assessed for apoptosis after 24 and 48 h of treatment. LC neurons were positively identified using the neuronal marker TUJ1 (βIII tubulin antibody). Terminal deoxynucleotidyl transferase-mediated DNA nick end labeling (TUNEL) was used to identify DNA fragmentation. As TUNEL-positive cells can sometimes represent cells undergoing necrosis, the DNA-binding fluorescent Hoechst dye was used to identify and validate chromatin condensation and DNA fragmentation. These were visualized by fluorescence microscopy to identify apoptosis after 24 and 48 h, in the absence of rTNF-α and cocaine, respectively, (a and g) and in the presence of rTNF-α (b and c), and cocaine (h and i). Abundant TUNEL-positive cells (green; arrows indicate some of the TUNEL-positive cells), co-localized with Hoechst (blue) and TUJ1 (βIII tubulin) staining (red) was observed, 24 and 48 h after rTNF-α (b and c) and cocaine treatment in LC neurons (h and i). Quantitative analysis indicated a significant cell death effect induced by rTNF-α (ANOVA: $^*p < 0.0001$; $n = 3$; f) and cocaine (ANOVA: $^*p < 0.0002$; $n = 3$). In contrast, apoptosis induced by rTNF-α was significantly attenuated in combination with nTNF-α, at 24 and 48 h (d, e, and f; ANOVA: $^\#p$'s < 0.0001). Apoptosis induced by cocaine was also significantly attenuated in combination with nTNF-α at 24 and 48 h (j, k, and l; ANOVA: $^\#p$'s < 0.0001).

respective cocaine treatments; Fig. 3j, k, and l). In addition, nTNF-α showed a significant decrease in the percent of cell death compared with controls at both time points studied ($F = 2.87$; $p < 0.074$).

Cocaine and TNF-α increase caspase-3 enzyme activity, which is attenuated by nTNF-α

As cocaine up-regulates TNF-α expression and activates caspase-3, it stood to reason that TNF-α might activate



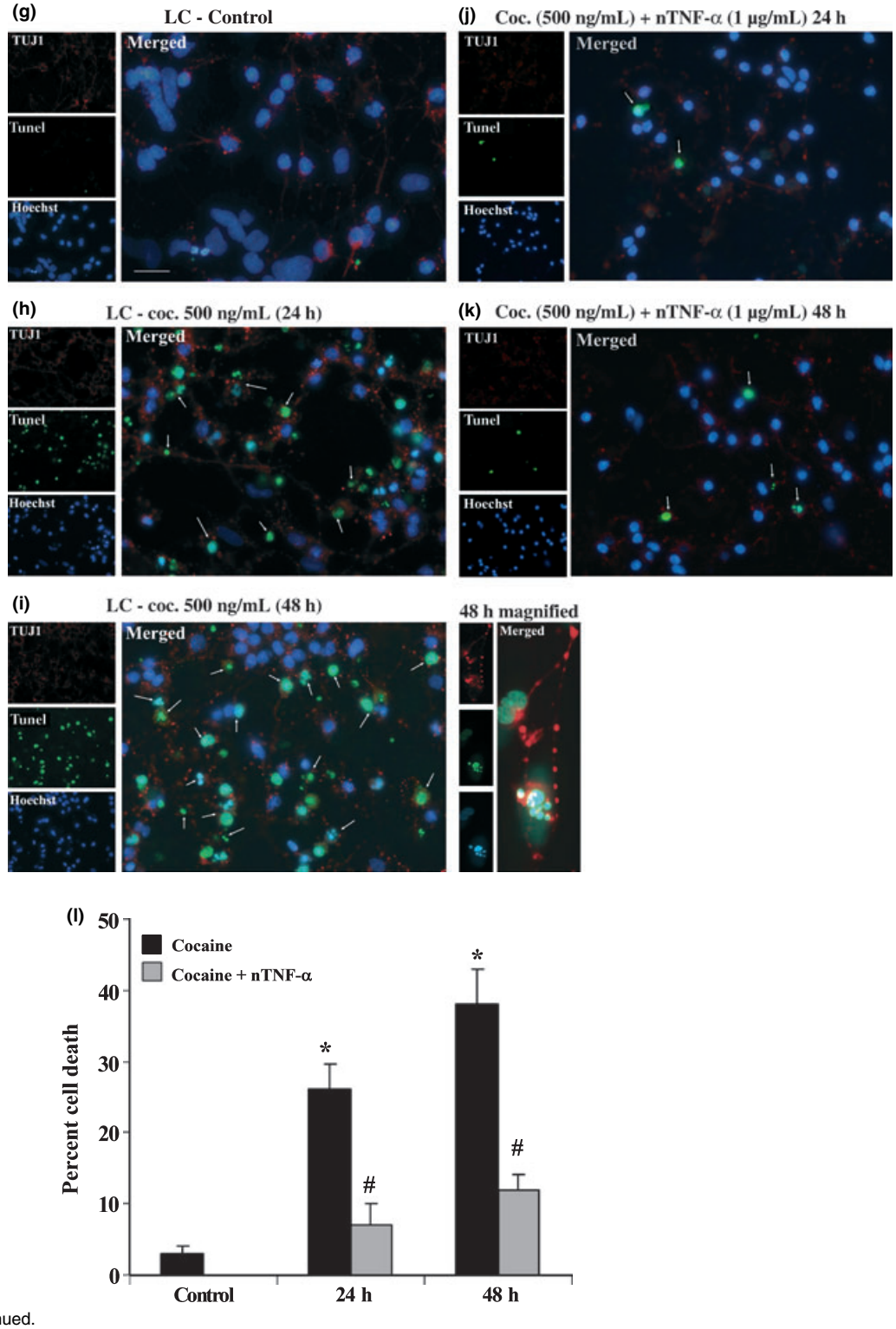


Fig. 3 Continued.

caspase-3 directly. Thus, LC neurons were treated *in vitro* with cocaine alone (500 ng/mL), cocaine in combination with neutralizing rat TNF-α (nTNF-α; 1 μg/mL), recombinant rat TNF-α alone (rTNF-α; 10 ng/mL), and rTNF-α in combination with nTNF-α. Caspase-3 activity was measured at 30 min and 1 h, two critical time points at which cocaine showed peak activation of caspase-9 and caspase-3 (Dey *et al.* 2007). In this study, as previously, cocaine

exposed LC neurons showed a significant induction of caspase-3 activity at both time points ($F = 6.41$; $p < 0.012$; Fig. 4a). Neutralizing TNF-α in combination with cocaine significantly reduced cocaine-induced caspase-3 activity ($F = 13.84$; $p < 0.0001$; Fig. 4a). Surprisingly, LC neurons treated with rTNF-α alone did not show a significant induction of caspase-3 activity ($F = 0.35$; $p = 0.711$). However, nTNF-α in combination with TNF-α significantly attenuated basal levels of rTNF-α-expressed caspase-3 at both time points ($F = 4.56$; $p < 0.017$; Fig. 4a).

Additional experiments were conducted to study caspase-3 expression triggered by exogenous application of rTNF-α at earlier time points (10, 15, and 20 min), in addition to previously studied time points (30 min and 1 h). Results revealed that rTNF-α significantly induced caspase-3 at 15 min and 20 min (F's > 55.0; p's < 0.0003). As previously (Fig. 4a), TNF-α-induced caspase-3 activity was reduced to basal levels by 1 h (Fig. 4b). The rTNF-α-induced caspase-3 activity was attenuated when combined with nTNF-α (F's > 25.0 p's < 0.001; Fig. 4b).

Cocaine and TNF-α show concomitant induction of JNK phosphorylation, but not phosphorylation of p38-MAPK in fetal LC neurons as assayed by western blotting

As TNF-α is an important activator of the members of the MAPK family, JNK and p38, which are important regulators of apoptosis, we investigated the expression levels of phosphorylated JNK (PJNK) and p38 using western blot analysis. LC neurons exposed to cocaine *in vitro* showed significant induction of phospho-p38 (Pp38) at 30 min ($F = 295.91$; $p < 0.0001$), but did not show a significant induction at 1 h, compared with controls (Fig. 5a and b). However, nTNF-α did not attenuate cocaine-induced Pp38, when in combination with rTNF-α at both time points studied. In contrast, LC neurons exposed to rTNF-α did not induce Pp38 protein expression ($F = 3.767$; $p = 0.087$). In addition, rTNF-α when combined with nTNF-α failed to attenuate levels of Pp38 ($F = 2.12$; $p = 0.17$; Fig. 5a and b). [Data presented as ratio of phosphorylated/(phosphorylated + total p38)].

In contrast, an increase in PJNK was observed in LC neurons exposed to cocaine at 30 min and 1 h ($F = 16.38$; $p < 0.003$), when compared with controls (Fig. 6a and b). Cocaine-induced activation of JNK was reduced by nTNF-α at both time points studied ($F = 44.28$; $p < 0.0001$) (Fig. 6a and b). Similar to cocaine treatment, exposure to rTNF-α increased phosphorylation of JNK ($F = 18.64$; $p < 0.0027$), at 30 min (d: $p < 0.007$), which returned to basal levels after 1 h (d: $p = 0.438$). nTNF-α significantly reduced rTNF-α-induced PJNK ($F = 23.88$; $p < 0.0002$) at both time points studied (Fig. 6a and b). These data show that cocaine exposure induces phosphorylation of JNK and p38 at early time periods in fetal LC neurons.

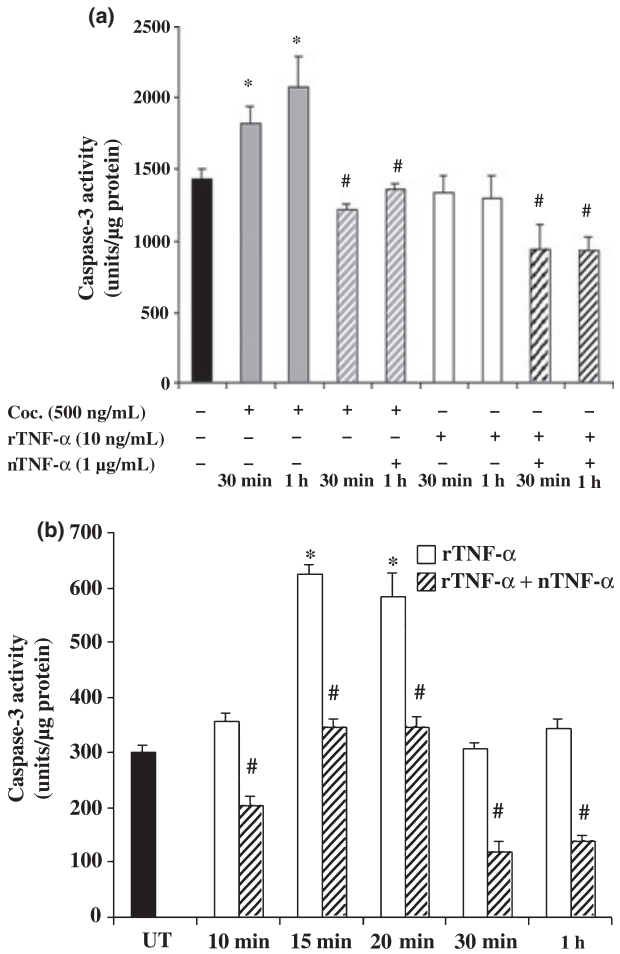


Fig. 4 Tumor necrosis factor alpha (TNF-α) and cocaine-induced caspase-3 activation. Fetal locus coeruleus (LC) neurons were analyzed for effector caspase-3 activity, using the caspase-3 substrate, Ac-DEVD-7-amino-4-methylcoumarin. Cells were treated with (i) cocaine alone (500 ng/mL), or (ii) cocaine in combination with nTNF-α (1 μg/mL), or (iii) recombinant (r)TNF-α alone (10 ng/mL), or (iv) rTNF-α in combination with nTNF-α. Cell lysates obtained from control and treated LC neurons, normalized for protein content, were incubated with caspase-3 substrate following the protocol in the Materials and methods section. The caspase-3 activity assay measured the enzyme-catalyzed release of the fluorogenic 7-amino-4-methylcoumarin moiety, which was quantified using a Wallac Victor III fluorimeter. Caspase-3 activity was significantly induced in cocaine-exposed LC neurons at 30 min and 1 h (gray bars; ANOVA: $^{*}p < 0.012$) and activity was attenuated when cocaine was combined with nTNF-α (gray striped bars, ANOVA: $^{\#}p < 0.0001$) (a). In contrast, treatment with rTNF-α did not induce caspase-3 activity in LC neurons at 30 min and 1 h (white bars, ANOVA: $p = 0.711$) (a). However, rTNF-α in combination with nTNF-α significantly reduced basal levels of caspase-3 activity (black striped bars, ANOVA: $^{\#}p < 0.017$; $n = 3$). However, additional experiments show that rTNF-α-induced caspase-3 activity at earlier time points of 15 and 20 min (white bars, ANOVA: $^{*}p < 0.0003$) that was reduced to basal levels by 1 h (b). The rTNF-α-induced caspase-3 activity was attenuated when combined with nTNF-α (black striped bars, ANOVA: $^{\#}p < 0.001$; $n = 3$) (b).

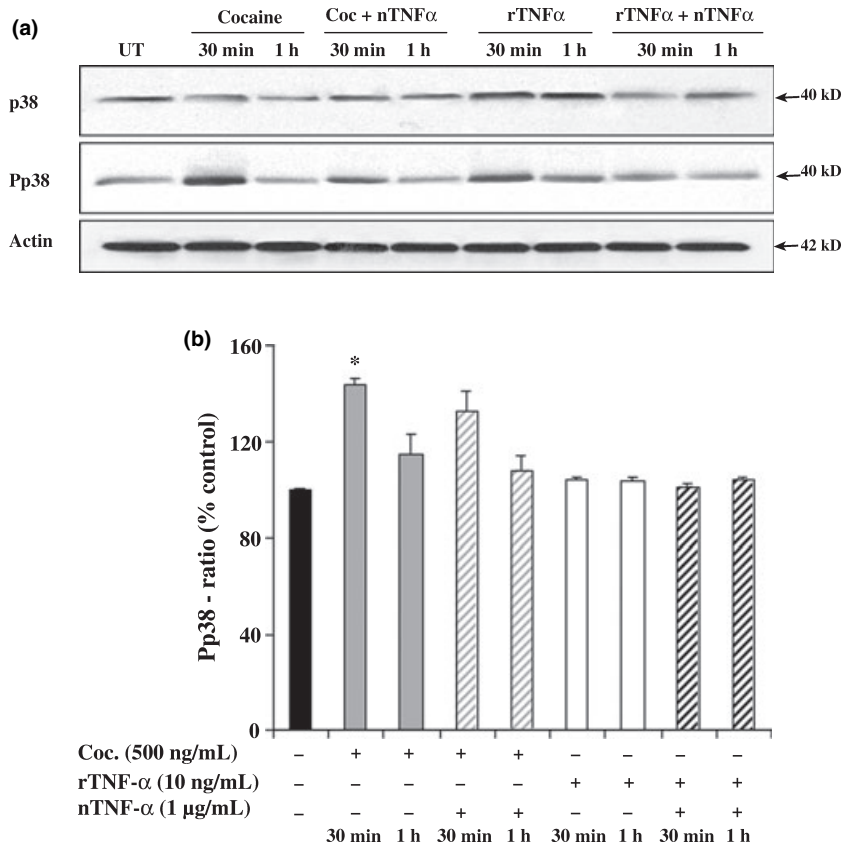


Fig. 5 Cocaine-induced phosphorylation of p38-mitogen-activated protein kinases activity in fetal locus coeruleus (LC) neurons. LC neurons were treated with (i) cocaine alone (500 ng/mL), or (ii) cocaine in combination with neutralizing tumor necrosis factor alpha (nTNF-α; 1 μg/mL), or (iii) recombinant (r)TNF-α alone (10 ng/mL), or (iv) rTNF-α in combination with nTNF-α. Cell lysates were extracted from control and treated LC neurons at 30 min and 1 h after treatment, and 15 μg of cell lysates normalized for protein content was loaded and run on a 12.5% sodium dodecyl sulfate–polyacrylamide gel. Western blot analyses revealed that cocaine exposure in embryonic LC neurons-induced Pp38 protein levels (gray bar, ANOVA: $^*p < 0.0001$), showing a significant induction at 30 min (d: $^*p < 0.0001$), compared with controls (a and b). However, nTNF-α did not reduce cocaine-induced Pp38 protein levels (gray striped bars). In contrast, rTNF-α treated LC neurons did not show a significant induction of Pp38 (white bars, ANOVA: $p = 0.087$) (a and b), and basal levels were not attenuated by nTNF-α when in combination with rTNF-α (black striped bars, ANOVA: $p = 0.81$) (a and b). Quantitative analysis of Pp38 was performed on blots represented in (a). Data is presented as ratio of [phosphorylated/(phosphorylated + total p38)]. A mouse anti-actin monoclonal antibody was used as an internal loading control (a) ($n = 3$).

Recombinant TNF-α did not induce Pp38, but given that nTNF-α attenuated both cocaine-induced and rTNF-α-induced JNK phosphorylation, the data show that TNF-α is sufficient to mediate apoptosis in cocaine exposed fetal LC neurons via a JNK-induced apoptotic pathway *in vitro*. [Data presented as ratio of phosphorylated/(phosphorylated + total p38)].

TNF-α and cocaine show concomitant induction of Bax protein levels in fetal LC neurons as assayed by western blotting

Cocaine exposure *in vitro* (500 ng/mL) significantly increased Bax protein levels at 30 min and 1 h ($F = 63.92$; $p < 0.0001$), but did not show a significant change in Bcl-2 levels ($F = 0.241$; $p = 0.79$; Fig. 7a, b, and c). To determine the potential role of TNF-α in prenatal cocaine-mediated changes in Bax and Bcl-2 protein levels, we studied the effect of nTNF-α in combination with cocaine *in vitro*, on LC neurons. Cocaine-induced Bax protein levels were significantly reduced when combined with nTNF-α ($F = 46.15$; $p < 0.0001$) at both time points studied (Fig. 7a and b). No changes were observed under these conditions in Bcl-2 levels (Fig. 7a and c). Similar to cocaine treatment, exposure of LC neurons to rTNF-α alone resulted in a significant induction of Bax levels in comparison with controls at 30 min and 1 h ($F = 7.33$; $p < 0.024$), which was attenuated in combination with nTNF-α ($F = -5.25$; $p < 0.027$) (Fig. 7a and b). Significant changes in Bcl-2 levels were not observed in LC neurons treated with rTNF-α. However, when cells were treated with rTNF-α in combination with nTNF-α, Bax levels were attenuated ($F = 6.78$; $p < 0.013$), only at 1 h (d: $p < 0.004$; Fig. 7a and c).

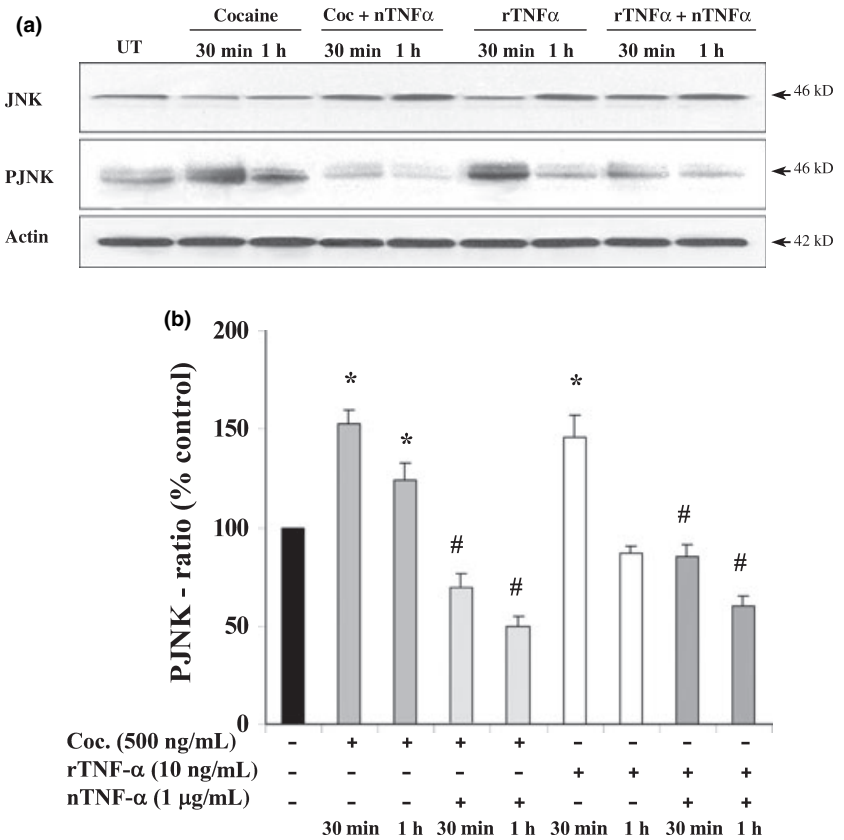


Fig. 6 Recombinant tumor necrosis factor alpha (rTNF-α) and cocaine showed a concomitant induction of c-Jun NH_2-terminal kinase (JNK) phosphorylation in fetal locus coeruleus (LC) neurons. LC neurons were treated with (i) cocaine alone (500 ng/mL), or (ii) cocaine in combination with neutralizing (n)TNF-α (1 μg/mL), or (iii) recombinant (r)TNF-α alone (10 ng/mL), or (iv) rTNF-α in combination with nTNF-α. Cell lysates were extracted from control and treated LC neurons at 30 min and 1 h after treatment, and 15 μg of cell lysates normalized for protein content was loaded and run on a 12.5% sodium dodecyl sulfate–polyacrylamide gel. Western blot analyses revealed that cocaine exposure in embryonic LC neurons-induced phosphorylated JNK (PJNK) protein levels (gray bars, ANOVA: $^{*}p < 0.003$) (a and b), compared with controls, which was reduced when combined with nTNF-α (gray striped bars, ANOVA: $^{\#}p < 0.0001$) (a and b). Similar to cocaine effects, exposure to rTNF-α alone also induced PJNK protein levels (white bars, ANOVA: $^{*}p < 0.0027$) in LC neurons, at 30 min after treatment (d: $^{*}p < 0.007$) (a and b), however, returned to basal levels at 1 h (d: $p = 0.438$), compared with controls, and was attenuated by nTNF-α (black striped bars, ANOVA: $^{\#}p < 0.0002$) (a and b). Quantitative analysis of PJNK (b) was performed on blots represented in (a). Data is presented as the ratio of [phosphorylated/(phosphorylated + total JNK)]. A mouse anti-actin monoclonal antibody was used as an internal loading control (a) ($n = 3$).

Discussion

Previously, we demonstrated both *in vitro* and *in vivo* that cocaine has deleterious effects on the development (Foltz *et al.* 2004; Snow *et al.* 2004; Dey *et al.* 2006) and survival (Snow *et al.* 2001; Dey *et al.* 2007) of embryonic LC neurons. Further, we showed that cocaine exposure induced pro-apoptotic Bax levels and decreased anti-apoptotic Bcl-2 levels, followed by activation of initiator caspase-9 and effector caspase-3 and cleavage of its target proteins, α-fodrin and poly (ADP-ribose) polymerase, all of which define an apoptotic scenario for cocaine exposed LC neurons (Dey *et al.* 2007). The effects were compared with those of cocaine on dopaminergic substantia nigra neurons to address specificity, given that both the dopaminergic and noradrenergic systems are compromised by prenatal cocaine exposure (Nicholson *et al.* 1995; Ignatowski *et al.* 1996, 1997; Oliver *et al.* 1998; Jones *et al.* 2000; Stanwood *et al.* 2001; Dey *et al.* 2007).

The present study is a continuation in these efforts, designed to now identify the molecular underpinnings for the deleterious effects of cocaine on LC neurons by defining the specific signal transduction pathways involved. In this study, we examined: (i) whether cocaine-induced TNF-α expression might underlie decreased LC survival *in vitro* and (ii) whether TNF-α might trigger apoptotic pathways in cocaine-exposed LC neurons.

Tumor necrosis factor-α is a pleiotropic pro-inflammatory cytokine having a critical role in both cell differentiation and apoptosis (Wu *et al.* 2003; Gupta and Gollapudi 2005). TNF-

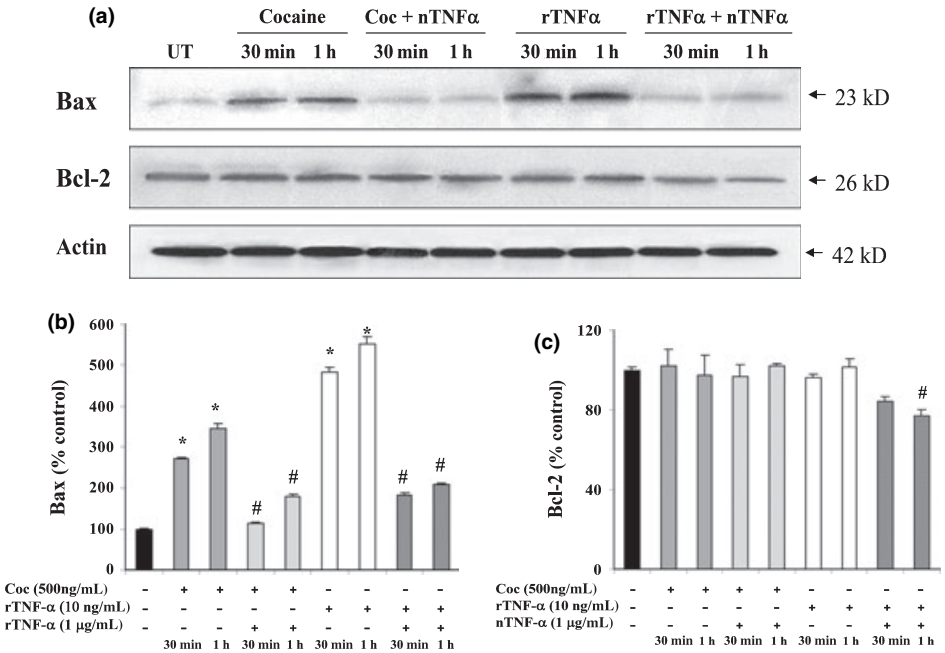


Fig. 7 Tumor necrosis factor alpha (TNF-α) and cocaine show concomitant induction of Bax protein levels in fetal locus coeruleus (LC) neurons. LC neurons were treated with (i) cocaine alone (500 ng/mL), or (ii) cocaine in combination with neutralizing (n)TNF-α (1 μg/mL), or (iii) recombinant (r)TNF-α alone (10 ng/mL), or (iv) rTNF-α in combination with nTNF-α. Cell lysates were extracted from control and treated LC neurons at 30 min and 1 h after treatment, and 15 μg of cell lysates normalized for protein content was loaded and run on a 12.5% sodium dodecyl sulfate–polyacrylamide gel. Western blot analyses revealed that cocaine exposure in embryonic LC neurons-induced Bax protein levels (gray bars, ANOVA: $^{*}p < 0.0001$), compared with controls, which was reduced when in combination with nTNF-α (gray striped bars, ANOVA: $^{\#}p < 0.0001$) (a and b). There were no significant changes in Bcl-2 levels (ANOVA: F's <1.0) (a and c). Similar to results following cocaine treatment, LC neurons exposed to recombinant TNF-α significantly induced Bax protein levels (white bars, ANOVA: $^{*}p < 0.024$), compared with controls, which was reduced when combined with nTNF-α (black striped bars, ANOVA: $^{\#}p < 0.027$) (a). nTNF-α reduced basal levels of rTNF-α expressed Bcl-2, at 1 h (d: $^{\#}p < 0.004$). Quantitative analysis of Bax (b) and Bcl-2 expression (c) was performed on blots represented in (a). A mouse anti-actin monoclonal antibody was used as an internal loading control (a) ($n = 3$).

α activates a variety of signaling pathways by binding to type 1 (TNFR1) and type 2 receptors (TNFR2) (Darnay and Aggarwal 1997; Ashkenazi and Dixit 1998). TNFR1 contains the cytoplasmic death domain that triggers the cell death cascade by activating members of the MAPK family and caspases (Ashkenazi and Dixit 1998; Grell *et al.* 1999; Johnson and Lapadat 2002; Wada and Penninger 2004). Our results show that TNF-α is an important modulator of apoptotic cell signaling in cocaine-exposed LC neurons, as treatment *in vitro* with rTNF-α (rTNF-α) showed a dose-dependent decrease in LC metabolic activity, indicating an overall decreased neuron viability (MTT assay, DNA fragmentation, and TUNEL assay). Similarly, Barker *et al.* (2001) reported that TNF-α induces cell death of nerve growth factor-deprived sympathetic and sensory neurons. Many of these neurons were rescued from cell death by function-blocking antibodies against either TNF-α or TNFR1. Further, inflammatory pathways triggered by cocaine via activation of redox-sensitive transcription factors and induction of inflammatory cytokines such as TNF-α in human brain microvascular endothelial cells may contribute to the cerebrovascular insults observed in patients with a history of cocaine abuse (Lee *et al.* 2001). In addition to these examples in neural systems, TNF-α may induce apoptosis in non-neural systems as well (Meldrum 1998; Zhu *et al.* 2006). Importantly, in our culture system, glia could release factors such as TNF-α (Muller 1997; Koistinaho and Koistinaho 2002), which could contribute to cocaine toxicity in LC neurons.

Caspase-3 activation is a well-established indicator of apoptosis (Xiao *et al.* 2001; Bae and Zhang 2005; Novikova *et al.* 2005b), and cocaine-induced cell death in fetal CNS has been demonstrated by studies both *in vitro* and *in vivo* (Nassogne *et al.* 1995, 1997, 1998). We showed previously that LC neurons exposed to cocaine rapidly increased caspase-3 activity, which was confirmed by significantly reduced forms of inactive pro-caspase-3 (Dey *et al.* 2007). In the current study, we investigated the effects of TNF-α on various apoptotic parameters. As caspase-3 activity was induced significantly at 30 min, with a peak activity at 1 h (Dey *et al.* 2007), and functions downstream of all the parameters studied (bax, bcl-2, p38, and JNK), subsequent studies were conducted at 30 min and 1 h time points. The

use of these time points was in consideration of the fact that TNF-α expression was reduced to its basal levels by 1 h. Surprisingly, rTNF-α did not induce caspase-3 activity at 30 min and 1 h. However, additional experiments revealed that, rTNF-α directly showed a significant induction of caspase-3 activity at 15 and 20 min returning to basal levels by 1 h. Further, the levels of caspase-3 were significantly attenuated by nTNF-α at all time points, when combined with cocaine and rTNF-α, respectively, indicating that both exogenously and endogenously expressed TNF-α *can* regulate caspase-3 activity in a time-dependent manner, in addition to regulating other apoptotic components in LC neurons. For example, Bax/Bcl-2, JNK, and p38 are affected by cocaine exposure at appropriate time points to suggest causation. Further, the role of TNF-α in regulating caspase-3 was clearly demonstrated by the fact that nTNF-α in combination with cocaine-attenuated apoptosis (Fig. 3). Thus, apoptosis via induction of TNF-α may be one of the most common mechanisms for the teratogenic effects of cocaine-induced toxicity (Fiala *et al.* 1998; Gan *et al.* 1999; Lee *et al.* 2001; Wang *et al.* 2001, 2002). The apoptotic pathway converges at the downstream cysteine family of proteases, caspase-3 (Green 1998; Haeberlein 2004). caspase-3 activation then triggers the final proteolytic phase of apoptosis. Cocaine induction of apoptotic proteins, death receptors, the Bcl-2 family of proteins, caspases and their substrates, cytochrome *c*, and JNK-MAPK, lead to decreased survival in several neural (Nassogne *et al.* 1998; Novikova *et al.* 2005a,b) and non-neural systems as well (Zhang *et al.* 1999; Bae and Zhang 2005; Li *et al.* 2005a).

Tumor necrosis factor-α is a potent inducer of the members of the MAPK family, JNK, and p38, which are important regulators of apoptosis (Chang and Yang 2000; Park *et al.* 2002; Li *et al.* 2005b). Yanase *et al.* showed that phosphorylation of JNK-1 via up-regulation of TNF-related apoptosis-inducing ligand mediates interferon-α-induced apoptosis in Daudi B lymphoma cells (Yanase *et al.* 2005). Further, treatment of U937 cells with TNF-α with or without MAP and ERK kinase inhibitors (p38 and MEK) enhanced apoptosis and cleavage of caspase-3, as well as enhanced phosphorylation of JNKs (Nakada *et al.* 2001). Thus, it was intriguing to learn that cocaine activates members of the MAPK family, JNK and p38, by increasing their phosphorylated forms. Also intriguing was that nTNF-α significantly attenuated the levels of phospho-JNK alone, indicating that TNF-α may regulate cocaine-induced phosphorylation of JNK, but not p38. In addition, cocaine and rTNF-α elevated Bax levels at concurrent periods of time (30 min and 1 h), which was attenuated in combination with nTNF-α, implicating a potential role for TNF-α in the activation of these critical apoptotic proteins in cocaine-exposed LC neurons *in vivo*.

Evidence for a potential relationship between cocaine and TNF-α in triggering the cascade of apoptotic proteins is based on the findings that: (i) cocaine and rTNF-α increased caspase-3 activity, and nTNF-α attenuated both cocaine-induced and TNF-α-induced caspase-3 activity, at all time points; (ii) Cocaine and rTNF-α concomitantly induced phosphorylation of JNK at 30 min, which was attenuated when combined with nTNF-α; and (iii) Bax levels were elevated by cocaine and rTNF-α independently at concurrent time periods (30 min and 1 h), which was reduced by nTNF-α, without a change in Bcl-2 levels. This third effect results in altering the Bax/Bcl-2 ratio, which signals apoptosis in neurons. This current data is indicative of a potential role for TNF-α in regulating caspase-3 activity, in addition to the role of regulating other pro-apoptotic proteins (Dey *et al.* 2007). These data also indicate that TNF-α could play an important role in cocaine-induced apoptosis via induction of Bax, thereby altering the Bax/Bcl-2 ratio, and induction of members of the MAPK family, and suggests the potential for this effect *in vivo*.

Based on our previous data (Dey *et al.* 2007), reports in the literature for both neural and non-neural tissues (He *et al.* 2000a,b; Wang *et al.* 2001; Xiao *et al.* 2001; Li *et al.* 2005a), and our current results, we propose the following potential mechanism for cocaine-induced apoptosis in embryonic LC neurons (Fig. 8). Cocaine exposure to embryonic LC neurons could activate TNF-α. Cocaine exposure would alter the Bax to Bcl-2 ratio by up-regulation of Bax protein levels (potentially via the activation of TNF-α). This could then lead to activation of the downstream initiator caspase-9 and effector caspase-3 (Dey *et al.* 2007). Subsequently, the activation of caspase-3 downstream target proteins, poly (ADP-ribose) polymerase and α-fodrin (Nicholson *et al.* 1995; Oliver *et al.* 1998; Dey *et al.* 2007), could initiate apoptosis. In addition, concurrent induction of phospho-JNK by cocaine and TNF-α could trigger apoptosis in cocaine-exposed LC neurons via regulation of JNK. Cocaine-induced phosphorylation of p38 may involve other signaling pathways, which are independent of TNF-α. Whether there might be crosstalk between activated caspase-3 and JNK/P38 in LC neurons exposed to cocaine *in vitro* is unclear and will require further study using respective inhibitors of these proteins.

The accumulating evidence given here indicates a wide variety of CNS effects caused by TNF-α. There are other TNF-α-mediated pathways that will require testing in the future. Roles related to the noradrenergic identity of LC neurons may be paramount. For example, in the presence of the antidepressant desipramine, TNF-α inhibits norepinephrine release. Further, α_2-adrenergic receptor activation regulates TNF-α-mediated norepinephrine release, suggesting an interaction between neuronally derived TNF-α and α_2-adrenergic receptors that may lead to potential adrenergic dysfunction (Ignatowski and Spengler 1994; Ignatowski *et al.* 1996). Thus, it will be critical in future studies to determine if cocaine induces the interaction of TNF-α with adrenergic

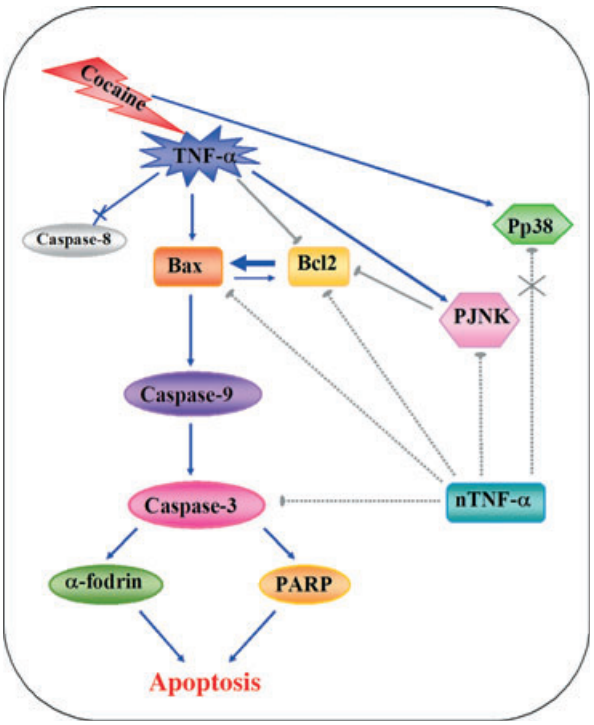


Fig. 8 A potential molecular mechanism for cocaine-induced apoptosis in locus coeruleus (LC) neurons. Based on our previous and current results, we propose a potential mechanism for cocaine-induced apoptosis in embryonic LC neurons. Cocaine exposure to embryonic LC neurons could activate tumor necrosis factor alpha (TNF-α). Cocaine exposure could alter the Bax to Bcl-2 ratio by up-regulation of Bax protein levels (potentially via the activation of TNF-α). This could then lead to activation of the downstream initiator caspase-9 and effector caspase-3. Subsequently, the activation of caspase-3 downstream target proteins, poly (ADP-ribose) polymerase (PARP) and α-fodrin could initiate apoptosis. In addition, TNF-α may trigger apoptosis in cocaine-exposed LC neurons via phosphorylation of c-Jun NH_2-terminal kinase (JNK). Further, cocaine-induced phosphorylation of p38 may involve other signaling pathways independent of TNF-α.

receptors and subsequent G protein-mediated second messenger signaling, which could mediate developmental demise of LC neurons (Snow *et al.* 2004), such as decreased neurite outgrowth and survival.

In summary

Cocaine exposure to noradrenergic LC neurons has selective, deleterious effects on the development of these neurons, under the conditions employed (e.g. 500 ng/mL; see Materials and methods for rationale). These effects are specific to early gestation, where critical aspects of neuronal development are occurring. The elucidation of signal transduction pathways leading to malfunction of developing neurons following cocaine exposure *in vitro* may reveal therapeutic targets for the development of pharmacotherapeutic intervention strategies.

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