Cocaine-induced alterations in nucleus accumbens ionotropic glutamate receptor subunits in human and non-human primates

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Abstract

Chronic cocaine and withdrawal induce significant alterations in nucleus accumbens (NAc) glutamatergic function in humans and rodent models of cocaine addiction. Dysregulation of glutamatergic function of the prefrontal cortical–NAc pathway has been proposed as a critical substrate for unmanageable drug seeking. Previously, we demonstrated significant up-regulation of NMDA, (±)- -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptor subunit mRNAs and protein levels in the ventral tegmental area (VTA), but not the substantia nigra, of cocaine overdose victims (COD). The present study was undertaken to examine the extent of altered ionotropic glutamate receptor (iGluR) subunit expression in the NAc and the putamen in cocaine overdose victims. Results revealed statistically significant increases in the NAc, but not in the putamen, of NMDA receptor subunit (NR)1 and glutamate receptor subunit (GluR)2/3 wit trends in GluR1 and GluR5 in COD. These results extend our previous finding and indicate pathway-specific alterations in iGluRs in COD. In order to determine that changes were related to cocaine intake and not to other factors in the COD victims, we examined the effects of cocaine intravenous self-administration in rhesus monkeys for 18 months (unit dose of 0.1 mg/kg/injection and daily drug intake of 0.5 mg/kg/session). Total drug intake for the group of four monkeys was 37.9 ± 4.6 mg/kg. Statistically significant elevations were observed for NR1, GluR1, GluR2/3 and GluR5 ($p < 0.05$) and a trend towards increased NR1 phosphorylated at serine 896 ($p = 0.07$) in the NAc but not putamen of monkeys self-administering cocaine compared with controls. These results extend previous results by demonstrating an up-regulation of NR1, GluR2/3 and GluR5 in the NAc and suggest these alterations are pathway specific. Furthermore, these changes may mediate persistent drug intake and craving in the human cocaine abuser.

Keywords

cocaine; glutamate; nucleus accumbens; protein expression; putamen

Studies in rodent models of chronic cocaine administration indicate persistent or even permanent biochemical alterations in regions associated with the mesolimbic dopamine pathway that may compromise neuronal function, including up-regulation of the cAMP pathway (Nestler *et al.* 1990; Terwilliger *et al.* 1991; Striplin and Kalivas 1992; Miserendino and Nestler 1995; Carlezon *et al.* 1998; Self *et al.* 1998; Pliakas *et al.* 2001) and activator protein 1 family members (Hope *et al.* 1992; Nye *et al.* 1995; Hiroi *et al.* 1997; Pich *et al.* 1997; Haile *et al.* 2001). More recently, attention has focused on significant alterations in glutamatergic transmission in the ventral tegmental area (VTA) and nucleus accumbens (NAc) following cocaine administration in rodents and humans associated with the neuroplasticity of cocaine addiction (White *et al.* 1995; Fitzgerald *et al.* 1996; Zhang *et al.* 1997; Churchill *et al.* 1999; Ungless *et al.* 2001; Tang *et al.* 2003). A further delineation of the neural contributions and alterations of addictive behaviors has been postulated recently which identifies the dysregulation of prefrontal glutamatergic projections to NAc as an essential component. Briefly stated, prefrontal cortical dopamine alterations lead to preferential responding for drug-related stimuli, whereas accumbal glutamatergic alterations underlie the unmanageable aspects of drug-seeking behaviors (Kalivas *et al.* 2003, 2005).

As stated, one premise of the hypothesis for altered prefrontal–glutamatergic pathway activity emanates from studies utilizing a reinstatement model of self-administration in rodents, whereby responding maintained by cocaine is extinguished and then 'reinstated' by the presentation of stimuli previously associated with responding (e.g. cocaine, environmental cues, stress, etc.). Using this paradigm, studies have demonstrated elevated extracellular levels of NAc glutamate during reinstatement (McFarland *et al.* 2003, 2004) and attenuation of reinstatement responding by intra-NAc application of (±)- -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptor agonists (Cornish *et al.* 1999; Cornish and Kalivas 2000; Di Ciano and Everitt 2002; Park *et al.* 2002). In parallel, several studies have indicated significant elevations in glutamate receptor subunit (GluR)-1, GluR2/3 and NMDA receptor subunit (NR)-1 protein levels in the NAc during withdrawal from chronic cocaine administration (Churchill *et al.* 1999; Kelz *et al.* 1999; Lu *et al.* 2002; Lu *et al.* 2003). Alterations in these subunits may underlie, in part, the increased responsivity of glutamate receptors to stimulation in the NAc (Zhang *et al.* 1997) and provide a potential neural correlate of the unmanageable aspects (i.e. craving) observed in human cocaine abusers (Kalivas *et al.* 2005).

Extrapolation and validation of results from rodent models in humans is difficult yet essential to provide guideposts for evaluating drug-induced biochemical alterations. Recently, we reported significant elevations in mRNA and protein levels of NR1, GluR1, GluR2/3 and KA2 in the VTA of cocaine overdose victims (Tang *et al.* 2003) which compliments the aforementioned studies in rodents. Such studies provide important information on the neurobiochemical mechanisms induced by chronic cocaine administration which may, in turn, affect subsequent drug intake. Whereas rodent models have provided significant information on drug-induced alterations, non-human primate models of drug abuse more closely approximate the neuroanatomy and biochemical milieu of the human brain. For instance, differences between rodents and primates in frontal lobe anatomy (Preuss 1995) are likely to be reflected in prefrontal–accumbal glutamatergic neurotransmission. In addition, different midbrain dopamine projections in rodents have been ascribed to different midbrain nuclei; however, studies in primates suggest a more complex pattern (Lynd-Balta and Haber 1994; Williams and Goldman-Rakic 1998). Moreover, neuronal projections in the rhesus monkey have been shown to be a good model for human neural circuits in other systems (Burkhalter and Bernardo 1989). The use of nonhuman subjects that are similar to humans will allow us to develop a clear and clinically relevant characterization of behavioral and biochemical changes associated with cocaine use in a well-controlled laboratory setting. An animal model for the effects of cocaine self-

administration is needed as studies with human subjects typically contain many confounding variables, e.g. lifestyle characteristics and multidrug histories that are correlated with cocaine use. The use of non-human primates will provide the experimental control necessary to obtain and document the effects of cocaine exposure independent of major confounding variables.

To this end, the present study was undertaken to evaluate the effects of chronic cocaine use in humans on specific ionotropic glutamate receptor subunits previously shown to be altered in cocaine overdose victims (COD). One of the limitations of human post-mortem studies is the inability to delineate state versus trait effects – in other words, do the observed effects reflect a biochemical predisposition to cocaine intake or do the effects manifest as a consequence of chronic cocaine intake? Assessment of protein changes in the rhesus monkeys with extensive cocaine self-administration histories enables the determination of whether such changes may be related to cocaine intake in the primate brain.

Experimental procedures

Experiment 1 – protein assessment in post-mortem human brain tissue: cocaine overdose victims and controls

Tissue was obtained at autopsy from age-matched and drug-free control individuals and cocaine overdose victims by the University of Miami Brain Endowment Bank™ (Table 1). Gross and microscopic diagnostic neuropathologic examinations, which included examination of multiple cortical and subcortical regions, were performed in all cases and no neuropathological abnormalities relevant to mental status were found. All cases were retrospectively accrued based on toxicological data at the time of autopsy. Classification as death resulting from cocaine overdose was based on toxicology and circumstances surrounding the death, including review of prior arrest records and treatment admissions, as well as pathological indications (e.g. perforation of the nasal septum, needle track marks) were reviewed carefully before classifying a cocaine intoxication case. Cocaine and benzoylecgonine concentrations in brain and blood were assessed using gas chromatography/mass spectroscopy as described previously (Hernandez *et al.* 1994). All cases were evaluated for common drugs of abuse including alcohol and positive urine screens were confirmed by quantitative analysis of blood to exclude cases from the study based on evidence of polydrug or alcohol use prior to death. Furthermore, neuroadaptive changes in the human post-mortem brain reflect chronic cocaine abuse, as death in a naive user is a rare occurrence, and this cohort of post-mortem subjects have many surrogate measures of chronicity (Ruttenber *et al.* 1997).

Following removal, brains were photographed and cut into 1.5-cm coronal blocks using a plexiglass holder. Blocks were rapidly frozen in 2-methyl butane on dry ice at −30°C and subsequently stored at −80°C. One hundred-milligram punches were dissected from the blocks containing the NAc and putamen – one hemisphere for RNA and the contralateral hemisphere for protein analysis. Possible neuronal loss, ischemic cell changes and reactive gliosis were assessed using semiquantitative ratings by the neuropathologists and found to be negligible in all cases used in the present study.

Protein preparation and western blot analysis—Tissue samples were homogenized in 10 mM HEPES, 10 mM NaCl, 1 mM KH_2PO_4, 5 mM $NaHCO_3$, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM EDTA and the following protease inhibitors (PI): 1 mM phenylmethylsulfonylfluoride, 10 mM benzamidine, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 1 μg/mL pepstatin and centrifuged using a Beckman Coulter SW55Ti swinging bucket rotor at 5333 *g* for 5 min. Supernatant (cytosol and crude membrane) was removed and centrifuged at 59 255 *g* for 30 min at 4°C and the pure cytosolic supernatant

was removed and stored at −80°C. The pellet containing the crude plasma membrane was re-suspended in 20 mM Tris HCl, 1 mM EDTA (pH = 8.0) and 300 mM sucrose with PIs and centrifuged at 5333 *g* for 5 min. This procedure was repeated twice and the pellet was re-suspended in phosphate-buffered saline and stored at −80°C (crude plasma membrane fraction). The pellet from the initial centrifugation was re-suspended in 10 mM Tris (pH = 7.5), 300 mM sucrose, 1 mM EDTA (pH = 8.0), 0.1% NP40 and PIs and centrifuged at 2370 *g* for 5 min at 4°C. The supernatant was discarded and the pellet was re-suspended in the buffer and washed three times before re-suspension in the PI buffer and storage of samples at −80°C (nuclear fraction), as described previously (Tang *et al.* 2003).

Protein concentrations were calculated using the bicinochoninic acid protein assay kit (Pierce, Rockford, IL, USA) and diluted in Laemmli sample buffer to achieve the equivalent final protein concentrations. Ten micrograms of protein was loaded into 10% sodium dodecyl sulfate–polyacrylamide gels (SDS–PAGE), electrophoresed and transferred to nitrocellulose by electroblotting (30 V, overnight at 4°C) in 1 × transfer buffer (Bio-Rad Laboratories, Hercules, CA, USA). Nitrocellulose membranes were blocked in 0.5% w/v non-fat dry milk and 0.1% v/v Tween 20 in phosphate-buffered saline (pH 7.4, 0.12 M) for 1 h at 23°C prior to being incubated with primary antibodies in blocking buffer (Bio-Rad Laboratories) overnight at 4°C, followed by secondary antibody for 1 h at room temperature. Protein bands were visualized on Kodak XAR-5 film with enhanced chemiluminescence (ECL plus, Amersham Biosciences, Piscataway, NJ, USA). Equal protein loading was confirmed by stripping the blots and re-probing them with a monoclonal -tubulin antibody (Upstate USA, Inc., Waltham, MA, USA; 1 : 5000 v/v) followed by incubation with secondary antibody and visualization as described above. No significant differences were detected in -tubulin abundance between the groups for any of the blots, indicating that any differences in protein abundance between the groups was not because of unequal loading of the gels. Protein abundances were calculated by optical densitometry with a Scan Jet 2200C and imported into NIH Image 1.61 software for analysis.

Primary antibodies were as follows: mouse monoclonal antibodies directed against NR1 (no. MAB363; Chemicon International, Temecula, CA, USA) and rabbit polyclonal antibodies directed against GluR1, GluR2/3, GluR5 and KA2 (nos 06–306, 06–307, 07–258, and 06–315; Upstate USA, Inc.). Secondary antibodies were as follows: HRP-conjugated anti-rabbit IgG and HRP-conjugated anti-mouse IgG (nos 12–348 and 12–347; Upstate USA, Inc.).

Experiment 2 – protein assessment in rhesus monkeys: cocaine self-administration and control

Surgery and self-administration procedures—Four adult male rhesus monkeys (Macaca mulatta) were surgically prepared with chronically indwelling venous catheters according to the general procedure described previously (Herd *et al.* 1969; Byrd 1979). Animal care procedures strictly followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Emory University and Wake Forest University. Using appropriate anesthesia, either isoflurane alone or ketamine in combination with diazepam, and under aseptic conditions, one end of a silicone catheter was passed by way of a jugular or femoral vein to the level of the right atrium and vena cava, respectively. The distal end of the catheter was passed under the skin and attached to a vascular access port (Access Technologies, Skokie, IL, USA) which remained subcutaneous in the center of the back for easy access. The 0.25-mL unit was accessed during testing with special right-angle Huber needles (Access Technologies) that minimize damage to the port membrane and allow for repeated punctures over a year or more. Catheters were flushed periodically with heparinized saline (0.9%). Daily experimental sessions were conducted within a ventilated,

sound-attenuating chamber with each monkey seated in a standard primate chair of the type commercially available (Primate Products, Redwood City, CA, USA). A panel equipped with a response lever and stimulus lights was mounted on the front of the chair. During drug self-administration experiments, the vascular access port was connected via polyvinyl–chloride tubing to a motor-drive syringe located outside the test chamber to yield a precise injection volume of 2.0 mL. Drug dose was determined by the concentration of drug solution in the syringe. Experimental procedures were controlled online by microprocessor and electromechanical programming systems, and data was monitored and recorded during daily sessions.

Rhesus monkeys were trained to self-administer cocaine by pressing a response key while seated in a primate chair. Responding was initiated using a one-response fixed-ratio schedule (FR 1) so that each response in the presence of a red light will produce an intravenous drug injection and the brief illumination of a white light followed by a timeout. The ratio value was increased gradually as responding increases. When the schedule value reached FR 20, drug injection no longer followed completion of each FR and, instead, was arranged to follow an increasing number of FR components. Ultimately, the schedule was a second-order schedule of FR 20 components with drug injection following the first component completed after 10 min had elapsed [FI 600-s (FR 20 : S)]. A 2-s white light was presented upon completion of each FR 20 component. Drug administration was accompanied by a change in the stimulus light from red to white for 15 s, followed by a 1-min timeout. Daily sessions consisted of five consecutive 10-min intervals. The unit dose of cocaine remained constant at 0.1 mg/kg/injection (Howell and Wilcox 2001; Lindsey *et al.* 2004). By using this second-order procedure and limiting the daily session to approximately 1 h, total drug intake was standardized to 0.5 mg/kg/session.

Following completion of the drug self-administration studies, monkeys were restrained with Telazol, given intravenous heparin and then an overdose of intravenous sodium pentobarbital. After the confirmed absence of brainstem reflexes was established, the monkeys were transcardially perfused with phosphate-buffered saline to evacuate brain vasculature. Brains were blocked using a rhesus monkey brain matrix that allows 4-mm coronal blocks at various AP locations (Electron Microscopy Sciences, Ft. Washington, PA, USA). Blocks were divided into their two-component hemispheres – one for fresh frozen sections at −80°C and the other for dissection of blocks for paraffin embedding. Brain tissue from these monkeys was frozen within 40 min of the time of death.

Western blot analysis—For western blot analysis, membrane protein fractions were isolated using Mem-Per kits (Pierce) and concentrations were calculated using the bicinochoninic acid protein assay (Pierce). Samples were diluted in Laemmli sample buffer to achieve the equivalent final protein concentrations. Following denaturation, 10 μg of protein from each subject from each region was be separated on Tris-HCl SDS–PAGE gels and transferred to polyvinylidene difluoride membranes. After blocking membranes in blocking buffer, primary antibodies were incubated with membranes for 18 h. Primary antibodies were as follows: mouse monoclonal antibody directed against NR1 (no. MAB363; Chemicon International) and rabbit polyclonal antibodies directed against GluR1, GluR2/3, GluR5 and KA2 (nos 06–306, 06–307, 07–258, and 06–315; Upstate USA, Inc.), and phospho-NR1 (Ser896), phospho-NR1 (Ser897) and phospho-GluR2 (Ser880) (nos 06–640, 06–641 and 07–294; Upstate USA, Inc.). Equal protein loading was confirmed by stripping the blots and re-probing them with a monoclonal -tubulin antibody (Upstate Biotechnology Cell Signaling Systems; 1 : 5000 v/v) followed by incubation with the appropriate secondary antibody and visualization as described above. No significant differences were detected in -tubulin between cocaine-treated and control groups for any of the blots, indicating that differences in ionotropic glutamate receptor (iGluR) protein

abundance between the groups was not because of unequal loading of the gels. Visualization was accomplished with AlexFluor680- and IRDye800-labeled secondary antibodies (0.06 μg/mL) and scanned with a Licor Odyssey infrared scanner quantified with Odyssey version 1.2 software. All antibodies were tested across a range of protein concentrations to determine linearity of antigen to signal.

Phosphorylation state specificity of the pNR1 antibodies was evaluated by treating 10 μg of putamen membrane extract with 400 U of protein phosphatase (Upstate USA, Inc.) for 30 min at 37°C. protein phosphatase (PPase) dephosphorylates proteins and has been previously shown to dephosphorylated phosphorylated NR1 protein (Guerguerian *et al.* 2002). Aliquots of PPase-treated and untreated protein were electrophoresed, incubated with pNR1896 antibody, followed by secondary antibody and imaged as indicated previously to demonstrate attenuated signal intensity for the dephosphorylated proteins.

Data analysis

Background was subtracted from the density values to give the density value for each subject. All assays were conducted under conditions in which signal intensity was linear with protein concentrations determined in preliminary experiments. Data were analyzed using Students *t*-tests and were expressed graphically as per cent of control levels (mean ± SEM). Null hypotheses were rejected when $p < 0.05$.

Results

Experiment 1 – assessment of iGluR subunits in human post-mortem tissue

There was no significant difference in age between cocaine overdose victims and age-matched, non-drug controls [COD = 34.4 ± 2.6 year; controls (CTRL) = 36.9 ± 2.2 years; t = 0.736, d.f. = 14, p = 0.474], post-mortem interval (PMI) (COD = 12.5 ± 1.0 h; CTRL = 12.9 ± 0.7 h; t = 0.353, d.f. = 14, p = 0.730), or brain pH (COD = 6.5 ± 0.08; CTRL = 6.5 ± 0.05; t = −0.830, d.f. = 14, p = 0.421) indicating these factors did not significantly influence the observed changes in protein expression.

Western blot analysis was performed on membrane protein fractions from individual subjects to assess levels of previously described cocaine-regulated proteins. In membrane fractions, NR1 immunoreactive protein in the NAc was increased 133.7% (t = 2.882; d.f. = 13; p = 0.013) and GluR2/3 by 72.0% (t = −2.857; d.f. = 14; p = 0.013) in the NAc membrane fractions of COD (Fig. 1). No statistically significant changes were observed for GluR1 (85%; t = 2.060; d.f. = 14; p = 0.058), GluR5 (197.5%; t = 2.121; d.f. = 14; p = 0.052) or KA2 (5.8%; t = 0.261; d.f. = 14; p = 0.798). In the putamen, no significant differences were observed between COD and controls for NR1 (11.7%; t = −0.483; d.f. = 13; p = 0.637), GluR2/3 (33.0%; t = 0.939; d.f. = 13; p = 0.365), or GluR5 (32.3%; t = 1.319; d.f. = 13; p = 0.210) protein levels.

Experiment 2 – protein assessment in rhesus monkeys: cocaine self-administration and control

Three of four subjects readily acquired drug self-administration during the first month of training. The fourth subject required approximately 4 months to establish reliable drug self-administration and drug intake was very erratic during this extended training period. Once stable self-administration behavior was established, all subjects reliably received all scheduled injections during daily sessions. Total drug intake for the group of four subjects over the 18-month period was 37.9 ± 4.6 mg/kg.

Western blot analysis was performed on membrane protein fractions from individual monkeys in the cocaine and control groups to assess levels of cocaine-regulated proteins identified in human COD. In membrane fractions from the NAc, cocaine increased levels of GluR1 ($t = 3.040$; d.f. = 9; $p = 0.014$), GluR2/3 ($t = 2.304$; d.f. = 9; $p = 0.047$) and GluR5 ($t = 2.285$; d.f. = 9; $p = 0.048$) as well as NR1 ($t = 3.309$; d.f. = 9; $p = 0.009$), compared with control animals (Figs 2 and 3, respectively). In contrast, in the putamen, no statistically significant changes were observed in abundances of NR1 ($t = -0.098$; d.f. = 7; $p = 0.925$), GluR1 ($t = 1.858$; d.f. = 7; $p = 0.106$), GluR2/3 ($t = 0.115$; d.f. = 8; $p = 0.911$) or GluR5 ($t = -0.292$; d.f. = 7; $p = 0.779$) protein levels in monkeys with cocaine self-administration histories (data not shown). Thus, similar patterns of changes were observed in human COD victims and monkeys that self-administered cocaine.

The functioning of glutamatergic receptors can be altered, not only by changing receptor level/subunit composition, but also by altering the phosphorylation state of iGluR subunits. This is difficult to evaluate in human post-mortem samples because of the action of endogenous phosphatases during the PMI. However, the short PMI in our monkey sample allowed us to investigate alterations in the phosphorylation state of iGluR subunits in this model system.

A trend towards elevated NR1 phosphorylated at serine 896 was observed in the NAc of these monkeys as well ($t = 2.034$; d.f. = 9; $p = 0.072$; Fig. 3) although no significant alteration was observed in the putamen (data not shown). Moreover, the band corresponding to phosphorylated NR1 at serine 896 was abolished by the application of PPase. We could not reliably detect the abundance of KA2, NR1 phosphorylated at serine 897 or phosphorylated GluR2 at serine 880 in the NAc of either control or cocaine-treated monkeys. These proteins were not evaluated in the putamen.

Discussion

In the present study, Western blot analysis was used to examine the effect of cocaine intake on the expression of iGluR subunits in the NAc of cocaine overdose victims and rhesus monkeys with chronic cocaine self-administration histories. We observed significant increases in specific ionotropic glutamate receptor subunits that exhibited regionally specific expression patterns in both human COD and rhesus monkeys self-administering cocaine. Previously, we reported significant elevations NR1, GluR2/3, GluR5 and KA2 mRNA and protein levels were increased in the VTA, but not the substantia nigra of COD victims (Tang *et al.* 2003). In the present study, NR1 and GluR2/3 protein levels were significantly increased in the Nac, while GluR1 and GluR5 trended towards an increase in this region in COD. Interestingly, no changes were observed in the putamen of these subjects.

A major finding of the present study was the similar up-regulation of NR1 and GluR2 glutamate receptor subunit protein levels that were specific to the NAc in COD victims and rhesus monkeys with cocaine self-administration histories. In addition, GluR1 and GluR5 were significantly elevated in monkey NAc (trend in human COD) and phospho-NR1 at serine 896 was elevated. No significant alterations were observed in the putamen of either humans or rhesus monkeys. These findings are parsimonious with the hypothesis detailed by Kalivas and colleagues, wherein accumbal glutamatergic alterations underlie the unmanageable aspects of drug-seeking behaviors (Kalivas *et al.* 2003, 2005). Reinstatement of responding maintained by cocaine results in elevated NAc extracellular glutamate concentrations (McFarland *et al.* 2003, 2004) and is mediated in part by AMPA/kainate receptors in the NAc (Cornish *et al.* 1999; Cornish and Kalivas 2000; Di Ciano and Everitt 2002; Park *et al.* 2002). In parallel, several studies have indicated significant elevations in GluR1, GluR2/3 and NR1 protein levels in the NAc during withdrawal from chronic cocaine

administration (Churchill *et al.* 1999; Kelz *et al.* 1999; Lu *et al.* 2002; Lu *et al.* 2003). Similar alterations in the human COD victims may suggest that these changes are not as a result of the withdrawal per se, but rather are related to chronic cocaine use and persist into withdrawal. Alterations in these subunits may underlie, in part, the increased responsivity of glutamate receptors to stimulation in the NAc (Zhang *et al.* 1997) and provide a potential neural correlate of the unmanageable aspects (i.e. craving) observed in human cocaine abusers (Kalivas *et al.* 2005). Previous studies in rodents suggest that up-regulation of ionotropic glutamate receptor subunits are associated with augmented dopamine–glutamate interactions in the mesolimbic pathway (White and Kalivas 1998; Churchill *et al.* 1999; Ghasemzadeh *et al.* 1999; Cornish and Kalivas 2001). Although one should be cautious regarding direct comparisons between rodent models and human cocaine intake patterns, the present results offer confirmatory evidence in primate brain from human COD victims and rhesus monkeys with cocaine self-administration histories. Together with the present finds, regulation of ionotropic glutamate receptors in the NAc may contribute to the pathophysiological effects of cocaine use in humans.

Surprisingly, there is little information on the relative abundances of ionotropic glutamate receptor subunits in primate brain. In human striatum, NR1 mRNA exhibits relatively uniform distribution in the caudate and putamen (Kosinski *et al.* 1998), however, information on cellular and subcellular localization of NR1 in the striatum in the primate brain is lacking. NR1 and other iGluR subunits including AMPA and kainate receptors appear to be located both pre-and postsynaptically (Krebs *et al.* 1991; Ouagazzal *et al.* 1994; Tarazi *et al.* 1998; Meredith 1999). GluR1 and -2/3 immunoreactivities are abundant in the NAc and caudate–putamen of rhesus monkeys, with GluR1 exhibiting higher abundance in the NAc (Martin *et al.* 1993). GluR1 is enriched in dendrite spines of the NAc and striosomes of the dorsal striatum (Martin *et al.* 1993). Given the significant reciprocal influence of glutamate–dopamine neurotransmission in the NAc, the up-regulation of the iGluR subunits in the present study may provide a mechanism to influence dopamine excitability in the NAc.

In addition to altering the abundance of specific receptor subunits, subunit function is also modulated by kinase-specific phosphorylation and post-transcriptional modifications (Smart 1997; Dingledine *et al.* 1999; Borges and Dingledine 2002). In the present study, cocaine self-administration by rhesus monkeys was also shown to increase the abundance of NR1 phosphorylated at serine 896 in the NAc. In contrast, GluR2 was increased in the NAc of human COD and monkeys self-administering cocaine, but phosphorylation of GluR2 at serine 880 was not detected. Interestingly, phosphorylation of this subunit at serine 880 disrupts the interaction glutamate receptor interacting protein and AMPA binding protein with GluR2 and leads to internalization of the subunit from the synapse (Matsuda *et al.* 1999; Chung *et al.* 2000; Seidenman *et al.* 2003). The lack of detectable phosphorylation of GluR2 in monkey subjects indicates stabilization of GluR2 subunits. Combined with the increases in the abundance of GluR2, along with NR1, and the trends of increased GluR1 and GluR5, these findings support the possibility of enhanced glutamatergic neurotransmission in primate NAc as a consequence of chronic cocaine intake.

Increased NR1 and GluR2/3 subunits in the NAc of CODs and rhesus monkeys corroborate previous studies in which cocaine self-administration led to increased NR1, GluR2/3 mRNA levels in the NAc of rats (Crespo *et al.* 2002; Lu *et al.* 2003; Sutton *et al.* 2003) and extends our previous finding of increased NR1 and GluR2/3 protein levels in the VTA of COD victims (Tang *et al.* 2003). GluR1 was not significantly elevated in the NAc of human COD victims or rhesus monkeys self-administering cocaine in the present study and in contrast to effects reported in rats self-administering cocaine following 90 days of abstinence from cocaine (Lu *et al.* 2003). The reasons for this difference are unclear but may involve several

factors including dissection areas, species differences in regional abundances of the subunits or the extent and frequency of drug intake. Up-regulated GluR2 may not be specific to individuals with histories of cocaine use, as others have shown significant up-regulation of this subunit in the hippocampus of alcoholics (Breese *et al.* 1995), possibly reflecting a shared pathological marker for substance abuse. Similarly, up-regulation of GluR5 extends previous studies demonstrating elevated levels in the VTA of COD (Tang *et al.* 2003) and medial prefrontal cortex of rats self-administering cocaine (Toda *et al.* 2002). Alterations in iGluRs in the NAc suggest increased Ca^{2+} flux in this region and may be indicative of the increased excitability underlying long-term biochemical and behavioral effects of cocaine in humans which, in turn, may affect subsequent drug intake.

Post-mortem human brain studies are inherently difficult and potentially confounded with variables such as lifestyle characteristics, multiple drug histories, and comorbidity with Axis I psychiatric disorders (DSM-IV 1994). However, the use of human post-mortem tissue is essential to examine the neuropathophysiological changes associated with drug abuse, in that drug abuse is a quintessential human condition. The use of a non-human primate model of human drug intake enables a clinically relevant characterization of the behavioral and neurochemical changes associated with cocaine use, while controlling for some of the potential confounding variables in the human cocaine abuser. Coupling studies of human brain with a non-human primate model of drug abuse is a promising avenue for investigating cocaine abuse as a brain disorder.

Acknowledgments

Abbreviations used

AMPA	(±)- -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
COD	cocaine overdose victim
CTRL	control
GluR	glutamate receptor subunit
Ppase	protein phosphatase
iGluR	ionotropic glutamate receptor
NAc	nucleus accumbens
NR	NMDA receptor subunit
PMI	post-mortem interval
VTA	ventral tegmental area
SDS–PAGE	sodium dodecyl sulfate–polyacrylamide gels

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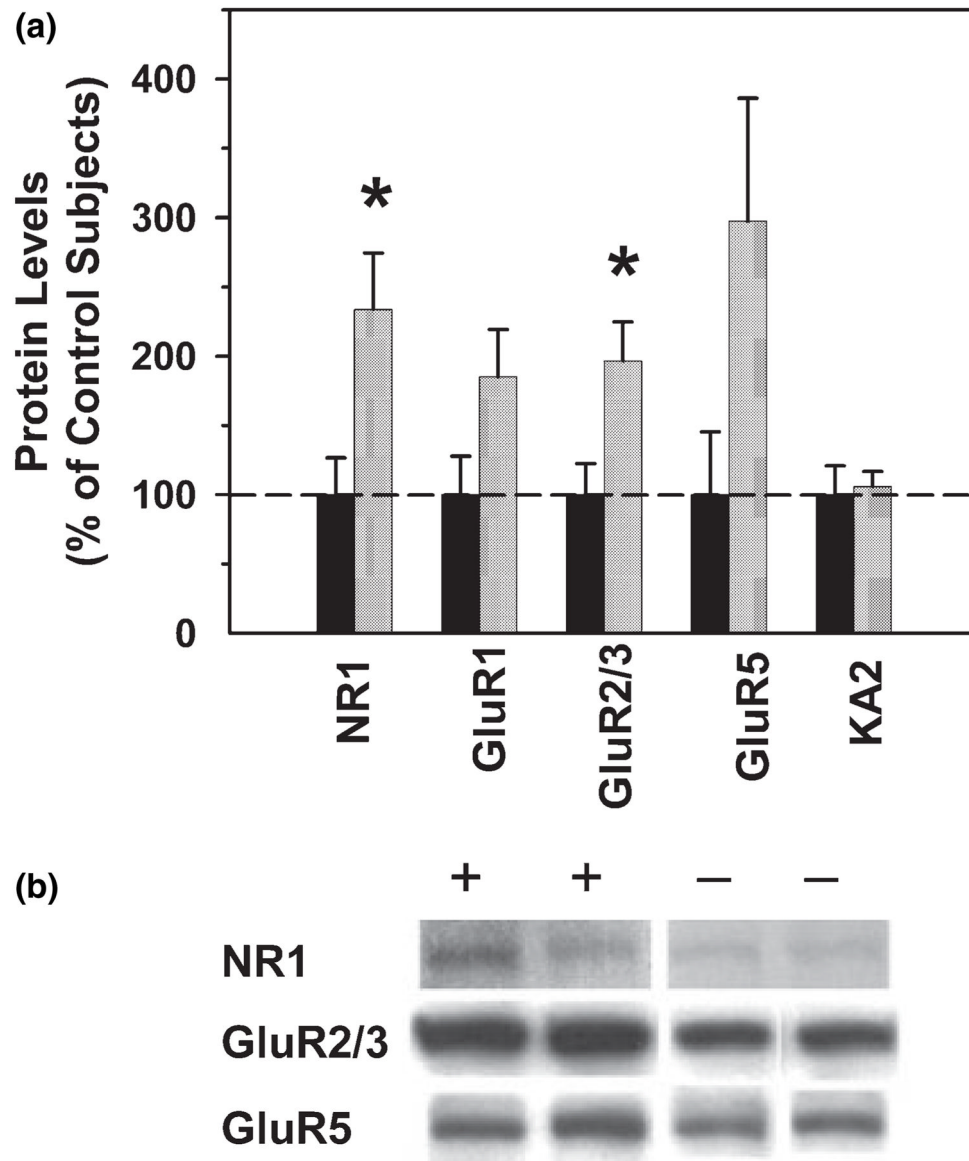
Fig. 1.
Ionotropic glutamate receptor subunit protein levels in NAc of cocaine overdose victims (n = 8) and controls (n = 8). Membrane fractions were isolated as described in Materials and methods and 10 µg were separated on 10% SDS–PAGE to assess glutamate receptor subunit immunoreactivity. Data are expressed as mean (± SEM) of the per cent of control values per amount of protein loaded. Asterisks indicate a significant difference ($p < 0.05$). Lower panel: representative bands from two cocaine overdose victims (+) and two control subjects (−) for each subunit.
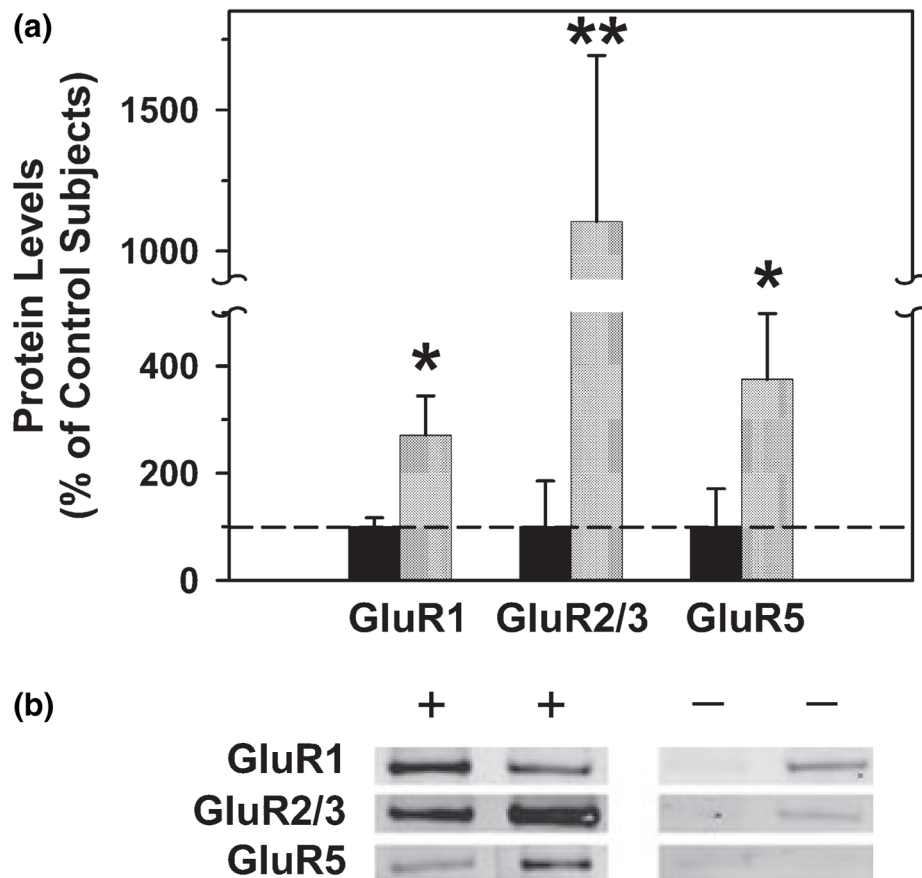
Fig. 2.
(a) Ionotropic glutamate receptor subunit protein levels in rhesus monkeys with cocaine self-administration histories ($n = 4$) versus controls ($n = 7$). Membrane fractions were isolated as described and 5 μg were separated on 10% SDS–PAGE to assess glutamate receptor subunit immunoreactivity. Data are expressed as mean (± SEM) of the per cent of control values per amount of protein loaded. Asterisks indicate a significant difference ($p < 0.05$). (b) Representative bands from two cocaine self-administration monkeys (+) and two control subjects (−) for each subunit are shown.
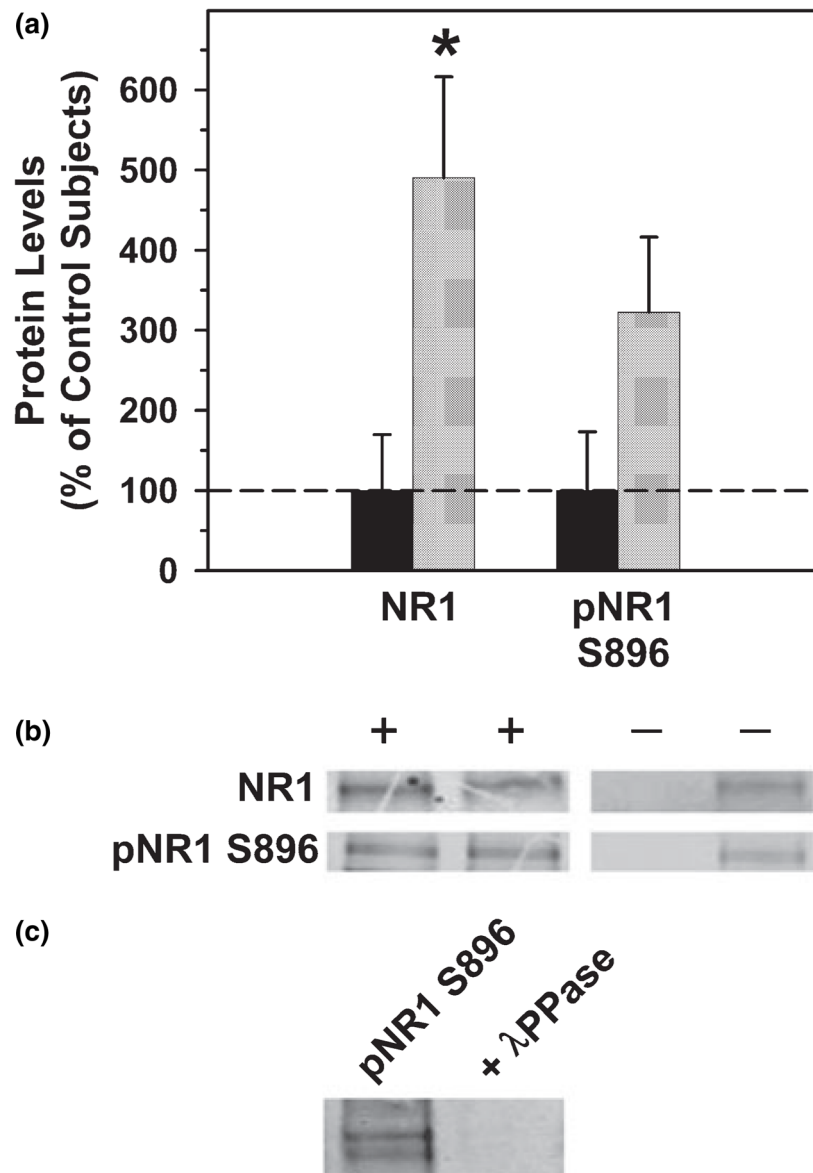
Fig. 3.
(a) NR1 subunit protein levels in rhesus monkeys with cocaine self-administration histories ($n = 4$) versus controls ($n = 7$). Membrane fractions were isolated as described and 5 μg was separated on 10% SDS–PAGE to assess NR1 and phosphorylated NR1 (serine 896) subunit immunoreactivity. Data are expressed as mean (± SEM) of the per cent of control values per amount of protein loaded. Asterisks indicate a significant difference ($p < 0.05$). (b) Representative bands from two cocaine self-administration monkeys (+) and two control subjects (−) for each subunit are shown. (c) Representative band corresponding to phosphorylated NR1 at serine 896 without PPase and with prior treatment of PPase.

Table 1

Clinical characteristics of control and cocaine subjects

	Sex/race	Age	PMI	pH	COD	Toxicology	Blood		Brain	
							COC (mg/L)	BE (mg/L)	COC (mg/kg)	BE (mg/kg)
CTRL1	W/M	35	16.0	6.6	Calcific aortic stenosis	No drugs detected	Neg	Neg	Neg	Neg
CTRL2	W/M	44	11.5	6.2	Acute myocardial infarction	No drugs detected	Neg	Neg	Neg	Neg
CTRL3	W/H/M	26	14.0	6.4	Idiopathic cardiac conduction system disease	No drugs detected	Neg	Neg	Neg	Neg
CTRL4	W/M	46	11.0	6.5	Aortic aneurysm	No drugs detected	Neg	Neg	Neg	Neg
CTRL5	W/M	34	14.0	6.1	Coronary arteriosclerosis	No drugs detected	Neg	Neg	Neg	Neg
CTRL6	W/F	39	11.0	6.7	Multiple sharp/blunt force injuries	No drugs detected	Neg	Neg	Neg	Neg
CTRL7	W/M	34	11.5	6.6	Acute asthmatic bronchitis	No drugs detected	Neg	Neg	Neg	Neg
CTRL8	W/M	37	14.5	6.6	Atherosclerotic cardiovascular disease	No drugs detected	Neg	Neg	Neg	Neg
COD1	W/M	40	11.5	6.5	Cocaine intoxication	COC, BE, diphenhydramine	0.05	0.28	0.12	< 0.05
COD2	W/M	41	12.0	6.6	Cocaine intoxication	COC, BE, ME	6.70	10.0	22.8	2.8
COD3	W/M	23	8.0	6.7	Cocaine intoxication	COC, BE, ME	0.05	0.38	0.11	0.15
COD4	W/M	42	10.5	6.4	Cocaine intoxication	COC, BE, CE	0.05	1.3	0.1	0.73
COD5	B/M	25	12.0	6.5	Cocaine intoxication	COC, BE	1.03	0.73	1.03	0.72
COD6	W/M	38	14.0	6.3	Acute cocaine intoxication	COC, BE	8.90	7.0	>20	3.7
COD7	B/M	34	14.0	6.7	GSW, cocaine related	COC, BE, ME, lidocaine	0.49	2.4	NA	NA
COD8	B/M	32	18.0	6.6	Cocaine intoxication	COC, BE, nicotine	0.2	3.1	1.11	0.89

B, black; BE, benzylecognine; CE, cocethylene; COC, cocaine; F, female; H, hispanic; M, male; ME, methylecognine; NA, not available; W, white.