THE EFFECTS OF COCAINE TREATMENT AND WITHDRAWAL ON NMDA RECEPTOR SUBUNITS AND ASSOCIATED NEURONAL PROTEINS

by

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CERTIFICATE OF APPROVAL

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LIST OF ABBREVIATIONS

AP-5; dl-2-amino-5-phosphonovaleric acid

CaMKII; calcium-calmodulin dependent protein kinase II

COC; cocaine

DA; dopamine

EPSC; excitatory postsynaptic current

GK; guanylate kinase

HEK; human embryonic kidney

Kd; kilo Dalton

KO; knockout

LTD; long-term depression

LTP; long-term potentiation

MAGUK; membrane-associated guanylate kinases

NOS; nitric oxide synthase

PDZ; PSD-95, Discs-large, ZO-1

PKA; cAMP-dependent protein kinase A

PKC; protein kinase C

PP1; type 1 protein phosphatase

PSD; postsynaptic density

PTP; protein tyrosine phosphatase

RT-PCR; reverse transcriptase-polymerase chain reaction

SAL; saline

SAP; synapse associate protein

SH; Src homology

VTA; ventral tegmental area

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ABSTRACT

Pharmacological, behavioral and neurochemical evidence suggests that glutamatergic neurotransmission plays a critical role in mediating some of the effects of cocaine on brain function. Repeated exposure to cocaine results in altered expression of NMDA receptor subunits. The following series of studies characterizes the effects of withdrawal from cocaine on the expression, phosphorylation and interaction of NMDA receptor subunits, splice variants and associated neuronal proteins. Specifically, changes in the expression of neuronal nitric oxide synthase (nNOS), postsynaptic density protein, PSD-95, synapse associated protein, SAP102, and FosB/)FosB were measured as potential molecular mechanisms for the cocaine-induced alterations in NMDA receptor subunit expression. Rats were treated with either saline or cocaine for 7 consecutive days and sacrificed at various time points following their last drug injection (*e.g.*, 24 hours, 72 hours or 14 days). Brain regions putatively involved in mediating the behavioral and neurochemical effects of cocaine, such as the frontal and parietal cortices, neostriatum, nucleus accumbens, ventral tegmental area (VTA) and hippocampus were assessed. Chapter one discusses a set of experiments that used immunohistochemistry to assess the effects of withdrawal from cocaine on the expression of NMDA receptor subunits (NR1, NR2B), nNOS and FosB/)FosB. Optical density analyses of rat brain sections revealed significant up-regulation of NR1 in the VTA at 72 hours and 14 days withdrawal. Structure-specific and withdrawal time-dependent alterations in NR2B expression were also found. For example, after 24 hours of withdrawal, cocaine-induced decreases in NR2B expression were observed in the nucleus accumbens shell whereas increases in NR2B expression were found in medial cortical areas. Two weeks of withdrawal from cocaine caused a ~50% increase in NR2B subunit expression in regions of the cortex, neostriatum, and nucleus accumbens. In contrast, cocaine-induced up-regulation of nNOS was transient and evident in cortical areas only at 24 hours after the last drug injection. Chapter two presents data which utilized immunoblotting to show that after 2 weeks of

withdrawal from cocaine, NR1 subunit expression in the neostriatum was down-regulated ~27%, as compared to saline-treated control rats. Further, at 24 hours but not 14 days of withdrawal, phosphorylation of serine residues 896 and 897 was reduced ~34% in the frontal cortex of rats treated with cocaine, as compared to controls. Results suggest that early changes in kinase or phosphatase activity may contribute to prolonged cocaine-induced alterations in NR1 expression. Chapter three focuses on the characterization of cocaine withdrawal-induced changes in the pattern, frequency and interaction of protein expression. Specifically, immunoblotting and confocal microscopy were used to compare the co-localization of NR2B with nNOS, SAP102 and FosB/)FosB following repeated cocaine or saline treatment and withdrawal. Results indicated that NR2B protein expression was up-regulated in the frontal cortex of rats treated with cocaine and subjected to 14 days of withdrawal. In addition, western blotting data suggested that for rats treated with cocaine, NR2B was transiently up-regulated in the neostriatum at 24 hours of withdrawal. SAP102 expression was also altered in the neostriatum of rats treated with cocaine. Specifically, immunoblotting revealed a ~20% decrease in the level of SAP102 for animals withdrawn for 24 hours, as compared to saline-treated control rats. Co-localization analyses revealed no cocaine withdrawal-induced changes in the co-expression of NR2B with nNOS, SAP102 or FosB/)FosB. Collectively, these data support a role for the NMDA receptor complex in mediating some of the neurochemical effects of cocaine. Identification of the drug-induced changes on specific NMDA receptor proteins and the potential mechanisms associated with those effects has implications for understanding the cellular and neuroanatomical foundation for drug actions at receptor-gated ion channels.

INTRODUCTION

NMDA receptors

The NMDA receptor represents an example of a heteromeric ligand-gated ion channel that interacts with multiple intracellular proteins by way of the different subunits (McBain and Mayer, 1994). NMDA receptors are concentrated at postsynaptic sites, however, a fraction appear to be presynaptic (Liu et al., 1994). Neurotransmission involving NMDA receptors has been implicated in a variety of unique roles: 1) NMDA receptor activation associated with long-lasting changes in synaptic strength (Ali and Salter, 2001), 2) organization of afferent fibers with respect to target neurons during development (Collingridge and Singer, 1990), and 3) participation in glutamate neurotoxicity (Choi and Rothman, 1990). NMDA receptors are derived from at least seven known subunit genes. One of these genes codes for the NR1 subunit, a ubiquitous and necessary component of functional NMDA receptor channels. A number of genes encoding NMDA receptor subunits have been identified: NMDAR1 {Moriyoshi et al., 1991}, NMDAR2A-2D (Kutsuwada et al., 1992), NMDAR3A (formerly NMDAR-L) (Sucher et al., 1995; Das, et al., 1998), and NMDAR3B (Nishi et al., 2001). The diversity of the NMDA receptor NR1 subunit is created by alternative splicing of the NMDAR1 subunit gene to yield eight functional splice forms which arise *via* the insertion or deletion of three short exon cassettes in the N-terminal (Morikawa, 1998) and C-terminal (C1, C2) domains of the subunit protein (Hollmann et al., 1993). Conversely, the heterogeneity of NR2 subunits occurs from the existence of multiple, related genes, NMDAR2A-2D, whose specific expression profiles in the brain are developmentally and regionally regulated (Ishii et al., 1993;Laurie et al., 1997). Understanding the compositional and distributional differences among the NMDA receptor proteins is important as individual NMDA receptor subunits and splice variant combinations have differing receptor functions, such as electrophysiological activity (Ishii et al.,1993),

pharmacological specificity (Buller et al., 1994), and cellular and subcellular localization (Ehlers et al., 1995; Aoki et al., 1994).

The studies described in this dissertation examined individual NMDA receptor NR1 splice variants, subunit proteins (NR2B), and interactions of neuronal elements by using newly available, specific antibodies to examine the effects of repeated cocaine administration and withdrawal on the NMDA receptor complex. Based on the information currently available in the literature and the need to focus the scope of the project, the NMDA receptor NR2 subunit, NR2B was selected for investigation, as opposed to other NR2 subunits. Over the last several years an increasing number of reports have demonstrated the importance of the NR2B subunit in a variety of synaptic signaling events. NR2B has been implicated in modulating functions such as learning, memory processing, and feeding behaviors as well as being involved in a number of human disorders. The following introduction provides a summary of recent findings regarding the structure, stoichiometry, localization, functional properties, and regulation of NMDA receptor subunits and associated neuronal proteins. The introduction concludes with a section discussing the role of the NMDA receptor complex in cocaine use and abuse.

Structural features. The NMDAR1 gene consists of 22 exons. Alternative RNA splicing of this gene creates 8 functional receptor isoforms that result from splicing in or out three alternative exons that correspond to exons 5, 21, and 22. Exon 5 encodes a 21 amino acid sequence that can be inserted into the N-terminal, while exons 21 and 22 encode 37 and 38 amino acids, respectively, that correspond to two locations at the end of the C-terminal. The splicing out of exon 22 removes a stop codon and thus generates an unrelated C-terminal sequence of 22 amino acid residues (Nakanishi and Masu, 1994). A ninth splice variant has also been identified. However, this isoform is truncated and contains only the first 180 N-terminal amino acids, consequently, the transmembrane domains required to make a functional ion channel are absent (for review see McBain and Meyer, 1994). The NR1 subunit possesses some of the structural features typically associated with ligand-gated ion channels. NR1 has four transmembrane

domains, an intracellular C-terminal (Raymond et al., 1994) and a large, extracellular N-terminal with 10 glycosylation sites. In addition, the N-terminal transmembrane domain 1 has been suggested to play a role in ligand binding to the receptor (for review see Hollmann and Heinemann, 1994).

The primary structures of the NMDA NR2 subunits were revealed for both mouse (Kutsuwada et al., 1992) and rat (Monyer et al., 1992) using independent cloning strategies. The predicted amino acid sequences for the ε2 (mouse terminology) and NR2B subunits show a sequence of 45 amino acids, specifically residues 1,362-1,406, which differ between the mouse and rat homologues (McBain and Mayer, 1994). The four NR2 subunits share considerable homology with each other. For example, the proposed proteins are 55% (NR2A and NR2C) and 70% (NR2A and NR2B) identical but are only about 20% homologous to the NR1 subunit (Monyer et al., 1992).

The NR2B subunit is composed of 1,456 amino acids with an approximate molecular mass of 170-180 kD. Hydropathy analysis of amino acid sequences predicted from the cDNA for the NR2B subunit suggests the presence of an extracellular N-terminal signal peptide and four putative transmembrane domains (M1-M4) (Kutsuwada et al., 1992). M2 forms a reentrant loop that lines the channel. Although the NR2 subunits have the same basic structure as NR1 and other glutamate-gated ion channels (see Hollmann and Heinemann, 1994 for review), they differ in that they possess intracellular C-terminal domains, which are in excess of 600 amino acids. These strikingly large C-terminal tails contain scattered regions of conserved sequences and are located distal to the proposed fourth transmembrane segments. The size of these C-termini is larger than the extracellular N-terminal segment preceding the first transmembrane region (Monyer et al., 1992). However, for all NMDA receptor subunits, the N-terminal extracellular domain is greater than twice the size of that for other receptors (~ 50 kD) (McBain and Mayer, 1994). Finally, the NR1 and NR2 subunits possess an asparagine residue (amino acid residue 589 for NR2B) in the second transmembrane region. This domain is the putative pore-forming region

for the NMDA receptor subunits. Thus, the asparagine residue may play a role in the high Ca^{2+} permeability of the channel.

Receptor stoichiometry. As will be discussed below, although relatively comprehensive, the localization data available for NMDA receptor subunits is not always able to differentiate between subunit expression and the presence of functional NMDA receptors. Questions about the distribution of NMDA receptor-gated ion channels are further complicated by conflicting reports regarding NMDA receptor stoichiometry and subunit assembly.

Premkumar and Auerbach (1997) inferred the stoichiometry of recombinant NMDA receptor channels from single-channel current patterns recorded from mouse NR1-NR2B receptors. The authors concluded that NMDA receptors are pentamers composed of three NR1 and two NR2 subunits. In contrast, Laube et al. (1998) presented evidence for a tetrameric structure of recombinant NMDA receptors. Using solubilized mouse forebrain the following subunit distribution of NMDA receptors was observed: NR1 17%, NR1/NR2A 37%, NR1/NR2B 40% and NR1/NR2A/NR2B 6% (Chazot and Stephenson, 1997). Further, in rat tissue, anti-NR2B antibodies immunoprecipitates NR2A, NR2B and NR1 subunits from the cortex and thalamus. NR2D is assembled with NR1, NR2A and NR2B subunits in the rat thalamus (Dunah et al., 1998).

The NR2A and NR2B subunits also appear to co-localize in other brain regions and *in vitro*. For example, immunohistological methods reveal that in hippocampal subfields (CA1-CA3), the NR2A- and NR2B-immunopositive cells exhibit extensive co-localization in the stratum radiatum and oriens (Fritschy et al., 1998). *In vitro* studies using human embryonic kidney (HEK) 293 cells transfected with NR1 and NR2A NMDA receptor subunits in combination with FLAG- and c-Myc epitope tagged NR2B subunits support and extend these findings by providing data regarding the number of NR2 subunits present per receptor complex. Specifically, results show co-assembly of three NR2 subunits $NR2A/NR2B_{FLAG}/NR2B_{c\text{-}myc}$ within the same NMDA receptor (Hawkins et al., 1999). Although characterization of NMDA receptor

composition continues, specific subunit assembly and receptor stoichiometry is regionally and developmentally regulated.

NR1 and NR2B protein expression. Luo et al. (1996) characterized the developmental expression profile of the NR1 subunit. Using an antiserum that recognized NR1 amino acids 656-811, results revealed that in all brain regions investigated, including olfactory bulb, cortex, hippocampus, midbrain and cerebellum, the levels of NR1 were low at birth. NR1 protein levels appeared to increase similarly across brain areas during the first three weeks after birth. Although the ontogenic expression patterns are comparable, significant differences were observed in the absolute amounts of NR1 protein. The levels of NR1 abundance are as follows: hippocampus > cortex > olfactory bulb > midbrain > cerebellum. In agreement with these findings, Benke et al. (1995) used NR1 N-terminal- and NR1 C-terminal-specific antisera and immunoblotting to examine the developing and adult rat brain. In adult rats, NR1-N immunoreactivity was the strongest in the hippocampus, cortex, striatum and thalamus. Weaker signals were detected in the tectum, brain stem and cerebellum. NR1-C expression levels were similar except that in thalamus, tectum, brain stem and cerebellum, staining was weak or absent. To more specifically characterize NR1 expression, Chen et al. (1996) used immunolabeling to reveal NR1-containing cells types in the neostriatum of rats. Results suggest that most cholinergic and somatostatin interneurons (83.3% to 100%) and over 97% of parvalbumin interneurons were labeled for NR1. Further, among striatal projection neurons, enkephalin-, substance P- and calbinidin-positive matrix neurons, 95-100% were labeled for the NR1 subunit.

Laurie et al. (1997) used immunoblotting techniques with rat, mouse, frog, rabbit and human brain to assess the pattern of protein expression for the NR2B subunit. Using an antibody that recognizes a portion of the C-terminal, NR2B subunit protein was observed in all species tested, except frog. In rat and human, authors found that the NR2B subunit is primarily expressed in forebrain structures, such as cortex, hippocampus, striatum, thalamus and olfactory bulb. Moderate levels of NR2B subunit expression are evident in the midbrain, such as hypothalamus,

colliculi and brain stem (rat only), and low expression occurs in the cerebellum and spinal cord. In agreement, Wang et al. (1995) reported that in the adult rat the NR2B subunit is expressed at its highest levels in the olfactory tubercle, hippocampus, olfactory bulbs, and cerebral cortex. Interestingly, regional subunit expression differences have been reported between rats and mice. Using an NR2B-specific antibody, Thompson et al. (2000) reported the presence of NR2B subunits in the mouse cerebellum. Specifically, prominent immunoreactivity was found in Purkinje cell bodies and dendrites but not in the granule cell layer.

Although Laurie et al. (1997) were only able to detect moderate amounts of NR2B protein expression in the hypothalamus, NR2B subunits in the diencephalon participate in various physiological functions. Thus, to broaden their understanding of the role of the NR2B subunit in homeostatic processes, Khan et al. (2000) localized the NR2B subunit in the diencephalon of adult male rats using immunoperoxidase, immunogold, and immunofluorescence techniques. In the hypothalamus, the highest levels of NR2B immunoreactivity were found in the paraventricular and supraoptic nuclei. Further, intense NR2B immunoreactivity was seen in the nucleus circularis, anterior fornical nucleus, and scattered clusters of lateral hypothalamic cells. NR2B immunoreactivity was also visible within the arcuate nucleus, the median eminence, and the tuberal nucleus, and light immunostaining was observed in all other hypothalamic nuclei examined. In the thalamus, the highest amount of NR2B immunoreactivity was found in the medial habenula and the anterodorsal, paraventricula, rhomboid, reticular, and dorsal lateral geniculate nuclei. Thus, the NR2B subunit appears to be widely distributed throughout the hypothalamus and thalamus, supporting its participation in a variety of regulatory functions.

NMDA receptors are found mostly in neurons, but some evidence reports the presence of NR1 and NR2A/B immunoreactivity in distal astrocytic processes and rarely in astrocytic cell bodies (Conti et al., 1996). It was speculated that NMDA receptors located on glial cells could potentially function to monitor glutamate levels in the extracellular space. In terms of cell surface expression, almost all NR2B subunits are reportedly found in the plasma membrane, as compared

to less than half of total NR1 subunits. Thus, these data suggest that neurons possess a large intracellular pool of NR1 subunits that await assembly with the NR2 subunits prior to expression at the plasma membrane (Hall and Soderling, 1997). Regulation of the insertion of NMDA receptor subunits into specific postsynaptic sites has been demonstrated and may be an important mechanism for mediating the activity of NMDA receptors. The distribution and potential stimulus-induced redistribution of receptor subunits is also a critical consideration for experimental methods (see Materials and Method: Tissue preparation section).

Functional properties. When expressed as homomeric receptors in transfected cell systems, NR1 responds to a number of potential endogenous agonists, including but not limited to glutamate, NMDA and L-aspartic acid. Heteromeric assembly is not necessary to obtain a functional ion channel, however, current amplitudes of homomeric NR1 receptors are small suggesting that *in vivo* other subunits are required to endow the receptor with its complete functional capacity (for review see Hollmann & Heinemann, 1994). In spite of its shorter length (~100 amino acids), the C-terminal tail of NR1 has been shown to interact with several different PSD proteins. For example, α-actinin-2, an actin-binding protein, interacts with the C0 segment common to all functional NR1 isoforms and may serve to provide cytoskeletal stability for the receptor complex (Wyszynski et al., 1998). Calmodulin (CaM) interacts with both C0 and C1 C-terminal inserts potentially causing splice variant-specific differential regulation of ion channel gating and receptor desensitization (Ehlers et al., 1996). Yotiao and neurofilament L interact with the C-terminal region of NR1 receptors containing the alternatively spliced C1 segment, however, the functions of these proteins are less clear (Lin et al., 1998; Ehlers et al., 1998; see also Postsynaptic density proteins section, p. 11). None of the four NR2 subunits, NR2A-NR2D, assemble into functional channels. However, when co-expressed with NR1 subunits, receptor activation can occur. Heteromeric combinations of NR1 and NR2B subunits are highly permeable to Ca^{2+} and are blocked by Zn^{2+} and MK-801 (dizocilpine maleate; open channel blocker) and by Mg^{2+} in a voltage-dependent manner and are co-activated by glutamate and

glycine (McBain and Mayer, 1994). As previously described, although NR2A-NR2D subunits have the same basic structure as the NR1 subunit (Hollmann and Heinemann, 1994), they differ in possessing strikingly large intracellular C-terminal domains of 627, 644, 404 and 461 amino acids, respectively. As subsequent sections will discuss, the C-terminal of the NR2B subunit has been suggested to play a role as the target domain for modulatory or accessory proteins in promoting receptor assembly, sorting or targeting. Similarly, this region of the subunit may contribute to different channel conformations and subsequently modulate receptor function.

Studies of NMDA receptor kinetics and related functional properties reveal that the mechanisms of ion channel activation and inactivation are extremely complex (McBain and Mayer, 1994). NR2B subunits dominate over other NR2 subunits in determining the functional properties of NMDA receptors in certain cell types [GABAergic medial septal neurons (Plant et al., 1997)]. These authors studied functional and molecular properties of septal neurons of rat forebrain using patch clamp, fluorometric Ca^{2+} measurements and single-cell RT-PCR. However, using transfected HEK293 cells and whole-cell patch-clamp recordings, NR1/NR2A-mediated peak current densities are approximately four times larger than NR1/NR2B. Further, peak channel open probability is significantly higher for NR1/NR2A than for NR1/NR2B, using two different open channel antagonists, MK801 and 9-aminoacridine. Results suggest that expression levels of NR2A and NR2B can regulate peak amplitude of NMDA receptor-mediated excitatory postsynaptic potentials and therefore play a role in mechanisms underlying synaptic plasticity (Chen et al., 1999).

Accompanying changes in channel activation, subunit-specific differences are similarly observed for the transition from the open to the closed state (i.e., deactivation kinetics). Heteromeric HEK cells expressing NMDA receptors were investigated using whole-cell recordings. NR1/NR2B showed no significant inactivation with application of extracellular Ca^{2+}. Ca^{2+}- and glycine-independent desensitization was less pronounced in NR1/NR2B receptors as compared with NR1/NR2A receptors (Krupp et al., 1996). Further, NMDA receptors transiently

transfected into HEK293 cells were characterized with subunit-specific antibodies and electrophysiological recordings. Recovery from desensitization was slower for NR1/NR2B than for NR1/NR2A channels (Vicini et al., 1998).

In addition to cell expression systems, the use of genetically altered mouse models has also provided important information about the role of the individual receptor subunits in modulating functional properties of NMDA receptors. Over expression of NR2B in the forebrains of transgenic mice leads to enhanced activation of NMDA receptors, facilitating synaptic potentiation in response to stimulation at 10-100 Hz (Tang et al., 1999). In contrast to overexpression, Tovar et al. (2000) studied fast NMDA receptor-mediated synaptic currents in neurons from mice lacking the NR2B subunit. In voltage-clamp recordings, excitatory postsynaptic currents (EPSCs) from NR2B knockout (KO) neurons expressed an NMDA receptor-mediated EPSC that was apparent as soon as synaptic activity developed. However, compared with wild-type neurons, NMDA receptor-mediated EPSC deactivation kinetics were much faster and were less sensitive to glycine, but were blocked by Mg^{2+} or the reversible antagonist, dl-2-amino-5-phosphonovaleric acid (AP-5). Whole cell currents from mouse KO neurons were also more sensitive to block by low concentrations of Zn^{2+} and much less sensitive to the NR2B-specific antagonist ifenprodil than wild-type currents. The rapid NMDA receptor-mediated EPSC deactivation kinetics and the pharmacological profile from NR2B neurons are consistent with the expression of NR1/NR2A heteromeric receptors in excitatory hippocampal neurons from mice lacking the NR2B subunit. Thus, NR2A can substitute and potentially provide some compensation for the absence of the NR2B subunit at synapses.

Behavioral assays, often in combination with pharmacological or genetic manipulation, have demonstrated a role for the NR2B subunit in mediating feeding and related physiological functions, learning, memory, pain and synaptic plasticity. Kutsuwada, et al. (1996) investigated the physiological significance of the NMDA receptor diversity and created NR2B subunit defective mice. These mice show no suckling response and die shortly after birth. Similarly,

mice expressing the NR2B-subunit in a C-terminally truncated form die perinatally. This is the most severe phenotype compared with other KO mice (NR2A and NR2C). The lethal phenotype of NR2B mice is speculated to result from an impairment in intracellular signaling due to the missing intracellular receptor domain (Sprengel et al., 1998). In Sprague Dawley rats the NR2A/B selective antagonist, ifenprodil, attenuates NMDA-elicited feeding. Lateral hypothalamic NMDA receptors, some of which contribute to feeding control, are composed of NR2A and/or NR2B subunits and thus implicates NR2A- and/or NR2B-linked signal transduction in feeding behavior (Khan et al., 1999; see also Stanley et al., 1996; (Stanley et al., 1997). Accompanying the NR2B subunit involvement in feeding behaviors, reports also document hypothalamic NR2B-containing receptor participation in the control of circadian rhythms and the activity of magnocellular neuroendocrine cells (for reviews see Brann, 1995; Ebling, 1996; Rea, 1998).

Mice overexpressing NR2B subunits exhibit superior ability in learning and memory in various behavioral tasks (novel object recognition task, fear conditioning, fear extinction, and water maze). Authors suggested that NR2B is critical in gating the age-dependent threshold for plasticity and memory formation (Tang et al., 1999). In contrast to mice overexpressing the NR2B subunit, a different, yet consistent phenotype is observed in mice with deficient levels of NR2B. Expression of the mutation created by the NR2B subunit defective mice impairs the formation of whisker-related neuronal barrelette structure and the clustering of primary sensory afferent terminals in the brain stem. Further, in the hippocampi of these mutant mice, synaptic NMDA receptor responses and long-term depression (LTD) are absent (Kutsuwada, Sakimura, Manabe, Takayama, Katakura, N, Kushiya, Natsume, Watanabe, Inoue, Yagi, Aizawa, Arakawa, Takahashi, Nakamura, Mori, and Mishina, 1996). Taken together, these results suggest that NR2B plays an essential role in learning, memory and neuronal pattern formation.

Interestingly, Rosenblum et al. (1997) used a conditioned taste aversion (CTA) paradigm to investigate the role of NR2B in behavioral aspects accompanying both feeding and learning.

Blockade of NMDA receptors with the reversible antagonist AP-5 during training impaired CTA memory. When rats taste an unfamiliar flavor and hence learn about it, either incidentally or in the context of CTA training, the tyrosine phosphorylation of NR2B in the insular cortex is increased. Specifically, the level of tyrosine phosphorylation of NR2B appears to be a function of the novelty and quanity of the taste substance consumed. Thus, NR2B-containing receptors are involved in taste learning in the insular cortex and tyrosine phosphorylation of NR2B subserves encoding the saliency shortly after an unfamiliar flavor is tasted.

In addition to its demonstrated role in mediating specific physiological and cognitive functions, the NR2B subunit has also been implicated in pain perception. Wei et al. (2001) studied the effects of forebrain-targeted overexpression of NR2B on the response of mice to peripheral tissue injury. Transgenic mice overexpressing NR2B in the anterior cingulate and insular cortices exhibit enhanced responsiveness to hind paw injection of inflammatory stimuli. Thus, these results provide further support for the development and use of NR2B-selective compounds for the treatment of pain management.

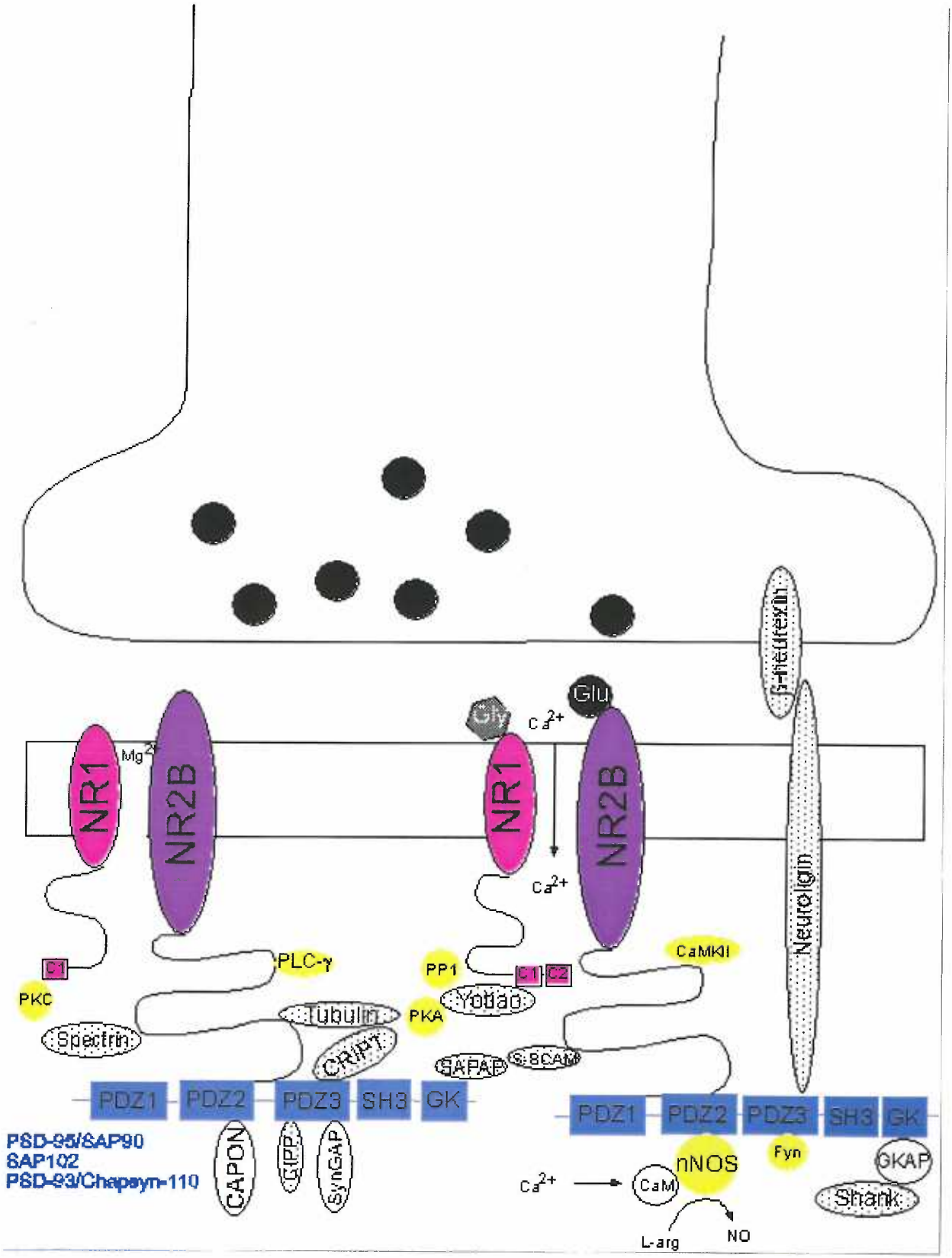
Postsynaptic density proteins

The postsynaptic density (PSD) is a small structure located beneath the postsynaptic membrane at synapses in the central nervous system. Until recently, the molecular composition and function of the PSD were largely speculatory. New tools and molecular methods have enabled the identification of proteins in the PSD; NR2B, PSD-95, SAP102, and nNOS are several of the recently located proteins. The NMDA subtype of glutamate receptor is modulated by a variety of ions, amino acid neurotransmitters, and second messengers (*e.g.*, Mg^{2+}, Ca^{2+}, glycine, Zn^{2+}, H^{+}, and NO) (McBain and Meyer, 1994). NMDA receptors are also regulated (often in a subunit- and splice variant-dependent manner) by intracellular components such as PSD proteins, enzymes, and growth factors. The potential molecular mechanisms for the regulation of NMDA

receptor subunit expression, distribution and function involve but are not limited to: 1) changes in the assembly of specific NR1 splice variants and/or other NR2 subunits, 2) posttranslational modifications such as phosphorylation, and 3) alterations in the interaction of NMDA receptor subunits with associated proteins such as PSD-95 or SAP102. The following section reviews the regulation of NMDA receptor subunits (*i.e.*, NR1 and NR2B) by endogenous proteins. Given the breadth of the topic, the discussion focuses on the role of neuronal elements associated with the PSD including cytoskeletal proteins and enzymes. The immediate early gene product, FosB/)FosB is also introduced as cocaine-induced expression of FosB/)FosB was used as a positive control for experiments discussed in Chapters 1 and 3. In addition, drug-related increases in FosB/)FosB expression were considered as a possible mechanism for cocaine withdrawal-induced changes in NMDA receptor subunit expression. Finally, a brief discussion of the ontogenic profile of some of the proteins is included below as it contributes to information about the developmental and functional significance of individual NMDA receptor subunits.

The developing mouse brain was examined for the expression of PSD-95 and SAP102, which are two PDZ (PSD-95, Discs-large, ZO-1) domain-containing proteins that associate with NMDA receptors (Fig. 1). Using *in situ* hybridization with antisense oligonucleotide probes, PSD-95 and SAP102 mRNA are shown to be expressed at embryonic day 13 in the mantle zone of various brain regions, where NR2B mRNA is also expressed at high levels. In the early postnatal period, mRNA for both PSD-95 and SAP102 is elevated and concentrated in the telencephalon and cerebellar granular layer, where NR2A and NR2B mRNA are abundantly expressed (Fukaya et al., 1999). Thus, the regional and temporal expression profile in the brain suggests that members of the PSD-95/SAP90 protein family can interact with NMDA receptor subunits to facilitate the formation of ion channel complexes, during and after synaptogenic stages.

Figure 1: Schematic representation of NR2B-containing NMDA receptor complex and associated neuronal elements. Blue proteins represent PSD-95 and related family members, kinases and phosphatases are shown in yellow, and other structural proteins are stippled. Please refer to text for information regarding specific protein-protein interactions and putative functions.


NR1
NR2B
Mg2+
C1
PKC
PLC-γ
Spectrin
Tubulin
CRIPT
PDZ1
PDZ2
PDZ3
SH3
GK
CAPON
GIPP1
SynGAP
PSD-95/SAP90
SAP102
PSD-93/Chapsyn-110
Gly
Glu
Ca2+
PP1
C1
C2
Yotiao
PKA
SAPAP
S-SCAM
CaMKII
Neuroligin
β-neurexin
PDZ1
PDZ2
PDZ3
SH3
GK
Fyn
GKAP
Shank
nNOS
CaM
Ca2+
L-arg
NO

To more specifically characterize the relationship of NR2B and cytoskeletal proteins during development, Sans et al. (2000) investigated the developmental changes in expression of PSD-93, PSD-95 and SAP102 in rat hippocampus using biochemical analyses and quantitative immunogold electron microscopy. At postnatal day 2, SAP102 is highly expressed, whereas the abundance of PSD-93 and PSD-95 is low. Interestingly, SAP102 expression increases during the first week after birth when active synaptogenesis putatively takes place. SAP102 protein expression remains stable through postnatal day 35, but shows reduced expression by 6 months of age. From immunoblots of hippocampus and immunogold analysis of CA1 synapses, the high level of NR2B expression at postnatal day 2 appears to coincide with the high level of synaptic SAP102 expression. To determine whether the developmental changes in PSD-93/PSD-95 and SAP102 reflect a preferred association with NR2A and NR2B subunits, respectively, Sans et al. (2000) measured co-immunoprecipitation in the adult hippocamupus. These studies suggest that there is a preference for complexes of NR2B/SAP102 and NR2A/PSD-93/-95. Thus, individual cytoskeletal proteins form specific interactions with NMDA receptor subunits and participate in functions that are critical to synapse development.

As described above, the NMDA receptor plays an important role in synaptic plasticity, neural development, and other physiological functions. Although much has been learned in recent years about the neurochemical and biological properties of glutamate receptors, understanding the molecular mechanisms including receptor targeting, clustering, and anchoring at the synapse is only now beginning to develop. Recent findings concerning the discovery of novel proteins potentially involved in the regulation of NR1 and NR2B *via* specific synaptic protein interactions are reviewed below.

Kornau et al. (1995) used a yeast 2-hybrid system to show that the cytoplasmic tails of NMDA receptor subunits interact with the 95 kD, PSD protein, PSD-95. PSD-95 is a component of the PSD at excitatory synapses. It contains three PDZ domains, a Src homology 3 (SH3) domain, and a guanylate kinase (GK) domain and has been characterized as a putative structural

or scaffolding protein. The PDZ domain appears to be important for the localization of select NMDA receptor subunits. Specifically, the second PDZ domain in PSD-95 binds to the C-terminal containing the tSXV (S, serine; X, any amino acid; V, valine) motif common to NR2 subunits and certain NR1 splice variants, and the NR2B subunit colocalizes with PSD-95 in cultured rat hippocampal neurons. In support of its role as a potential scaffolding protein for NR2B, PSD-95 also binds to the cytoplasmic C-terminal of neuroligins in mouse forebrain (neuroligins are neuronal cell adhesion molecules that interact with β-neurexins and form intercellular junctions). In contrast to NR2B, neuroligins interact with the third PDZ domain of PSD-95 (Irie et al., 1997). PSD-95 thus not only localizes NR2B at synaptic sites but also helps to form the architecture of a multi-protein signaling complex (Fig. 1).

To further characterize the interactions of NMDA receptor subunits with PSD proteins, Niethammer et al. (1996) used a yeast 2-hybrid system to screen a rat brain cDNA library. *In vitro* binding assays identified an interaction between NR2A and NR2B and three distinct members of the PSD-95/SAP90 family [SAP97/hdlg, PSD-95 and Clone 5 (Kim et al., 1995)]. In partial agreement with the findings of Kornau et al. (1995), the interactions appear to be mediated *via* the binding of C-terminal NMDA receptor subunits to the first two PDZ domains of PSD-95. Finally, Muller et al. (1996) described a novel 102 kD synapse associated protein, SAP-102 detected in dendritic shafts and spines of asymmetric synapses. Like PSD-95 and related proteins, it has three PDZ domains and an SH3 and GK domain. All three PDZ domains in SAP102 bind the cytoplasmic tail of NR2B *in vitro*. Masuko et al. (1999) provided further characterization of SAP102. These authors identified the CaM binding site on SAP102 (located near the SH3 domain of SAP102) and demonstrated that binding of CaM to SAP102 does not alter the interaction between SAP102 and NR2B. Further, SAP102 interacts with PSD-95 in the presence of Ca^{2+} and CaM thus revealing the potential for differential regulation of membrane-associated guanylate kinase (MAGUK) proteins at excitatory synapses. Al Hallaq et al. (2001) used immunoblotting of rat cortical and cerebellar tissue to measure the prevalence of NMDA

receptor subunits and PDZ domain-containing proteins. NR2A and NR2B subunits, despite previous electrophysiological evidence (Rumbaugh and Vicini, 1999), are enriched equally in the PSD fraction. In addition, PSD-95 and chapsyn-110 (another MAGUK family member) are both strongly expressed, but SAP102 to a lesser extent, in the PSD. Consistent with these findings, there is evidence which reveals that while PSD-95 and chapsyn-110 are present only at postsynaptic sites, SAP102 is located at axonal sites as well as postsynaptically (El Husseini et al., 2000).

The literature reveals that a number of the enzymes critical for signal transduction at excitatory synapses are linked to NMDA receptor subunits either directly or *via* their interactions with the cytoskeletal proteins previously described. Outlined below is information that illuminates how a number of kinases, phosphatases and other enzymes are involved in second messenger signaling and receptor regulation. Smart (1997) reviewed experiments that demonstrate how neurotransmitter-induced activation of serine/threonine, tyrosine and other kinases can result in the modulation of glutamate receptors. However, studies described in this section specifically emphasize the role of such enzymes on the regulation of NR1 and NR2B subunits.

In addition to tyrosine phosphorylation, phosphorylation of serine and threonine residues by cAMP-dependent protein kinase A (Colledge et al., 2000), protein kinase C (PKC), and CaMKII have also been shown to modulate receptor function. For example, primary hippocampal cultures were used to examine NR2A/2B phosphorylation and phosphorylation of serine but not of threonine or tyrosine was detected. Further, metabolic labeling with ^{32}P reveals that NR2 subunits are highly phosphorylated under basal conditions and exhibit (more) modest increases in response to stimulation, as compared with NR1 subunits (Hall and Soderling, 1997). The regulation by specific kinases can occur, if not directly by phosphorylation of the NR1 or NR2B subunit, then *via* their association with other NMDA receptor subunits or cytoskeletal proteins. PKA and various isoforms of PKC phosphorylate the NMDA receptor *in vitro*.

Immunoprecipitation experiments show that all three NMDA receptor subunits (NR1, NR2A, NR2B) are substrates for PKA as well as PKC (Leonard and Hell, 1997). PKC can affect NR1/NR2B receptor currents by direct phosphorylation of the NR2B C-terminal at residues serine-1303 and -1323 (Liao et al., 2001). Grosshans and Browning (2001) extended these findings and demonstrated that PKC activation can also induce tyrosine phosphorylation of the NR2B subunit. Further, CamKII directly binds to the NR1 and NR2B subunits (Leonard et al., 1999). Strack et al. (2000) showed that residues 1290-1309 in the cytosolic tail of NR2B are critical for CaMKII binding, and identified by site-directed mutagenesis several key residues (lysine-1292, leucine-1298, arginine-1299, arginine-1300, glutamine-1301, serine-1303). For example, phosphorylation of NR2B at serine-1303 by CaMKII inhibits binding and promotes slow dissociation of CaMKII/NR2B complexes. Importantly, NR2B but not NR2A or NR1 subunits are responsible for autophosphorylation-dependent targeting of CaMKII in intact cells. Specifically, autophosphorylation induces direct high-affinity binding of CaMKII to a 50 amino acid domain in the NR2B C-terminal (Strack and Colbran, 1998). Collectively, these findings illustrate that specific co-localization of CaMKII with NR2B-containing NMDA receptors depends on receptor activation, Ca^{2+} influx, and CaMKII autophosphorylation. Thus, translocation of CaMKII because of the specific interaction with the NMDA receptor ion channel may potentiate kinase activity and provide spatial and temporal control of postsynaptic substrate phosphorylation.

Another kinase that has repeatedly been shown to modulate receptor function is PKA. Westphal et al. (1999) isolated cDNAs encoding fragments of yotiao [a protein that binds NMDA receptor subunit NR1 and interacts with the C-terminal C1 exon cassette, (Lin et al., 1998)] by an interaction cloning strategy to identify A-kinase anchoring proteins and confirmed that the protein binds the NR1 subunit. Yotiao also binds to the type 1 protein phosphatase (PP1) and cAMP-dependent PKA. Anchored PP1 is constitutively active, limiting channel activity, whereas PKA activation overcomes PP1 activity and enables rapid enhancement of NMDA currents. Yotiao is

a scaffold protein that physically attaches PP1 and PKA to NMDA receptors to regulate channel activity. Taken together, these studies strongly support the importance of second messenger signaling *via* kinase and phosphatase activity for the regulation and function of NMDA receptors.

Another example of a second messenger molecule that is critically involved with NMDA receptor function is nitric oxide (NO) (Garthwaite, 1991), and its related enzyme, neuronal nitric oxide synthase (nNOS) (NOS: EC 1.14.13.39) (Hevel and Marletta, 1994). Neuronal NOS is a Ca^{2+}/CaM-dependent enzyme that is functionally coupled to Ca^{2+}influx through NMDA-type glutamate receptors (Garthwaite and Boulton, 1995) and is thus regulated by the gradients of Ca^{2+} that occur in the vicinity of open Ca^{2+} channels (Brenman and Bredt, 1997). Stimulation of NMDA receptors results in the activation of nNOS that catalyzes the formation of NO from L-arginine (Garthwaite et al., 1989). The mechanism for selectively coupling nNOS to Ca^{2+} influx through NMDA receptors appears to involve the targeting of nNOS to the PSD (Brenman and Bredt, 1997). Specifically, the N-terminal PDZ domain of nNOS links the enzyme to the PDZ domains of PSD-95 and PSD-93 (Brenman et al., 1996). Review of the literature indicates numerous roles for nNOS-dependent NO, including but not limited to stimulation of cyclic guanosine 5'-monophosphate synthesis *via* activation of guanylate cyclase (Garthwaite, 1991), enhancement of CaM-dependent phosphorylation of PSD proteins (Wu et al., 1996), and modulation of synaptic vesicle exocytosis (Meffert et al., 1996).

Weiss et al. (1998) investigated the localization of NMDA receptor, NR1 splice variants with nNOS using dual-labeled immunofluorescence and found that in the neostriatum, neocortex, and hippocampus, nNOS labeled neurons exhibit strong NR1 immunoreactivity. Surprisingly, however, in all three regions, nNOS positive neurons failed to show immunoreactivity for the NR1 splice variants containing C1 exon cassettes. Experiments described in this dissertation directly investigated the effects of repeated cocaine administration and withdrawal on nNOS expression. Results from these studies were used to relate regionally specific cocaine

withdrawal-induced changes in NMDA receptor subunit and splice variant expression with alterations observed in nNOS immunohistochemical staining.

During the past decade, substance abuse research has focused on transcription factor products of the *fos* family of immediate early genes. These transcription factors are induced rapidly and transiently in the brain in response to cocaine exposure and are subsequently hypothesized to influence the expression of specific late-response genes, which could bring about some of the neurochemical and behavioral adaptations observed following chronic drug treatment. Repeated cocaine exposure upregulates ΔFosB, and elevated levels of ΔFosB persist in the striatum long after the end of drug treatment (Hope et al., 1994). Specifically, ΔFosB accumulates in dynorphin-expressing striatonigral neurons (Hope et al., 1992). ΔFosB is a truncated splice variant of the *fos* B gene which results in the expression of an isoform of FosB that is missing the C-terminal 101 amino acids. The discovery of specific target genes *via* which ΔFosB produces its physiological effects is an important step in understanding the function played by this transcription factor. Two glutamate receptor subunits have been identified as possible targets for ΔFosB: NR1 (Fienberg et al., 1998) and GluR2 of AMPA receptors (Kelz et al., 1999). Cyclin-dependent kinase 5 is also a putative downstream target of ΔFosB as overexpression of ΔFosB or chronic cocaine administration raised levels of cyclin-dependent kinase 5 mRNA, protein and activity in the neostriatum of rats (Bibb et al., 2001).

Glutamatergic system and cocaine

The widespread recreational use of cocaine combined with its highly addictive and potentially fatal properties creates a societal and scientific problem of significant magnitude. Cocaine is a potent psychostimulant drug that acts by blocking dopamine uptake, thus increasing dopamine neurotransmission. The actions of cocaine, however, appear to be contingent not only upon the dopaminergic system but also upon the glutamatergic system. Review of the cocaine abuse literature suggests that numerous chemically-induced alterations in NMDA receptor-

mediated neurotransmission and receptor expression exist. It is further hypothesized that NMDA receptors mediate the adaptive mechanisms that are involved in the development, maintenance, and expression of drug addiction. Evidence for the role of altered NMDA receptor subunit expression in cocaine abuse is discussed below.

Cocaine administration alters the expression of NMDA receptor subunits. Fitzgerald et al. (1996) showed that chronic cocaine administration up-regulates the NR1 subunit in the ventral tegmental area (VTA) of rat brain. Cocaine-induced changes in NR2A/B expression were not observed, however the antibodies used were not able to differentiate between NR2A and NR2B subunits (Fitzgerald et al., 1996). Using a different method (immunohistochemistry) and more selective antibodies, Loftis and Janowsky (2000) sought to replicate the earlier findings and to further characterize the time course of potential cocaine-induced alterations of neuronal protein levels. Structure-specific and withdrawal time-dependent alterations in NR2B expression were found. After 24 hours of withdrawal, cocaine-induced decreases in NR2B expression were observed in the nucleus accumbens shell whereas increases in NR2B expression were found in medial cortical areas. Further, two weeks of withdrawal from cocaine causes an approximate 50% increase in NR2B subunit expression in regions of the cortex, neostriatum, and nucleus accumbens. In another study, NR1 expression levels in the nucleus accumbens and VTA were quantified from immunoblots of brain tissue at 24 hours and three weeks after withdrawal from seven days of daily cocaine injections. Rats exhibiting sensitized locomotor responses to the drug showed a significant increase in NR1 at 24 hours but not at three weeks of withdrawal in the VTA. Authors note that the functional importance of the altered subunit expression levels is suggested by the fact that changes were observed only in rats that manifested sensitized locomotor behavior (Churchill et al., 1999). In contrast, using a different drug treatment regime, Ghasemzadeh et al. (1999) observed somewhat dissimilar cocaine-induced effects. The expression of glutamate receptor subunit mRNA was examined three weeks after discontinuing one week of daily injections of saline or cocaine. In the dorsolateral striatum and VTA, an acute

cocaine challenge dose given after three weeks of withdrawal from cocaine or saline resulted in a significant reduction in mRNA for NR1. Collectively, these results suggest that regionally specific, time-dependent changes in NMDA receptor protein expression may underlie neuronal adaptations following repeated cocaine administration and may therefore contribute to withdrawal-related symptomology.

Research cited above and evidence discussed in subsequent sections demonstrates the importance in determining, with greater specificity, the effects of cocaine administration and withdrawal on NMDA receptor expression, distribution, and function. C-terminals of NMDA subunits appear to be critically involved in receptor function (Sprengel et al., 1998) and assembly (Niethammer et al., 1996). Thus, differentiation among splice variants and subunits is important for understanding the effects of cocaine administration. In addition to the VTA, previous research implicates a number of different brain areas potentially involved in mediating the effects of cocaine *via* glutamatergic systems. The nucleus accumbens (White et al., 1995), hippocampus (Smith et al., 1993), dorsolateral septal nucleus (Shoji et al., 1997), and prefrontal cortex (Reid et al., 1997) represent a few of the critically involved regions. Consequently, in combination with newly available antibodies that permit increased assay specificity, the experiments described in this dissertation used a combination of immunological techniques with tissue from cocaine- and saline-treated animals to more comprehensively characterize drug affected brain areas.

Receptor subunit- and kinase-specific phosphorylation is likely an important regulator of the cocaine-induced changes in neurotransmission. Review of the literature finds little information on the effects of cocaine treatment on NR1 or NR2B phosphorylation. However, experiments have been conducted to assess the influence of protein kinases on the expression of behavioral sensitization to cocaine. Prior to a challenge dose of cocaine, inhibitors of CaMKII (KN93), PKA (H-89), or PKC (bisindolymaleimide-I) were microinjected into the medial nucleus accumbens of rats repeatedly administered either cocaine or saline. None of the kinase inhibitors influence the behavioral response induced by cocaine in saline-pretreated rats. Among cocaine-

sensitized animals, KN93 or bisindolymaleimide-I blocks the expression of behavioral sensitization to cocaine whereas H-89 has no effect (Pierce et al., 1998). Thus, specific kinases are hypothesized to promote the expression of behavioral sensitization to cocaine as well as to influence the neurochemical adaptations accompanying repeated cocaine exposure.

In addition to characterizing NMDA receptor NR2B subunit and NR1 splice variant changes associated with cocaine administration, the proposed experiments are designed to assess the effects of cocaine on neuronal elements, nNOS, PSD-95 and SAP102, seemingly critical for synaptic transmission and potentially involved in modulation at the NMDA receptor. Detailed information describing the effects of cocaine administration on nNOS expression and activity is limited, and direct evidence regarding the effects of cocaine treatment on nNOS associated with specific NMDA receptor subunits and splice variants is absent. Pharmacological blockade of NOS reduces the reinforcing properties of cocaine (Pulvirenti et al., 1996), selective inhibition of nNOS blocks the induction of behavioral sensitization to cocaine (Haracz et al., 1997), inhibition of nNOS prevents the development of cocaine-induced conditioned locomotion (Itzhak, 1997), and chronic administration of cocaine is associated with increases in NOS activity in the cerebral cortex, cerebellum, midbrain, hypothalamus, hippocampus, amygdala and spinal cord (Bhargava and Kumar, 1999). Thus, these findings suggest a role for nNOS in cocaine abuse and dependence. However, given the diversity of potential influences nNOS activity has on neuronal functioning and the paucity of information regarding cocaine's effects on nNOS, it is important to more comprehensively and directly investigate nNOS expression and function associated with cocaine-induced changes in specific NMDA receptor subunits and splice variants. Brenman and Bredt (1997) suggest that in order to better understand NO actions in brain, the identification of the functional connection of nNOS with NMDA receptors is required. This dissertation project was designed to further characterize the association between nNOS and NMDA receptor subunits by assessing the effects of repeated cocaine administration and withdrawal on the expression and function of these critical neuronal elements. There is currently no published information

available describing the effects of cocaine treatment or withdrawal on the expression of PSD-95 or SAP102. Consequently, the use of splice variant-, subunit- and PSD protein-specific antibodies in combination with immunoblotting, light and confocal microscopy will provide new information about the mechanisms involved in cocaine-induced changes in the NMDA receptor complex and associated proteins.

Specific aims

The primary goal of these studies was to characterize time-dependent and regionally specific effects of repeated cocaine treatment and withdrawal on the expression, phosphorylation and interaction of NMDA receptor subunits, splice variants, and associated neuronal elements. The experiments in this dissertation were designed to test the hypothesis that cocaine treatment alters NMDA receptors in a time, subunit, splice variant, and region specific manner. Evidence supports a role for NMDA receptors in mediating behavioral (Pierce et al., 1997), electrophysiological (Smith et al., 1993), and biochemical (Nestler et al., 1993) responses to cocaine. Lau et al. (1996), however, suggest that data on protein interactions with native NMDA receptors is lacking. The specific aims are as follows: 1) to characterize cocaine-induced changes in the expression and localization of NMDA receptor subunit and splice variant protein, 2) to explore the relationship among neuronal nitric oxide synthase (nNOS), postsynaptic density protein (PSD-95), NMDA receptor subunits and phosphorylation to better understand the mechanisms associated with cocaine-induced changes in NMDA receptor expression, and 3) to assess cocaine withdrawal-induced changes in the localization and co-expression of NMDA receptor subunits and associated proteins as measured by confocal microscopy.

Specific Aim #1: ***To characterize cocaine-induced changes in the expression and localization of NMDA receptor subunit and splice variant proteins.*** The experiments used repeated cocaine administration, immunohistochemistry and immunoblotting of sections from cocaine-treated and

control animals to assess subunit regulation by labeling with antibodies that specifically differentiated among NR1 splice variants and other NMDA receptor subunits (*e.g.*, NR2B). Studies considered drug effects at two or three different withdrawal time points to determine if identified synaptic changes are short term or if they reflect a more permanent adaptation.

Specific Aim #2: ***To explore the relationship among neuronal nitric oxide synthase (nNOS), postsynaptic density protein (PSD-95), NMDA receptor subunits and phosphorylation to better understand the mechanisms associated with cocaine-induced changes in NMDA receptor expression.*** To investigate the possible involvement of phosphorylation on the regulation of NMDA receptors, the effects of withdrawal from cocaine administration on the expression and phosphorylation of NR1 splice variants were characterized using immunohistochemistry and immunoblotting. To determine possible mechanisms associated with cocaine withdrawal-induced changes in the phosphorylation of NMDA receptor subunits, the expression of nNOS and PSD-95 were also evaluated.

Specific Aim #3: ***To assess cocaine withdrawal-induced changes in the localization and co-expression of NMDA receptor subunits and associated proteins as measured by laser scanning confocal microscopy.*** Results from specific aim #3 provide information about the effects of cocaine withdrawal on the co-expression and localization of NMDA receptor subunit, NR2B and postsynaptic density proteins, PSD-95, SAP102 and nNOS. Immunochemical localization of protein expression was visualized using a confocal microscope. For a given brain region, saline-treated rats were used to establish the degree and relative abundance of protein co-expression per brain region of interest. Cocaine withdrawal-induced changes in the pattern and frequency of protein expression were subsequently measured. In addition, data obtained for specific aim #3 shows withdrawal time-dependent changes thus confirming alterations induced by cocaine

exposure and providing further evidence to support a mechanism for the regulation of NMDA receptor expression.

MATERIALS AND METHODS

Antibodies

A rabbit NR1 polyclonal antibody selective for splice variants NR1-1a, NR1-1b, NR1-2a, and NR1-2b [also referred to as C2 splice variant selective (NR1-C2); McBain and Mayer, 1994] was used in immunohistochemistry and immunoblotting experiments (Chemicon, Temecula, CA). A rabbit anti-NMDA receptor C1 exon polyclonal antibody (NR1-C1) selective for splice variants NR1-1a, NR1-1b, NR1-3a and NR1-3b with no cross reactivity to other glutamate receptor subunits was used for immunoblotting experiments (Chemicon, Temecula, CA). Anti-phospho-NR1 Ser896/897, Ser896, and Ser897 affinity purified rabbit antibodies (Upstate Biotechnology, Lake Placid, NY) were also used. The antibody specificity information available from Upstate Biotechnology states that anti-phospho-NR1 Ser896/897 is specific for NR1 dually phosphorylated on serine residues 896 and 897; anti-phospho-NR1 Ser896 is specific for NR1 phosphorylated on serine 896 and it also recognizes NR1 that is dually phosphorylated on serine residues 896 and 897. Anti-phospho-NR1 Ser896 does not recognize the subunit if only residue 897 is phosphorylated. Similar specificity information is also true for anti-phospho-NR1 Ser897. A NR2B rabbit polyclonal antibody (Molecular Probes, Eugene, OR) recognizing amino acid residues 984-1104 of the C-terminal region of rat brain subunit NR2B was also used. An anti-NR2B rabbit polyclonal antibody (Molecular Probes, Eugene, OR) recognizing amino acid residues 984-1104 of the C-terminal region of rat brain subunit NR2B or anti-NR2B, a mouse monoclonal antibody recognizing amino acid residues 892-1051 (Transduction Laboratories, Lexington, KY) were used for immunohistochemistry, immunoblotting and confocal experiments. A rabbit polyclonal antibody for nNOS that recognizes C-terminal amino acid residues 1383-1398 with specificity for rat nNOS was also used in immunohistochemistry, immunoblotting and confocal experiments (Transduction Laboratories, Lexington, KY). Anti-PSD-95, a mouse

monoclonal antibody (Transduction Laboratories) and rabbit anti-SAP102, a purified polyclonal antibody (Chemicon, Temecula, CA; Alomone Labs, Jerusalem, Israel) were used for immunoblotting and confocal microscopy experiments. Given cocaine's putative effects on immediate early gene products (Hope et al., 1994; Rosen et al., 1994; Chen et al., 1997; Moratalla et al., 1996), FosB immunoreactivity was additionally assessed to confirm that the cocaine treatment protocol (described below) was influencing brain protein expression. The FosB rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) that was used in immunohistochemistry and confocal experiments recognizes an epitope corresponding to amino acids 75-150 of the human FosB gene product. It is specific for FosB/)FosB and non cross-reactive with c-Fos, Fra-1 or Fra-2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). All antibodies were initially tested using a range of dilutions in order to assess detection limits. The dilutions selected were based on optimal signal-to-noise ratios.

Cocaine treatment regime

Male Sprague-Dawley rats (initial weights 175-225 g; Harlan, Indianapolis, IN) were group housed 2-3 per cage at the Portland Veteran's Affairs Medical Center Veterinary Medical Unit. Rats were maintained on a 12-hour light/dark cycle with continuous access to food (Purina rat chow) and water. The Institutional Animal Care and Use Committee approved all procedures involving animals according to NIH guidelines. Body weights were monitored during drug treatment to assure that saline- and cocaine-treated rats maintained health and all animals appeared to be of comparable health at the time of euthanasia. No significance differences in weight gain were observed for rats treated with cocaine as compared to saline. Rats were injected daily with either saline (0.9%) or cocaine HCl (RBI, Natick, MA) 20mg/kg intraperitoneally for 7 consecutive days. Rats subjected to a similar drug administration paradigm manifested up-regulation of the NMDA receptor subunit, NR1 in the VTA (Fitzgerald et al., 1996) behavioral sensitization (Churchill et al., 1999), and related neurochemical alterations, such as increased

adenylyl cyclase and PKA in the nucleus accumbens (Nestler et al., 1993). Robust changes in the expression of NR1 subunit (Fitzgerald et al., 1996) and FosB (Hope et al., 1994) protein levels are not apparent after only a single dose of cocaine. In addition, given cocaine's short half-life (Pan and Hedaya, 1999), a specific and appropriate 0-hour time point to sacrifice animals and assess protein levels was difficult to determine. Because the primary objective of this study was to compare potential neuronal changes that accompany different stages of withdrawal (*e.g.*, absence of cocaine bioavailability), no 0-hour time point was included. Saline-treated rats were used as controls, and because immunostaining their specified proteins reflects baseline expression levels, comparison of sections from cocaine-treated animals with sections from saline-treated animals was used to assess the extent to which withdrawal from drug administration influenced changes from baseline. For the immunohistochemistry experiments described in the first chapter, subjects were assigned to either one of six groups: saline administration followed by 24 hours of withdrawal (SAL24), cocaine administration followed by 24 hours of withdrawal (COC24), saline administration followed by 72 hours of withdrawal (SAL72), cocaine administration followed by 72 hours of withdrawal (COC72), saline administration followed by 14 days of withdrawal (SAL14d), and cocaine administration followed by 14 days of withdrawal (COC14d). For all other experiments, animals were assigned to one of four groups: saline or cocaine administration followed by 24 hours of withdrawal (SAL24 or COC24, respectively), and saline or cocaine administration followed by 14 days of withdrawal (SAL14d or COC14d, respectively). During withdrawal, animals remained in their home cages and were not injected with saline or cocaine. Group sample sizes are indicated in the figure legends.

Tissue preparation for immunohistochemistry and confocal microscopy

Following drug treatment and withdrawal, all rats were administered 0.5 cc rat anesthetic cocktail [5 ml ketamine (100 mg/ml), 2.5 ml zylazine (20 mg/ml), 1 ml acepromazine (10 mg/ml), 1.5 ml sterile saline] and transcardially perfused with 7 ml of heparin [diluted 1:1000

with 0.1M phosphate buffer (PB)], immediately followed by 300 ml of 2% paraformaldehyde, w/v in 0.1M PB at room temperature. Brains were rapidly removed and placed in ice cold 2% paraformaldehyde for 1 hour to postfix. The brains were then placed in ice cold 30% sucrose w/v, in 0.1M PB and stored at 4°C for 24-48 hours. Frozen tissue was coronally or saggitally (for experiments involving hippocampal regions only) sectioned on a cryostat at 30 μm. Vigilant attention to brain alignment and placement of the cut sections within the appropriate storage wells was given to facilitate subsequent analyses of comparable brain regions. Cut sections were kept in cryoprotectant [0.1M PB, 60% ethylene glycol, v/v, 60% sucrose, w/v, and 2% polyvinylpyrolidone (PVP-40), w/v] and stored at -20°C until assayed.

Tissue preparation for immunoblotting

Membrane enriched protein samples were prepared by modifications of the methods of Dunah et al. (2000). Cortical, neostriatal, or hippocampal regions were homogenized in 100 volumes (original wet weight) of ice-cold P2 buffer [10 mM Tris-HCl pH 7.5, 5 mM EDTA, supplemented with protease inhibitors (Complete™ tablet, Boehringer Mannheim, Germany), 1 μM microcystin, 1 μM KN-93, 1 μM H-89 and 1 μM bisindolymaleimide I]. The homogenate was centrifuged at 700 x g for 10 min at 4° C, and the resulting supernatant was again centrifuged at 10,000 x g at 4° C for 20 min. The pellet or P2 fraction containing total protein homogenate was re-homogenized in P3 buffer (same as above, with the addition of 320 mM sucrose) and centrifuged at 25,000 x g at 4° C for 30 min. Alterations in phosphorylation and receptor subunit expression occur primarily in synaptosomal membrane fractions (Dunah et al., 2000). Thus, membrane enriched protein samples were prepared to assess changes in receptors and proteins associated with neuronal membranes. The final membrane enriched pellet was resuspended in P2 buffer and stored at -80° C. Protein concentrations were determined using the BCA protein assay (Pierce, Rockford, IL).

Immunohistochemistry

Using an orbital shaker set at 60 RPM, free-floating tissue sections received 3, 20-minute washes in 0.1M PB at room temperature to remove cryoprotectant. Sections were treated with ImmonoPure Peroxidase Suppressor (Pierce, Rockford, IL) for 30 minutes at room temperature to inactivate endogenous peroxidases. Blocking nonspecific reaction sites on the sections was accomplished by incubation with normal goat serum (Vector Laboratories, Burlingame, CA) blocking solution (1% normal goat serum, v/v, 0.1% Triton X-100, v/v in 0.1M PB) for 1 hour at room temperature. After blocking, the sections were incubated overnight at 4°C with the primary antibody for either NR1, NR2B, nNOS, FosB, phospho-NR1 Ser896 or -NR1 Ser897 at dilutions of 1:500, 1:1000, 1:2000, 1:5000, or 1:500 respectively. All primary antibodies were diluted in the normal goat serum blocking solution. Following incubation with the primary antibody, sections were washed 3 times in 0.1M PB prior to treatment with the secondary antibody. The sections were incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories) in 5% normal goat serum, v/v in 0.1M PB for 30 minutes at room temperature, after which they received 3 washes in 0.1M PB. Protein staining was visualized using the Vectastain Elite ABC and DAB (diaminobenzidine) substrate kits (Vector Laboratories). Sections were mounted on gelatin-coated slides and allowed to dry overnight prior to dehydration in 70%, 90%, and 100%, v/v ethanol washes. After dehydration, the slides were placed in xylenes and coverslipped. For each experiment, some sections were incubated without the primary antibody to assess level of background staining and antibody specificity.

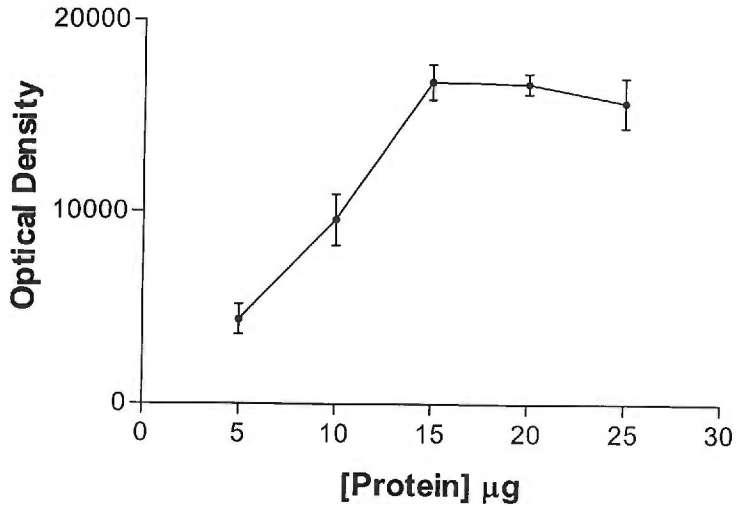
Gel electrophoresis and immunoblotting

SDS-sample buffer (Laemmli, Sigma, St. Louis, MO) was added to each vial and tissue samples were boiled for 5 min at 95° C prior to loading onto gels. Equal protein amounts were used in each lane of the gel for a given antibody and a given brain region. Because different

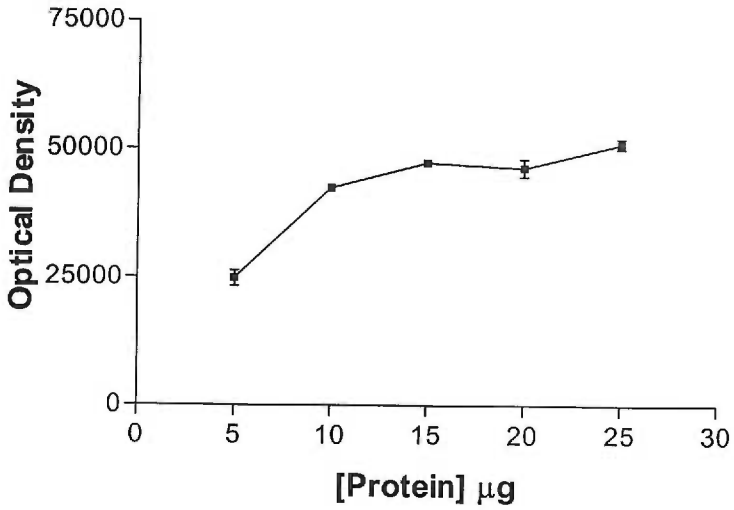
concentrations of protein were often needed for optimal detection when using the different antibodies (*e.g.*, anti-NR-C2 required 20 μg of protein/lane whereas anti-phospho-NR1 Ser896 required 15 μg/lane; Fig. 1), the blots could not always be stripped and re-probed with different antibodies. The order in which protein from different treatment groups was loaded into the lanes was randomized across gels to minimize possible order effects. Proteins were separated using 7.5% SDS-PAGE (Criterion precast gels; Bio Rad, Hercules, CA) and transferred to PDVF membranes (Millipore, Bedford, MA). Membranes were incubated in blocking buffer (5% nonfat dry milk in Tris-buffered saline [20 mM Tris-HCL (pH 7.4), 140 mM NaCl]) for 1 h at RT and washed for 30 min with 4 intermediate changes of Tris-buffered saline containing 0.1% Tween-20. The membranes were incubated (2-3 h at RT) in Tris-buffered saline with primary antibodies at appropriate dilutions and then incubated (1 h at RT) with species-appropriate secondary antibody, AP-conjugated (Bio Rad; 1:2000). Membranes were washed before and between incubations that included primary and secondary antibodies.

Figure 1: Representative protein concentration curves for immunoblotting experiments. To determine detection sensitivities for the various antibodies and brain regions investigated, protein concentration curves were generated. Samples were run in triplicate for each different protein concentration. The optimal concentrations of protein to load for anti-phospho-NR1 serine897 (A) and anti-NR2B (B) immunodetection were 15 μg and 10 μg per gel lane, respectively.

A


20000
10000
0
Optical Density
0
5
10
15
20
25
30
[Protein] µg

B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Confocal microscopy

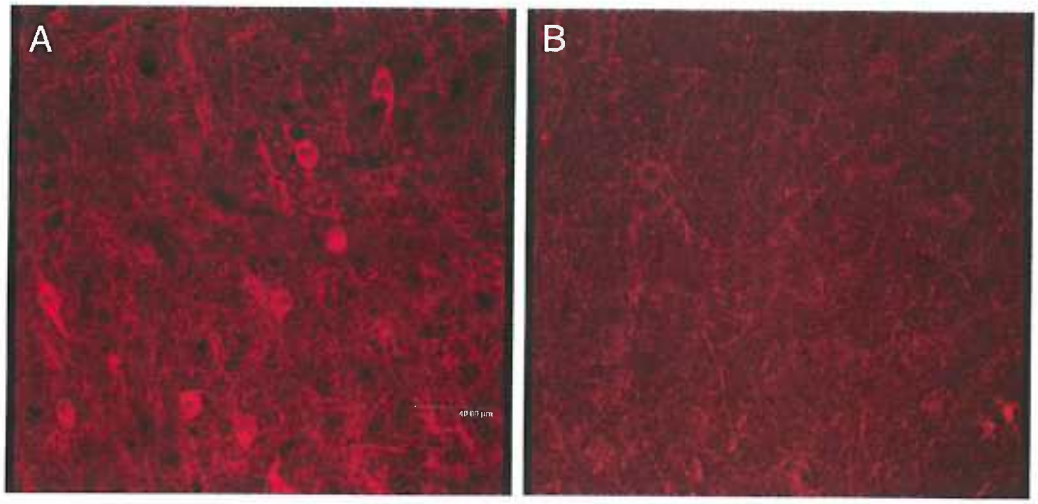
Using an orbital shaker set at 60 RPM, free-floating tissue sections received 3, 20-min washes each in 0.1M PB at RT to remove cryoprotectant. Blocking nonspecific reaction sites on the sections was accomplished by incubation with the appropriate normal animal serum for 1 h at RT. After blocking, the sections were incubated overnight at 4°C with the primary antibody for NR2B, nNOS, PSD-95, SAP102 or FosB/)FosB. All primary antibodies were diluted in 1% normal animal serum/0.1% TritonX-100/0.1M PB. Following incubation with the primary antibody, the sections received 3 washes (5 min each) in 5 ml 0.1M PB prior to treatment with the secondary antibody. The sections were incubated with fluorochrome-linked secondary antibody (Alexa Fluor 488 goat anti-rabbit IgG or 594 goat anti-mouse IgG, Molecular Probes) in 5% normal animal serum/0.1M PB for 30 min at RT, after which they received 3 washes (5 min each, 5 ml 0.1M PB). Sections were mounted on gelatin-coated slides and treated with an antifade reagent (ProLong Antifade, Molecular Probes) and coverslipped. For each experiment, some sections were incubated without the primary antibody to assess level of background staining and antibody specificity. Figure 2 illustrates the specificity of NR2B immunofluorescence as indicated by the regional differences in protein expression.

Data analysis for immunohistochemistry

Samples from different treatment conditions (*e.g.*, SAL24, COC24, SAL72, and COC72) were used in the same assays so that the effects of different incubations and reactions did not confound the effects of drug administration time or treatment group. Images were captured using an Olympus BX60 System Microscope and charge-coupled device camera. Image analyses were performed using the computer program, ImagePro Plus (Media Cybernetics, Silver Spring, MD). Intensity calibration was done prior to image analysis to establish baseline measurements of black

and incident light levels. Intensity values of a sample were thus calibrated to a standard density curve.

Figure 2: **NR2B immunofluorescence: rostral *vs.* caudal brain sections.** Panels A-B illustrate NR2B immunolabeling in the frontal cortex and posterior hypothalamic area (approximate Bregma coordinates 1.20 and -4.3 mm, respectively). Panel C shows data comparing total areas of immunofluorescence for rostral (frontal cortex) and caudal (posterior hypothalamus) sections (t-test, P=0.0015; n=3/brain region). Results indicated that the confocal microscopy techniques used in this dissertation project were sufficiently sensitive such that they were able to detect known differences in the regional distribution of NR2B protein expression (Laurie et al., 1997).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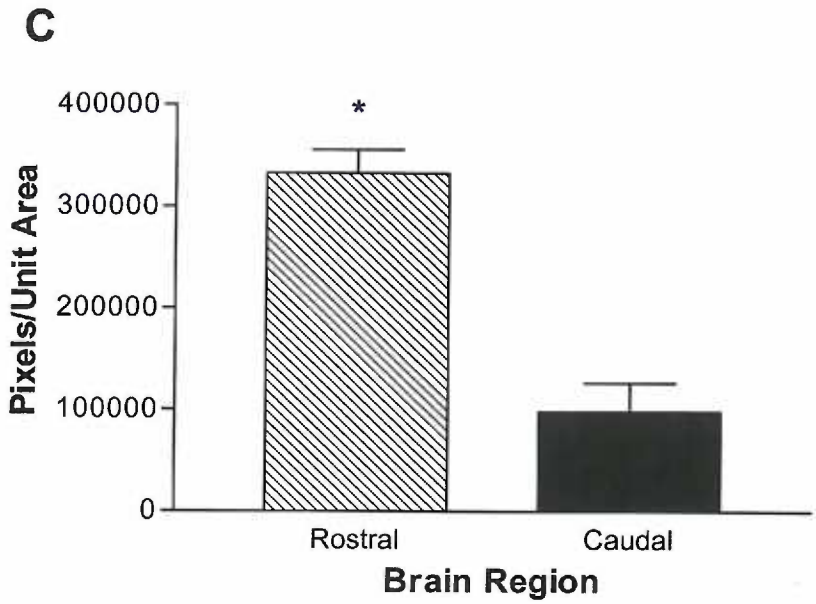
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Rostral
Caudal
Brain Region

Three sections per animal were used to calculate a mean individual score for specified analyses; variability among individual sections was typically < 15% of the mean. FosB, phospho-NR1 Ser896 and -NR1 Ser897 immunoreactivity was analyzed using computer-assisted counting of immunopositive cells. NR1, NR2B and nNOS immunoreactivity was measured using the average optical density values for a defined area of interest. Optical density difference scores were calculated by subtracting the mean optical densities of the various anatomical areas of the saline treatment groups from individual cocaine-treated sections within a particular withdrawal-time treatment group. Section-to-section variability of cocaine-treated rats was therefore included in the statistical analyses of optical density data. Because not all sections could be reacted on the same day, or in the same experiment, difference scores were calculated to minimize interexperimental variation. Common landmarks (*e.g.*, anterior commissure) were identified on the tissue sections to assure grouping of comparable brain regions. Approximate bregma coordinates for the areas of interest are indicated in the figure legends (Paxinos and Watson, 1998). FosB cell counts were analyzed using an ANOVA and optical density values were compared using Bonferroni corrected, two-tailed t-tests. Difference scores across withdrawal time points were compared using a one-way ANOVA followed by Tukey *post hoc* tests when appropriate. All other statistical analyses for the immunohistochemical experiments used unpaired, two-tailed t-tests. Statistical calculations were performed using GraphPad Prism software. Results for the cocaine-treated animals are presented as percentage of withdrawal time-matched saline controls.

Data analysis for immunoblotting

Each protein sample was analyzed in triplicate, as tissue from each animal was run three times on two or three gels. Proteins were visualized with enhanced chemiluminescence using the Storm 840 phosphorimaging system and quantified with ImageQuaNT (Molecular Dynamics, Sunnyvale, CA). Background correction values were subtracted from each lane to minimize the

variability across membranes. Bonferroni corrected, two-tailed *t*-tests were conducted to determine statistically significant differences between treatment groups and to test the hypotheses that the ratios of phospho-NR1 serine (896/897)/NR1-C1, phospho-NR1 serine 896/NR1-C1 and phospho-NR1 serine 897/NR1-C1 protein expression were significantly different than unity (*i.e.*, 1/1) for brain regions showing cocaine withdrawal-induced changes. Where specified, results are presented as percent change from appropriate saline control rats (*e.g.,* SAL24 or SAL14d). All saline groups represent baseline expression values (and are thus shown as 100%). Values from treated animals were compared to values from saline animals on the same gel.

Data analysis for confocal microscopy

Samples from different treatment conditions (*e.g.,* SAL24, COC24, SAL14d, and COC14d) were used in the same assays so that the effects of different incubations and reactions did not confound the effects of drug administration time or treatment group. Images were captured using a Leica confocal microscope. The sections were examined at 40x magnification. Fluorophore-labeled secondary antibodies were excited at 488 nm and detected within the range of 499-558 nm and 568 nm and detected within the range of 579-654 nm. Images were acquired separately in each channel (sequential scan mode) to eliminate the possibility of signal cross talk from one channel to the other. Analyses were performed using the computer program, ImagePro Plus. Three sections per animal were used to calculate a mean individual score for the specified region of interest (frontal cortex or dorsolateral neostriatum; bregma coordinates are indicated in the figure legends). Protein expression was measured using the average total area of immunofluorescence per fluorophore for the defined brain region. Co-expression of proteins was calculated by keeping analysis parameters constant (*e.g.*, gain and offset values) and comparing single-protein expression percentages. More specifically, ImagePro Plus utilized Boolean arithmetic to calculate the "AND" function from two overlayed images (red and green channels).

A new image was thus created based on the degree of pixel overlap. Results were analyzed using unpaired t-tests and $p < 0.05$ was considered statistically significant.

Chapter 1: Regulation of NMDA receptor subunits and nitric oxide synthase expression during cocaine withdrawal

INTRODUCTION

Cocaine is a potent psychostimulant that blocks dopamine (DA) re-uptake and increases neurotransmitter availability in the synapse. However, the neuroadaptations caused by repeated cocaine administration are also contingent upon a functional glutamatergic system (Karler et al., 1994). Pharmacological and behavioral evidence suggests the existence of multiple interactions among DA, glutamate, and neuronal nitric oxide synthase (nNOS: EC 1.14.13.39) in brain structures associated with psychomotor stimulation (Itzhak, 1997).

The NMDA subtype of glutamate receptor is a heteromeric ligand-gated ion channel composed of multiple receptor subunits (NR1, NR2A-2D and NR3A) that interact with various synaptic proteins (McBain and Mayer, 1994). Evidence supports a role for NMDA receptors in mediating behavioral (Pierce and Kalivas, 1997), electrophysiological (Smith et al., 1993), and biochemical (Nestler et al., 1993) responses to cocaine. For example, sensitization to the toxic, locomotor stimulant and convulsant effects of cocaine is blocked by a number of NMDA receptor antagonists such as MK-801 (Pierce et al., 1997), ifenprodil, and 1-[1-(2-thienyl)-cyclohexyl]piperidine (TCP) (Itzhak and Stein, 1992; Wolf, 1998; Witkin et al., 1999). Repeated administration of cocaine alters rat NR1 mRNA levels in the nucleus accumbens, VTA, and striatum (Ghasemzadeh et al., 1999) and increases rat NR1 protein levels in the VTA (Churchill et al., 1999; Fitzgerald et al., 1996). Further, cocaine exposure and withdrawal are often associated with time dependent effects that can result in enduring changes in the nervous system (Pierce and Kalivas, 1997). Characterizing the effects of cocaine on the composition and distribution of NMDA receptor proteins is important because individual NMDA receptor subunit and splice variant combinations have differing receptor functions (Ishii et al., 1993; Buller et al., 1994; Ehlers et al., 1995).

Repeated cocaine administration alters a number of intracellular components critical to signal transduction [G proteins (Striplin and Kalivas, 1992), PKA (Beitner-Johnson and Nestler, 1992), nNOS (Bhargava and Kumar, 1997)], cytoskeletal stability [neurofilament-like protein (Beitner-Johnson and Nestler, 1992)], gene expression [activating protein-1 binding activity (Hope et al., 1994)], and protein synthesis [tyrosine hydroxylase (Beitner-Johnson and Nestler, 1991)]. One important example of a second messenger molecule that is critically involved with NMDA receptor function is nitric oxide (NO) and its related enzyme, nNOS (Garthwaite, 1991; Sattler et al., 1999). Neuronal NOS is a Ca^{2+}/CaM-dependent enzyme that is functionally and selectively coupled to Ca^{2+} influx through NMDA-type glutamate receptors (Garthwaite and Boulton, 1995). Stimulation of NMDA receptors results in the activation of nNOS, which catalyzes the formation of NO from L-arginine (Garthwaite et al., 1989). The enzyme is linked to the NR2B subunit *via* PSD-95 (Brenman and Bredt, 1997). Because of the cytoskeletal association between NMDA receptor subunits and nNOS, and the putative cocaine-induced enhancement of NOS enzymatic activity (Bhargava and Kumar, 1997), experiments were performed to evaluate changes in the expression of nNOS during withdrawal from repeated cocaine administration.

The present study characterized time-dependent and regionally specific effects of cocaine treatment and withdrawal on the expression of NMDA receptor subunits, NR1 and NR2B, and associated neuronal elements. A member of the Fos-related antigen family, FosB/ΔFosB, was also assessed during cocaine withdrawal to confirm previously demonstrated effects of chronic drug treatment on protein expression (Hope et al., 1994; Moratalla et al., 1996). NR1 expression in the VTA was selected for study in order to replicate and extend findings that were previously reported by Fitzgerald et al., (1996). NR2B (as opposed to other NMDA receptor subunits) expression was characterized to focus on the pathways where DA, nNOS and NMDA receptor expression might contribute to the maintenance of behavioral or physiological features accompanying withdrawal from cocaine. NR2B subunit expression was not assessed in the VTA

as its abundance in midbrain structures is limited (Laurie et al., 1997). In rats, NR2B is preferentially expressed in forebrain structures (Laurie et al., 1997; Charton et al., 1999) and is hypothesized to play a role in synaptic plasticity (Herkert et al., 1998; Tang et al., 1999) and modulation of receptor function (Lau and Huganir, 1995). The NR2B subunit confers unique properties to the receptor complex *via* modulation of receptor responses by phosphorylation of subunit tyrosine, serine, and threonine residues (Kennedy, 1998; Anders et al., 1999; Kalluri and Ticku, 1999). Further, the NR2B subunit is the most tyrosine-phosphorylated protein in the PSD (Moon et al., 1994) and tyrosine phosphorylation of NR2B is up-regulated following ethanol treatment in the hippocampus of C57BL mice (Miyakawa et al., 1997). In addition, NR2B, but not NR2A or NR1 subunits are responsible for autophosphorylation-dependent targeting of CaMKII in intact cells (Strack and Colbran, 1998). Thus, NR2B phosphorylation could be important for the activation of pathways that are substrates for the effects of cocaine exposure and withdrawal. Therefore, protein levels for FosB/ΔFosB, NR1, NR2B, and nNOS were assessed in brain regions that have been putatively identified in mediating the behavioral and neurochemical effects of cocaine, including areas of the cortex, neostriatum, nucleus accumbens, hippocampus, and VTA using immunohistochemical methods.

RESULTS

Effects of repeated cocaine treatment on FosB immunoreactivity

As an initial assessment of cocaine's influence on protein expression, rats that had been treated with cocaine or saline and sacrificed at 24 hours or 72 hours after their last drug injection were used to measure FosB expression. Immunopositive cell staining for FosB was measured in dorsal neostriatal fields. Three sections per animal per treatment group were reacted and averaged to obtain mean cell counts. Table 1 illustrates treatment group means. Two-way ANOVA for treatment by withdrawal time yielded a significant main effect of treatment [$F_{(1,16)} = 22.19$, $p = 0.0002$]. Thus, at 24 hours and 72 hours after repeated cocaine treatment, FosB

immunoreactivity was increased in regions of the rat dorsal neostriatum as compared with saline treated animals. Analyses indicated no significant main effect for withdrawal time.

Table 1. FosB immunopositive cells identified in the neostriatum for cocaine- and saline-treated rats. For each treatment group (n = 5), labeled cells were counted in 300 x 300 pixel areas from comparable regions of the dorsal neostriatum, located approximately 0.20 mm anterior to bregma (Paxinos and Watston, 1998). Values represent means ± SEM (*p < 0.001, relative to saline-treated controls).

	WITHDRAWAL TIME	
TREATMENT	24 h	72 h
Saline	**135.0 ± 18.58**	**146.4 ± 30.37**
Cocaine	**247.0 ± 17.9***	**258.4 ± 25.93***

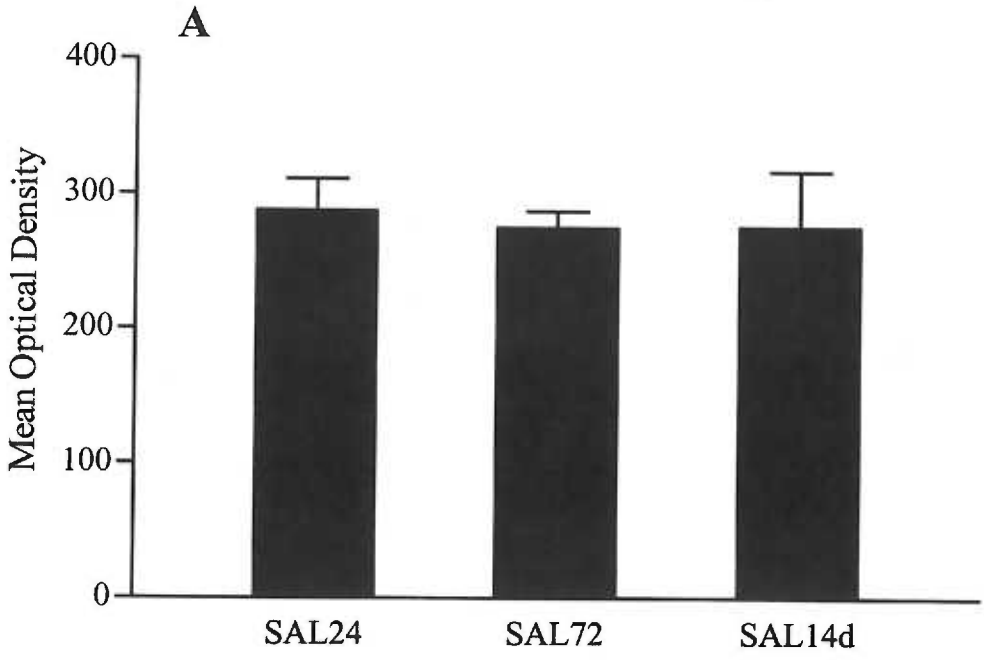
Effects of withdrawal from repeated cocaine treatment on the expression of NMDA receptor subunit, NR1

VTA. NR1 immunoreactivity was analyzed in regions of the VTA for rats treated with cocaine or saline and sacrificed at 24 hours, 72 hours, or 14 days following their last injection. NR1 protein levels were measured only in the VTA to demonstrate the specificity of immunolabeling techniques used in this study and to more comprehensively characterize the time course of putative, cocaine withdrawal-associated changes (Fitzgerald et al., 1996). Animals treated with saline exhibited similar and consistent NR1 protein expression levels at the three different withdrawal-time points (Fig. 1A). Optical density analyses revealed significant differences between SAL72 and COC72 ($t = 2.97$, $p < 0.05$) and SAL14d and COC14d ($t = 3.33$, $p < 0.05$) (Fig. 1B). Following 24 hours of withdrawal, the cocaine-treated group exhibited an increase in NR1 immunoreactivity as compared to the saline-treated group. However, the up-regulation in NR1 subunit expression did not reach statistical significance (Fig. 1B). Although a trend toward increased expression over time was evident, the one-way ANOVA of difference scores across the three time points was not significant ($F = 2.26$, $p = 0.11$).

Effects of withdrawal from repeated cocaine treatment on the expression of NMDA receptor subunit, NR2B

NR2B immunoreactivity was measured in a number of forebrain and limbic structures putatively involved in mediating the effects of cocaine (Fig. 2). Areas of investigation included the CA1, CA3 and dentate gyrus of the hippocampal formation, neostriatum, nucleus accumbens, and regions of the cortex. As observed for NR1 expression in the VTA, NR2B subunit protein expression levels were stable across all time points for rats treated with saline, thus permitting reliable analyses of difference scores (Fig. 3A). Optical density analyses illustrated withdrawal-time dependent alterations in NR2B expression in a region-specific manner.

Figure 1: NR1 up-regulation in the VTA during withdrawal from repeated cocaine administration. Analyzed VTA regions were located approximately 5.2 mm posterior to bregma (Paxinos and Watson, 1998). SAL24 and COC24 n = 7/treatment group; SAL 72 and COC72 n = 8/treatment group; SAL14d and COC14d n = 5/treatment group. A) Mean optical density values for saline-treated rats across three withdrawal-time points. B) Optical density percent change for cocaine-treated rats as compared to saline-treated controls. Significant differences were observed at 72 hours and 14 days after withdrawal from cocaine as compared to saline-treated animals (*$p < 0.05$).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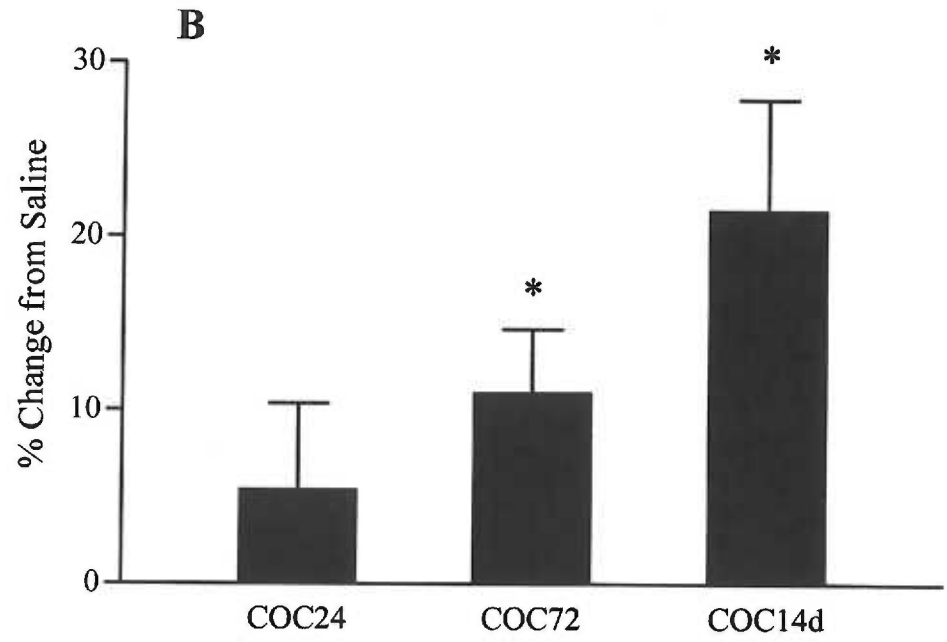
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Hippocampal formation. No significant changes in NR2B immunoreactivity were observed following 24 or 72 hours of withdrawal from the last drug injection in any of the hippocampal regions analyzed: CA1, CA3, or dentate gyrus (data not shown).

Dorsolateral neostriatum. At 24 and 72 hours following repeated cocaine exposure, NR2B subunit expression in dorsolateral regions of the neostriatum did not appear to be affected. Two weeks of withdrawal from cocaine, however, resulted in increased NR2B immunolabeling in areas of the neostriatum, as compared to animals that received saline ($t = 6.03$, $p < 0.05$) (Fig. 3B).

Nucleus accumbens. One day after the last drug injection, cocaine-treated rats had significant decreases ($t = 3.06$, $p < 0.05$) in NR2B immunolabeling in the shell of the nucleus accumbens as compared to saline-treated controls. Following 72 hours of withdrawal, the reduction in NR2B subunit expression was attenuated, as no significant differences in immunostaining were observed between saline- and cocaine-treated animals. No differences in subunit expression were observed in the core of the accumbens at the 24 or 72 hour withdrawal time points. In contrast, 14 days of withdrawal from cocaine exposure resulted in significant increases in NR2B expression in the nucleus accumbens shell ($t = 4.77$, $p < 0.05$) and core ($t = 4.36$, $p < 0.05$) (Fig. 3C).

Cortex. Rats treated with cocaine had significant increases in NR2B immunoreactivity in regions of the medial frontal cortex (anatomical region described in legend for Fig. 2) at 24 hours ($t = 3.32$, $p < 0.05$), 72 hours ($t = 3.93$, $p < 0.05$) and 14 days ($t = 3.36$, $p < 0.05$) after the last drug injection (Fig. 3B). Areas of the lateral frontal cortex (anatomical region described in legend for Fig. 2) failed to show significant cocaine-induced protein expression differences at the 24-hour withdrawal time point (Fig. 3D). Up-regulation of NR2B in the lateral frontal cortex was, however, observed at both the 72-hour ($t = 2.22$, $p < 0.05$) and the 14-day ($t = 7.31$, $p < 0.05$) withdrawal time points (Fig. 3D). Similarly, significant cocaine-induced increases in NR2B

Figure 2: Representative sections illustrating cocaine withdrawal-induced alterations in immunostaining for NR2B. A) NR2B immunolabeling in regions of the medial frontal cortex [located approximately 1.5 mm anterior to bregma just off the midline and identified as M2, secondary motor cortex (Paxinos and Watson, 1998)] from rats treated with saline (left) or cocaine (right) and exposed to 14 days of withdrawal. Arrows identify cells with NR2B immunoreactivity. All layers of the adult cerebral cortex contain immunoreactive structures, but the most intensively stained neurons are located in layers II/III, V and VI (Charton et al., 1999). In the pyramidal neurons the immunostaining extends also into apical dendrites. B) NR2B immunolabeling in regions of the lateral frontal cortex [located approximately 1.5 mm to bregma just lateral to the medial frontal cortex and identified as M1, primary motor cortex (Paxinos and Watson, 1998)] from rats treated with saline (left) or cocaine (right) and exposed to 14 days of withdrawal. C) Increased magnification illustrating NR2B immunolabeling of cortical layer V from a rat treated with cocaine and withdrawn for 72 hours. White arrow indicates immunopositive cell body and black arrow indicates dendritic labeling. CC, corpus callosum.

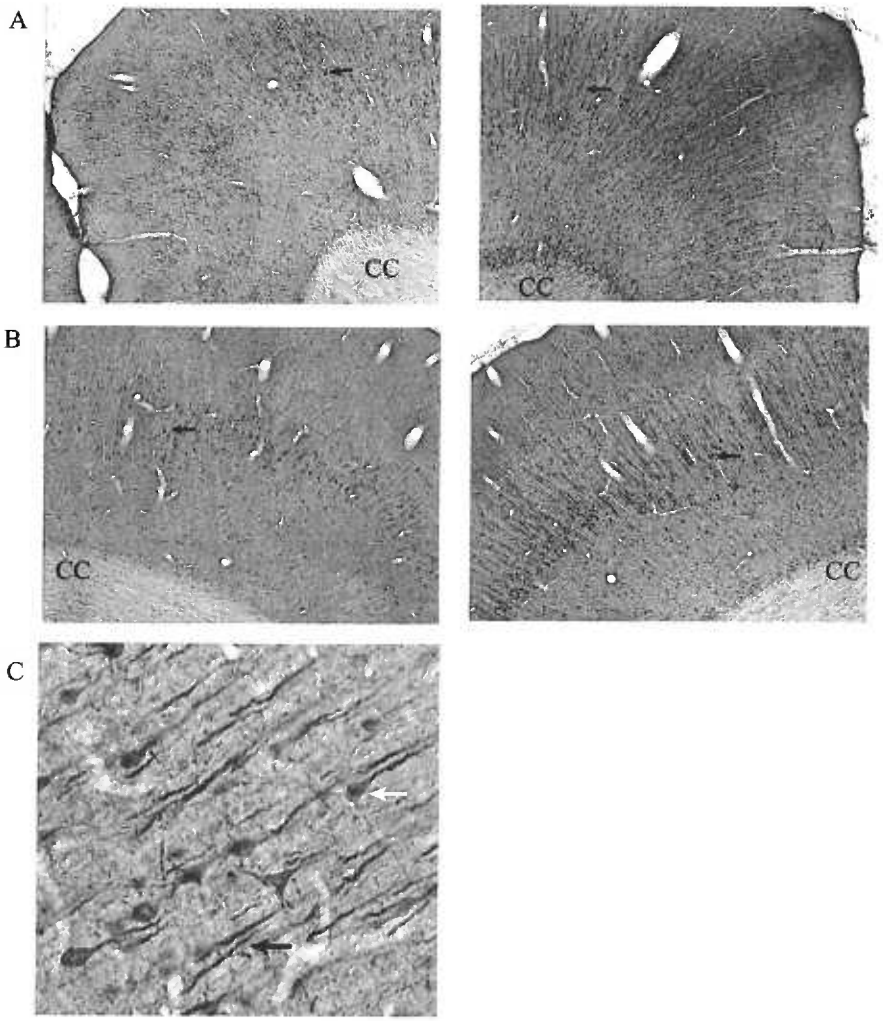
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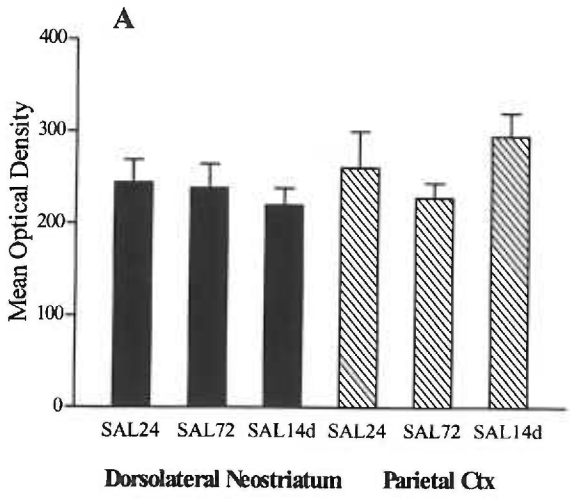
Figure 3: Cocaine withdrawal-induced time-dependent changes in NR2B expression. COC24 and SAL24, n = 5/treatment group; COC72 and SAL72, n = 6/treatment group; COC14d and SAL14d, n = 5/treatment group. For panels A-C data are expressed as percent change from saline-treated control rats (*$p < 0.05$). A) Histogram illustrating the relatively stable mean optical density values for saline-treated rats across the three withdrawal time points in the dorsolateral neostriatum (solid bars) and parietal cortex (hatched bars). B) Solid bars represent NR2B cocaine withdrawal-induced increases in the medial frontal cortex and hatched bars represent optical density percent changes from saline-treated rats in the dorsolateral quadrant of the neostriatum located approximately 1.5 mm anterior to bregma (Paxinos and Watson, 1998). C) Cocaine withdrawal-induced changes in regions of the nucleus accumbens shell (solid bars) and core (hatched bars) [approximately 1.5 mm anterior from bregma (Paxinos and Watson, 1998)]. D) Solid bars represent NR2B up-regulation in the lateral frontal cortex. Hatched bars represent NR2B up-regulation in the parietal cortex approximately 1.5 mm anterior to bregma and identified as S1 cortex (Paxinos and Watson, 1998).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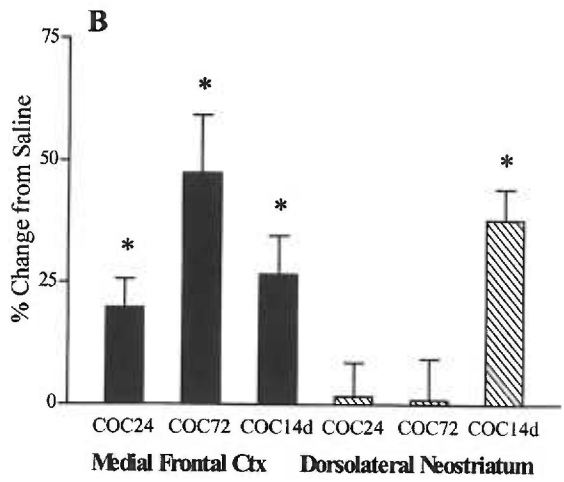
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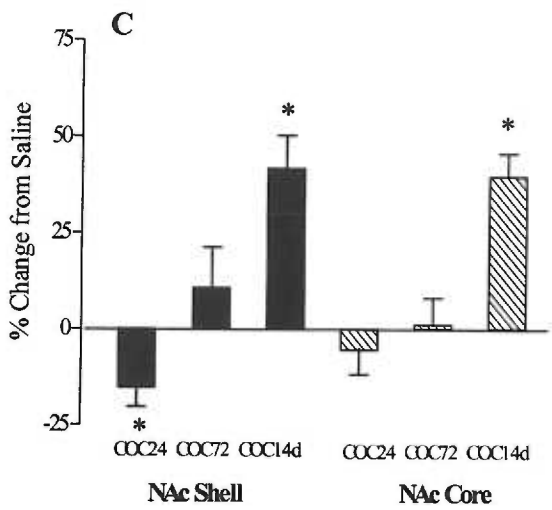
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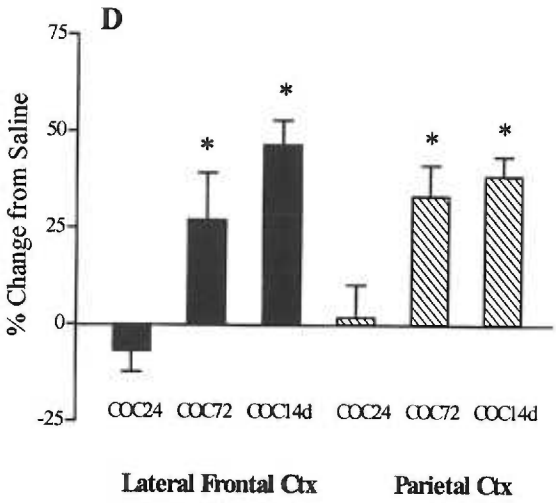
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Lateral Frontal Ctx
Parietal Ctx

immunoreactivity were observed in regions of the parietal cortex at 72 hours ($t = 4.08$, $p < 0.05$) and 14 days ($t = 7.54$, $p < 0.05$) but not at 24 hours after the last drug injection (Fig. 3D).

One-way ANOVA of difference scores across the three withdrawal time points suggested that, unlike NR1 expression, NR2B protein levels were significantly affected by the length of time since last drug administration. Table 2 illustrates results from *post hoc* analyses for areas of the lateral frontal ($F = 8.75$, $p = 0.0006$) and parietal ($F = 8.11$, $p = 0.001$) cortices, dorsolateral neostriatum ($F = 6.518$, $p = 0.0033$), and nucleus accumbens shell ($F = 17.11$, $p < 0.0001$) and core ($F = 8.42$, $p = 0.0008$). The ANOVA for regions of the medial frontal cortex was not significant across withdrawal times.

Effects of withdrawal from repeated cocaine treatment on the expression of nNOS

Dorsolateral neostriatum and nucleus accumbens. Significant changes in nNOS protein levels were not observed in the dorolateral neostriatum or nucleus accumbens shell or core at any time during withdrawal (24 hours, 72 hours, or 14 days; data not shown).

Cortex. At 24 hours after the last injection, sections from rats treated with cocaine had increased nNOS expression in areas of the medial frontal cortex ($t = 3.33$, $p < 0.05$) and parietal cortex ($t = 2.69$, $p < 0.05$) (Fig. 4). Interestingly, the nNOS up-regulation observed after 24 hours of withdrawal was transient and did not follow corresponding increases in NR2B expression, as there were no significant differences in nNOS expression following 72 hours or 14 days of withdrawal for either cortical region (Fig. 5). Significant changes in nNOS subunit expression were not observed in the lateral frontal cortex for any of the withdrawal time points (data not shown). As with the NMDA receptor subunits (Fig. 1A), the expression of nNOS remained relatively stable and consistent across the different withdrawal time points for saline-treated rats (data not shown).

One-way ANOVA of difference scores confirmed that the increase in nNOS observed at 24 hours in the frontal cortex did not persist at the subsequent withdrawal time points ($F = 4.01$, p

= .027). Tukey's *post hoc* test for multiple comparisons revealed significant differences between COC24 and COC14d ($p < 0.05$) in cortical tissue, but not between COC24 and COC72 or between COC72 and COC14d. Although the ANOVA failed to affirm statistically significant differences between COC24 and COC72 in the cortex, the significant increase in nNOS expression observed in the cortex after 24 hours of withdrawal from cocaine, as compared to saline-treated controls, was not seen following 72 hours of withdrawal from drug administration.

Table 2. Effects of cocaine withdrawal time on NR2B expression. Values in the table represent results from Tukey's multiple comparison tests that were conducted on ANOVA findings yielding $p < 0.05$. Asterisks denote significant results obtained from Tukey *post hoc* analyses.

Brain Region	Mean Difference	Tukey Test P Values
Lateral frontal cortex		
COC24 vs COC72	-88.37*	< 0.05
COC24 vs COC14d	-143.0*	< 0.001
COC72 vs COC14d	-54.68	> 0.05
Parietal cortex		
COC24 vs COC72	-70.64*	< 0.05
COC24 vs COC14d	-108.7*	< 0.001
COC72 vs COC14d	-38.03	> 0.05
Neostriatum		
COC24 vs COC72	1.78	> 0.05
COC24 vs COC14d	-79.20*	< 0.05
COC72 vs COC14d	-80.99*	< 0.01
Accumbens shell		
COC24 vs COC72	-42.7	> 0.05
COC24 vs COC14d	-135.1*	< 0.001
COC72 vs COC14d	-92.41*	< 0.001
Accumbens core		
COC24 vs COC72	-18.61	> 0.05
COC24 vs COC14d	-96.07*	< 0.01
COC72 vs COC14d	-77.46*	< 0.01

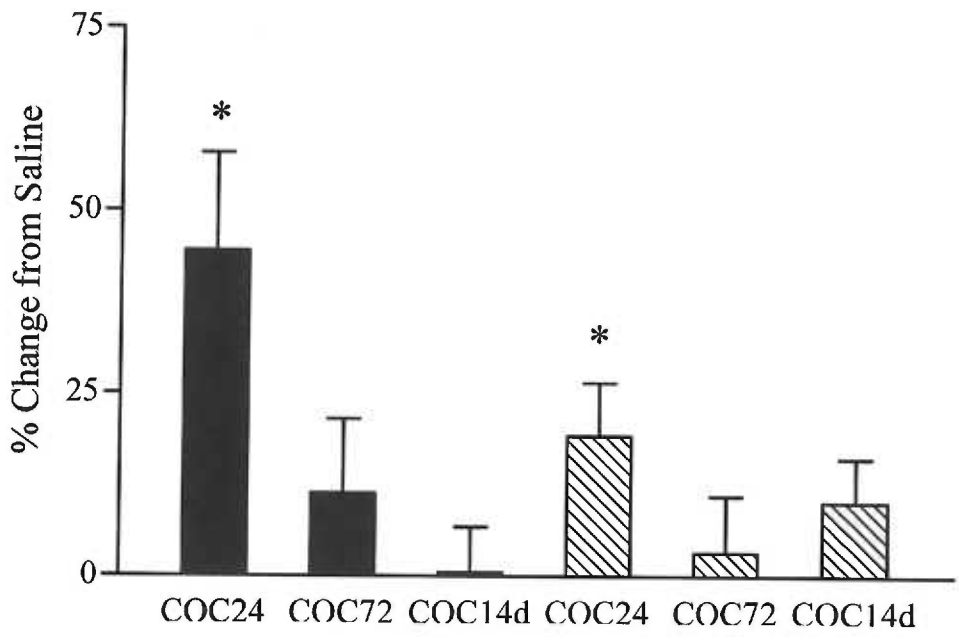


Figure 4: nNOS time-dependent upregulation following repeated cocaine administration. COC24 and SAL24, n = 4, COC72 and SAL72, n = 4, COC14d and SAL14d, n = 5. A) Solid bars represent nNOS up-regulation in the medial frontal cortex approximately 1.5 mm to bregma and just off the midline (Paxinos and Watson, 1998). Hatched bars represent nNOS up-regulation in the parietal cortex approximately 1.5 mm to bregma. Data are expressed as percent change from saline-treated control rats (*$p < 0.05$).

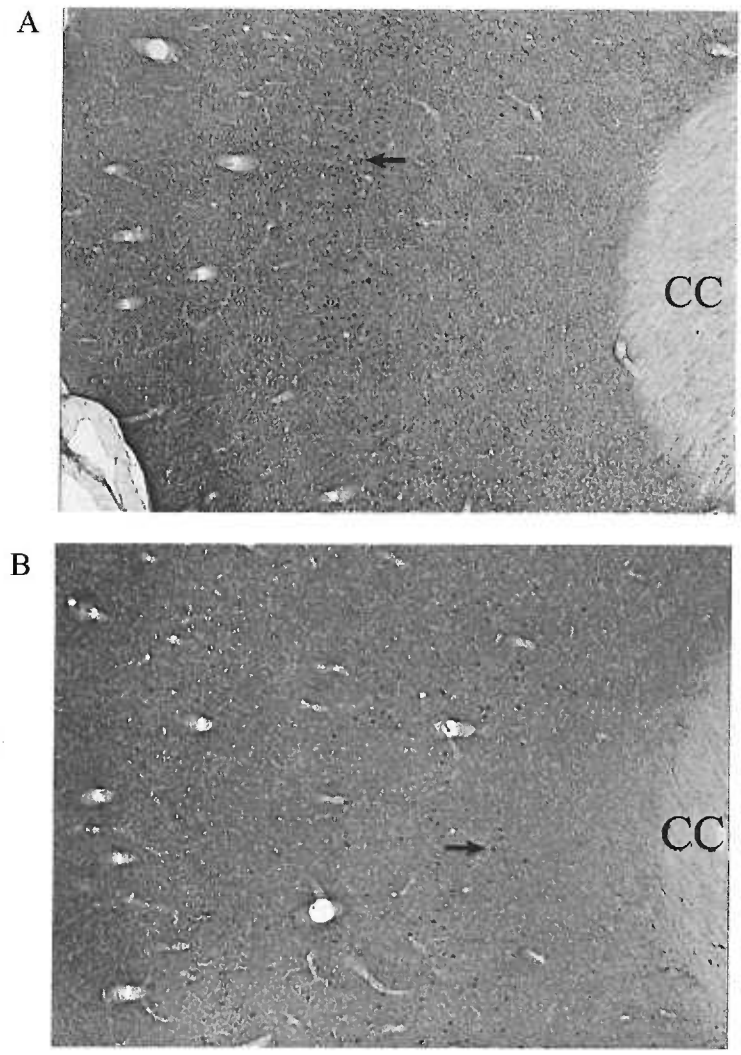


Figure 5: Representative sections showing transient cocaine withdrawal-induced increase in immunostaining for nNOS. Panels illustrate nNOS immunoreactivity in the parietal cortex of a rat treated with cocaine and withdrawn for 24 hours (A) as compared to a rat withdrawn for 72 hours (B). Arrows identify examples of nNOS immunopositive cells in comparable brain sections.

DISCUSSION

FosB expression is altered during withdrawal from cocaine. In the present study, increases in FosB immunoreactivity were observed in regions of the neostriatum at 24 and 72 hours after withdrawal of rats from cocaine, as compared to saline-treated control animals. These findings are consistent with previous reports, which demonstrate that following repeated cocaine administration; FosB expression persists above control levels in areas of the neostriatum and nucleus accumbens for up to 7 days of withdrawal (Hope et al., 1994; Moratalla et al., 1996). Taken together, these results support the reliability of repeated cocaine treatment in producing elevated numbers of FosB-positive neurons and suggest that the induction of transcription factors including Fos-related antigen such as FosB, may represent a general response that is associated with chronic perturbations that may not be limited to long-term cocaine exposure (Chen et al., 1995; Chen et al., 1997).

NMDA receptor subunit-specific plasticity during withdrawal from cocaine. Repeated cocaine administration altered the expression of the NMDA receptor subunits, NR1 and NR2B, in a withdrawal-time and region-specific manner. These results are generally in agreement with Fitzgerald et al. (1996) who, using immunoblotting of brain tissue from rats that were repeatedly treated with cocaine, observed an increase in NR1 expression only in the VTA after 18 hours of withdrawal. Cocaine-induced changes in NR2A/B were not found by those investigators, however the antibodies used were not able to differentiate between NR2A and NR2B subunits. Using a different method (immunohistochemistry) and more selective antibodies, the present study sought to replicate the earlier findings and to further characterize the time course of potential cocaine-induced alterations of neuronal protein levels. Experiments included drug effects at three different withdrawal time points to determine if previously identified changes were short-term, or if they reflected a more permanent adaptation. Although the increase in NR1 expression at 24 hours of withdrawal did not reach statistical significance, a trend was observed. However, the results presented here demonstrate that at both 72 hours and 14 days after the last

injection of cocaine, there were significant increases in NR1 expression in the VTA as compared to NR1 expression in saline-treated animals (Fig. 1B). Results recently published by Churchill et al. (1999) suggest that animals that develop behavioral sensitization to cocaine have increased NR1 protein expression levels in the VTA at 1 day but not at 3 weeks of withdrawal, although the reported elevation in NR1 levels at 3 weeks came close to statistical significance ($p = 0.056$). Both the VTA and NMDA receptors have been implicated in the development of sensitization (Pierce and Kalivas, 1997) and drug-induced changes in the VTA are often transient and evident at earlier withdrawal time points (Wolf, 1998). However, results presented here suggest that neuroanatomical changes in the VTA may persist beyond the early stages of withdrawal from cocaine, and may thus be involved not only in the initiation of psychostimulant-induced sensitization but also in the maintenance of sensitization or other withdrawal-related behaviors.

In addition to changes in NR1 expression, rats withdrawn from repeated cocaine treatment had significant changes in NR2B immunoreactivity in the frontal cortex and nucleus accumbens as early as 24 hours after the last drug injection. At 72 hours of withdrawal from cocaine, further increases were observed in regions of the frontal and parietal cortices, as compared with saline-treated control animals. Two weeks of withdrawal from cocaine resulted in significant NR2B up-regulation in the frontal cortex, parietal cortex, neostriatum, and nucleus accumbens (Fig. 3). The delayed increase in NR2B subunit expression observed in regions of the dorsolateral neostriatum suggests the involvement of this area and the corticostriatal pathway in the manifestation of more enduring changes associated with cocaine treatment, such as locomotor sensitization. Components of the mesoaccumbens DA system and the ventral striatal glutamatergic system, which projects from the hippocampus and amygdala to the nucleus accumbens, may also be involved in cocaine-induced changes in protein expression (McGinty, 1995). Specifically, decreased expression of NR2B in the shell of the accumbens at 24 hours was replaced by significant up-regulation in the shell as well as the core at 14 days of withdrawal, suggesting that different phases of withdrawal may be differentially regulated by NMDA

receptor-mediated neurotransmission. In addition, significant up-regulation of NR2B in the nucleus accumbens following 14 days of withdrawal from cocaine is consistent with the suggestion that changes in this brain region are often more persistent and evident only after longer withdrawal times (Wolf, 1998).

Withdrawal from cocaine administration causes region-specific, short-term changes in nNOS expression. Results presented here indicate that withdrawal from repeated administration of cocaine results in transient, but not long-term increases of nNOS expression in regions of the cortex. The significant cocaine-induced up-regulation observed at 24 hours was absent at 72 hours and 14 days of withdrawal in the frontal and parietal cortices (Fig. 4). The regulation of NO formation appears to be mediated by the compartmentalization of nNOS with NMDA receptors at specific synaptic sites (Weiss et al., 1998). The mechanism for coupling nNOS to Ca^{2+} influx through NMDA receptors involves the targeting of nNOS to the PSD (Brenman and Bredt, 1997). Specifically, the N-terminal PDZ domain of nNOS links the enzyme to the second PDZ repeat of PSD proteins, PSD-95 and PSD-93 (Brenman et al., 1996). PDZ domains of PSD-95 also interact with the C-terminal domain containing the tSXV motif (S, serine; X, any amino acid; V, valine) common to NMDA receptor NR2 subunits and certain NR1 splice variants. Interestingly, the development of sensitization to cocaine can be influenced by NOS. Pharmacological blockade of NOS reduces the reinforcing properties of cocaine (Pulvirenti et al., 1996) and selective inhibition of nNOS blocks the induction of behavioral sensitization to cocaine (Haracz et al., 1997). Chronic administration of cocaine followed by 1 hour of "withdrawal" increases NOS activity in the cerebral cortex, cerebellum, midbrain, hypothalamus, hippocampus, amygdala and spinal cord, and 48 hours after withdrawal from cocaine NOS activity remains elevated only in the cortex (Bhargava & Kumar, 1997). The findings reported here thus suggest that early, time-limited cocaine-induced alterations in nNOS expression may reflect part of the progression of molecular signaling changes necessary for the expression of long-term consequences that are associated with cocaine withdrawal, such as behavioral sensitization.

The findings presented here support the hypothesis that changes in NMDA receptor subunit expression underlie neuronal adaptations to repeated cocaine treatment and suggest that altered interaction among proteins may represent putative mechanisms for cocaine-induced changes in NMDA receptor function. Further, the results are unique in that they demonstrate for the first time, the presence of persistent cocaine-induced elevations of specific NMDA receptor subunits in discrete brain regions.

CHAPTER 2: Cocaine withdrawal-induced alterations in the expression and serine phosphorylation of the NR1 NMDA receptor subunit

INTRODUCTION

Cocaine treatment alters the expression of NMDA receptor subunits in a region- and withdrawal time-dependent manner (Fitzgerald et al., 1996; Loftis and Janowsky 2000). Pharmacological and behavioral evidence suggests a relationship between NMDA receptor-mediated glutamatergic transmission and psychostimulant-induced locomotor hyperactivity (Gainetdinov et al., 2001). These effects may be mediated by subunit- and kinase-specific phosphorylation, which modulate subunit function, expression and regulation (Lu et al., 2000; Westphal et al., 1999; Kalluri and Ticku, 1999; Smart, 1997). However, the functional consequences of cocaine withdrawal-induced alterations in amino acid-specific phosphorylation of individual NMDA receptor NR1 splice variants are not known.

The NMDA subtype of glutamate receptor is a heteromeric ligand-gated ion channel composed of multiple receptor subunits (NR1, NR2A-B and NR3A). The diversity of the NMDA receptor NR1 subunit is created by alternative splicing of the NMDAR1 gene to yield eight functional splice variants that include insertions or deletions of three exon cassettes in the N-terminal (N1) and C-terminal (C1, C2) domains of the subunit protein (McBain and Mayer, 1994; Fig. 1). Although reports regarding NMDA receptor stoichiometry are somewhat ambiguous, studies indicate that receptor complexes may contain two or three different NR1 splice variants (Blahos and Wenthold, 1996; Premkumar and Auerbach, 1997). The C1 cassette present in selected splice variants contains all known sites of phosphorylation on the NR1 subunit (Tingley et al., 1997). Activation of protein kinase C (PKC) and cyclic AMP-dependent protein kinase A (Colledge et al., 2000) together lead to the dual phosphorylation of the NR1 subunit serine residues 896 and 897, respectively (Tingley et al., 1997). Changes in PKC activity in the VTA may play a role in the initiation of drug-induced behavioral sensitization whereas changes in PKC

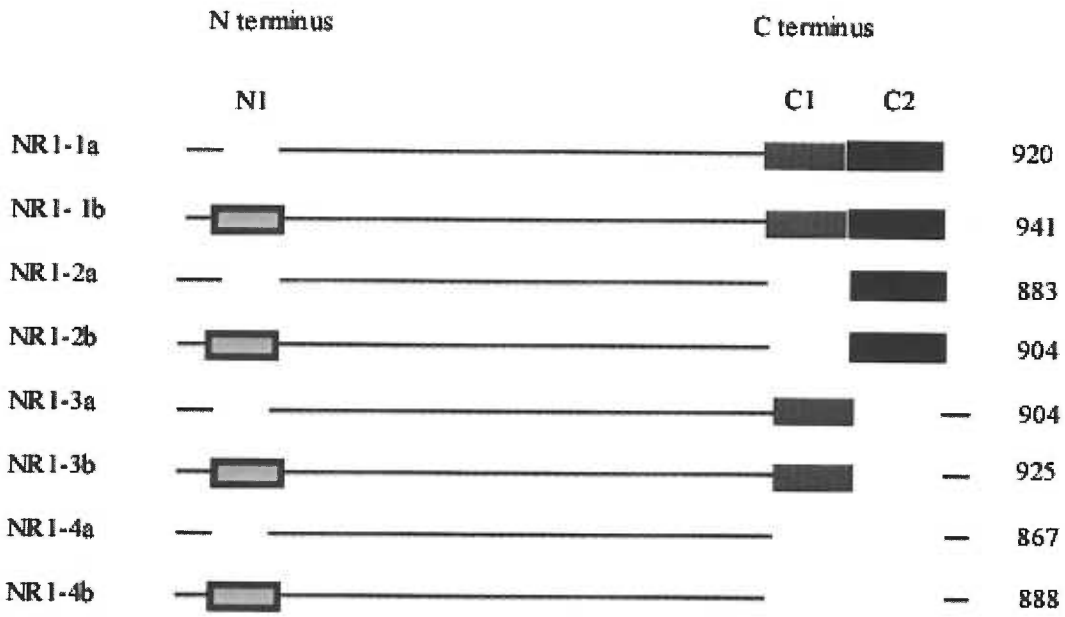


Figure 1: Schematic structure of the eight functional splice variants for the NR1 subunit. Numbers to the right of the figure denote the mature protein size. There are three different deletions at the C-terminal end: 1) 111 base pair deletion (nucleotides 2536-2646), 2) 356 base pair deletion (nucleotides 2647-3002), and 3) 467 base pair deletion (nucleotides 2536-3002; combination of 111 and 356 deletions) (Hollmann, Boulter, et al. 1993 312 /id). Absence (1-4a) or presence (1-4b) of a 21 amino acid sequence close to the N-terminus is also illustrated. Note that the NR1-2a and NR1-2b splice variants are selectively labeled by the NR1-C2 antibody (and not the NR1-C1 antibody) whereas the NR1-3a and NR1-3b splice variants are selectively labeled by the NR1-C1 antibody (and not the NR1-C2 antibody).

activity in the medial prefrontal cortex may be related to the expression of the sensitized response to cocaine (Steketee et al., 1998).

Recent findings suggest that the 95 kDa postsynpatic density protein, PSD-95, acts as a scaffold for associating NMDA receptors and protein kinases (Tezuka et al., 1999), and PSD-95 appears to play a role in controlling kinase modulation of NMDA receptor currents (Liao et al., 2000). Further, A-kinase anchoring proteins (AKAPs) such as AKAP79/150 provide a scaffold for signaling enzymes, PKA and PKC (Klauck et al., 1996). AKAP79/150 and its associated kinases assemble with PSD-95 and can be recruited to glutamate receptor complexes (Colledge et al., 2000). Thus, phosphorylation mediated by kinase-specific interactions with cytoskeletal proteins could regulate NMDA receptor characteristics that are linked to the down-stream manifestation of drug withdrawal-induced symptoms.

To investigate the possible involvement of phosphorylation on cocaine-induced regulation of NMDA receptors, the effects of drug withdrawal on the expression and phosphorylation of the NR1 subunit were characterized. In addition, expression of PSD-95 was evaluated to determine if structural changes, such as alterations in receptor complex assembly might contribute to possible mechanisms associated with cocaine withdrawal-induced effects on the phosphorylation of NR1 splice variants.

RESULTS

Effects of cocaine withdrawal on NR1-C2 subunit expression

There were no differences in NR1-C2 expression between the saline- and cocaine-treated rats at 24 hours or 14 days of withdrawal in the frontal cortex (Fig. 2A). There were also no cocaine withdrawal-induced effects in NR1-C2 expression in the neostriatum at the 24-hour withdrawal time point. However, at 14 days of withdrawal from cocaine there was a significant (~27%) decrease in the expression of the NR1 C2-containing splice variants in the neostriatum, as compared to saline-treated controls.

In addition to cortical and neostriatal regions, the hippocampus is involved in mediating the behavioral and neurochemical effects of cocaine *via* altered glutamatergic functioning. However, immunoblotting techniques used to assess the expression of the NR1 subunit in hippocampal regions indicated that at 24 hours and 14 days after the last drug administration, there were no significant differences between rats treated with saline or cocaine (Fig. 2A).

Effects of cocaine withdrawal on NR1-C1 subunit expression

Cocaine withdrawal-induced changes in the expression of NR1 splice variants not recognized by the NR1-C2 antibody (specifically, NR1-3a and NR1-3b) as well as NR1-1a and NR1-1b (Fig. 1) were assessed using a NR1-C1 selective antibody. Immunoblotting experiments were performed on tissue from rats treated for 7 days with either cocaine or saline and withdrawn for 24 hours or 14 days. Comparison of the saline- and cocaine-treated animals yielded no significant differences in the frontal cortex, neostriatum or hippocampal regions at 24 hours or 14 days after the last drug administration (Fig. 2B). Thus, cocaine administration and subsequent withdrawal does not appear to alter the expression of NR1 C1-containing subunits in the identified brain areas.

Serine phosphorylation of NR1 during cocaine withdrawal

To more comprehensively characterize cocaine-withdrawal effects on receptor subunit phosphorylation and to investigate possible mechanisms for cocaine withdrawal-induced changes in the expression of the NR1 subunit, anti-NR1-C1 phosphoserine antibodies were used to evaluate potential drug-induced changes in the phosphorylation state of the receptor subunit. Rats treated with cocaine and withdrawn for 24 hours had (~34%) decreased serine 896/897 phosphorylation of the NR1 C1-containing splice variants in the frontal cortex, as compared to saline-treated controls (Figs. 3A, 5). This was a transient effect, however, because two weeks following the last drug administration no significant changes in serine 896/897 phosphorylation

of the NR1 C1-containing splice variants were detected (Fig. 3A). However, in addition to being withdrawal-time dependent, this drug-induced effect was regionally specific, as NR1 serine 896/897 phosphorylation was not altered in the neostriatum following 24 hours or 14 days of withdrawal from cocaine, as compared to cortex and saline-treated controls. Similarly, immunoblot analyses indicated that dual phosphorylation of serine residues 896 and 897 on the NR1 subunit was not significantly different in the hippocampus following 24 hours or 14 days of withdrawal from cocaine, as compared to the hippocampi of saline-treated animals (Fig. 3A).

For a more detailed characterization of the cocaine withdrawal-induced alterations in NR1 phosphorylation, antibodies specific for phospho-NR1 serine 896 and for phospho-NR1 serine 897 were used. Precise kinase involvement was targeted by using these probes to assess potential changes in the presence or absence of serine 896 or serine 897 phosphorylation. Immunoblotting experiments were performed on cortical tissue from rats treated for 7 days with either cocaine or saline and withdrawn for 24 hours. Comparison of the saline- and cocaine-treated animals yielded no significant difference between the two groups (Table 1). There were however, modest trends toward cocaine withdrawal-induced decreases in the phosphorylation of both serine 896 (12.7% decrease as compared to saline controls) and serine 897 (18.8% decrease as compared to saline controls), suggesting alterations in both PKC and PKA activity at these NR1-C1 residues.

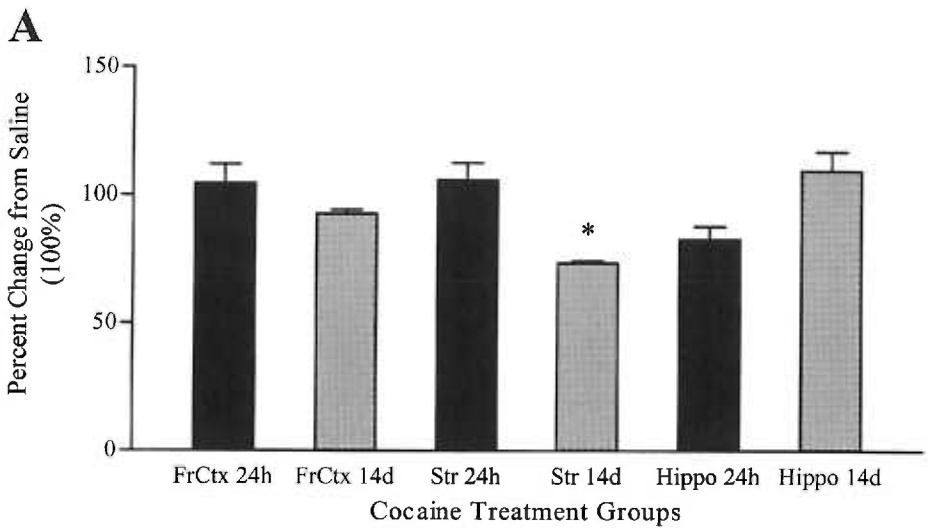
Cocaine withdrawal-induced changes in the relative proportion of NR1 subunit phosphorylation

Figure 3B illustrates the relative proportions of phospho-NR1 serine 896/897 and NR1-C1 expression during withdrawal from cocaine, and across different brain regions. Bonferroni-corrected *t*-tests indicate that PKC- and PKA-mediated phosphorylation of NR1-C1 was reduced relative to the expression of the NR1 C1-containing subunits at 24 hours and 14 days of

Table 1. Relative amounts of NR1 phosphorylation as measured by immunoblotting of frontal cortex tissue from rats treated with cocaine or saline and withdrawn for 24 hours. Unpaired Student's *t*-tests yielded no significant differences between the two treatment groups (SAL24h and COC24h) for either antibody. Percentages represent (non-significant) change from appropriate saline treatment groups.

Treatment group	Antibody	Mean relative optical density ± SEM	Percent (%)
SAL24h	Anti-phospho-NR1 Ser896	4253 ±433.2	100
COC24h		3714 ±296.5	87.3
SAL24h	Anti-phospho-NR1 Ser897	2793 ±233.2	100
COC24h		2266 ±294.4	81.2

Figure 2: Changes in NR1-C2 and NR1-C1 expression during withdrawal from repeated cocaine administration. A. *NR1-C2.* In frontal cortex, no cocaine withdrawal-induced differences in NR1-C2 expression were found in cocaine-treated rats at 24 hours or 14 days of withdrawal, as compared to saline-treated controls. Similarly, there were no cocaine withdrawal-induced differences in NR1 expression between the saline- and cocaine-treated rats at 24 hours withdrawal in the neostriatum. However, at 14 days of withdrawal from cocaine, there was a significant decrease in the expression of the NR1 C2-containing splice variants in the neostriatum ($^*p < 0.05$). In hippocampus at 24 hours and 14 days after the last drug administration, no significant differences were found between rats treated with saline and cocaine.
B. *NR1-C1.* In frontal cortex, no cocaine withdrawal-induced differences in NR1-C1 expression between the saline- and cocaine-treated rats at 24 hours or 14 days of withdrawal were found. There were also no significant cocaine withdrawal-induced differences in NR1-C1 expression between the saline- and cocaine-treated rats after 24 hours or 14 days of withdrawal in the neostriatum. Similarly, in hippocampus at 24 hours and 14 days after the last drug administration, no significant differences were found between rats treated with saline or cocaine. FrCtx, frontal cortex; Hippo, hippocampus; Str, neostriatum.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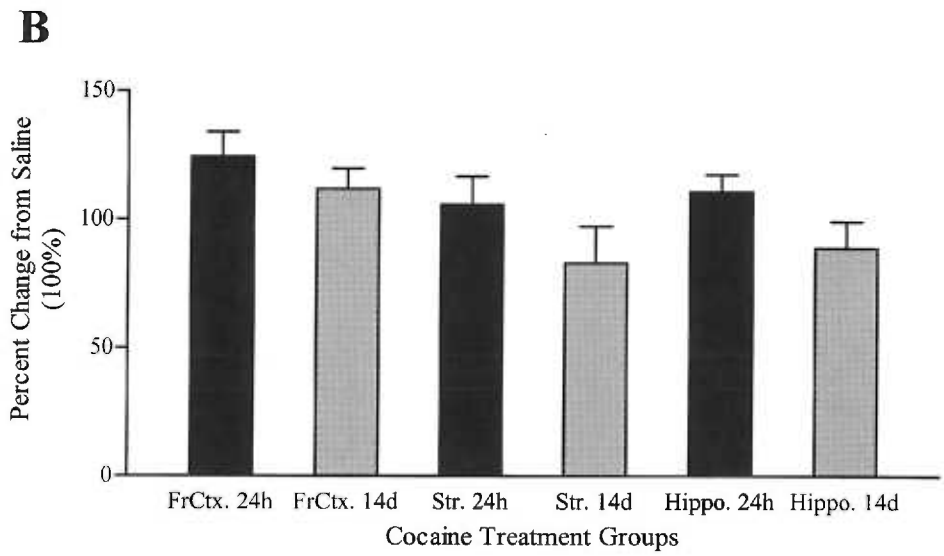
Cocaine Treatment Groups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Cocaine Treatment Groups

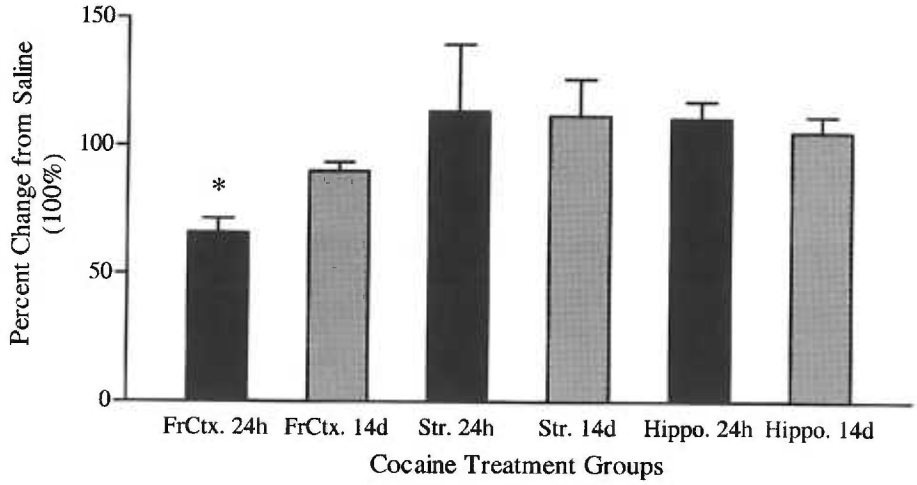
FIGURE 3: Cocaine withdrawal-induced alterations in the phosphorylation of NR1

A. *Phospho-NR1 serine 896/897.* Rats treated with cocaine and exposed to 24 hours of withdrawal showed decreased phosphorylation of the NR1 subunit in frontal cortex, as compared to saline-treated controls (*$p < 0.05$). Two weeks following the last drug administration, no significant changes in phosphorylation of the NR1 C1-containing splice variants were detected. In neostriatum and hippocampus, NR1 serine phosphorylation was not altered following 24 hours or 14 days of withdrawal for animals treated with cocaine as compared to saline-treated controls.

B. *Ratio of phospho-NR1 serine (896/897)/NR1-C1.* Bonferroni-corrected, two-tailed *t*-tests indicated that the ratio of phospho-NR1 serine (896/897)/NR1-C1 expression was significantly less than unity in frontal cortex regions of COC24h and COC14d rats (*$p < 0.008$). FrCtx, frontal cortex; Hippo, hippocampus; Str, neostriatum.

C. *Ratio of phospho-NR1 serine 896/NR1-C1 and phospho-NR1 serine 897/NR1-C1.* Sequential analysis was used to assess specific kinase changes associated with the cocaine withdrawal-induced decrease in phospho-NR1 serine 896/897 (Fig. 3A). Bonferroni-corrected, two-tailed *t*-tests revealed that the ratios of phospho-NR1 serine 896/NR1-C1 and phospho-NR1 serine 897/NR1-C1 were significantly less than unity in the frontal cortex at 24 hours of withdrawal (*$p < 0.025$).

A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Cocaine Treatment Groups

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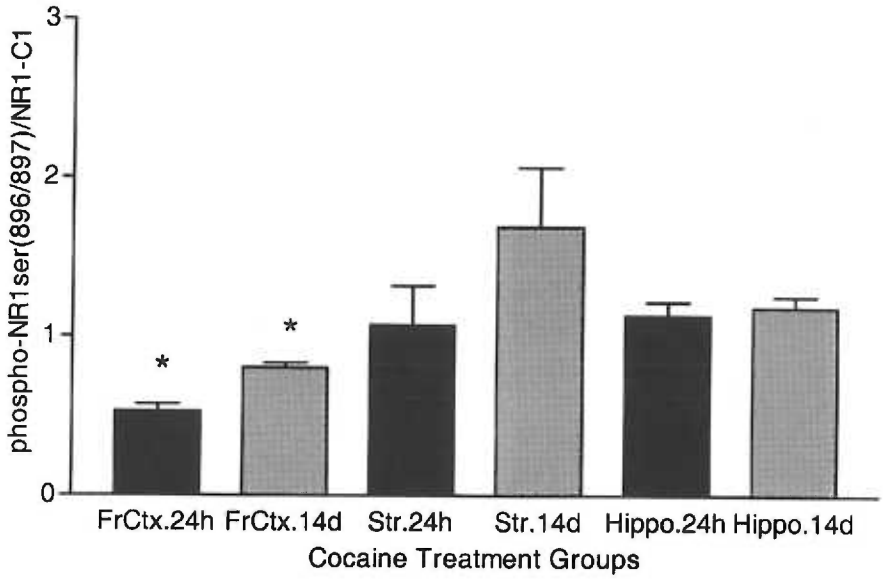
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Cocaine Treatment Groups

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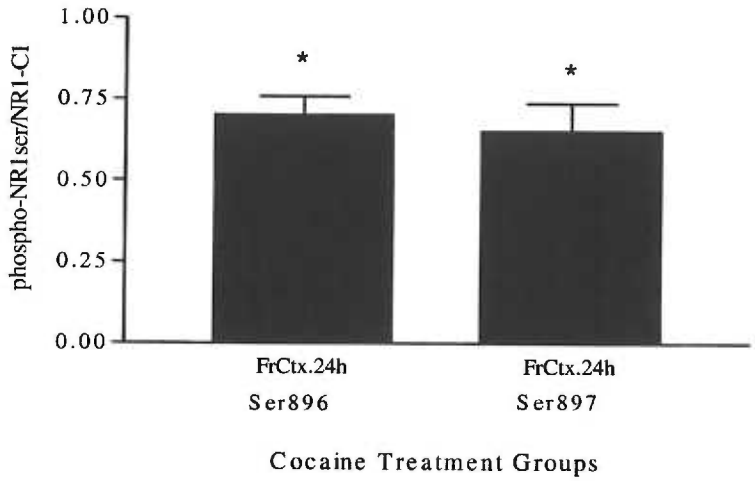
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FrCtx.24h
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Cocaine Treatment Groups

withdrawal in the frontal cortex of cocaine-treated animals. In addition, the ratios of phospho-NR1 serine 896/NR1-C1 and phospho-NR1 serine 897/NR1-C1 were calculated and found to be significantly less than one (Fig. 3C). Thus, the down-regulation of phospho-NR1 serine 896/897 expression (Fig. 3A) might coincide with decreased kinase activity, and altered serine phosphorylation could be important for modulating NMDA receptor drug-withdrawal effects.

PSD-95 expression during withdrawal from cocaine

NMDA receptor potentiation is mediated by PKA and PKC (Raman et al., 1996; Leonard and Hell, 1997). However, PKC-induced potentiation of NMDA receptor activity does not occur by direct phosphorylation of NR1 but rather may occur *via* associated postsynaptic density proteins that are putatively involved in functions such as targeting, anchoring or signaling (Lin et al., 1998; Zheng et al., 1999). Thus, PSD-95 expression was investigated for it's possible role in controlling cocaine withdrawal-induced kinase modulation of the NMDA receptor. No significant changes in PSD-95 expression were detected in the frontal cortex, neostriatum or hippocampus at 24 hours or 14 days after withdrawal from cocaine, as compared with saline-treated controls (data not shown; see Chapter 3).

DISCUSSION

The experiments described above systematically assessed the cocaine withdrawal-induced changes in the expression and serine phosphorylation of NR1 splice variants in the frontal cortex, neostriatum, and hippocampus. After two weeks of withdrawal from cocaine, NR1 C2-containing splice variant expression in the neostriatum was significantly down-regulated ~27%, as compared to saline-treated control rats. No other cocaine-induced changes in NR1-C2 subunit expression were detected, suggesting that the decrease observed was withdrawal time- and region-specific. In addition, immunolabeling with the anti-NR1-C1 antibody yielded no

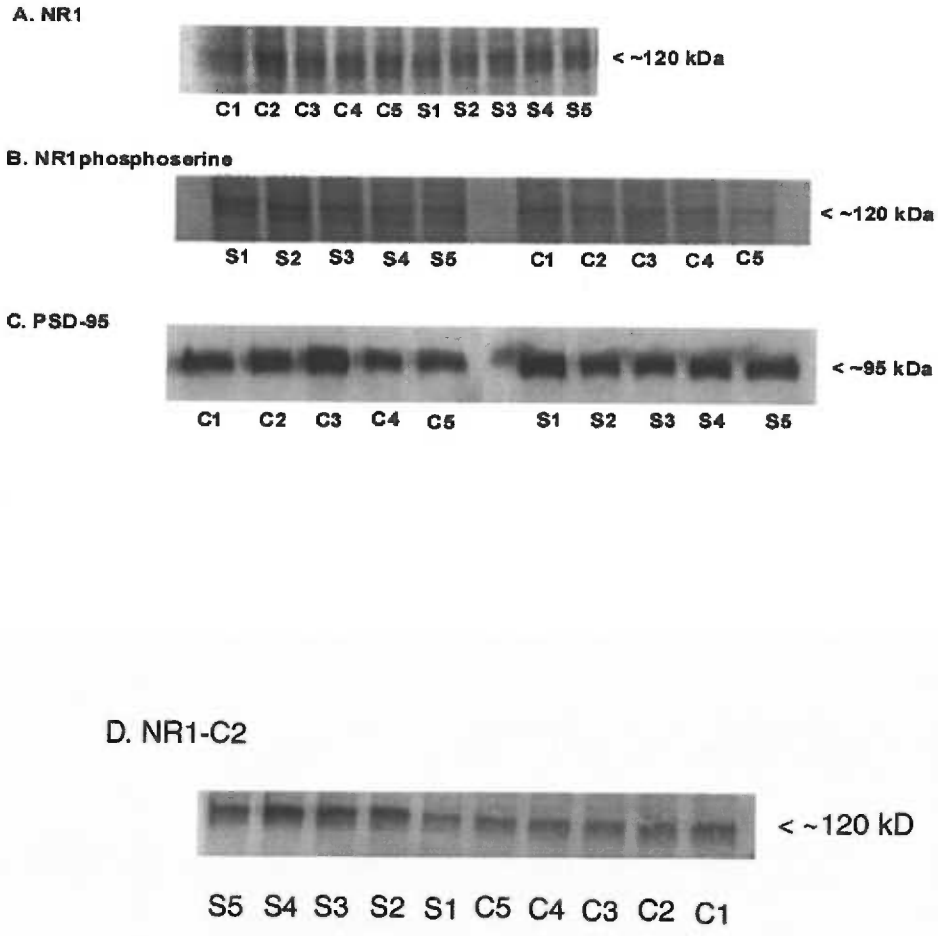
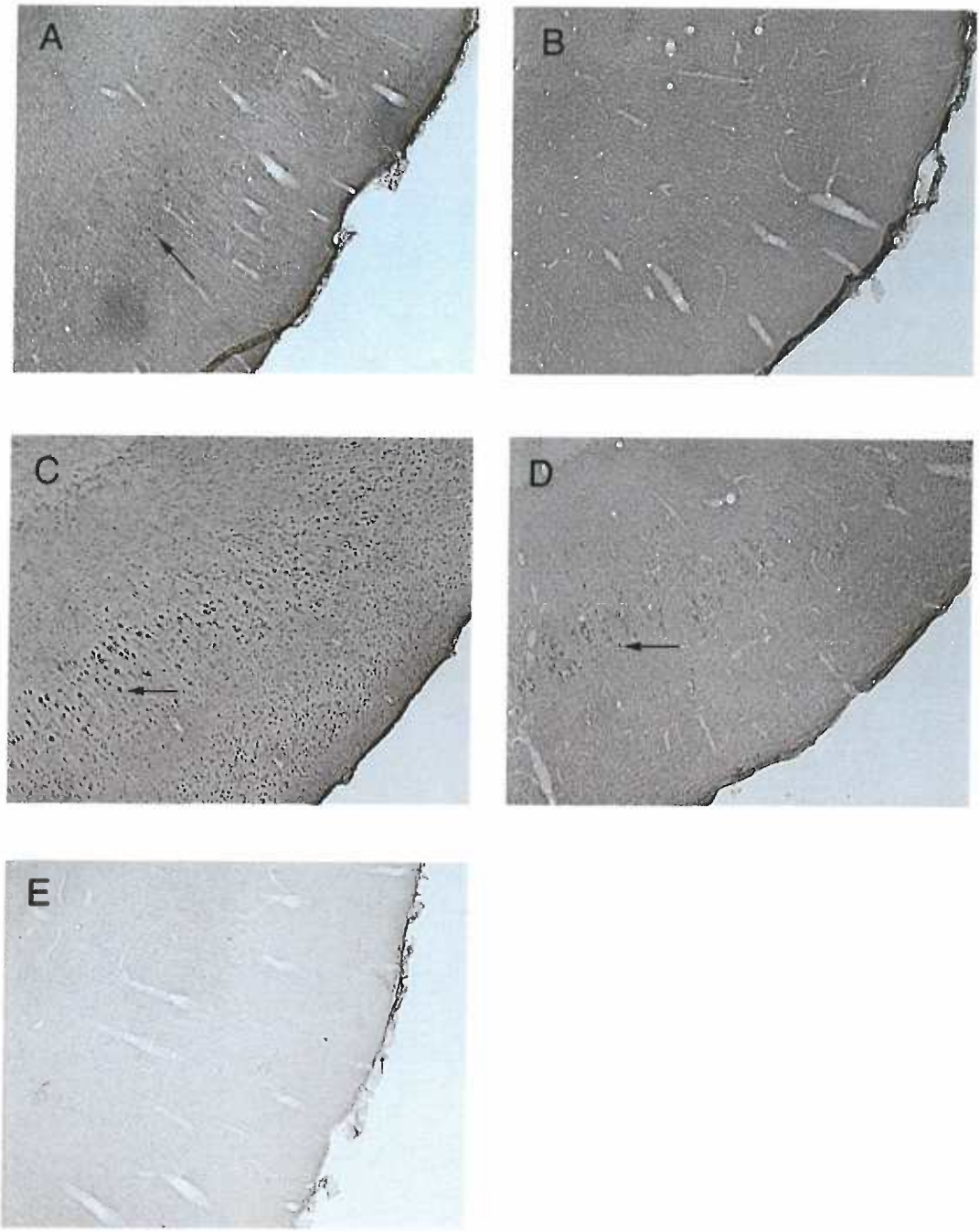


FIGURE 4: Representative immunoblots. A. Expression of the NR1 C2-containing splice variants for saline- and cocaine-treated rats in frontal cortex at 24 hours of withdrawal. B. Expression of NR1 dually phosphorylated on serine 896 and 897 for saline (S)- and cocaine (C)-treated animals in frontal cortex, 24 hours of withdrawal. C. Detection of PSD-95 in frontal cortex for rats treated with saline and cocaine at withdrawn for 24 hours D. NR1-C2 expression in the neostriatum at 14 days of withdrawal. C1-C5, COC-treated rats; S1-S5, SAL-treated rats.

Figures 5: Photomicrographs illustrating cocaine withdrawal-induced decreases in immunostaining for phospho-NR1 Ser896 and -NR1 Ser897. Panels illustrate phospho-NR1 Ser896 immunolabeling in regions of the frontal cortex [located approximately 1.2 mm anterior to bregma and identified as M1, primary motor cortex (Paxinos and Watson, 1998)] from rats treated with saline (A) or cocaine (B) and exposed to 24 hours of withdrawal. Immunopositive cell counts were obtained for 400x500 pixel regions of the cortex and are as follows: saline-treated rats 405.9 +/- 115.2 and cocaine-treated rats 250.4 +/-67.68, n = 5/treatment group. Phospho-NR1 Ser897 immunolabeling is shown in regions of the frontal cortex [located approximately 1.2 mm anterior to bregma and identified as M1, primary motor cortex (Paxinos and Watson, 1998)] for rats treated with saline (C) or cocaine (D) and exposed to 24 hours of withdrawal. Immunopositive cell counts were similarly acquired and are as follows: saline-treated rats 309.8 +/-45.77 and cocaine-treated rats 235.9 +/-25.06, n = 5/treatment group. Panel (E) shows a control section incubated without primary antibody. Arrows indicate immunopositive cell bodies.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significant differences between cocaine- or saline-treated animals across brain regions or withdrawal time points. Taken together, these results suggest splice variant-selective changes that reveal, by inference, the expression of NR1-2a and NR1-2b to be decreased in the neostriatum two weeks after cocaine administration.

Although previous studies have reported increases in NMDA receptor subunit expression following cocaine treatment (Fitzgerald et al., 1996; Loftis and Janowsky, 2000), the present findings highlight the potential diversity of the NR1 subunit created by the eight functional splice variants. Recombinant NMDA receptor channels containing different splice variants exhibit distinct functional properties and pharmacological profiles (Hollmann et al., 1993; for review see Sucher et al., 1996). This paper is the first to report cocaine withdrawal-induced changes in the expression of the NR1 receptor subunit that were revealed using antibodies that differentiated among several of the NR1 splice variants. Further, not all reported drug-related changes in NMDA receptor expression are associated with up-regulation. For example, acute administration of cocaine reduced the level of mRNA encoding NMDAR1 in the striatum (Ghasemzadeh et al., 1999) and repeated cocaine treatment resulted in a small (11%) reduction of NR1 expression in the nucleus accumbens one day after cocaine exposure (Churchill et al., 1999).

At 24 hours but not 14 days of withdrawal, phosphorylation of serine residues, 896 and 897 was reduced ~34% in the frontal cortex of rats treated with cocaine, as compared to controls (Fig. 5). Other brain regions investigated, including the hippocampus and neostriatum, failed to show drug-associated changes in serine phosphorylation. The use of newly available antibodies that individually labeled phosphorylated NR1 serine 896 and serine 897 detected no significant cocaine withdrawal-induced changes. However, small reductions (12.7% and 18.8%, respectively) in serine phosphorylation were found in the frontal cortex at 24 hours of withdrawal, implying potential cocaine-induced alterations in kinase activity at these residues (Table 1). Consequently, when combined with the data on NR1-C1, these results suggest that

only the phosphorylation and not the expression of NR1 C1-containing splice variants is altered at the early withdrawal time point (Fig. 3C).

NMDA receptor function is modulated by both tyrosine and serine protein kinases. PDZ domain-containing proteins play a role in the efficient trafficking and anchoring of NMDA receptor subunits and signaling molecules to discrete intracellular sites. PSD-95 regulates the activity of the NMDA receptor complex by inhibiting the PKC-mediated potentiation of the channels (Yamada et al., 1999). Consequently, PSD-95 expression was investigated for it's possible role in controlling cocaine withdrawal-induced kinase modulation of the NMDA receptor. No significant changes in PSD-95 expression were detected in the frontal cortex, neostriatum or hippocampus at 24 hours or 14 days after withdrawal from cocaine as compared with saline-treated controls (data not shown; see Chapter 3). In addition to PSD-95, there are a number of other postsynaptic density anchoring proteins to consider for further investigation of drug-related receptor regulation. For example, yotiao, an NMDA receptor-associated protein that binds type I protein phosphatase (PP1) and PKA (Westphal et al., 1999), participates in the regulation of NMDA receptors and may thus play a role in the molecular mechanisms accompanying cocaine exposure and withdrawal.

There are at least two possible explanations for the reduction in the serine 896/897 phosphorylation of the NR1 subunit at 24 hours of withdrawal in the frontal cortex (Figs. 3A, 5). The decrease in NR1 phosphorylation could have resulted from a cocaine withdrawal-induced down-regulation of the NR1 subunit. However, data illustrated in figures 2A and 2B indicate that there were no significant differences in NR1-C2 or -C1 subunit expression in the frontal cortex between cocaine- and saline-treated rats. These results suggest that 24 hours of withdrawal from cocaine caused a transient decrease of either or both PKC- and PKA-mediated phosphorylation of neighboring NR1 serine residues in rat frontal cortex. However, in addition to being withdrawal-time dependent, this drug-induced effect was regionally specific, as NR1 serine 896/897 phosphorylation was not altered in the neostriatum following 24 hours or 14 days of withdrawal

from cocaine, as compared to cocaine- and saline-treated controls. Similarly, densitometric analyses of immunolabeling indicated that phosphorylation of serine residues 896 and 897 on the NR1 subunit was not significantly different in the hippocampus following 24 hours or 14 days of withdrawal from cocaine, as compared to saline-treated animals (Fig. 3A). Thus, posttranslational factors such as phosphorylation and protein-protein interactions may be important in regulating NMDA receptor drug-withdrawal sensitivity.

Figure 3B illustrates the relative proportion of phospho-NR1 serine (896/897)/NR1-C1 expression during withdrawal from cocaine and across different brain regions. PKC- and PKA-mediated phosphorylation of NR1 was reduced relative to the expression of the NR1 C1-containing splice variants at 24 hours and 14 days of withdrawal in the frontal cortex of cocaine-treated animals. Changes in PKC and PKA activity at NR1 C1-containing splice variants were specifically evaluated by calculating the ratios of phospho-NR1 serine 896/NR1-C1 and phospho-NR1 serine 897/NR1-C1, respectively (Fig. 3C). The phospho-NR1 serine 896 and 897 antibodies were used to follow-up on the significant finding obtained with the phospho-NR1 serine 896/897 antibody (Fig. 3A). Results suggest that in the frontal cortex, protein kinase activity was attenuated at 24 hours after the last drug treatment.

Taken together, the results presented in this chapter indicate that 24 hours after repeated drug exposure, the phosphorylation of serine residues 896 and 897 on the NR1 subunit was decreased in the frontal cortex of cocaine-treated rats. Using 3xCRE-*luciferase* constructs in primary striatal cultures, Leveque et al. (2000) demonstrated that mutation of serine 896 and 897 to alanine residues, which prevents phosphorylation at both sites, inhibits forskolin-mediated gene expression. A cocaine withdrawal-induced transient reduction in phosphorylation of the NR1 subunit could therefore alter the expression of down-stream effector proteins and contribute to drug-related changes in neurophysiological functioning and subsequent behavioral features. Thus, cocaine-withdrawal induced alterations in the phosphorylation and expression of the NMDA receptor could be important for the short-term neurochemical changes associated with

cocaine exposure as well as for the more enduring responses accompanying psychostimulant abuse or treatment.

Chapter 3: Regulation of postsynaptic density proteins during cocaine withdrawal

INTRODUCTION

Cocaine is one of the most reinforcing drugs of abuse known in both humans (Franken et al., 2000) and animals (Sutton et al., 2000). Repeated cocaine use can induce a profound state of addiction in humans characterized by compulsive drug taking and an inability to stop using despite significant deleterious physical, social and economic consequences. In recent years progress toward understanding the neural substrates of addiction to cocaine has been substantial. Neurotransmitter systems including dopaminergic (Fang and Ronnekleiv, 1999; Garris et al., 1999), cholinerigic (Zocchi & Pert, 1994; Mark et al., 1999) and glutamatergic (White et al., 1995; Wolf, 1998) systems have been (and are currently being) investigated for their role in contributing to cocaine abuse. However, there has been limited research focused on studying the effects of psychostimulant treatment on postsynaptic density (PSD) proteins. NMDA receptor subunits are trafficked, assembled and localized to discrete membrane locations *via* specific PSD proteins (Sheng and Pak, 2000). Further, PSD proteins are critically involved in maintaining the receptor complex at synapses and facilitating efficient signaling by keeping enzymes, such as nNOS in close proximity to ion channels (Craven and Bredt, 1998). Thus, understanding the relationship among these neuronal elements may help elucidate possible mechanisms related to cocaine-induced changes in neurochemical functioning.

The PSD is an electron-dense, submembraneous region that contains an elaborate network of cytoskeletal proteins and associated enzymes (Kennedy, 1997). Within the PSD are several members of the membrane-associated guanylate kinase (MAGUK) family of proteins [also described as synapse-associated proteins (SAP)]. Two such members are PSD-95 and the less well-characterized protein, SAP102. PSD-95 was initially detected in PSD fractions prepared from rat brain (Cho et al., 1992) and subsequently in presynaptic nerve terminals of

symmetric type 2 inhibitory synapses (Kistner et al., 1993) and in PSDs of asymmetric type 1 excitatory synapses of rat forebrain (Hunt et al., 1996). PSD-95 is expressed throughout the brain and appears especially enriched in forebrain structures. Subcellular localization studies indicate that PSD-95 is most dense in the neuropil and dendrites (Cho et al., 1992). SAP102 was initially detected in dendritic shafts and spines of excitatory synapses (Muller et al., 1996). SAP102 is found most prominently in rat cerebral cortex, hippocampus, cerebellum and olfactory bulb. It is enriched in preparations of synaptic junctions, where it functions as a component of the cytoskeleton. SAP102 is apparent along dendrites and its patchy distribution is very similar to that of NMDA receptors (Muller et al., 1996).

MAGUKs can form homomeric and heteromeric oligomers by means of their PDZ domains (Sheng and Sala, 2001). PDZ regions (80-90 amino acid motif) are novel protein-protein binding motifs that interact with cytoplasmic tail sequences (tSXV motifs) of transmembrane proteins. PSD-95 interacts with the NR2B subunit *via* its C-terminal sequence - ESDV. This short motif mediates binding to the first two PDZ domains of PSD-95 (Sheng and Pak, 2000). PDZ domains of PSD-95 also interact with a similar motif within the N-terminus of nNOS (Brenman et al., 1996). In mutant mice, nNOS lacking a PDZ motif does not associate with PSD-95 in brain (Brenman et al., 1996). PSD-95 and SAP102 can also interact in the presence of both Ca^{2+} and calmodulin (Masuko et al., 1999). All three PDZ domains of SAP102 bind NR2B *in vitro* and recombinant proteins containing the C-terminal of the NR2B subunit associate with SAP102 in rat brain homogenates (Muller et al., 1996). Thus, SAP102 is also hypothesized to be involved in linking NMDA receptor subunits to the cytoskeleton at excitatory synapses.

Using immunoblotting and confocal microscopy, the following study sought to provide information about the effects of cocaine treatment and withdrawal on the co-expression and localization of the NMDA receptor subunit, NR2B, as well as its associated PSD proteins, PSD-95, SAP102 and nNOS. The immediate early gene product, FosB/ΔFosB was also assessed.

Cocaine-induced changes in the expression of FosB/ΔFosB were used as a positive control for the confocal microscopy experiments. In addition, drug-related increases in FosB/ΔFosB expression were considered as a possible mechanism for cocaine withdrawal-induced changes in NMDA receptor subunit expression.

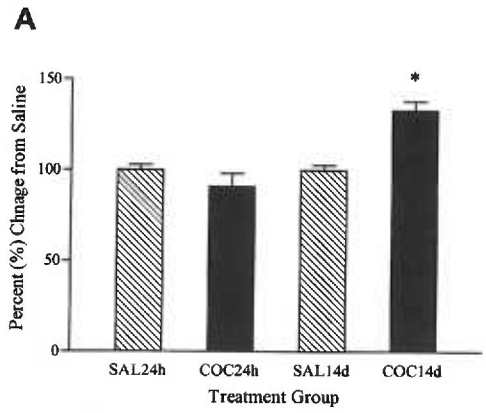
RESULTS

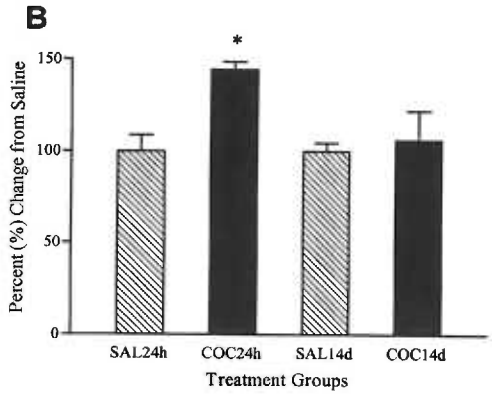
Cocaine withdrawal-induced changes in NR2B and SAP102 as measured by immunoblotting experiments

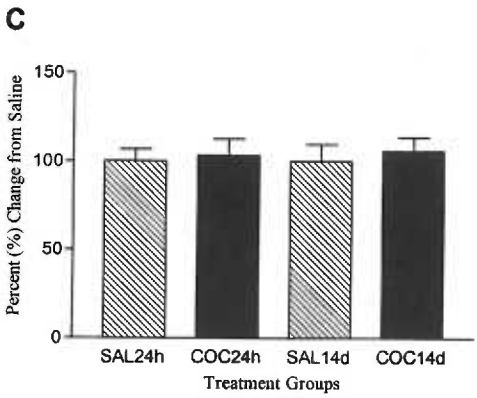
NMDA receptor subunit, NR2B, expression was investigated in the frontal cortex and neostriatum of rats at 24 hours and 14 days after cocaine administration. At 24 hours of withdrawal, no change in NR2B protein expression was detected in the frontal cortex. However, 24 hours following the last drug treatment, NR2B expression was up-regulated 44.6% in the neostriatum of rats treated repeatedly with cocaine, as compared with saline-treated control animals. Similarly, two weeks of withdrawal from cocaine resulted in a 33.1% cocaine withdrawal-induced increase in NR2B expression in the frontal cortex. However, after 2 weeks of withdrawal NR2B expression in the neostriatum had returned to control levels (Figs. 1A, 1B, 3).

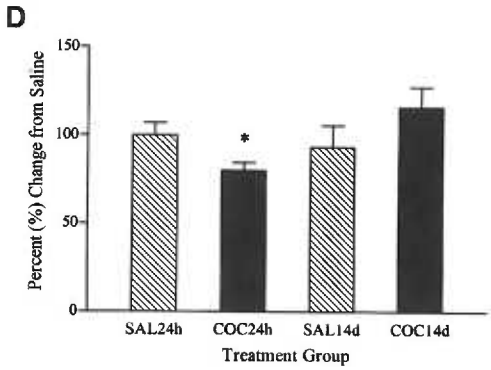
SAP102 was also investigated for potential cocaine withdrawal-induced changes in protein expression. At 24 hours and 14 days of withdrawal, no significant change in SAP102 expression was observed in the frontal cortex for rats that were administered cocaine or saline. However, 24 hours of withdrawal from cocaine yielded an approximate 20% decrease in SAP102 expression in the neostriatum, as compared with saline-treated animals. At two weeks after the last drug treatment, SAP102 expression in the neostriatum had returned to baseline levels (Figs. 1C, 1D).

Figure 1: NR2B and SAP102 expression levels are differentially altered during withdrawal from cocaine. **A**. NR2B. In frontal cortex, no cocaine withdrawal-induced differences in NR2B expression were found in cocaine-treated rats at 24 hours of withdrawal, as compared with saline control animals (100%). At 14 days of withdrawal from cocaine there was a significant increase in the expression of the NR2B subunit in the frontal cortex (*, $p < 0.05$). **B**. NR2B. 24 hours after the last drug administration there was a significant up-regulation of NR2B in the neostriatum of cocaine-treated rats, as compared with saline-treated animals. In neostriatum, there were no significant cocaine withdrawal-induced differences in NR2B expression between saline- and cocaine-treated rats at 14 days of withdrawal. **C**. SAP102. In frontal cortex, 24 hours and 14 days after the last drug administration, no significant differences were found between treatment groups. **D**. SAP102. At 24 hours of withdrawal from cocaine, there was a significant decrease in the expression of the SAP102 in the neostriatum (*$p < 0.05$). However, at two weeks following the last cocaine injection, differences in SAP102 expression were no longer apparent.


A
Percent (%) Chnage from Saline
150
100
50
0
*
SAL24h
COC24h
SAL14d
COC14d
Treatment Group


B
Percent (%) Change from Saline
150
100
50
0
*
SAL24h
COC24h
SAL14d
COC14d
Treatment Groups


C
Percent (%) Change from Saline
150
100
50
0
SAL24h
COC24h
SAL14d
COC14d
Treatment Groups


D
Percent (%) Change from Saline
150
100
50
0
*
SAL24h
COC24h
SAL14d
COC14d
Treatment Group

PSD-95 and nNOS expression levels appear stable during withdrawal from cocaine, as indicated by immunoblotting experiments

PSD-95 expression was assessed in the frontal cortex and neostriatum following repeated cocaine administration and withdrawal (24 hours and 14 days). Immunoblot analyses revealed no significant withdrawal-induced differences in protein expression for any of the brain regions investigated (Figs. 2A, 2B). Thus, in combination with data described in chapter 2, it appears that PSD-95 is a relatively stable protein since cocaine treatment and withdrawal did not affect its regional or cellular expression profile.

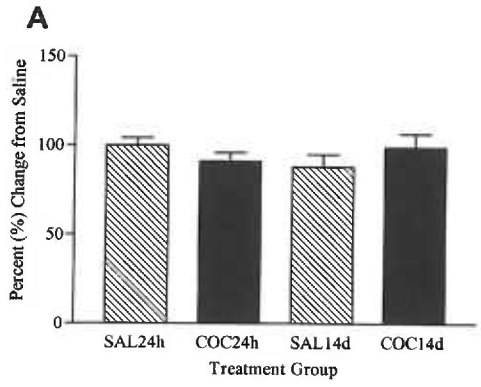
Immunoblot analyses indicated that nNOS, a protein linked to the NMDA receptor complex *via* PSD-95 and potentially involved in drug-induced changes in signal transduction, expression was not altered in the frontal cortex or neostriatum at 24 hours or 14 days after the last cocaine exposure (Figs. 2C, 2D).

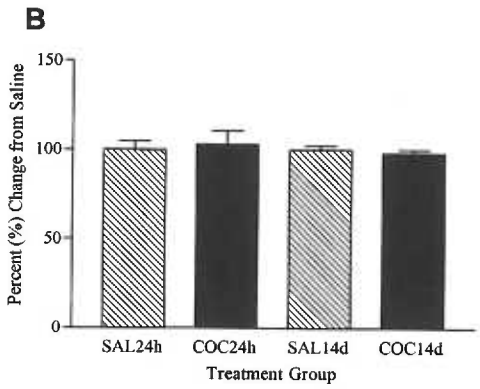
Cocaine withdrawal-induced changes in protein expression as measured by quantitative confocal microscopy

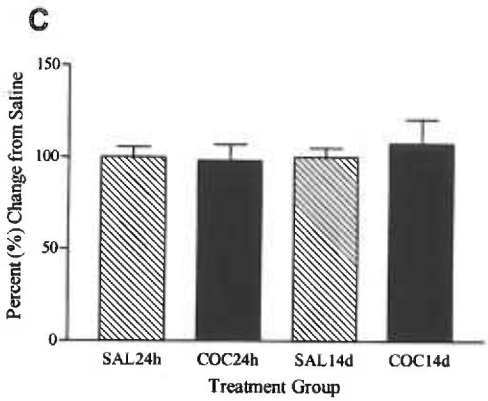
Brain sections from rats treated with cocaine or saline and withdrawn for 24 hours or 14 days were used to assess the effects of drug withdrawal on specific regional expression and co-expression protein levels. Dual labeling experiments investigated NR2B/nNOS, NR2B/SAP102 and NR2B/FosB/ΔFosB expression in regions of the frontal cortex and dorsolateral neostriatum.

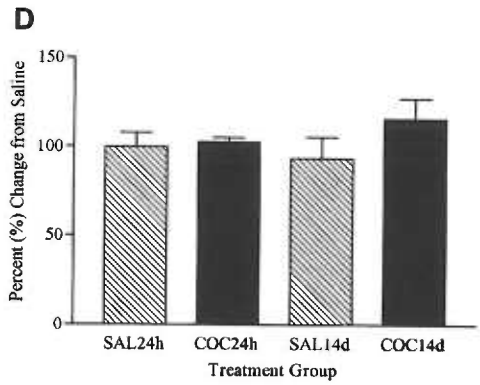
Frontal Cortex. At 24 hours of withdrawal, NR2B subunit expression in the frontal cortex was not statistically different for cocaine- and saline-treated animals (Fig. 4A). However, results confirmed that NR2B expression was significantly up-regulated in the frontal cortex after two weeks of withdrawal from cocaine administration (Figs. 4B-4D). As with the immunoblotting experiments, at 24 hours and 14 days of withdrawal, nNOS expression in the frontal cortex of rats treated with cocaine did not differ from saline-treated animals (Figs 5A, 5B).

Figure 2: PSD-95 and nNOS expression levels are not affected during withdrawal from cocaine. Results illustrate PSD-95 protein expression in the frontal cortex (A) and neostriatum (B) and nNOS protein expression in the frontal cortex (C) and neostriatum (D), as measured by immunoblotting (n = 5/treatment group).


A
Percent (%) Change from Saline
150
100
50
0
SAL24h
COC24h
SAL14d
COC14d
Treatment Group


B
Percent (%) Change from Saline
150
100
50
0
SAL24h
COC24h
SAL14d
COC14d
Treatment Group


C
Percent (%) Change from Saline
150
100
50
0
SAL24h
COC24h
SAL14d
COC14d
Treatment Group


D
Percent (%) Change from Saline
150
100
50
0
SAL24h
COC24h
SAL14d
COC14d
Treatment Group

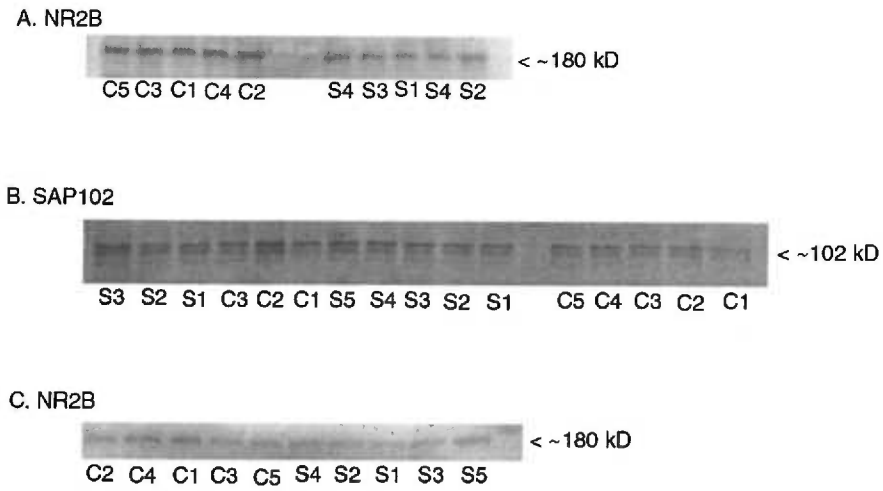
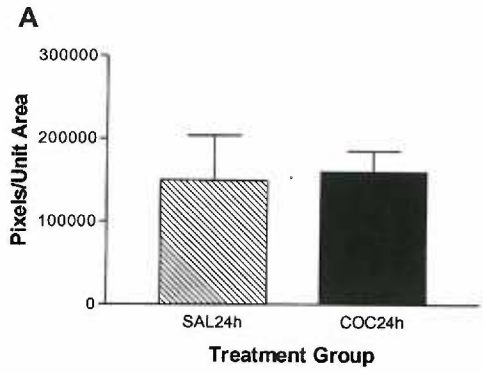
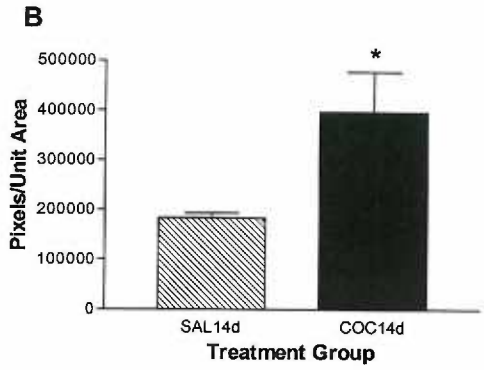
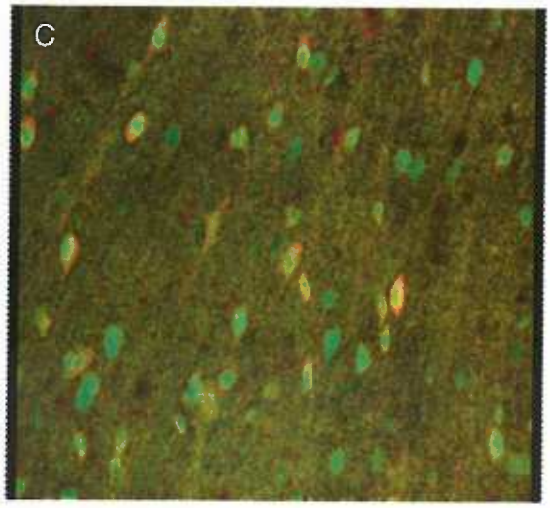


Figure 3: Representative immunoblots. **A**. Expression of NR2B in the frontal cortex at 14 days of withdrawal. **B.** Expression of SAP102 in the neostriatum at 14 days of withdrawal. **C.** Expression of NR2B in the neostriatum at 24 hours of withdrawal

Figure 4: Withdrawal time-dependent NR2B up-regulation in the frontal cortex as revealed by confocal microscopy. **A**. 24 hours of withdrawal from repeated cocaine administration did not alter the expression of NR2B in the frontal cortex. **B**. However, at 14 days after the last drug injection, NR2B was significantly up-regulated in the frontal cortex of rats treated with cocaine, as compared to saline-treated animals (*, $p < 0.05$). Photomicrographs illustrate NR2B immunofluorescence in the frontal cortex of a rat treated with cocaine and withdrawn for two weeks (C) as compared to a rat treated with saline (D). NR2B immunopositive cells and processes are shown in red and nNOS immunopositive cells are shown as green. Putative co-expression of NR2B with nNOS is illustrated in yellow. Immunopositive cell bodies are predominantly located in cortical layers III and V (approximate anatomical location: 1.0 mm anterior to bregma).


A
300000
200000
100000
0
Pixels/Unit Area
SAL24h
COC24h
Treatment Group


B
500000
400000
300000
200000
100000
0
Pixels/Unit Area
*
SAL14d
COC14d
Treatment Group


C

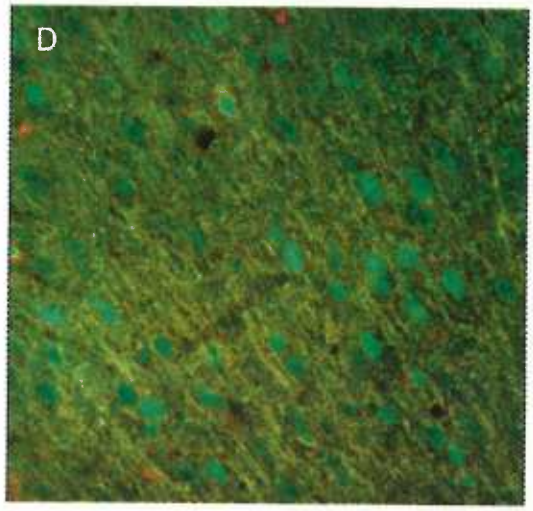
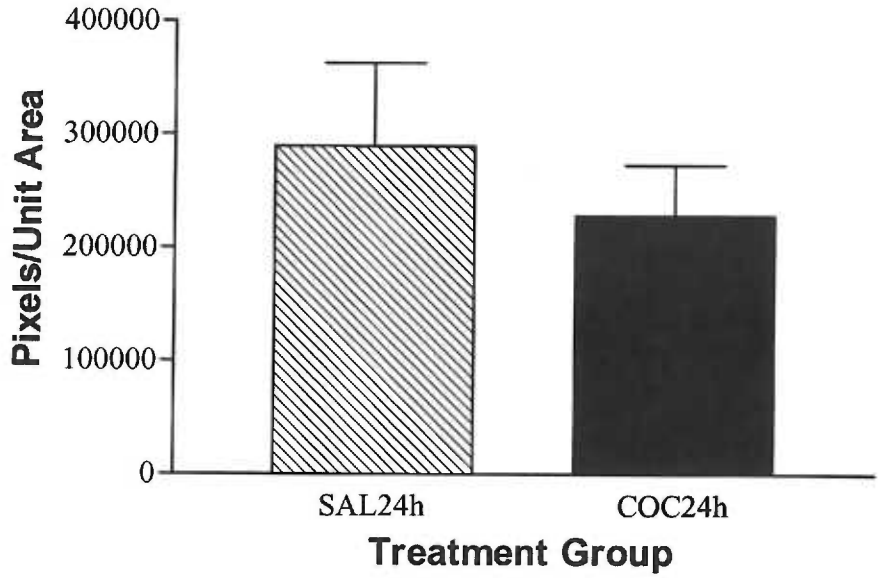
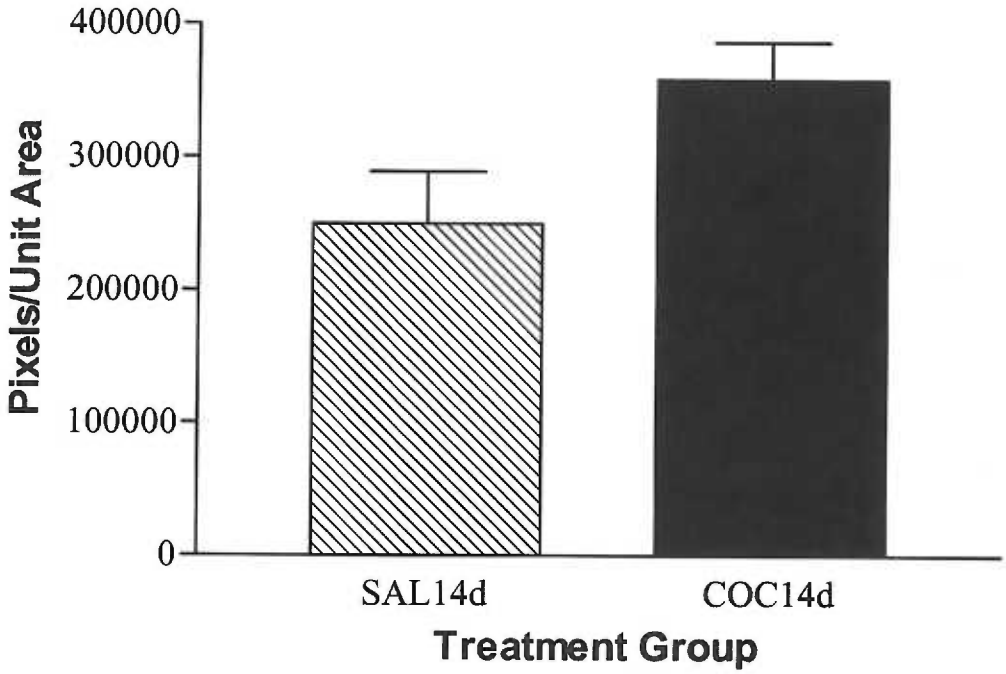

D

Figure 5: nNOS expression after 24 hours and 14 days of withdrawal from cocaine. Levels of nNOS protein abundance in the frontal cortex were not affected during withdrawal from repeated cocaine treatment, as measured by quantitative confocal microscopy. (n = 6/treatment group for the 24 hour withdrawal data shown in panel A; n = 5/treatment group for the 14 day withdrawal data illustrated in panel B)

A


400000
300000
200000
100000
0
Pixels/Unit Area
SAL24h
COC24h
Treatment Group

B

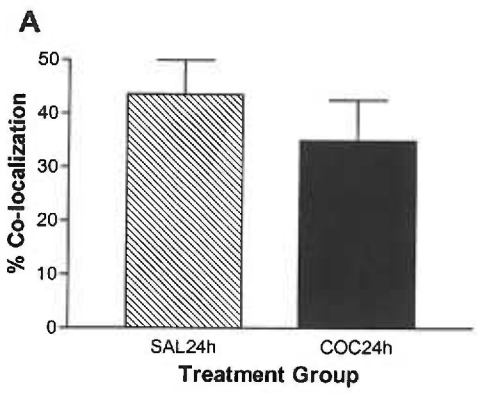

400000
300000
200000
100000
0
Pixels/Unit Area
SAL14d
COC14d
Treatment Group

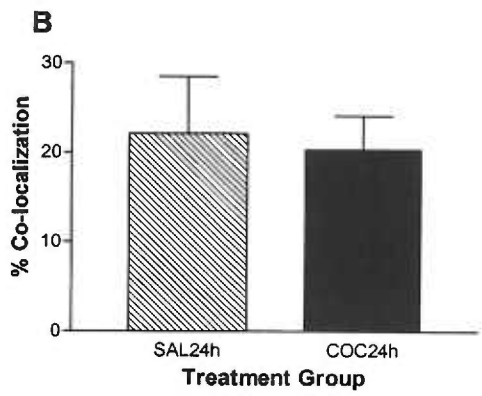
Co-localization analyses indicated that there were no changes in the co-expression of NR2B with nNOS or nNOS with NR2B in the frontal cortex of rats exposed to cocaine and subsequently subjected to 24 hours (Figs. 6A, 6B) or 14 days of withdrawal, as compared to control animals (Figs. 6C, 6D).

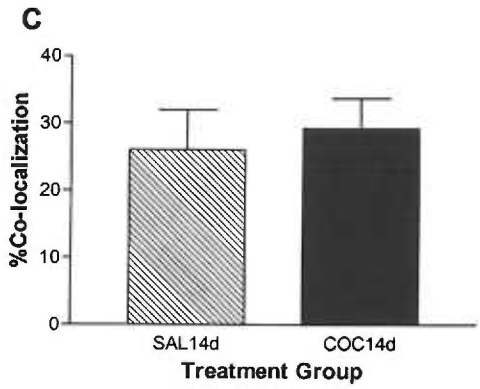
Neostriatum. To assess the sensitivity of the assay and to probe for potential mechanisms associated with cocaine withdrawal-induced alterations in protein expression, FosB/ΔFosB immunolabeling was performed. Previous data suggests that cocaine treatment induces the expression of FosB/ΔFosB (Nestler et al., 1999; Loftis and Janowsky, 2000). Using brain tissue from rats exposed to cocaine or saline and withdrawn for 72 hours, confocal microscopy revealed an upregulation of FosB/ΔFosB protein expression in cocaine-treated animals, as compared to controls (Fig. 7). NR2B, nNOS and SAP102 protein abundance was also evaluated in this brain region. There were no significant cocaine withdrawal-induced alterations in the expression of the NR2B subunit in the dorsolateral neostriatum at either 24 hours or 14 days following the last drug administration (Table 1). Similarly, nNOS expression appeared to remain stable across withdrawal time points. The total area of nNOS immunoreactivity for cocaine-treated rats was not remarkably different than that observed for saline-treated rats at either 24 hours or 14 days post-injection (Table 1). SAP102 immunofluorescence was evaluated in the dorsolateral region of the neostriatum for cocaine- and saline-treated rats at 24 hours after the last drug dose. Results revealed that there were no significant cocaine withdrawal-induced changes in SAP102 expression (Table 1).

Dual labeling experiments of FosB/ΔFosB with NR2B were performed to assess the co-localization of these proteins in the dorsolateral neostriatum. The goal was to provide evidence of a potential mechanism for cocaine withdrawal-induced changes in protein expression. FosB/ΔFosB immunofluorescence was prominent within the cell bodies of striatal neurons; however, NR2B labeling in this region of the brain appeared to be limited to neuronal processes.

Figure 6: Co-localization of NR2B with nNOS in the frontal cortex across different withdrawal time points. No significant differences in the co-expression of NR2B with nNOS were detected in the frontal cortex at 24 hours (A and B) or 14 days (C and D) of withdrawal. Panels A and C show (NR2B "AND" nNOS)/NR2B revealing the percentage of nNOS co-localized with NR2B immunopositive cells. (NR2B "AND" nNOS)/nNOS are shown in panels B and D illustrating the percentage of NR2B co-expressed with nNOS immunopositive cells.


A
% Co-localization
50
40
30
20
10
0
SAL24h
COC24h
Treatment Group


B
% Co-localization
30
20
10
0
SAL24h
COC24h
Treatment Group


C
%Co-localization
40
30
20
10
0
SAL14d
COC14d
Treatment Group

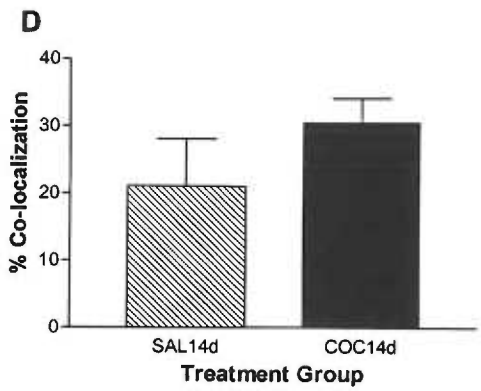
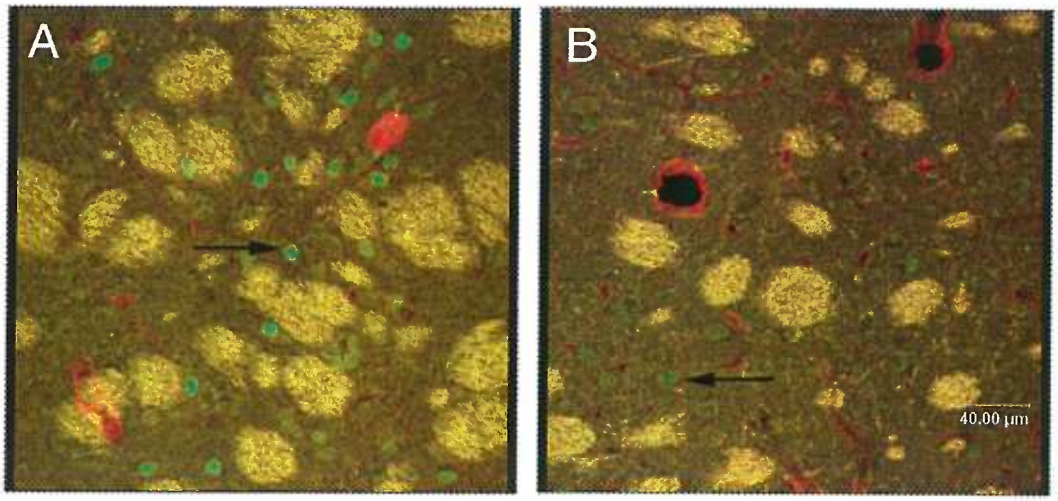

D
% Co-localization
40
30
20
10
0
SAL14d
COC14d
Treatment Group

Figure 7: Photomicrographs of rat brain tissue labeled for FosB/ΔFosB in the dorsolateral neostriatum. Panels illustrate FosB/ΔFosB immunofluorescence in regions of the dorsolateral neostriatum (located approximately 1.2 mm anterior to bregma) from rats treated with cocaine (A) and saline (B). Arrows indicate FosB/ΔFosB immunopositive cell bodies. **C.** Total area of FosB/ΔFosB immunofluorescence in areas of the dorsolateral neostriatum for saline- and cocaine-treated rats withdrawn for 72 hours (n = 5/treatment group).


A
B
40.00 µm

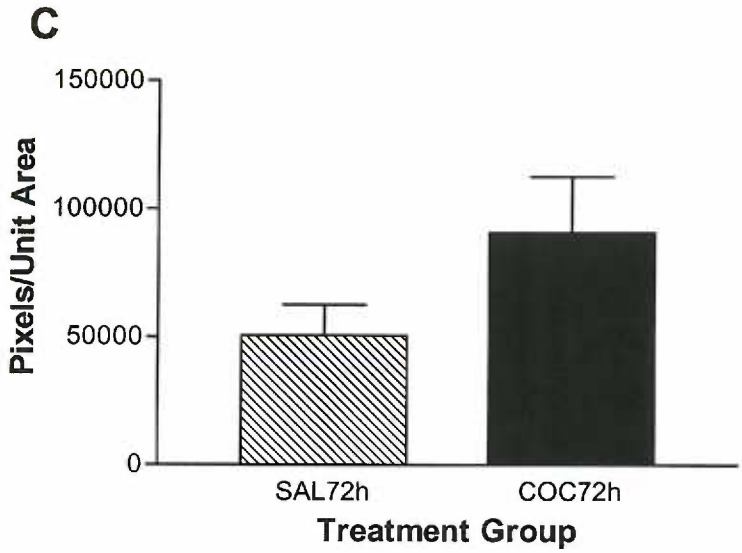

C
150000
100000
50000
0
Pixels/Unit Area
SAL72h
COC72h
Treatment Group

Table 1: Total area of immunofluorescent labeling for NR2B, nNOS and SAP102 in the dorsolateral neostriatum of rats treated with cocaine or saline. Values in the table are pixels per unit area and they represent total areas of immunofluorescence (± SEM) as revealved by computer-assisted analysis of confocal microscope images (n = 6/treatment group for the 24 hour withdrawal time point; n = 5/treatment group for the 14 day withdrawal time point). SAP102 expression levels were not determined (N.D.) as western blotting data indicated that there were no cocaine withdrawal-induced changes in SAP102 at 14 days after the last drug injection.

Treatment Group	Protein Expression		
	NR2B	nNOS	SAP102
SAL24h	453,400 ± 56,260	490,000 ± 62,660	244,000 ± 53,710
COC24h	443,600 ± 27,640	510,500 ± 41,190	296,500 ± 66,330
SAL14d	555,600 ±52,340	598,200 ± 50,970	N.D.
COC14d	637,200 ± 94,580	646,700 ± 81,130	N.D.

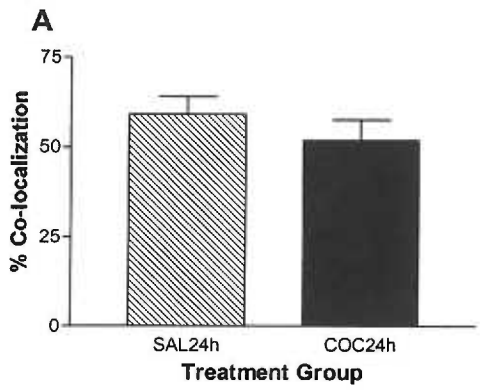
Consequently, using the AND function (Boolean arithmetic) to assess co-expression of these proteins yielded results to suggest that NR2B and FosB/ΔFosB were not co-localized in either the cocaine- or saline-treated rats (Figs. 7A, 7B). Further, observations from a visual assessment of immunolabeled brain sections did not show that there was co-expression of NR2B and FosB/ΔFosB within the same neuron. It thus remains unclear whether or not FosB/ΔFosB serves to regulate NMDAR2B gene expression under both baseline and drug-altered conditions.

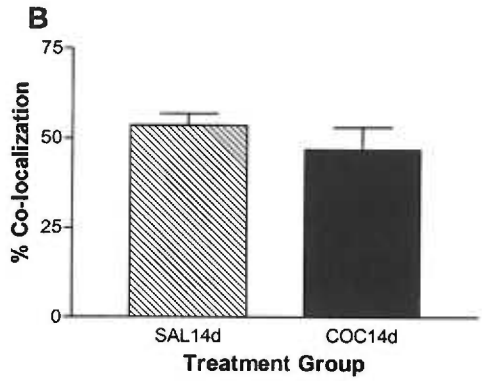
Co-localization comparisons indicated that psychostimulant treatment and withdrawal did not affect the co-expression of NR2B with nNOS or nNOS with NR2B (Fig. 8). Interestingly, there appeared to be scant co-expression of NR2B with SAP102 in either the cocaine- or saline-treated rats (Fig. 9A, 9B). To confirm that detection of the co-localization of these proteins was possible using the antibodies and fluorophores selected, cortical regions were visually assessed with the confocal microscope. Co-expression of SAP102 with NR2B is apparent and extensive in the frontal cortex (Fig. 9C).

DISCUSSION

The major findings from the studies described above are that cocaine withdrawal alters the expression of NR2B and SAP102 in a regional- and withdrawal time-dependent manner. Results from immunoblotting and confocal microscopy experiments indicated that NR2B protein expression was up-regulated in the frontal cortex of rats treated with cocaine and subjected to 14 days of withdrawal (Fig. 1A). In addition, western blotting data suggested that NR2B was up-regulated in the neostriatum at 24 hours of withdrawal (Fig. 1B). This latter finding was not confirmed by confocal microscopy, however; only the dorsolateral region of the neostriatum was assessed using this method. SAP102 expression was also altered in the neostriatum of rats treated with cocaine. Specifically, immunoblotting revealed an approximate 20% decrease in the level of SAP102 for animals withdrawn for 24 hours, as compared to saline-treated control rats (Fig. 1D).

Figure 8: Co-localization of NR2B with nNOS in the dorsolateral neostriatum across different withdrawal time points. No significant differences in the co-expression of NR2B with nNOS were detected in regions of the dorsolateral neostriatum at 24 hours (A and B) or 14 days (C and D) of withdrawal. Panels A and C show (NR2B "AND" nNOS)/NR2B revealing the percentage of nNOS co-localized with NR2B immunopositive cells. (NR2B "AND" nNOS)/nNOS are shown in panels B and D illustrating the percentage of NR2B co-expressed with nNOS immunopositive cells in the neostriatum.


A
75
50
25
0
% Co-localization
SAL24h
COC24h
Treatment Group


B
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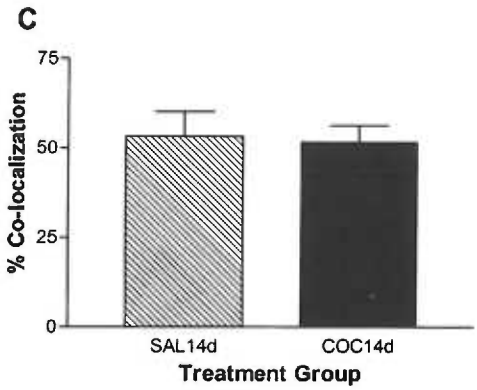
COC14d
Treatment Group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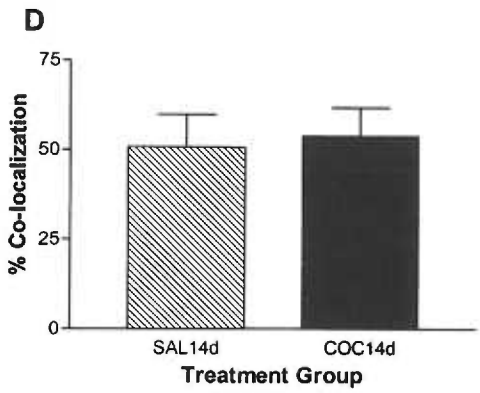
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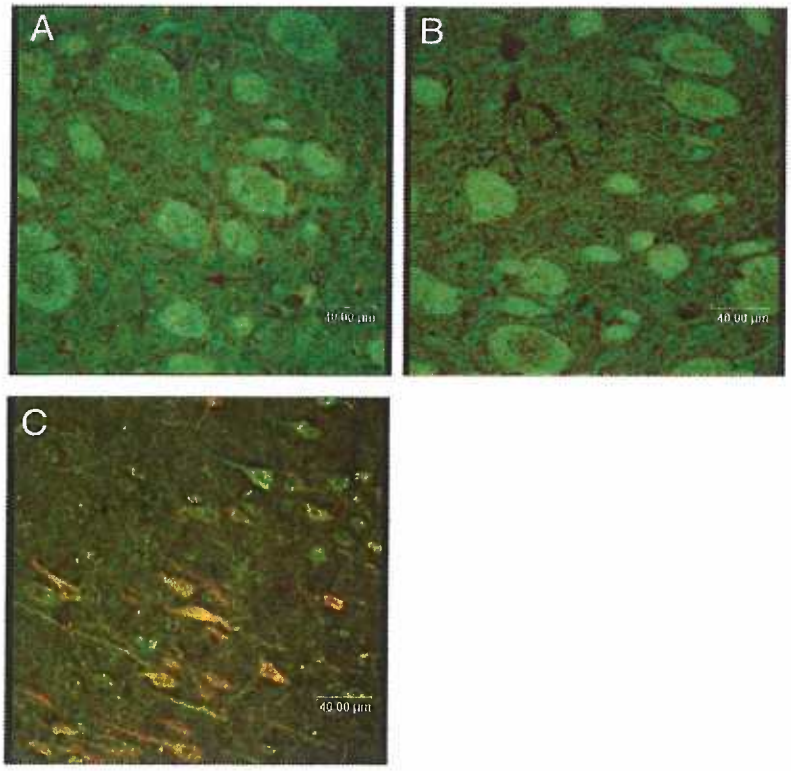

Figure 9: Photomicrographs of rat brain tissue dually labeled for NR2B and SAP102. Brain sections from cocaine- (A) and saline-treated (B) rats in areas of the dorosolateral neostriatum are shown. Panel C illustrates co-expression of NR2B with SAP102 predominantly in layers III and V of the frontal cortex. NR2B immunofluorescence appears as red and SAP102 as green. Areas of overlap and putative co-localization are shown in yellow.

As with NR2B expression, the cocaine withdrawal-induced reduction in SAP102 was not observed using immunofluorescent labeling. Given that FosB/ΔFosB expression showed a cocaine-induced upregulation in the dorsolateral neostriatum (Fig. 7), it is less likely that the confocal microscopy method was ineffectual at detecting changes in protein abundance. Thus, the inability to detect cocaine withdrawal-induced alterations in NR2B and SAP102 were probably more a function of the significantly smaller region of assessment rather than an issue of assay sensitivity.

Review of the literature reveals scant information regarding the effects of cocaine on PSD-95 or SAP102. PSD-95 expression was not significantly different for saline- or cocaine-treated rats at either withdrawal time point or across brain regions, including the frontal cortex, neostriatum and hippocampus (Figs 2A, 2B; see also Ch. 2). Studies investigating the effects of ethanol on PSD-95 expression had similar outcomes. In rat cortical neuronal cultures, the effects of chronic ethanol exposure on the expression of NMDA receptor subunit proteins and PSD-95 were studied. Exposure to 100 mM ethanol for 4 days significantly increased the levels of NR1, NR2A and NR2B, but had no effect on levels of PSD-95 expression (Chandler et al., 1999). Similarly, the effects of gestational ethanol exposure on protein interactions among the NMDA receptor complex were characterized. Prenatal ethanol exposure had no effect on PSD-95 protein expression. Further, anti-PSD-95 immunoprecipitates revealed an abundance of NR1 and NR2B subunits, and these complexes were unaffected by gestational ethanol exposure. Authors concluded that gestational ethanol exposure does not appear to alter the composition of the PSD-95-linked NMDA receptor complex (Hughes et al., 2001). Thus, PSD-95 appears to be a relatively stable protein that is not altered by certain drugs of abuse.

Questions regarding effects of cocaine on the expression and function of nNOS have been more widely investigated. Results presented in this chapter showed that there were no cocaine withdrawal-induced changes in the expression of nNOS at either 24 hours or 14 days following drug treatment (Figs 2C, 2D, 5). However, a number of studies have suggested that

nNOS does play a role in mediating certain behavioral features and symptoms associated with repeated cocaine exposure. The development of cocaine-induced stereotypy is completely blocked by daily intra-VTA pretreatment with 7-nitroindazole (Wiley, 1998). Further, attenuation of the locomotor stimulant effects of cocaine is evident in animals that have received daily intra-VTA 7-nitroindazole infusions (Byrnes et al., 2000). Itzhak et al. (1998) found that homozygous nNOS (-/-) mice are resistant to cocaine-induced behavioral sensitization. Importantly, nNOS inhibition decreases cocaine self-administration. Collins et al. (2002) found that 7-nitroindazole reduces responses maintained by the cocaine-training dose and produces a downward shift in the cocaine dose-response curve. Taken together, the data presented in this chapter and the studies described above suggest that more work needs to be done to better understand the relationship among NMDA receptor activation, NO signaling and cocaine use.

The PSD is a highly dynamic structure, which is reorganized in an activity-dependent manner. Synaptic activity can alter the composition of the complex and consequently affect the function of the receptors localized within the PSD (Wyneken et al., 2001). Although there has been limited substance abuse research attention focused on such cytoskeletal proteins, it is known that the molecular actions of cocaine are not limited to transcription factors and neurotransmitter system gene products. Cocaine administration causes a significant yet transient increase in activity-regulated, cytoskeleton-associated (arc) mRNA levels in the rat striatum (Fosnaugh et al., 1995). Further, immunolabeling revealed that Arc protein appears to be a component of the cytoskeleton and is expressed in neuronal cell bodies and dendrites. Cocaine-induced changes in Arc and other potential proteins may play a role in modifying cytoskeletal dynamics. Thus, drug-induced alterations in the neuronal morphologic structure may contribute to some of the neurochemical changes and behavioral features associated with cocaine use.

PSD-95, SAP102 and nNOS are only three of numerous proteins and enzymes associated with NMDA receptor subunits at the PSD. There exist many other potential targets through which cocaine could act to alter neuronal functioning. The list of PSD proteins is long and new

proteins are continually being identified. For example, a novel amidohydrolase superfamily protein, termed nedasin, which interacts with the PDZ domains of SAP102 both *in vitro* and *in vivo* may regulate NMDA receptor clustering and the structure of the synaptic junction. Specifically, one isoform, nedasin S, competitively inhibits the binding between NR2B and SAP102 (Kuwahara et al., 1999). Given that MAGUKs, such as SAP102 play a role in synaptic organization by linking interacting proteins to signaling molecules, cocaine-induced changes in the levels of these proteins could have profound consequences on downstream events.

The experiments discussed in this chapter provide support for the role of the NMDA receptor complex in the etiology of symptoms related to cocaine addiction. It remains to be determined what the functional consequences of changes in NR2B and SAP102 expression are and further, how these changes relate to symptoms associated with cocaine abuse (*i.e.*, compulsive use) and withdrawal (*i.e.*, anxiety and depression).

DISCUSSION

Cocaine addiction is often accompanied by a high frequency of relapse that can appear after years of abstinence from drug use (Jaffe, 1989). The DSM-IV reports that; "The essential feature of substance abuse is a maladaptive pattern of substance use manifested by recurrent and significant adverse consequences related to the repeated use of substances." It is further stated that with substance dependence, "there is a persistent desire or unsuccessful efforts to cut down or control substance use" (DSM-IV, pp.181-182). Relapse to cocaine abuse is characterized by uncontrollable drug cravings that can be triggered by a single exposure to cocaine or an environmental stimulus such as a stressor or drug-associated cue (Childress, 1999). Understanding the molecular and neuroanatomical changes associated with cocaine exposure and withdrawal could lead to the development of therapeutics that would ameliorate such cravings and thus decrease the rate of relapse. Experiments conducted for this dissertation project revealed several putative cocaine withdrawal-induced changes in the composition and potentially the function of the NMDA receptor complex. The regulation of proteins by cocaine administration provides a model in which to study the molecular mechanisms by which repeated exposure to drugs of abuse alters levels of specific proteins and leads to changes in target neurons that underlie aspects of drug addiction (Nestler et al., 1993). A single model is currently not available to account for the diversity of experimental findings regarding the specific mediators involved in neurotransmission, signal processing, and synaptic organization associated with the NMDA receptor complex. The major findings described in this dissertation indicated that the expression of NMDA receptor subunits, NR1 and NR2B, nNOS, SAP102 and FosB/ΔFosB was altered in a withdrawal time- and brain region-dependent manner. In addition, changes in the serine phosphorylation of specific amino acid residues on a select number of NR1 isoforms were observed. The significance and potential implications of these results are discussed below,

including: 1) a brief review of other biochemical and neuranatomical changes occurring in the brain during similar withdrawal times, 2) potential mechanisms to explain how cocaine treatment and withdrawal could cause changes in protein expression, and 3) corresponding behavioral features present during cocaine withdrawal.

Many neurochemcial and molecular components in addition to more direct or well-characterized targets, such as dopaminergic or glutamatergic systems may influence cocaine-induced changes in protein expression. Given the number of newly discovered proteins associated with the PSD [*e.g.*, Shank (Naisbitt et al., 1999), Yotiao (Westphal et al., 1999), and CAPON (Jaffrey et al., 1998)], it is likely that the interaction of other regulatory elements is involved in the effects of cocaine but have yet to be characterized. For example, withdrawal from chronic cocaine self-administration results in the induction of a novel mRNA transcript named NAC-1 because it is currently known to be expressed only in accumbal neurons (Cha et al., 1997). In addition, NMDA receptors are regulated (often in a subunit- and splice variant-dependent manner) by intracellular components such as protein kinases (Hall and Soderling, 1997), PSD proteins (Wyszynski et al., 1997), second messengers [*e.g.*, NO (McBain and Mayer, 1994)], and neurotrophins (Bai and Kusiak, 1997). A summary of relevant finding described herein and in the literature as well as potential mechanisms associated with cocaine-induced regulation of protein expression, localization and function are discussed below.

Initial cocaine withdrawal-induced changes in protein expression. Cocaine administration causes a transient up-regulation of nNOS activity that persists in the frontal cortex during the early phases of withdrawal from the drug (Bhargava and Kumar, 1997). Twenty-four hours after the last cocaine treatment increases in nNOS expression were observed in regions of the cortex (Ch.1, Fig. 5). These changes are hypothesized to be discrete and localized to defined cortical layers, as significant cocaine withdrawal-induced alterations in nNOS protein expression were not seen in the lateral cortical region (Ch.1). Further, western blotting using tissue obtained from whole frontal cortex or immunfluorescence labeling of slightly different cortical regions

also failed to show cocaine-induced changes in nNOS protein expression (Ch.3, Figs. 2, 5). The effects of cocaine on the expression of nNOS may thus result from specific alterations in neurotransmission and signal transduction that are cell-type and region-specific.

There appear to be numerous roles for NO, such as stimulation of cyclic guanosine 5'-monophosphate synthesis *via* activation of guanylate cyclase (Garthwaite, 1991), enhancement of CaM-dependent phosphorylation of PSDs (Wu et al., 1996), and importantly, modulation of synaptic vesicle exocytosis (Meffert et al., 1996). Thus, cocaine exposure could serve to further stimulate dopamine (DA) release *via* NO-mediated increases in exocytosis (Hanbauer et al., 1992). Although there is limited information regarding the effects of cocaine treatment and withdrawal on DA release in the specific cortical regions investigated, DA release in the VTA is enhanced during early withdrawal from repeated treatment with cocaine (Kalivas and Duffy, 1993). According to Kalivas (1995) the increased DA in the VTA is hypothesized to activate D1 receptors located on excitatory amino acid afferent terminals, which then cause cortical fiber excitatory amino acids to be released and subsequently stimulate NMDA receptors. Increased stimulation of NMDA receptors in the cortex during early withdrawal could contribute to the potential compensatory up-regulation of NR2B protein expression observed at 24 hours following cocaine treatment in regions of the frontal cortex (Ch. 1, Fig. 3). As with the changes observed in nNOS expression, these cocaine-induced alterations in NR2B are likely dependent on specific and regionally discrete cortical inputs since western blotting and confocal microscopy failed to reveal significant differences in the expression of NR2B in frontal cortex at 24 hours of withdrawal. In the shell of the nucleus accumbens, the transient down-regulation of NR2B that is observed (Ch.1, Fig. 3C) might similarly be associated with the decreased DA concentrations that have been measured in the accumbens (Kalivas and Duffy, 1993). Cocaine and NO-mediated alterations in synaptic concentrations of DA could thus indirectly contribute to changes in NR1 and NR2B expression observed at 24 and 72 hours after drug administration.

Findings suggest that the NR2B subunit was also up-regulated in the neostriatum at 24 hours after cocaine treatment, while the expression of SAP102 appeared to be decreased in rats treated with cocaine, as measured by immunoblotting (Ch.3, Fig.1). Investigation of the possible cocaine withdrawal-induced perturbations in the interaction of NR2B with SAP102 was described in chapter 3. Dual labeling immunofluorescent confocal microscopy did not detect co-localization of these proteins in regions of the neostriatum (Ch.3, Fig. 9). However, co-expression was observed in the frontal cortex (Ch. 3, Fig. 9C). It is hypothesized that since NR2B cell body immunolabeling was not seen in the neostriatum, potential interactions of NR2B with SAP102 could be occurring on neuronal processes located within the neuropil that were not detected using immunofluorescent labeling. Thus, it is yet to be determined whether cocaine withdrawal alters the interaction of SAP102 with NR2B in the neostriatum. A role for SAP102 in the contribution of synapse formation by clustering neurotransmitter receptors and other molecules at synaptic sites has been proposed (Muller et al., 1996). More recently it has become clear that regulation of receptor function can occur through changes in the targeting of receptor subunits. The amino acid sequences in the C-terminus of selected NR1 splice variants contribute to the subcellular distribution (Ehlers et al., 1995) and surface expression (Okabe et al., 1999) of the receptor proteins. In addition to the putative clustering of NR2B with SAP102 and its related family of proteins, the synaptic association with such cytoskeletal elements may also regulate the expression and function of NR2B-containing receptors. For example, co-expression of PSD-95 with NR1/NR2A results in a decreased sensitivity to L-glutamate and an enhanced expression of NR2A and NR2B subunits (Rutter and Stephenson, 2000). Further, Roche et al. (2001) demonstrated that PSD-95 inhibits NR2B-mediated internalization and that deletion of the PDZ-binding domain of NR2B increases internalization in cultured hippocampal neurons. Thus, characterization of the effects of cocaine withdrawal on the expression of NMDA receptor subunits and associated PSD-95 family member proteins is complex since alterations in the interaction of NR2B with SAP102 could serve to further regulate protein expression. Another

example of the interrelatedness among PSD proteins is provided by the suggestion that the linking of NMDA receptors to nNOS by PSD-95 may explain why Ca^{2+} influx following NMDA receptor activation leads to tightly coupled nNOS activation. Cocaine-induced changes in the cellular distribution, protein abundance or interaction of NR2B, SAP102 or nNOS could have a significant impact on glutamatergic function and Ca^{2+}-dependent second messenger systems.

Repeated cocaine exposure upregulates ΔFosB, which could mediate compensatory changes in Ca^{2+}-dependent gene expression. In addition, there is evidence of down-regulation of the DA transporter gene and functional reorganization of striatal immediate early gene products after cocaine withdrawal (Kuhar and Pilotte, 1996; Cerruti et al., 1994; Moratalla et al., 1996). Although FosB/ΔFosB was up-regulated in the neostriatum (Ch.1, Table 1; Ch. 3, Fig. 7), it is not known whether this early cocaine withdrawal-induced increase can be associated with similarly observed increases in NR2B expression in the neostriatum. Confocal microscopy and dual labeling of NR2B and FosB/ΔFosB in the dorsolateral neostriatum revealved minimal co-expression of these proteins (Ch.3, Fig. 7). It thus remains unclear how ΔFosB serves to regulate NMDAR2B gene expression during cocaine withdrawal.

Cocaine treatment and 24 hours of withdrawal resulted in a transient decrease in the phosphorylation of specific serine residues on select NR1 splice variants in the frontal cortex (Ch.2, Figs. 3, 5). Consistent with these findings, Steketee et al. (1998) demonstated that repeated cocaine treatment followed by 24 hours of withdrawal significantly reduced the activity of PKC in the medial prefrontal cortex. Protein phosphorylation is a major mechanism for the regulation of NMDA receptor activity (Zheng et al., 1999). PKC activation and phosphorylation of NR1 can inhibit the clustering of NR1, and phosphorylation by PKA and PKC can antagonize its interaction with spectrin, a structural protein linked to NMDA receptor subnits (see Introduction, Fig. 1; Wechsler and Teichberg, 1998). Further, activation of PKC induces a rapid dispersal of NMDA receptor subunits from synaptic to extrasynaptic plasma membranes in cultured rat hippocampal neurons (Fong et al., 2002). Thus, a cocaine-related reduction in the

serine phosphorylation of NR1 subunits could attenuate phosphorylation-induced decreases in clustering and interacting with cytoskeletal proteins (*i.e.*, spectrin) and reduce translocation to the extracellular space, thereby increasing NR1 stability in the membrane and potentially its functionality as a component of NMDA receptor-gated ion channels.

Presynaptic NMDA receptor function is also regulated through changes in subunit expression and association with modulatory proteins (Premkumar and Auerbach, 1997; Yamada et al., 1999). Interestingly, Crump et al. (2001) demonstrated that blockade of the NMDA receptor channel (*via* the NMDA receptor antagonist, APV) results in receptor redistribution to the synapse, which takes place without new protein expression but requires PKA-mediated phosphorylation. The role of phosphorylation in the regulation of glutamate receptor function awaits further investigation. However, characterization of the effects of cocaine on the NMDA receptor complex may serve as a useful model for understanding drug-induced changes in receptor expression and function. Specifically, phosphorylation and alternative splicing of the NR1 subunit could operate as mechanisms for regulating the trafficking and membrane delivery of NMDA receptors and associated proteins. NR1 phosphorylation could be important for the activation of pathways that are substrates for the effects of cocaine exposure and withdrawal. Consequently, by altering the level or efficiency of NMDA receptor expression *via* cocaine exposure and subsequent withdrawal, such posttranslational mechanisms could modify the magnitude of NMDA receptor-mediated synaptic responses, thus influencing synaptic plasticity and down-stream signal transduction events.

Long-term and cocaine withdrawal-induced changes in protein expression. At 14 days of withdrawal nNOS expression is no longer up-regulated (Ch.1, Fig. 4; Ch. 3, Figs. 2, 5, Table 1) and evidence suggests that nNOS activity has returned to baseline (Bhargava and Kumar, 1997). However, two weeks of withdrawal from cocaine resulted in significant up-regulation of NR2B in regions of the cortex, dorsolateral neostriatum (IHC only), and nucleus accumbens (Ch.1, Fig. 3;

Ch.3, Fig. 1) and NR1 in the VTA (Ch.1, Figs. 1). Consistent with these findings, Robinson and Kolb (1999) observed a 13.5% increase in the density of dendritic spines on medium spiny neurons at 4 weeks following cocaine exposure. Further, the changes observed were located on the distal ends of the neurons—putative sites of convergence for DA and glutamate inputs on these cells. Conversely, a cocaine withdrawal-induced decrease in NR1-C2 expression was observed in the neostriatum (Ch.2, Fig. 2). The differential regulation of NMDA receptor subunits is interesting yet not surprising given that these receptor subunits have very different levels of cell surface expression as well as both basal and stimulated phosphorylation states (Hall and Soderling, 1997). Specifically, only about 50% of total NR1 protein expression is found at the cell surface, as compared to more than 90% of NR2B subunit expression. Further, NR1-C2 protein abundance was measured using immunoblotting of rat brain preparations that consisted of membrane-enriched fractions, whereas the increase in NR2B expression was detected by densitometry analysis of rat brain slices. Thus, NR1 expression not localized to the membrane was likely not quantified using the immunoblotting techniques described in this dissertation (see Methods section).

Because nNOS expression and activity are only transiently up-regulated it is unlikely that these short-term neurochemical changes can, by themselves, maintain the longer-lasting physiological or behavioral changes evident after cocaine administration or during withdrawal. The persistent increases in receptor protein expression observed during cocaine withdrawal are hypothesized require multiple changes occurring downstream. For example, augmented DA release has been observed in rats exhibiting psychostimulant-induced behavioral sensitization (Kalivas and Duffy, 1993). Further, excitatory amino acids promote the release of DA in the striatum and accumbens (Galli et al., 1991) and cocaine-stimulated glutamate release is sensitized in the nucleus accumbens following chronic cocaine treatment and 10 days of withdrawal (Reid and Berger, 1996). Cocaine-induced changes in neurochemical functioning thus appear to depend

not only upon the brain regions involved but also upon the specific neuronal and intracellular interactions.

In addition to the cocaine-induced changes in protein expression identified in chapters 1-3, a number of concurrent behavioral alterations associated with cocaine withdrawal have been described. The identified drug-induced effects may therefore be important for the precipitation of short-term behavioral changes associated with cocaine withdrawal in animal models, such as decreases in locomotor activity, cognitive impairments and increases in anxiety levels (Fung and Richard, 1994). Murphy et al. (2001) assessed the effects of withdrawal from repeated cocaine treatment on latent inhibition of a conditioned fear response. After 3 days of withdrawal from drug administration, animals were tested for conditioned freezing to a footshock chamber, and susequently, for conditioned freezing to a tone. Rats treated with cocaine and not pre-exposed to the tone demonstrated increased latent inhibition compared to saline-treated animals suggesting that cocaine withdrawal is sassociated with heightened anxiety. Interestingly, this enhanced inhibition was not seen in rats that were pre-exposed to the tone indicating that cocaine withdrawal may also disrupt the retrieval of previously learned information (Murphy et al., 2001). The biochemical and neuroanatomical cocaine withdrawal-induced changes described in this dissertation might also contribute to the maintenance of more enduring responses associated with psychostimulant treatment such as behavioral sensitization (Kalivas and Alesdatter, 1993; Itzhak, 1997). In humans, such changes in glutamatergic function could play a role in the neurological, sleep and mood impairments (O'Malley et al., 1992; Watson et al., 1992) observed in cocaine users and individuals experiencing withdrawal and related symptoms.

CONCLUSION

The experiments described herein provide support for the role of the NMDA receptor complex in the etiology of symptoms related to cocaine addiction. The results suggest that region- and withdrawal time-specific changes in expression and interactions among proteins associated with the NMDA receptor complex may underlie neuronal adaptations following repeated cocaine administration. In combination with an expanding spectrum of available research tools and newly identified PSD proteins, further characterization of the specific molecular changes associated with cocaine exposure may ultimately suggest novel directions for the development of pharmacotherapeutic agents and relapse prevention strategies.

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