Ontogeny- and Sex-Dependent Contributions of the Neuronal Nitric Oxide Synthase (nNOS) Gene to Rewarding and Psychomotor Stimulating Effects of Cocaine

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ONTOGENY- AND SEX-DEPENDENT CONTRIBUTIONS OF THE NEURONAL NITRIC OXIDE SYNTHASE (nNOS) GENE TO THE REWARDING AND PSYCHOMOTOR STIMULATING EFFECTS OF COCAINE

By

Mara A. Balda

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Coral Gables, Florida

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ONTOGENY- AND SEX-DEPENDENT CONTRIBUTIONS OF THE NEURONAL NITRIC OXIDE SYNTHASE (nNOS) GENE TO THE REWARDING AND PSYCHOMOTOR STIMULATING EFFECTS OF COCAINE

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Multiple interactions between dopamine (DA), glutamate, and nitric oxide (NO) in mesolimbic and corticostriatal circuits suggest that NO may play a critical role in cocaine-induced behavioral and neural plasticity. Clinical and preclinical studies have revealed that females and adolescents display unique vulnerabilities to the behavioral and neurochemical effects of cocaine as a result of sex-dependent and ontogeny-dependent differences in dopaminergic systems. Thus, my research objectives were to investigate the contributions of the neuronal nitric oxide synthase (nNOS) gene, ontogeny, and gender on the rewarding and sensitizing effects of cocaine. I found that nNOS significantly influences the rewarding aspects of cocaine in adolescent mice and adult male mice (i.e., major deficits in several phases of cocaine conditioned place preference (CPP) were detected in nNOS knockout (KO) adolescent mice and nNOS KO adult male mice). However, the contribution of nNOS was sex-dependent as CPP phases were normal in KO adult females. In contrast to CPP, I found a major ontogeny-dependent contribution of nNOS to the sensitizing effects of cocaine. Namely, while nNOS is essential for the development of behavioral sensitization in adult males, this type of behavioral plasticity develops independently of nNOS during adolescence. The
contribution of nNOS was once again sex-dependent as behavioral sensitization was normal in adult KO females. Together, this line of investigation has revealed that the NO-signaling pathway has a) a sex-dependent role in the neuroplasticity underlying cocaine CPP and b) a sex-dependent and ontogeny-dependent influence on cocaine-induced behavioral sensitization.

Stereological and western blot analysis revealed that a sensitizing regimen of cocaine resulted in an increase in nNOS and tyrosine hydroxylase (TH) immunoreactivity in the dorsal striatum (dST) of adult, but not adolescent, wild-type (WT) male mice. In the absence of nNOS, dopaminergic neurons in the ventral tegmental area (VTA) were severely reduced and cocaine caused a downregulation of dST TH suggesting that nitrergic levels modulate TH. Thus, the finding that nNOS is essential for the development of sensitization in adulthood, but not adolescence, together with the fact that cocaine upregulated nNOS and TH in the dST in adult, but not adolescent mice, strongly suggest that the nitrergic system underlies behavioral sensitization through modulation of the dopaminergic system in adulthood. These findings suggest different approaches in the clinical treatment of drug craving and drug-seeking behavior in adolescent and adult patients.
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BST</td>
<td>Bed nucleus of the stria terminalis</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Calcium</td>
</tr>
<tr>
<td>CaM</td>
<td>Calmodulin</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CPP</td>
<td>Conditioned place preference</td>
</tr>
<tr>
<td>CR</td>
<td>Conditioned response</td>
</tr>
<tr>
<td>CS</td>
<td>Conditioned stimulus</td>
</tr>
<tr>
<td>DA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>DAT</td>
<td>Dopamine transporter</td>
</tr>
<tr>
<td>dST</td>
<td>Dorsal striatum</td>
</tr>
<tr>
<td>EDRF</td>
<td>Endothelial relaxing factor</td>
</tr>
<tr>
<td>EC</td>
<td>Extracellular</td>
</tr>
<tr>
<td>Glu</td>
<td>Glutamate</td>
</tr>
<tr>
<td>cGMP</td>
<td>Guanosine 3’:5’-cyclic monophosphate</td>
</tr>
<tr>
<td>sGC</td>
<td>Guanylyl cyclase</td>
</tr>
<tr>
<td>ir</td>
<td>Immunoreactive</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>L-NAME</td>
<td>N(omega)-nitro-L-arginine methyl ester</td>
</tr>
<tr>
<td>nNOS</td>
<td>neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NAC</td>
<td>Nucleus accumbens</td>
</tr>
<tr>
<td>PD</td>
<td>Postnatal day</td>
</tr>
<tr>
<td>PSD</td>
<td>Postsynaptic density</td>
</tr>
<tr>
<td>PFC</td>
<td>Prefrontal cortex</td>
</tr>
<tr>
<td>PKG</td>
<td>Protein kinase G</td>
</tr>
<tr>
<td>7-NI</td>
<td>7-nitroindazole</td>
</tr>
<tr>
<td>SNP</td>
<td>Sodium nitroprusside</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine hydroxylase</td>
</tr>
<tr>
<td>US</td>
<td>Unconditioned stimulus</td>
</tr>
<tr>
<td>VTA</td>
<td>Ventral tegmental area</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
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</table>
Chapter 1
Introduction

**Epidemiology of Cocaine Abuse**

Cocaine addiction is a serious public health concern associated with significant medical, social, and economic consequences. In 2007, an estimated 22.3 million persons (9.0 percent of the population aged 12 or older) were classified with substance dependence or abuse in the past year based on criteria specified in the *Diagnostic and Statistical Manual of Mental Disorders* (American Psychiatric Association, DSM-IV, 4th edition, 2000). Cocaine was reported as the specific illicit drug that had the third highest levels of past year dependence or abuse in 2007 (1.6 million) (SAMHSA 2007).

Substance dependence or abuse of illicit drugs are associated with many factors including age, gender, race, ethnicity, education, employment, criminal justice populations, and geographic area. For instance, the rate of substance dependence or abuse for males aged 12 or older in 2007 was about twice as high as the rate for females (12.5 vs. 5.7 percent) leading to the impression that females are less vulnerable to drug abuse than are men (SAMHSA 2007). Although the rates of cocaine abuse have historically been and currently are lower in women than in men, accumulating evidence from both clinical and preclinical studies suggests that females are not less vulnerable to drug abuse than males, and with regard to certain aspects of drug abuse, they may have an enhanced biological vulnerability (Becker and Hu 2008; Brady and Randall 1999; Lynch 2006; Lynch et al. 2002; Roth and Carroll 2004b). Among youths aged 12 to 17, however, the rate of substance dependence or abuse among males was the same as the rate among females (7.7 percent for both) suggesting that the trend for greater drug use
and abuse among males does not apply to younger populations (SAMHSA 2007). One main goal of this thesis was to investigate if specific populations, particularly female and adolescent mice, display differences in vulnerability to psychostimulant drugs.

**Molecular and cellular mechanisms of cocaine addiction**

The main characteristics of cocaine addiction are compulsive drug use despite adverse consequences and high rates of relapse during periods of abstinence. A current hypothesis is that compulsive cocaine use and cocaine relapse are due to drug-induced neuroadaptations in reward-related learning and memory processes. These changes cause hypersensitivity to cocaine-associated cues, impulsive decision making, and abnormal habit-like learned behaviors that are insensitive to adverse consequences (Thomas et al. 2008).

Cocaine, a drug with high abuse liability, causes increases in extracellular monoamine (dopamine, serotonin, norepinephrine) concentrations by blocking the reuptake of monoamines released from vesicular stores (Ritz et al. 1987). The defining mechanism action of cocaine, critical to reinforcing drug use, is blockade of the dopamine transporter (DAT), leading to an accumulation of dopamine in the synaptic cleft and enhanced dopaminergic signaling at postsynaptic dopamine receptors. Although the basic pharmacology of cocaine has been elucidated, understanding addiction to cocaine has been challenging, primarily because the action of cocaine at the DAT is only the initial step in a cascade of molecular events that ultimately leads to enduring pathological neuroplasticity in the central nervous system (CNS). One of the key neural circuits of reward and addiction thought to be perturbed by drug experience is known as the mesolimbic system (Figure 1.1) which originates in the ventral tegmental
area and projects to limbic structures including the nucleus accumbens (NAC), prefrontal cortex (PFC), and caudate putamen (dorsal striatum). All drugs of abuse increase dopamine-mediated transmission in the NAC (Di Chiara and Imperato 1988; Koob et al. 1998; Kuhar et al. 1991; Wise 1998) indicating that the VTA-NAC pathway is a site where virtually all addictive drugs converge to produce their acute reward signals. While the VTA-NAC pathway is considered a major rheostat of reward, other limbic circuits (e.g. dorsal striatum, prefrontal cortex, amygdala, and hippocampus) are thought to contribute to the long-lived features of addiction that are central to relapse (Baker et al. 2003; Burns et al. 1996; Kalivas and Nakamura 1999; Volkow and Fowler 2000; White and Kalivas 1998).

**The role of dopamine in drug-induced neuroadaptations**

Alterations in the expression of genes involved in dopaminergic neurotransmission were among the earliest cocaine-induced neuroadaptations to be discovered. The signaling cascade mediating such events include a generalized upregulation of the cAMP pathway and CREB (Carlezon et al. 1998; Miserendino and Nestler 1995; Nestler 1993; Pliakas et al. 2001; Self et al. 1998; Striplin and Kalivas 1992; Terwilliger et al. 1991), activator protein 1 family members (Couceyro et al. 1994; Hiroi et al. 1997; Hope et al. 1992; Nye et al. 1995; Pich et al. 1997), delta-Fos-B and preprodynorphin (Graybiel et al. 1990; Young et al. 1991). Interestingly, these molecular adaptations modulate sensitivity to the rewarding effects of subsequent drug exposures and the expression of behaviors potentially related to addiction, such as behavioral sensitization and conditioned place preference (Mackler et al. 2000). Other groups have reported additional adaptations in the mesolimbic and corticostriatal circuits including changes in
levels of G-protein subunits, tyrosine hydroxylase (the rate limiting enzyme in dopamine biosynthesis), neurofilaments proteins, and neuropeptide systems (Beitner-Johnson et al. 1992; Carlezon and Nestler 2002; Johnson et al. 1992; Ortiz et al. 1995; Striplin and Kalivas 1992).

**The role of glutamate in drug-induced neuroadaptations**

In addition to dopamine, several studies suggest that glutamate (Glu) transmission is involved in the behavioral and neurochemical effects of cocaine, as well as the critical pathological neuroadaptations induced by chronic cocaine administration. Repeated administration of cocaine results in an increase in the capacity of cocaine to elevate extracellular (EC) glutamate in both the NAC and VTA (Kalivas and Duffy 1998; Pierce et al. 1996; Reid and Berger 1996). Furthermore, drug seeking behavior (reinstatement of cocaine self-administration) produces a massive release of prefrontal glutamate into the nucleus accumbens that is not observed with natural rewards (McFarland et al. 2003; Timmerman and Westerink 1997). Perhaps as a consequence of cocaine-induced glutamate release, a number of changes in postsynaptic glutamate transmission have been reported including increased AMPA and NMDA receptor expression in the VTA and NAC leading to increased excitability of midbrain and accumbal neurons (White et al. 1995; Zhang et al. 1997). Finally, these and other neuroadaptations most likely give rise to stable structural changes in neurons of the reward pathway that underlie the life-long risk of relapse characterizing addiction including increases in structural protein such as actin cycling and formation of F-actin, expansion in the dendritic arborization of NAC neurons, and alterations in scaffolding proteins such as postsynaptic density (PSD)-95 proteins and Homer proteins (Swanson et al. 2001; Toda et al. 2006; Yao et al. 2004).
Preclinical evidence for cocaine-induced neuroadaptations in animal models has incited studies on alterations in the brains of patient afflicted with addiction, mainly through neuroimaging and postmortem studies. At rest, there is reduction in metabolic activity of the prefrontal cortex of psychostimulant addicts which has been taken to indicate a potential deficit in cognitive ability to resist relapse (Volkow et al. 2005). Interestingly, while hypoactivity is reported under basal conditions, the presentation of drug cues or low doses of psychostimulants to human cocaine addicts induced drug craving that was shown to correlate with the activation of the amygdala, limbic prefrontal cortical areas, and the dorsal striatum (Childress et al. 1999; Garavan et al. 2000; Grant et al. 1996; Volkow et al. 2006). Lastly, it has been shown that some of the adaptations that occur in animal models of drug abuse are applicable to the human brain as postmortem studies have shown alterations of ionotropic glutamate receptor subtypes and CREB following chronic cocaine use (Tang et al. 2003).

The cocaine-induced neural changes in animal models of addiction and in human postmortem brains, along with the pathological neuroimaging in addicts described thus far, together provide strong evidence that chronic cocaine use can exert long-term effects involving signaling molecules, gene transcription, and structural and functional reorganization of key circuitry ultimately culminating in addiction.

**Nitric oxide in the CNS**

As already noted research pertaining to cocaine addiction has traditionally focused on dopaminergic transmission and, in more recent times, on glutamatergic systems. As shown in Figure 1.2, NMDA receptor activation by glutamate leads to the Ca\(^{2+}\) influx responsible for the activation of nNOS (Brenman and Bredt 1997) and
subsequent formation of nitric oxide (NO) (Garthwaite 1991; Snyder 1992). The critical role of NMDA receptors in mediating addiction and its role in NO formation strongly suggest that NO signaling may contribute to cocaine-related addictive behaviors, a concept that is explored in depth in this thesis. Furthermore, substantial evidence for extensive NO/DA interactions strengthens the hypothesis of a significant role of NO in mediating the effects of drugs of abuse.

NO was first discovered and described as endothelial relaxing factor (EDRF), a free radical gas released by endothelial cells to signal smooth muscle relaxation and increase blood flow (Palmer et al. 1987). Since that time, NO has been implicated in many aspects of vertebrate CNS functions including neurotransmitter release, learning and memory (Devan et al. 2006; Golombek et al. 2004; Susswein et al. 2004), neural development, regeneration, synaptic plasticity, regulation of gene expression (Contestabile and Ciani 2004; Estrada and Murillo-Carretero 2005; Mize and Lo 2000), and neuropathology and/or neuroprotection (Calabrese et al. 2007; Dawson and Dawson 1998; Duncan and Heales 2005; Keynes and Garthwaite 2004).

Nitric oxide is produced by a group of isoenzymes, the nitric oxide synthases, referred to as neuronal (nNOS), endothelial (eNOS), and inducible (iNOS). The first two are considered constitutive and calcium dependent and the third is present primarily in microglia and macrophages (Bredt and Snyder 1994). The first NO synthase to be purified and cloned (Bredt et al. 1991; Snyder and Bredt 1991) and the most abundant isoform found in the central and peripheral nervous systems is nNOSα, which is activated by Ca2+ complexed with calmodulin (CaM) and has a wide but uneven distribution in the mammalian brain (Bredt et al. 1991; Vincent and Kimura 1992). nNOS assembles as a
homodimer of two identical reductase subunits, each composed of 686 amino acid residues. It belongs to a family of reductase proteins that share a conserved organization of FMN-binding, FAD-binding, and NADPH binding domains. Electron transfer proceeds from NADPH to FAD to FMN to the heme region of the trans-oxygenase domain, where production of NO is catalyzed. Transfer to the heme region is rate-limiting, and is induced by conformational changes following Ca\(^{2+}/CaM\) binding to the NOS enzyme (Garcin et al. 2004). The physical association of nNOS\(\alpha\) and the NMDA receptor subunit NR2B with PSD-95 through their specialized PDZ domains (Brenman et al. 1996) helps explain the link between NMDA receptors and NO production (Garthwaite 1985; Garthwaite et al. 1988).

One of the most recognized physiological NO signal transduction mechanisms is the activation of guanylyl cyclase (sGC) and subsequent production of the second messenger guanosine 3’:5’-cyclic monophosphate (cGMP) (Garthwaite et al. 1988; Miki et al. 1977; Palmer et al. 1987). The most widespread mechanism employed by cGMP is activation of protein kinase G (PKG). Several substrates for PKG have been identified (Schlossmann and Hofmann 2005) and many of its actions are exerted at the level of phosphatases, leading indirectly to increased or decreased levels of phosphorylation of effector proteins. One biologically relevant target is the phosphorylation of synaptic vesicle proteins by cGMP-dependent protein kinases associated with neurotransmitter release. In PC12 cells, the NO/sGC/cGMP pathway increased DA release in a calcium dependent manner (Serra et al. 2003).
**Nitric oxide and dopamine interactions**

In addition to the well-known pathways (e.g., sGC-cGMP system) involved in the CNS actions of NO, novel targets and mechanisms are emerging; out of which the most related to drugs of abuse is the inhibitory effect of NO on monoamine transporters. NO facilitates both tonic extracellular DA levels and phasic DA neuron spike activity (Grace 1991; Moore et al. 1999; West et al. 2002; West and Grace 2000). NO also facilitates glutamate release, and stimulation of glutamate receptors on dopaminergic nerve terminals may further stimulate DA release (Moore et al. 1999). In vitro and in vivo experiments suggest that endogenous NO exerts a tonic inhibitory effect on monoamine transporters (Kiss 2000). In vitro, NO inhibited DA reuptake into striatal (Lonart and Johnson 1994; Pogun et al. 1994) and hippocampal (Lonart and Johnson 1995) synaptosomes. *In vivo* microdialysis studies have shown that striatal release of DA is decreased in the presence of the NOS inhibitor, N(omega)-nitro-L-arginine methyl ester (L-NAME). This effect disappeared in the presence of the DA uptake inhibitor, nomifensine, indicating that the effect was mediated via the DA transporter (Kiss et al. 1999). While inhibiting nNOS led to increased uptake of DA, the NO generator sodium nitroprusside (SNP) (100 μM) decreased the uptake of [3H]DA in striatal slices (Kiss et al. 2004). It has been suggested that DAT activity may be modulated by interaction of NO with easily accessible DAT cysteine residues (Pogun et al. 1994; Zahniser and Doolen 2001).

Just as NO has been shown to regulate dopaminergic neurotransmission, ample also shows dopaminergic modulation of the nitrergic system; providing yet another mechanism by which NO may contribute to the addictive properties of cocaine.
Activation of striatal and hippocampal D1 receptors increased NO release (Morris et al. 1997; Sammut et al. 2006; Sammut et al. 2007b; Yang 1999) by stimulating NOS-positive interneurons (Centonze et al. 2002). 6-Hydroxydopamine lesions markedly reduced nNOS activity in the striatum and frontal cortex (de Vente et al. 2000) and decreased the numbers of NADPH-d/NOS-ir neurons in the nucleus accumbens (Gomes and Del Bel 2003). In vivo administration of haloperidol (DA receptor antagonist) attenuated expression of nNOS-ir neurons in the pituitary gland, while in vivo application of DA increased the expression (Carretero et al. 2003). Although nNOS is considered a constitutively expressed protein, these studies and others (Forstermann et al. 1998; Sasaki et al. 2000) suggest that nNOS is subject to expressional regulation in response to activity-dependent plasticity.

The role of NO in effects of psychostimulants

Several lines of evidence suggest that the nitrergic system undergoes psychostimulant-induced neuroplasticity. Acute cocaine administration significantly increased nNOS activity and NO efflux in the medial prefrontal cortex over an extended time period lasting at least 45 min (Sammut and West 2008). Chronic cocaine administration increases NOS activity in the cerebral cortex, cerebellum, midbrain, hypothalamus, hippocampus, amygdala, and spinal cord up to 48h after withdrawal (Bhargava and Kumar 1997). A significant cocaine-induced upregulation of nNOS expression was observed at 24h but not 72h or 14 days of withdrawal in the frontal and parietal cortices (Loftis and Janowsky 2000). A high dose of amphetamine increased striatal nNOS mRNA expression (Wang and Lau 2001) as well as striatal nNOS enzymatic activity (Lin et al. 1999). The results of the above studies are consistent with
the literature demonstrating that psychostimulant-induced behavioral sensitization, self-administration, and expression of conditioned responses are suppressed by administration of nNOS inhibitors and in nNOS KO mice (Haracz et al. 1997; Itzhak 1997; Itzhak et al. 1998a; Kim and Park 1995; Orsini et al. 2002; Pulvirenti et al. 1996). Taken together these studies indicate that NO and subsequent NO/DA/Glu interactions play a critical role in the neuroplasticity and behavioral plasticity associated with psychostimulant exposure.

Adolescence and psychostimulant abuse – clinical studies

Reports estimate that up to 50% of substance use disorders are initiated during adolescence (Kandel et al. 1992) suggesting that substance abuse may have early developmental origins. In support of this notion, individuals who initiate drug use in early adolescence show a faster progression from first use to addiction, a pattern of heavier lifetime consumption, and greater difficulty abstaining than those who start as older adolescents or young adults (Chambers et al. 2003; Clark and Lewis 1998; Mackesy-Amiti et al. 1997; Spear 2000). Although this early exposure effect has been suggested to be one of the most powerful predictors of drug abuse in adulthood (Spear 2000), the long term consequences of adolescent exposure to certain drugs, such as psychostimulants, are relatively unknown.

Adolescent dopaminergic plasticity

A current hypothesis is that adolescent vulnerability to drug addiction stems from the maturation of forebrain dopamine function. Evidence suggests that certain pre- and post-synaptic components of dopaminergic circuitry in young adolescents differ significantly from adults. A common developmental trend is that both D1 and D2 receptors attain maximal expression during mid-adolescence (Andersen and Teicher
2000; Schambra et al. 1994; Teicher et al. 1995). Studies have shown that striatal and PFC DA receptors are overexpressed before the onset of puberty, peak around postnatal day (PD) 40, and then undergo pruning prior to adulthood (Andersen et al. 1997; Andersen and Teicher 2000; Murrin and Zeng 1990; Tarazi et al. 1999; Teicher et al. 1995). Similarly, at the presynapse, DA transporter density in the striatum increases from PD25 through PD50, and then decreases continuously until old age (Moll et al. 2000). Such pruning has also been shown for dopamine receptors and the dopamine transporters in postmortem studies of adolescent human brain (Meng et al. 1999; Seeman 1999). As a result the CNS developmental changes may affect the way adolescents respond to cocaine. Additionally, exposure to such drugs during adolescence may interfere with ongoing neuroplasticity and cause long-term adaptations which render the organism more susceptible to drug abuse later in life.

Behavioral effect of psychostimulants in adolescence – preclinical studies

Behavioral sensitization: Presumably due to developmental changes in dopamine systems the locomotor activity in response to psychostimulants is age-dependent with adolescents differing from younger and older rodents (Adriani and Laviola 2000; Bolanos et al. 1998; Catlow and Kirstein 2005; Laviola et al. 1999; Laviola et al. 1995; Maldonado and Kirstein 2005a; b; Niculescu et al. 2005; Spear and Brake 1983). Adolescents displayed less locomotor activity than adults after acute cocaine administration (Bolanos et al. 1998; Laviola et al. 1995; Spear and Brake 1983) while others have shown adolescents to have greater cocaine-induced locomotor activity than preadolescents and adults (Catlow and Kirstein 2005; Maldonado and Kirstein 2005a; b; Niculescu et al. 2005). Interestingly, the age related differences may depend on cocaine
dose tested as young adolescents (PD28) have greater locomotor responses to acute low
dose cocaine (<15 mg/kg) rather than high doses (>25 mg/kg) (Badanich et al. 2006;
Caster et al. 2005; 2007; Parylak et al. 2008). In behavioral sensitization studies
examining the ability of repeated cocaine treatment to induce a progressive and enduring
increase in the motor stimulant effect of cocaine, some have found that adolescent rats,
unlike their adult counterparts, were not sensitized to cocaine (Collins and Izenwasser
2002; Guerriero et al. 2006). Others reported that cocaine-induced sensitization was
detectable at lower doses in adolescent animals (Laviola et al. 1995; Niculescu et al.
2005; Ujike et al. 1995).

**Conditioned place preference:** Age-related differences in the behavioral
response to psychostimulant have also been observed when using other paradigms of
drug abuse such as conditioned place preference. The place conditioning paradigm
includes Pavlovian learning and is used to evaluate approach or escape behavior;
conditioned place preference assesses drug and natural reward. Cocaine conditioned
place preference was greater in adolescent than in adult rats in response to a low dose of
cocaine (Badanich et al. 2006; Zakharova et al. 2009), but similar at higher doses
(Bolanos et al. 1996; Campbell et al. 2000; Laviola et al. 1992; Schramm-Sapyta et al.
2004) suggesting that adolescents are more sensitive to the conditioned rewarding
properties of low dose cocaine. Recently, it was shown that adolescent rats take longer
than adults to extinguish CPP to cocaine and that they exhibit a stronger reinstatement
upon priming, suggesting that the cocaine cue may have greater salience in the adolescent
rats (Brenhouse and Andersen 2008). In contrast to these studies, is the finding that there
are no differences between cocaine CPP in adolescent and adult rats in response to
conditioning with either 5 or 10 mg/kg cocaine (Campbell et al. 2000). The different results may be due to methodological differences such as pre-exposure handling used to reduce the stress of experimenter manipulation, habituation to a novel environment prior to drug challenge, specific adolescent age (early, mid, late adolescence), rodent strain, and differences in dosing regimens utilized. In spite of these inconsistencies these studies have been instrumental in characterizing the adolescent psychostimulant response profile mostly during adolescence. Yet, the lasting effects of early drug exposure into adulthood remain largely unknown.

**Ontogeny-dependent neuroadaptors following psychostimulant administration**

The biological vulnerability to addiction during adolescence may stem from unique molecular and cellular responses to drugs of abuse during this period. While adult mice exhibit greater peaks in extracellular DA in response to repeated cocaine, adolescent mice presented a more rapid onset of peak extracellular DA levels than adults (Badanich et al. 2006; Camarini et al. 2008; Philpot and Kirstein 1999) suggesting an earlier anticipatory dopaminergic response in younger animals. Repeated cocaine treatment also led to an upregulation of DA transporters in the adult striatum, an effect absent in adolescent rats (Collins and Izenwasser 2002). Similarly methamphetamine administration led to a decrease in dopamine transporter density, tyrosine hydroxylase, and dopamine uptake in adult, but not adolescent rats (Kokoshka et al. 2000; Pu and Vorhees 1993). On the other hand, an adolescent specific alteration is an upregulation in GluR1 in the mPFC after cocaine administration (Camarini et al. 2008). Major age related differences are observed when considering the induction of immediate early genes, markers of cocaine-induced neuronal activation thought to
involved in drug-induced learning process like CPP and behavioral sensitization. Low
dose cocaine induced more locomotor activity and striatal c-fos expression in adolescent
than adult mice. High dose cocaine induced more locomotor activity, striatal c-fos, and
striatal zif268 expression in adults suggesting that the coordinated expression of
transcription factors by cocaine continues to develop during adolescence (Caster and
Kuhn 2009).

Collectively, these studies clearly indicate that adolescents differ significantly
from adults in their response to psychostimulants. Not only are the neural substrates of
psychostimulants still experiencing developmental changes, but exposure to drugs during
this critical period affects these cocaine-sensitive neural circuits in an age-specific
manner. These naturally occurring and drug-induced changes in the CNS contribute to
ontogeny-dependent differences in the behavioral effects of psychostimulants. While
most of the studies thus far on the effect of abused substances on adolescent behavioral
and neurochemical responses have been enlightening, they have focused on relatively
short-term consequences of drug exposure. However, I consider it essential to investigate
the long-term consequences of adolescent exposure to cocaine on the susceptibility to
psychomotor stimulation and drug reward into adulthood. Moreover, investigations into
neuroadaptations underlying behavioral plasticity induced by cocaine in adolescence
need to be extended to include the nitrergic system.

**Sex differences in the effects of psychostimulants – clinical studies**

The rates of drug abuse are currently lower in women than in men. Nevertheless,
the number of women using and abusing prescription and illegal drugs is increasing.
Cocaine abuse in particular has increased in the last decade among women so that of the
1.8 million Americans who use cocaine, approximately 39.5% are female (SAMHSA 2007). Despite the finding that men are 2–3 times more likely than women to have a drug abuse/dependence disorder evidence suggests that women are more vulnerable to some aspects of psychostimulant abuse. Sex differences during the transition from controlled to uncontrolled drug use have been reported. Women enter treatment programs after fewer years of drug use and take less time to become addicted to drugs after initial use than do men. These data suggest that in women there is an accelerated transition from casual, controlled use to uncontrolled, “binge” patterns of use (Westermeyer and Boedicker 2000). Furthermore, once addicted to a drug, women find it more difficult to quit than men do and consistently show shorter or less frequent abstinence periods compared to men (Back et al. 2005; Perkins 1999). During abstinence women report higher levels of craving and increased depressive symptomatology compared to men (Elman et al. 2001). In a study comparing levels of cocaine cue reactivity, cues associated with cocaine use produced greater increases in craving in women compared to men (Robbins et al. 1999).

When prevalence rates are compared among adolescents, the gender differential disappears. An equal number of adolescent males and females are current illicit drug users (9.8% and 9.5%, respectively). It was recently demonstrated that cocaine use was higher in adolescent females than in adolescent males. Adolescent females were also more likely to use cocaine at an earlier age and with a greater frequency (Chen and Kandel 2002). Like adult females, they also reported more symptoms associated with substance dependence, such as inability to cut down and need for larger quantities (Chen and Kandel 2002). Thus, these sex-and age-specific differences in cocaine use patterns
and behavioral responses indicate that the biological basis of addiction is sexually
dimorphic as well as age-dependent.

Numerous studies suggest that gonadal hormones modulate behavioral
responsivity to cocaine. In women, the subjective effects of cocaine such as euphoria,
desire, and increased energy are potentiated during the follicular phase (when estradiol
levels are rising and progesterone levels are low) when compared to males (Evans et al.
2002) and when compared to females in the luteal phase (when estradiol levels are
moderate and progesterone levels are high) (Justice and de Wit 2000). Moreover,
administration of estradiol during the follicular phase further increases the subjective
effects of psychostimulants (Justice and de Wit 2000). In contrast, the subjective effects
of psychomotor stimulant drugs are negatively correlated with salivary progesterone
levels in women (White et al. 2002). Progesterone administered during the follicular
phase has been reported to attenuate the subjective response to repeated self-administered
cocaine (Evans et al. 2002). Collectively, these studies suggest that estradiol potentiates
while progesterone attenuates the subjective effects of cocaine.

**Sex differences in the effects of psychostimulants – preclinical studies**

Rodent studies also show sex differences in cocaine-induced behavioral activity.
Overall, female rats have greater locomotor and stereotypic behavior than male rats after
acute cocaine administration (Chin et al. 2002; Festa et al. 2004; Sell et al. 2000; van
Haaren and Meyer 1991; Walker et al. 2001). In terms of behavioral sensitization, the
progressive increase in motor behaviors after repeated cocaine injections, females exhibit
more robust sensitization after fewer cocaine injections than do males (Chin et al. 2002;
Glick et al. 1983; van Haaren and Meyer 1991). Female rats were also more sensitive to
the rewarding effects of cocaine through acquisition of conditioned place preference at lower doses and at shorter conditioning lengths (Russo et al. 2003). Similarly, female rats acquired cocaine self-administration faster than did males and at lower cocaine doses (Lynch and Carroll 1999). These results from rodent studies accurately reflect the overall sex differences in cocaine craving and intake patterns reported in the clinical literature. Similar to humans, gonadal hormones play a key role in modulating behavioral responses to cocaine in rodents. For instance, it was reported that hyperactivity induced by cocaine was greatest in female rats during proestrus (when estrogen levels peak) and estrus (when estrogen levels rapidly decline) (Sell et al. 2000). Ovariectomized (OVX) female rats treated with estrogen, or estrogen and progesterone showed enhanced acute behavioral responsiveness, sensitization to cocaine, and cocaine self-administration when they were compared to OVX rats treated with progesterone alone or vehicle (Hu et al. 2004; Lynch et al. 2001; Sell et al. 2000). In summary, in both humans and rodents, estrogen appears to play a role in the enhanced responsiveness of psychostimulants in females.

There is considerable preclinical evidence that sex and hormones affect neurotransmission in the mesolimbic and striatal dopaminergic system (Becker and Rudick 1999; Becker et al. 2001) that most likely contribute the sex differences in the behavioral response to psychomotor stimulants discussed above. Basal dopamine release and uptake in the striatum is higher in female than male rats (Walker et al. 2000) suggesting that there are functional sex differences in striatal dopaminergic transmission. Amphetamine-induced DA release is also greater in the striatum and NAC of female than male rats (Becker and Ramirez 1981; Becker and Rudick 1999; Castner et al. 1993). Administration of physiological concentrations of estradiol to striatal slices directly
stimulates DA release in vitro (Becker 1990). Furthermore, amphetamine-stimulated
dopamine release is greatest during the estrus phase of the cycle, a time when the
behavioral response is also greatest (Becker and Cha 1989). Together, the data suggest
that estradiol exerts its effects on dopaminergic systems, important circuitry for the
addictive properties of psychostimulants.

**Behavioral sensitization**

The behavioral sensitization paradigm as an animal model of addiction was a
major focus of the present thesis. Behavioral sensitization refers to the progressive and
enduring augmentation of behavioral responses to psychomotor stimulants that develops
during their repeated administration (Kalivas and Stewart 1991; Robinson and Becker
1986). This repeated administration produces gradual and incremental neuroadaptations
that render the animals hypersensitive to these agents. Behavioral sensitization is a robust
phenomenon that has been observed across several species. The mechanisms of neural
sensitization are thought to exhibit two distinct temporal domains: 1) the induction/
initiation, and 2) the expression of sensitization (Pierce and Kalivas 1997). The induction
of behavioral sensitization is defined as the transient sequence of cellular and molecular
events precipitated by the psychostimulant that lead to the enduring changes in neuronal
function responsible for behavioral augmentation. Induction of behavioral sensitization to
psychostimulants occurs in the ventral tegmental area (Cador et al. 1995; Hooks et al.
defined as the neuronal alteration arising from the initiating process that mediates the
augmented behavioral response and is mediated by the nucleus accumbens (Cador et al.
1995; Paulson and Robinson 1991; Perugini and Vezina 1994). Anatomical separation of
sites for initiation and expression implies ‘transfer’ of sensitization from VTA to NAC, presumably as a result of changes in the firing rate or pattern of mesoaccumbens DA neurons. Although sensitization is in essence a non-associative learning process, the context plays a major role in the development and expression of locomotor sensitization (Robinson and Berridge 2003). Thus, a higher degree of sensitization is usually observed when drug injections are administered in a context different from that of the home cage and very often subjects will show sensitization to the context when administered saline where they had previously received drug.

Although sensitization has been shown in humans (Robinson and Badiani 1998), most studies have focused on sensitization of locomotor activity in rodents. There is compelling evidence that behavioral sensitization provides a useful model for sensitization of drug craving. Both depend on VTA dopamine neurons projecting to limbic and cortical regions. Prior exposure to cocaine or amphetamine, resulting in behavioral sensitization, promotes drug self-administration and enhances stimulus-reward learning and responding for conditioned reward (Everitt and Wolf 2002). Finally, both sensitization in rats and drug craving in humans are strongly modulated by environmental stimuli, conditioning, and stress (Paulson et al. 1991; Shaham et al. 2000). Once established, sensitization is very long-lasting; amphetamine sensitization can last up to a year in rats, a species that lives about two years (Wolf 1998). Furthermore, behavioral sensitization is accompanied by dramatic changes in addiction related neuronal circuits at molecular, cellular, and systems levels (Wolf 1998). In a recent review, Robinson and Berridge proposed several specific hypotheses regarding the role of sensitization in establishing the neural and behavioral bases of drug craving that constitute the
“Incentive-Sensitization Theory of Addiction” (Berridge and Robinson 1998; Robinson and Berridge 2003). According to this theory, addictive drugs produce long-lasting adaptations in brain systems mediating incentive-motivational effects, leading to sensitization of these systems to drugs and, importantly, to cues that trigger pursuit of rewarding stimuli (Zhang et al. 1997). This ‘incentive sensitization’ has been proposed as a possible mechanism in explaining the transition from a regular pattern of voluntary drug intake to compulsive drug-seeking and -taking behavior (Berridge and Robinson 1998; Robinson and Berridge 2003). Basically, in the terms often used by these authors, drug addiction can be seen as a product of the sensitization in the drug ‘wanting’ with no change (or a decrease) of the drug ‘liking’.

**Conditioned place preference**

The conditioned place preference is a popular method that provides unique information about the rewarding/reinforcing effects of drugs of abuse (Carr et al. 1988; Schechter and Meehan 1993; Tzschentke 1998). In the conditioned place preference procedures, the drug’s effects (which serve as an unconditioned stimulus; US) and a previously neutral stimulus (context) are repeatedly paired. The US (drug) is administered contingent to the occurrence of an event (i.e. presence/absence of the stimuli). Through this process, which is thought to be Pavlovian in nature, this stimulus acquires the ability to act as a conditioned stimulus (CS). Thereafter, if the drug is found rewarding or aversive, the CS will be able to elicit approach or avoidance behavior, respectively. Therefore, CPP is unique in that it measures the learning processes about the rewarding effect of contextual cues associated with a drug stimulus.
Another advantage of CPP is that different phases of the addiction cycle (abstinence and relapse) can be modeled in CPP studies by examining the maintenance, extinction, and reinstatement of the conditioned response. Some of these procedures have already been used in rodents and it has been demonstrated that drug priming injections (Itzhak and Martin 2002; Kuzmin et al. 2003) can reinstate extinguished drug-seeking behavior in a CPP paradigm. Thus, habit formation, cue-reactivity, extinction and reinstatement of conditioned behavior are important elements of addictive behavior that are quantifiable in CPP experiments. Finally, another advantage of CPP is that it has considerable utility in probing the neural circuits involved in drug reward. Notably CPP involves learning and memory processes and substrates (e.g., amygdala, hippocampus, striatum, and nucleus accumbens) that overlap at least partially with those associated with the effects of drugs of abuse (McDonald and White 1993). Thus, I posit that the CPP paradigm is particularly relevant for the investigation of learning and memory processes involved in drug addiction.

**Research objectives and hypothesis**

Clinical and pre-clinical studies have revealed that certain populations, specifically females and adolescents, may display unique vulnerabilities to the effects of psychostimulants both at the behavioral and neurochemical levels (Roth and Carroll 2004a; Spear 2000; Spear and Brake 1983). The heightened vulnerability characteristic of adolescence is thought to be a result of the reorganization of the dopaminergic systems in the brain during this period. In females, the enhanced susceptibility to the addictive properties of cocaine has been attributed to the modulation of dopaminergic function by gonadal hormones (Walker et al. 2006). Given the extensive interactions between NO
and DA systems, the primary goals of this thesis are to investigate the roles of nNOS, ontogeny, and gender in the rewarding and psychomotor stimulating effects of cocaine using the conditioned place preference and behavioral sensitization paradigms, respectively. Adaptations of nNOS-immunoreactive (ir) neurons and TH-ir neurons will be explored as potential neural mechanisms contributing to variations at the behavioral level. I hypothesize that nNOS contributes to cocaine conditioned place preference and behavioral sensitization in an ontogeny- and sex-dependent manner. I further hypothesize that the neuroadaptations contributing to behavioral sensitization include the overexpression of nitrergic neurons after repeated cocaine exposure and their modulation of dopaminergic neurons in the mesolimbic and corticostriatal regions. These neuroadaptations may also occur in an ontogeny-dependent manner.

In Chapter 2 of this thesis, the roles of nNOS, ontogeny, and gender on the rewarding effects of cocaine and subsequent drug-seeking behavior are investigated using the CPP paradigm. Specifically, the acquisition, maintenance, extinction, and reinstatement of conditioned response to the reward stimulus are investigated. Results from this chapter were published in the following paper:


In Chapter 3 the roles of nNOS and ontogeny on the development and short- and long-term persistence of cocaine-induced behavioral sensitization are investigated in male mice. I also explore the influence of ontogeny on cocaine-induced
neuroadaptations in the nitrergic neurons in mesolimbic and striatal regions as a neural correlate to behavioral sensitization. Results from this chapter were published in the following paper:


In Chapter 4 I investigate the effects of cocaine treatment, the nNOS gene, and ontogeny on mesolimbic and striatal dopaminergic neurons as additional neural correlates to behavioral sensitization. A portion of the results from this chapter were published in the following paper.


In Chapter 5 the roles of nNOS and ontogeny in the development and short- and long-term persistence of cocaine-induced behavioral sensitization are investigated in female mice. Results from this chapter were published in the following paper:

Finally, Chapter 6 is a general discussion and review of the central findings in this thesis. The significance of my findings is discussed in the context of current addiction research. Lastly, future lines of research stemming from my current findings are discussed.
Figure 1.1. Key neural circuits implicated in addiction (Koob et al., 1992). Projections of the mesolimbic dopamine system that are thought to be a crucial substrate for drug reward are shown in red: this system originates in the ventral tegmental area (VTA) and projects to the nucleus accumbens (NAC) and other limbic structures, including the prefrontal cortex (PFC) and ventral domains of the caudate-putamen (C-P). Abbreviations: ARC, arcuate nucleus; Cer, cerebellum; DMT, dorsomedial thalamus; IC, inferior colliculus; LC, locus coeruleus; LH, lateral hypothalamus; PAG, periaqueductal gray; SC, superior colliculus; SNr, substantia nigra pars reticulata; VP, ventral pallidum.
Figure 1.2. Activation of neuronal nitric oxide synthase in the CNS. Release of glutamate stimulates NMDA receptors and causes an influx of Ca\(^{2+}\). Neuronal nitric oxide synthase is associated with the post-synaptic density protein (PSD-95) in the neuronal membrane. In response to increased intracellular Ca\(^{2+}\), nNOS interacts with CaM. The Ca\(^{2+}\)-CaM complex, in combination with BH4, binds to nNOS and induces its translocation from the plasma membrane to the cytoplasm. The dephosphorylation of nNOS by calcineurin initiates the production of NO. NO activates guanlyl cyclase (GC) and activates the various cGMP-regulated signaling pathways. nNOS is inactivated by phosphorylation by protein kinase A (PKA) or protein kinase C (PKC). Reproduced from http://www.sigmaaldrich.com.
Chapter 2

Adolescent and adult responsiveness to the incentive value of cocaine reward in mice: Role of neuronal nitric oxide synthase (nNOS) gene (Balda et al. 2006).

Summary

A major concern in adolescent psychostimulant abuse is the long-term consequence of this practice, because early drug exposure may cause long-term adaptations, which render the organism more susceptible to drug abuse later in life. The incentive value of drug and natural reward in rodents is commonly assessed by the conditioned place preference paradigm, which involves Pavlovian learning. The aims of the present study were to investigate: a) the acquisition, expression, maintenance and reinstatement of cocaine CPP from periadolescence (PD24–45) through adulthood (PD70); b) potential sexual dimorphism in adolescence and adulthood in response to cocaine-induced CPP; and c) the role of the nNOS gene in long-term neural plasticity underlying responsiveness to cocaine and cocaine-associated cues. Adolescent WT mice acquired significant cocaine (20 mg/kg) CPP that was maintained from PD24 through PD43. Upon extinction, CPP was reinstated in adulthood (PD70) following a priming injection of cocaine (5 mg/kg). In contrast, cocaine CPP acquired between PD26 and PD31 in adolescent nNOS KO mice, was neither maintained nor reinstated by cocaine. There was no sexual dimorphism in adolescent WT and KO mice. Genotype differences and sexual dimorphism were observed in adult mice. Cocaine CPP in adult WT males (PD89–94) was maintained for 4 weeks post training, and subsequently reinstated by cocaine priming; the magnitude of CPP in adult WT males was lower than in female counterparts. CPP in adult KO males (PD88–93) was neither maintained nor reinstated by cocaine priming; in contrast, CPP in adult KO females was not significantly different.
from adult WT females. Results suggest that the nNOS gene is essential during adolescence of both sexes for the development of long-term neural plasticity underlying responsiveness to the incentive value of cocaine reward. Sexual dimorphism in response to cocaine CPP emerges in adulthood; nNOS contribution to long-term plasticity is therefore sexually dimorphic and age-dependent in female but not in male subjects.

**Background**

Adolescence is a period characterized by increasing social behavior, risk-taking, and novelty seeking; the adaptive transition to adulthood may potentially contribute to a unique susceptibility to the abuse of drugs (Spear 2000). Animal and human studies demonstrated specific neural changes in certain brain regions during adolescence. Increase in PFC dopamine fiber density (Kalsbeek et al. 1988) and overproduction of striatal and PFC DA receptors in rats (Adriani and Laviola 2000; Andersen et al. 1997) and striatal DA receptors in humans (Seeman 1999) have been reported. Synaptic elimination of glutamatergic PFC afferents in adolescent humans (Huttenlocher 1984) and non-human primates (Zecevic et al. 1989) and reductions in glutamate and NMDA receptor binding in rat PFC (Guilarte and McGlothan 1998) have also been noted.

The corticostriatal and mesolimbic DA and glutamate systems hold major roles in the effects of psychostimulants, and exposure to such drugs during adolescence may interfere with ongoing neuroplastic events. The incentive value of drug and natural reward in rodents is commonly assessed by the conditioned place preference paradigm, which involves Pavlovian learning. Repeated pairing of a subject with unconditioned stimulus (US, e.g., psychostimulant) in a specific environment, to become conditioned stimulus (CS), elicits conditioned response (CR). Based on the CPP paradigm some
evidence suggests age-dependent changes in the incentive value of drug reward (Tirelli et al. 2003). For instance, in CD1 mice amphetamine CPP developed on PD14–28 but not PD30–49 (Adriani and Laviola 2003; Laviola et al. 1994). It is thought that enhanced DA activity in the PFC compared to the NAC during adolescence may render the organism less sensitive to psychostimulants (Spear 2000). However, cocaine CPP developed in mice (Laviola et al. 1992) and rats (Bolanos et al. 1996; Campbell et al. 2000) on PD21–34 as in adult subjects. Therefore, it is not entirely clear if the incentive value of psychostimulant reward changes from adolescence to adulthood. Most of the studies conducted thus far on the effect of abused substances on adolescent behavior have encompassed a wide adolescent age range (PD20 through PD35) and focused on relatively short-term consequences of drug exposure (e.g., one to two weeks following cessation of drug treatment). Hence, knowledge about the long-term consequences of adolescent exposure to psychostimulants is scarce. The first goal of the present study was to investigate: (a) the acquisition and expression of cocaine CPP during the periadolescent period (PD24–45); and (b) the vulnerability of the same subjects to cocaine challenge in adulthood (e.g., PD70). Additionally, given the relative paucity of studies on the effect of psychostimulants in female rodents, compared to studies in male rodents, the second goal was to investigate potential sex-dependent differences in response to cocaine CPP during adolescence and adulthood. The third goal was to investigate the role of nNOS in the neural plasticity underlying the acquisition, maintenance and reinstatement of cocaine CPP. The reinstatement of CPP following extinction is considered as a model for drug relapse.
In brain, nitric oxide is produced primarily by the conversion of L-arginine to L-citrulline via nNOS in response to stimulation of N-methyl-d-aspartate (NMDA) type of glutamate receptors (Garthwaite and Boulton 1995). The spliced variant nNOSα, which constitutes 95% of nNOS catalytic activity, is linked to the NR2B subunit of the NMDA receptors via a postsynaptic density protein (PSD95) (Brenman and Bredt 1997). NO has a major role in non-synaptic communication between glutamatergic and monoaminergic neurons (Kiss and Vizi 2001). NO exerts facilitatory influence on both tonic extracellular DA levels and phasic DA neuron spike activity (Grace 1991; West et al. 2002; West and Grace 2000). NO also facilitates glutamate release, and stimulation of glutamate receptors on dopaminergic nerve terminals may further stimulate DA release (Moore et al. 1999). In a series of studies we found that mice lacking the nNOS gene (nNOS KO) are less sensitive to the psychomotor, rewarding and neurotoxic effects of cocaine and methamphetamine, thus supporting the role of nNOS in the neural plasticity and neurotoxicity that underlie psychostimulants effects (Itzhak and Ali 2006; Itzhak et al. 2002).

Ontogeny studies of nNOS have shown that the expression of nNOS in the striatum and cerebellum nearly doubled between PD20 and 60 (Keilhoff et al. 1996) and that nNOS and tyrosine hydroxylase immunoreactive neurons appear within the same striatal patches at distinctive times during development (Murata and Masuko 2003). Therefore, nNOS may influence adolescent responsiveness to psychostimulants directly through intrinsic changes or via its effects on DA and glutamate systems which are in transition. Accordingly, the third goal of the present study was to investigate the role of nNOS in the various phases of cocaine CPP in adolescent and adult mice. Results suggest
that the nNOS gene is required in adolescence for the development of neuroadaptations that enable the maintenance and reinstatement of CPP, thus the nitrergic system may be critically involved in the development of persistent drug seeking behavior from adolescence through adulthood.

**Materials and Methods**

**Animals**

Adult male and female homozygote nNOS KO mice (B6;129S4-\(\text{Nos1}\); 6–8 weeks old) were purchased from Jackson Laboratories (Bar Harbor, Maine). These mice were generated on a mixed SV129 and C57BL/6 background as described previously (Huang et al. 1993). Because the breeding of nNOS KO mice that have been backcrossed to the C57BL/6 mouse strain was unsuccessful (personal communication with Jackson Laboratories and our own experience), B6;129SF2 mice (KO and WT) were used. Following the arrival of the nNOS KO mice to viral antibody free (VAF) facilities at the University of Miami Miller School of Medicine, animals were single-sex habituated to the new environment for 7–10 days before breeding. Routinely, each litter of newborns (6–8 mice) contained about equal numbers of males and females. Mice were weaned on PD21 and were housed in single-sex groups of 4–5 per cage. Behavioral testing of adolescent and adult KO mice began on PD26 and PD88, respectively. “Adolescence” in this context represents the periaadolescent period in mice and rats, which commences 7–10 days preceding the onset of puberty (PD38–40) and ends a few days thereafter (Spear and Brake 1983; Tirelli et al. 2003). Subsequently, each experimental group (\(n = 8–10\)) contained mice from 3–4 litters.
Adult SV129 males and C57BL/6 females (6–7 weeks old) were purchased from Jackson Laboratories. Following a habituation period, mice were bred to generate B6;129SF1 progeny. Following weaning (PD21), the mating of F1 × F1 offspring (>PD60) generated B6;129SF2 progeny (8–12 mice per litter). This latter progeny is considered the WT counterpart to nNOS KO mice, as it is congenic to the KO mouse strain (Itzhak et al. 1998a; Itzhak et al. 1998b; Itzhak et al. 1998c). WT mice were weaned on PD21 and segregated according to sex into groups of 4–5 per cage. Behavioral testing of adolescent and adult WT mice commenced on PD24 and PD89, respectively. Routinely, each experimental group (n = 8–10) contained mice from 3–4 litters. Since in the present study the KO and WT mice are considered inbred, the concern of the litter effect that pertains to outbred mice is insignificant. Nevertheless, a single group was comprised of no more than 3 mice from the same litter.

Animals were housed in a temperature- (22 ± 0.5 °C) and humidity- (50%) controlled room and maintained on a 12-h light/dark schedule with free access to food and water. Animal care was in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, National Academy Press, 1996) and was approved by the University of Miami Animal Care and Use Committee.

**Immunohistochemistry of nNOS**

Adult (PD92–94) WT and nNOS KO mice were anesthetized with a cocktail of ketamine (100 mg/kg) and xylazine (10 mg/kg). After loss of the foot-pinch response, mice were perfused transcardially via the left ventricle with sodium phosphate-buffered saline (PBS) followed by p-formaldehyde (4%) in PBS. The brain was removed and post-fixed overnight in the same fixative at 4 °C. Coronal sections (20 μm) were cut with a
Vibratome 1000 (TPI Inc., St. Louis, MO), collected in PBS and blocked overnight (4 °C) with normal goat serum (10%) in PB (0.1 M) containing Triton X-100 (0.3%). Sections were labeled with a rabbit polyclonal antibody to nNOS (1:750; Santa Cruz Biotechnology, CA). The antibody was diluted in a vehicle of normal goat serum (2%) with Triton X-100 (0.03%) in PB (0.1 M; 72 h; 4 °C). Sections were rinsed in PBS (2 × 15 min) and incubated with Alexa Fluor® 594 chicken anti-rabbit IgG (1:200; 45 min; 22 °C). Sections were rinsed in PBS (2 × 10 min), mounted onto slides, and coverslipped with Gel/Mount containing anti-fade agents. The sections were analyzed by a fluorescence compound microscope (Olympus BX51; C. Squared Co., USA) equipped with a cooled monochrome camera (Retiga 2000R) and Image Pro Plus software.

**Conditioning apparatus**

Place preference was monitored by a conditioning apparatus, the Opto-Max Activity Meter v2.16 (Columbus Instruments, Columbus, OH). The CPP cage (Plexiglas; 42 × 20 × 20 cm) was separated by a removable guillotine door into two compartments, one comprising 4 black walls and a smooth black floor and the other 4 white walls and a floor covered with a beige sandpaper (fine grit 150C, Norton). Thus, the two compartments had different visual and tactile cues. During the habituation period, and subsequently during testing of CPP the guillotine door remained open; during each training session the guillotine door remained closed. The apparatus was covered with a transparent Plexiglas lid perforated to allow adequate ventilation. The cage was equipped with matching pairs of horizontal sensors mounted alongside opposing lengths (42 cm long). The black and white compartments (21 × 20 × 20 cm) were each scanned at a rate of 10 Hz by 7 infrared beams, spaced at 2.54 cm intervals. A null zone 8 cm wide was
assigned at the interface of the black and white zones and was monitored by 2 beams to ensure that only full entry into each compartment was registered as real time spent in each compartment. Information collected from sensors, e.g., time spent in each compartment and horizontal locomotor activity was recorded and analyzed by the Opto-Max interface and software.

**Conditioned place preference training**

Behavioral experiments were conducted between 09:00 to 16:00 h in a sound-attenuated room that is temperature- (22 ± 0.5 °C) and humidity- (50%) controlled, and different from the housing room. The conditioning experiments of WT and nNOS KO, adolescent and adult, male and female mice were all uniform and consisted of the following phases: (a) habituation to the CPP cages; (b) acquisition; (c) expression; (d) maintenance/extinction; and (e) reinstatement of CPP. On the first day, mice were habituated to the CPP cage for 20 min and time spent in each compartment was recorded. For the next four days animals underwent two training sessions a day, 30 min each: during the morning session one compartment was paired with saline injection (0.1 ml/10 g body weight) and in the afternoon session the opposite compartment was paired with cocaine-HCl (Sigma; St. Louis, MO) injection (20 mg/kg; IP). The day following the four training days, time spent in each compartment and horizontal locomotor activity was recorded for 20 min. Expression of CPP was determined by the difference in time spent in drug- and saline-paired compartments, in a drug-free state but after saline injection. Based on our previous experience, administration of saline just before testing enhances the expression of CPP. This procedure also allows differentiation
between expression of CPP in response to injection stimulus (which may acquire properties of CS) and drug US (e.g., reinstatement experiments).

Although in most CPP experiments, as in our own previous work (Achat-Mendes et al. 2003; Itzhak et al. 1998c), subjects undergo a single training session a day for eight days (i.e., four-drug and four-saline sessions), the current experimental design was shorter (four days instead of eight) because the duration of periadolescence in mice is relatively short. Accordingly, the current design allowed us to investigate first, the acquisition, expression and maintenance of CPP during periadolescence (PD24–26 through PD43–45) and second, the reinstatement of CPP in adulthood (PD70). For uniformity, adult mice were trained in the same manner as adolescent mice.

Subsequent to the CPP expression test, mice were retested weekly for 4 weeks immediately after saline administration. The repeated testing allowed us to examine: (a) the maintenance; and (b) the extinction of CPP. Following extinction, reinstatement of CPP was determined by a single administration of priming injection of cocaine (5 mg/kg) just prior to testing. If reinstatement occurred, maintenance of CPP was retested after 7–10 days, immediately after saline administration.

During the pretraining phase all mice tested showed some degree of preference either to the black or the white compartment of the CPP cage. Analyses of the results showed that the preference for either the black or the white compartment was independent of: (a) age; (b) sex; and (c) genotype (i.e., nNOS KO and WT mice). This random pattern of pretraining preference in WT and KO mice was observed in several preliminary experiments, which included one or two pretraining habituation sessions (the latter were performed on two separate days), and habituation sessions in the absence and
presence of the sandpaper in the white compartment. The findings suggest inherent and persistent random preference in both WT and nNOS KO mice. Given that in a group of 8–10 mice of each age, sex and genotype there was a random 50% preference to one of the two cage compartments, in each experiment 50% of the subjects of each group were paired with cocaine in the least-preferred compartment (black and white). This training is neither entirely “biased” (e.g., pairing all subjects with the drug in a single least-preferred compartment) nor is it entirely “unbiased” (e.g., subjects do not show initial preference, and half of the subjects are paired with drug in each compartment). This experimental design was consistent in all groups and therefore it is unlikely that differences observed between specific groups during posttraining tests were influenced by this specific experimental design.

Data Analysis

Results of CPP experiments are presented as the mean ± SEM difference in time (seconds) spent in drug- and saline-paired compartments. Each experimental group consisted \( n = 8–10 \). Data of adolescent CPP experiments correspond to a 10-min period and data of adult CPP experiments correspond to a 20-min period. This difference is due to the finding that results of 20-min adolescent CPP were less consistent compared to the first 10-min interval. Therefore, the magnitude of CPP in adolescent and adult mice was not comparable. CPP for each age-group was analyzed by two-way ANOVA: sex \( \times \) time (time as a repeated measure) and post-hoc Bonferroni tests. Time in this context represents the magnitude of CPP (time in drug-paired minus time in saline-paired compartments) pre-CPP and thereafter at various time-intervals post-CPP. In addition, results of individual groups were analyzed by one-way repeated ANOVA with post-hoc
Bonferroni test. Routinely results of one-way repeated ANOVA confirmed the two-way ANOVA. Results of locomotor activity recorded during the first test for CPP expression are expressed as mean ± SEM ambulatory counts recorded for 10 min (adolescent) or 20 min (adults) and were analyzed by two-way ANOVA: genotype × sex, and post-hoc Bonferroni tests. A $P$ value of less than 0.05 was considered as statistically significant.

Results

Immunohistochemistry of striatal nNOS

Immunostaining of nNOS was performed in order to verify that KO mice lacked the enzyme. Fig. 2.1 shows representative images of striatal neurons of WT and KO adult male mice (PD92–94) that stain positively and negatively for nNOS, respectively. The intensity of nNOS immunoreactive neurons in dorsal regions of the striatum was higher than in other brain regions (VTA, amygdala and frontal cortex; data not shown) of WT mice. Additional studies in adult females and adolescent mice (PD25–PD35) yielded similar results, confirming the presence and absence of nNOS in WT and KO mice, respectively.

WT adolescent CPP

WT adolescent CPP experiments were initiated on PD24 (males and females) and the first test was on PD29. Thereafter, mice were retested for CPP maintenance until adulthood (PD60). Reinstatement of CPP by cocaine priming was tested on PD70, and maintenance of the reinstated conditioned response was tested on PD77 (Fig. 2.2A). Analysis of CPP in WT adolescent males and females by two-way ANOVA (sex × time) showed an overall significant time effect $F[6,112] = 6.37; P < 0.0001$, but a non-significant sex effect $F[1,112] = 0.01; P = 0.91$. 
Individual differences between the magnitude of posttraining CPP at various time points compared to pretraining place preference and sex-dependent differences were analyzed by group-specific two-way ANOVA and Bonferroni post hoc tests. On PD29 there was a significant difference between results of pre- and post-CPP (Fig. 2.2A) in both males (253 s; \( P < 0.01 \)) and females (454 s; \( P < 0.001 \)) demonstrating the development of CPP in both sexes; the difference in the magnitude of CPP in males and females was not statistically significant. The 2nd test on PD36 revealed that CPP was maintained in males (241 s; \( P < 0.05 \)) and females (387 s; \( P < 0.001 \)). The 3rd test on PD43 showed that CPP was maintained in females (358 s; \( P < 0.001 \)) but not males (70 s; \( P > 0.05 \)). The 4th test on PD60 showed no significant CPP in both WT males and females, suggesting extinction of CPP. A priming cocaine injection (5 mg/kg) on PD70 reinstated significant CPP in both males (244 s; \( P < 0.05 \)) and females (443 s; \( P < 0.001 \)). Notably, the magnitude of CPP after cocaine priming (PD70) was not significantly different from that observed on PD29, i.e., the 1st CPP test (Fig. 2.2A). One week after cocaine priming, on PD77, CPP was no longer apparent in either sex. Separate analyses of the data from male and female subjects by one-way repeated ANOVA showed the same results, thus confirming the results of the two-way ANOVA. Overall, results suggest no significant differences between male and female WT adolescents in the induction, expression, and maintenance of CPP (except on PD43); this pattern was preserved through adulthood in the reinstatement of CPP.

**nNOS KO adolescent CPP**

nNOS KO adolescent CPP experiments commenced on PD26 (males and females) and the first test was on PD31. Thereafter, mice were retested for CPP maintenance until
adulthood (PD60). Reinstatement of CPP by cocaine priming was tested on PD70 (Fig. 2.2B). Analysis of CPP in KO adolescent males and females by two-way ANOVA (sex × time) showed a significant time effect $F[5,104] = 5.39; P < 0.001$, but a non-significant sex effect $F[1,104] = 0.02; P = 0.88$.

Individual differences between the magnitude of posttraining CPP at various time points compared to pretraining place preference and sex-dependent differences were analyzed by group-specific two-way ANOVA and Bonferroni post hoc tests. In the 1st test (PD31) there was a significant difference between results of pre- and post-CPP (Fig. 2.2B) in both males (582 s; $P < 0.001$) and females (268 s; $P < 0.05$), demonstrating the development of CPP in both sexes; the difference in the magnitude of CPP in males and females did not reach statistical significance. Subsequent retesting on PD38, PD45 and PD60 revealed that CPP was extinguished, as the differences between pre- and post-CPP were non-significant (Fig. 2.2B). A priming cocaine injection (5 mg/kg) on PD70, which reinstated CPP in WT mice, had no significant effect on KO mice (male and female; Fig. 2.2B). Given that the adolescent KO mice failed to show reinstatement, maintenance of the conditioned response one week later was omitted. The major differences between adolescent nNOS KO and WT mice are in: (a) the maintenance; and (b) the reinstatement of CPP.

**Adolescents' locomotor activity**

To determine whether motor behavior might have influenced CPP results, locomotor activity in a drug-free state during CPP testing was simultaneously recorded for 10 min. Results in Fig. 2.3A represent ambulatory counts recorded during the first CPP test (WT: PD29; KO: PD31). Data were analyzed by two-way ANOVA
(genotype × sex). There was a non-significant genotype effect $F[1,33] = 1.534; P = 0.224$ and a non-significant sex effect $F[1,33] = 2.058; P = 0.160$. Ambulatory counts recorded in subsequent CPP tests in a drug-free state were similar to results presented in Fig. 2.3A. These findings suggest that the differences between WT and KO mice in CPP cannot be attributed to variations in motor behavior during testing.

**WT Adult CPP**

WT adult CPP experiments were initiated on PD89 (males and females); thereafter, expression, maintenance and reinstatement of CPP were investigated through PD137 (a total of 48 days). Analysis of CPP in WT adult males and females by two-way ANOVA (sex × time) showed a significant time effect $F[6,75] = 11.26; P < 0.0001$ and a significant sex effect $F[1,75] = 4.29; P = 0.041$.

Individual differences between the magnitude of posttraining CPP at various time points compared to pretraining place preference and sex-dependent differences were analyzed by group-specific two-way ANOVA and Bonferroni post hoc tests. In the 1st test (PD94; Fig. 2.4A), significant differences between results of pre- and post-CPP in both males (536 s; $P < 0.01$) and females (846 s; $P < 0.001$) were observed as well as a significant sex-dependent difference in the magnitude of CPP $F[1,24] = 5.138; P = 0.032$.

In the 2nd test (PD103), significant differences between results of pre- and post-CPP in males (460 s; $P < 0.001$) and females (747 s; $P < 0.001$) were observed as well as a sex-dependent difference $F[1,24] = 8.274; P = 0.0088$. In the 3rd test (PD114), significant differences between results of pre- and post-CPP in males (485 s; $P < 0.01$) and females (489 s; $P < 0.001$) were observed, but sex-dependent difference was non-significant ($P > 0.05$). In the 4th test (PD121), significant differences between results of pre- and
post-CPP in males (384 s; $P < 0.05$) and females (322 s; $P < 0.05$) were observed, but sex-dependent difference was non-significant. Results suggest that CPP was maintained in both sexes for a period of 27 days posttraining. However, the significant decrease in CPP magnitude in female WT mice on the 4th test compared to the 1st test ($P < 0.01$) suggests partial extinction of CPP (Fig. 2.4A).

A priming injection of cocaine (5 mg/kg) given 34 days posttraining resulted in significant CPP in males (561 s; $P < 0.001$) and females (738 s; $P < 0.001$); sex-dependent difference was non-significant ($P > 0.05$). Retesting one week after cocaine priming showed significant CPP in males (442 s; $P < 0.01$) and females (403 s; $P < 0.01$), suggesting that drug priming caused not only reinstatement but also preservation of the conditioned response (Fig. 2.4A).

**nNOS KO Adult CPP**

KO adult CPP experiments were initiated on PD88 (males and females); thereafter, expression, maintenance and reinstatement of CPP were investigated through PD134 (a total of 46 days). Analysis of CPP in KO adult males and females by two-way ANOVA (sex $\times$ time) showed a significant time effect $F[5,72] = 11.85; P < 0.0001$, a significant sex effect $F[1,72] = 5.79; P = 0.0187$ and a significant interaction $F[5,72] = 2.827; P = 0.021$.

Individual differences between the magnitude of posttraining CPP at various time points compared to pretraining place preference and sex-dependent differences were analyzed by group-specific two-way ANOVA and Bonferroni post hoc tests. In the 1st test (PD93), significant differences between results of pre- and post-CPP in both males (646 s; $P < 0.01$) and females (657 s; $P < 0.001$) were observed, suggesting the
development of CPP in both sexes (Fig. 2.4B). In the 2nd and 3rd tests, significant differences between results of pre- and post-CPP in females (491 s; \( P < 0.001 \) and 367 s; \( P < 0.05 \), respectively), but not in males, were observed. In the 4th test (PD120), no significant CPP was observed in either sex. Results show that in contrast to WT adult males, CPP in KO adult males was extinguished after the 1st test, similar to the results from KO adolescent males and females.

Cocaine priming (5 mg/kg) given 34 days posttraining resulted in significant CPP in KO females (689 s; \( P < 0.001 \)) but not in KO males (65 s; \( P > 0.05 \)) (Fig. 2.4B). Two-way ANOVA showed a significant drug effect \( F[1,23] = 14.09; P = 0.001 \), a significant sex effect \( F[1,23] = 5.775; P = 0.024 \) and a significant interaction \( F[1,23] = 9.60; P = 0.0051 \). Thus, in contrast to WT adult males, cocaine priming did not reinstate conditioned response in KO adult males, but it did in both WT and KO adult females. Retesting the KO females one week after cocaine prime showed significant CPP (370 s; \( P < 0.05 \)) (Fig. 2.4B), suggesting that conditioned response was maintained after cocaine prime, similar to the results of WT adult females. Retesting the KO adult males was not performed because of the absence of CPP reinstatement in this group.

**Adults’ locomotor activity**

To determine whether motor behavior might have influenced CPP results, locomotor activity in a drug-free state during CPP testing was simultaneously recorded for 20 min. Results in Fig. 2.3B represent ambulatory counts recorded during the first CPP test (WT: PD94; KO: PD93). Data were analyzed by two-way ANOVA (genotype × sex). There was a non-significant genotype effect \( F[1,34] = 0.809; P = 0.374 \) and a non-significant sex effect \( F[1,34] = 0.332; P = 0.567 \). Results of ambulatory counts
recorded in subsequent CPP tests in a drug-free state were similar to results presented in Fig. 2.3B. These findings suggest that the differences in the results of CPP cannot be attributed to variations in motor behavior during testing.

**Discussion**

The aims of the present study were to investigate: (a) the acquisition, expression, maintenance and reinstatement of cocaine CPP from periadolescence (PD24–45) through adulthood (PD70); (b) potential sexual dimorphism during adolescence and adulthood in the response to cocaine-induced CPP; and c) the role of the nNOS gene in the neural plasticity that underlie the acquisition, expression, maintenance and reinstatement of cocaine CPP.

In adolescent WT mice no significant sex-dependent differences in the acquisition, expression, and maintenance of cocaine CPP were observed. The only exception was that WT adolescent females maintained CPP 1 week longer than their male counterparts. In both sexes, however, CPP was extinguished following the second week of posttraining (PD43; Fig. 2.2A). The finding that WT adult females showed a greater CPP magnitude than their male counterparts (Fig. 2.4A), suggests that sexual dimorphism in response to cocaine in adulthood may be due to post-puberty sex-dependent hormonal changes.

One of the major findings of the present study is that exposure to cocaine during adolescence renders the organism sensitive to a priming injection of cocaine in adulthood (PD70). Notably, the magnitude of CPP induced by the low dose of cocaine (5 mg/kg) on PD70 was similar to the magnitude of CPP that developed in the same mice on PD29 (Fig. 2.2A). These findings corroborate our previous studies which showed that exposure
of adolescent Swiss Webster mice to other psychostimulants, such as methylphenidate and MDMA, caused sensitized responses to: (a) reinstatement of cocaine CPP; and (b) cocaine-induced psychomotor stimulation in adulthood compared to control mice that received saline in adolescence (Achat-Mendes et al. 2003). Taken together these findings imply the development of long-lasting neural adaptations from adolescence through adulthood that promote high vulnerability to cocaine priming. Given that the paradigm of CPP reinstatement is relevant to drug relapse, the results suggest that exposure to cocaine during adolescence facilitates susceptibility to future drug relapse in adulthood.

Major differences between adolescent WT and nNOS KO mice were observed. First, cocaine CPP in both male and females adolescent KO mice was extinguished following the first CPP test on PD31. Second, a priming injection of cocaine (5 mg/kg) did not reinstate CPP in either sex of KO mice. Notably, similar to adolescent WT mice, there was no significant sexual dimorphism in adolescent KO mice. The findings that CPP in adolescent KO mice was neither maintained nor reinstated suggest that the nNOS gene has a critical role, during adolescence, in neural plasticity that supports the persistence of conditioned response to cocaine and cocaine-associated cues. The differential response of adolescent WT and KO mice to cocaine CPP was not influenced by motor activity because results in Fig. 2.3A show that there was no significant difference in locomotor activity during CPP testing. Additionally, our previous studies have shown that cocaine pharmacokinetics in WT and nNOS KO mice are similar, suggesting that genotype-dependent differences in response to cocaine are not influenced by cocaine pharmacokinetics (Itzhak et al. 1998a).
The results of cocaine CPP in adult WT mice revealed sex-dependent differences, which were not observed in adolescent WT mice. Specifically, the magnitude of CPP in WT adult females was significantly higher than in their male counterparts (Fig. 2.4A). Several investigators reported sex-related differences in response to psychostimulants. Adult (Bowman and Kuhn 1996; Laviola et al. 1995; Walker et al. 2001) and adolescent (Cirulli and Laviola 2000) female rats were more sensitive to cocaine- and amphetamine-induced hyperactivity than males. Also, female rats self administer cocaine more readily than males; it was suggested that the sex differences are due to: (a) intrinsic sex differences in brain organization; and (b) the effects of circulating estradiol (Hu et al. 2004). Heightened basal striatal DA release and uptake in female compared to male rats (Walker et al. 2000) may reflect sexual dimorphism in striatal function and responsiveness to psychostimulants (Castner et al. 1993). Additional explanation of the sexual dimorphism in the response of WT adult mice to cocaine may be due in part to effects of sex hormones on NOS. Evidence suggests that estradiol increases mRNA expression and activity of nNOS and endothelial NOS (eNOS) in various brain regions of the rat including the preoptic area, hypothalamus and hippocampus (Chu and Faraci 2004; Grohe et al. 2004; Weiner et al. 1994); castration of rats also increased the number of nNOS positive neurons (Singh et al. 2000). In contrary, androgens decreased mRNA expression and the number of nNOS positive neurons (Singh et al. 2000). Thus, increased expression of CPP in adult female WT mice may be due in part to augmentation of NO production particularly in the hippocampus (Grohe et al. 2004), which has a major role in spatial learning of CPP.
Despite the sexual dimorphism in the expression of CPP, the conditioned response to cocaine-associated cues was maintained in both male and female adult WT mice 4 weeks following posttraining, with little evidence of extinction (Fig. 2.4A). This finding is different from results of adolescent WT mice in which CPP was extinguished following the second week of posttraining (Fig. 2.2A). Moreover, subsequent to reinstatement of CPP by cocaine priming, the conditioned response to drug-associated cues re-emerged in adult WT mice (PD137; Fig. 2.4A), but not in WT mice that were conditioned by cocaine in adolescence (PD77; Fig. 2.2A). Thus, the major difference between adults and adolescents conditioning by cocaine is that the conditioned stimulus (CS, e.g., drug-associated cues) has a greater influence on adults' behavior than adolescents' behavior. Given that learning and memory have a major role in the maintenance of conditioned response, this difference may be due in part to sub-optimal PFC function, which may weaken incentive learning and memory consolidation processes during adolescence compared to adulthood. Regardless, it appears that the responsiveness to drug unconditioned stimulus is the same in WT mice conditioned by cocaine either in adolescence or adulthood.

Results of cocaine CPP studies in adult nNOS KO mice revealed major sex-dependent differences. First, in KO males CPP was extinguished immediately after the first test, but in KO females CPP was maintained for 3 weeks posttraining (similar to WT adult females). Second, a priming injection of cocaine (5 mg/kg) had no effect on KO males but it reinstated CPP in adult KO females (similar to adult WT females). Thus, while the response of adult and adolescent KO males to cocaine CPP was the same, the response of adult and adolescent KO females was different. These findings suggest that in
male subjects, independently of age, the nNOS gene is required for long-term neural plasticity associated with responsiveness to cocaine and cocaine-related cues. However, in female subjects the role of nNOS in neural plasticity is age-dependent. Adolescent KO females (like their male counterparts) neither maintained CPP nor responded to cocaine priming. Adult KO females did not differ in their response to cocaine CPP from their WT counterparts, suggesting that neural adaptations in adult females may be nNOS-independent. The dichotomy between female and male KO mice is unclear, but the following are possible explanations: First, in adult KO females the stimulating effect of circulating gonadal hormones, estrogen and progesterone, on hippocampal eNOS and the sustained production of NO may improve cognitive performance (Grohe et al. 2004), and thus cocaine-conditioned behavior. Second, enhanced basal DA release and uptake in the striatum of female compared to male rats (Walker et al. 2000) may strengthen the incentive value of cocaine reward in females. Third, the estrous cycle, which causes a surge of estrogen and progesterone, was found to increase basal and amphetamine-stimulated DA in striatum and NAC of rats (Becker and Rudick 1999). Although adult females in the current study were conditioned by cocaine without monitoring of the estrous cycle, the influence of the latter on the strengthening of cocaine CPP cannot be ruled out. These processes in females may contribute in part to long-term neuroadaptations underlying responsiveness to cocaine and cocaine-associated cues, independently of nNOS function. Accordingly, nNOS contribution to long-term plasticity associated with the incentive value of cocaine reward is sexually dimorphic and age-dependent in females.
The results of the present study on cocaine CPP in adult KO mice differ from our previous study on cocaine CPP in adult nNOS KO mice (Itzhak et al. 1998c). In the latter study we reported that adult nNOS KO males and females did not develop CPP using the same training dose of cocaine (20 mg/kg). However, several major differences in the training procedures, the CPP apparatus, and the sources of the KO mice may have contributed to this apparent discrepancy: (a) The habituation period to the CPP cages in the present study was 1 day whereas in the previous study it was 2 days; extended habituation may cause “latent inhibition” which diminishes acquisition of conditioned response. (b) CPP training in the present study lasted for only 4 days but in the previous study it lasted for 8 days; the more “intense” training of the present study (two sessions a day) might have facilitated the conditioning process. (c) In the present study the first and last training sessions were saline and cocaine, respectively, whereas in the previous study the order of training sessions was reversed. Thus, in the present study CPP was tested 24 h after the last cocaine-session and in the previous study testing occurred 48 h after the last cocaine-session. (d) The current CPP apparatus (2 compartments) is different than the one previously used (3 compartments). (e) In the present study, routinely, half of the mice were paired with cocaine in the white compartment and the other half with the black compartment. In the previous study, all KO mice showed very strong preference to the black compartment and therefore they were all paired with cocaine in the white compartment; the initial biased preference to a single compartment (unlike the results of the present study) might have hindered the conditioning process with a single non-preferred compartment. (f) The source of the present KO mice we bred was Jackson Laboratories (see Section 2), while previously the KO mice we bred were obtained from
Dr. Paul Huang (Harvard Medical School, Charlestown, MA, USA). Overall, it appears that the training procedures and perhaps the sources of the initial generation of the KO mice may have significant effects particularly on the acquisition phase of Pavlovian conditioned behavior.

In summary, the present study demonstrates that conditioning by cocaine in the periadolescent period (PD24–45) has profound implications on susceptibility to cocaine priming in adulthood (PD70). Sexual dimorphism in response to cocaine conditioned place preference is apparent in adult but not in adolescent subjects, implicating post-puberty sex-dependent hormonal changes in this trend. The nNOS gene is essential for long-term neural plasticity underlying susceptibility to the incentive value of cocaine reward in male subjects (adolescent and adult). The nNOS gene, however, has a differential role in long-term plasticity in adolescent and adult females. In the latter it appears that nNOS-independent mechanisms may contribute to neural adaptations associated with the exposure to cocaine. Elucidation of the potential sex- and age-dependent differences in the expression of nNOS and dopamine containing neurons in mesocorticoliclimbic structures may provide insight into sexual dimorphism and age-related differences in response to psychostimulants such as cocaine.
Figure 2.1. Representative nNOS immunohistochemistry of coronal sections obtained from adult male mouse brain. A: nNOS-positive neurons in WT mice are localized in dorsal regions of the striatum. B: The absence of nNOS-positive neurons in striatum of KO mice. Bar represents 50 μm. Magnification: 20×. (Taken from Balda et al. 2006).
Figure 2.2. Induction, maintenance, extinction and reinstatement of cocaine CPP in adolescent wild-type (WT) and nNOS knockout (KO) mice. Adolescent male and female mice ($n = 9–10$ per group) were conditioned by cocaine (20 mg/kg) for 4 days and tested for CPP expression in a drug-free state 1, 8, 15 and 30–32 days posttraining. Results are presented as mean $\pm$ SEM of the difference in the time spent in the assigned drug-paired and saline-paired compartments pretraining (PRE), posttraining (POST), after cocaine priming (PRIME), and post-priming (POST). PD24 and 26 denote the age of the subjects on pretraining day. PD29 and 31 denote the 1st CPP test, PD36 and 38 denote the 2nd test, PD43 and 45 denote the 3rd test, PD60 denotes the 4th test, PD70 denotes testing of cocaine priming and PD77 denotes testing with saline injection post-cocaine priming. Note that while in WT male and female mice (panel A) CPP was maintained through PD36 and PD43, respectively, in KO male and female mice (panel B) CPP was extinguished. In adulthood, on PD70, a priming injection of cocaine (5 mg/kg) reinstated CPP in WT (panel A) but not in KO (panel B) males and females. *$P < 0.001$ (pre-CPP vs. post-CPP; see detailed statistics in text). (Taken from Balda et al. 2006).
Figure 2.3. Spontaneous locomotor activity of adolescent and adult, male and female, wild-type (WT) and nNOS knockout (KO) mice. Ambulatory counts of adolescent (A) and adult (B) mice were recorded for 10 and 20 min, respectively, during the test of conditioned place preference (CPP) and are presented as mean ± SEM. Analyses by two-way ANOVA (genotype × sex) revealed non-significant genotype-effect and non-significant sex-effect in both adolescent and adult subjects. (Taken from Balda et al. 2006).
Figure 2.4. Induction, maintenance, extinction and reinstatement of cocaine CPP in adult wild-type (WT) and nNOS knockout (KO) mice. Adult male and female mice ($n = 8–10$ per group) were conditioned by cocaine (20 mg/kg) for 4 days and tested for CPP expression in a drug-free state 1, 8–10, 20–21 and 28 days posttraining. Results are presented as mean ± SEM of the difference in the time spent in the assigned drug-paired and saline-paired compartments pretraining (PRE), posttraining (POST), after cocaine priming (PRIME), and post-priming (POST). PD88 and 89 denote the age of the subjects on pretraining day. PD93 and 94 denote the 1st CPP test, PD100 and 103 denote the 2nd test, PD112 and 114 the 3rd test, PD120 and 121 the 4th test, PD127 and 128 denote testing of cocaine priming, and PD134 and 137 denote testing post-cocaine priming. Note that while in WT males (panel A) CPP was maintained throughout the 28 days posttraining, in KO males (panel B) CPP was extinguished after the first test. WT females (panel A) maintained the expression of CPP throughout the 28 days posttraining, and KO females (panel B) throughout 20 days posttraining. A priming injection of cocaine (5 mg/kg) given 35 days posttraining reinstated CPP in WT males and females; CPP was maintained 9 days after cocaine priming (panel A). Cocaine priming had no significant
effect on KO adult males, but it reinstated CPP in KO adult females (panel B). Only the KO adult females were retested with saline 7 days later to assess maintenance of cocaine CPP. The major differences between WT and KO mice in the maintenance and reinstatement of CPP were gender-specific in adulthood. *$P < 0.001$ denotes comparisons between pre-CPP and post-CPP; *$P < 0.05$ denote comparisons between males and females (see detailed statistics in text). (Taken from Balda et al. 2006).
Chapter 3

Differential role of the nNOS gene in the development of behavioral sensitization to cocaine in adolescent and adult B6;129S mice (Balda et al. 2008).

Summary

Previous studies have suggested the involvement of nNOS in the development of behavioral sensitization to psychostimulants. Ontogeny-dependent differences in the response to psychostimulants have been reported. To investigate: a) the short- and long-term consequences of adolescent and adult cocaine exposure on behavioral sensitization, and b) the role of the nNOS gene in behavioral sensitization in adolescent and adult mice. Adolescent and adult WT and nNOS KO male mice received saline or cocaine (20mg/kg) for 5 days and then were challenged with cocaine (20mg/kg) after a drug-free period of either 10, 30, or 90 days. Locomotor activity was recorded by infrared beam interrupts. Using stereology and western blotting, nNOS immunoreactivity in the dorsal striatum, nucleus accumbens, and prefrontal cortex was quantified 24h and 10 days after repeated administration of cocaine. Repeated administration of cocaine to either WT or nNOS KO mice during adolescence resulted in locomotor sensitization which persisted into adulthood. WT but not KO adult mice developed long-term sensitization to cocaine. Twenty four hours after repeated cocaine administration there was a significant increase in the expression of nNOS-ir neurons and protein levels in the dorsal striatum of adult but not adolescent WT mice. The cocaine-induced upregulation in nNOS observed after a 24hr drug free period was transient and returned to pre-cocaine levels after 10 days. The nNOS gene is essential for the induction of behavioral sensitization to cocaine in adulthood but not in adolescence. The increased expression of nNOS-ir neurons in the dorsal striatum may underlie the induction of behavioral sensitization in
adulthood. Thus, the NO signaling pathway has an ontogeny-dependent role in the
neuroplasticity underlying cocaine behavioral sensitization.

**Background**

Cocaine is a potent psychostimulant that blocks dopamine reuptake and causes a
marked increase in extracellular DA (Ritz et al. 1987). Enhanced DA transmission in key
regions such as the nucleus accumbens, dorsal striatum, and prefrontal cortex is thought
to underlie behavioral sensitization to cocaine (Kalivas and Stewart 1991; Pierce and
Kalivas 1997). Behavioral sensitization refers to the phenomenon whereby repeated
administration of psychostimulants results in progressive augmentation of the initial
locomotor stimulant response to these drugs (Stewart and Badiani 1993). Once acquired,
behavioral sensitization is long-lasting, thus resembling long-term sensitivity to drugs
observed in human addicts (Kalivas and Duffy 1993). The neural and behavioral
plasticity caused by repeated cocaine administration are also dependent on glutamatergic
neurotransmission (Vanderschuren and Kalivas 2000). For instance, blockade of the
ionotropic glutamate receptor, the N-methyl-D-aspartate (NMDA) receptor, prevents the
development of behavioral sensitization to cocaine and amphetamine (Karler et al. 1989;
Pulvirenti et al. 1994; Wolf and Jeziorski 1993; Wolf et al. 1994).

Stimulation of the NMDA receptor opens channel pores that allow an influx of
calcium into the cell; binding of calcium to calmodulin activates the neuronal isoform of
nNOS which catalyzes the formation of NO from L-arginine and, subsequently, increases
the synthesis of cyclic GMP (Garthwaite 1991; Snyder 1992). The link between NMDA
receptor subunits and nNOS led to studies on the role of nNOS in the effects of
psychostimulants. Pharmacological blockade of nNOS attenuated cocaine- and
methamphetamine-induced behavioral sensitization (Itzhak 1997). Likewise, nNOS KO mice showed reduced behavioral sensitization to cocaine (Itzhak et al. 1998a). Recently, we reported that cocaine-induced conditioned place preference in male nNOS KO mice is short-lived and cannot be reinstated by cocaine priming (Balda et al. 2006; Itzhak and Anderson 2007). The hypothesis that NO plays a critical role in cocaine-induced behavioral plasticity is further supported by evidence describing multiple interactions between DA, glutamate, and nNOS in mesolimbic and corticostriatal circuits (Carlsson and Carlsson 1990; Kiss 2000).

Clinical and preclinical studies have shown that neuronal circuits, including the neural substrates of cocaine, continue to develop and mature during adolescence. Striatal DA receptors are several fold higher in adolescents than adults and undergo pruning prior to adulthood (Tarazi et al. 1999; Teicher et al. 1995). DA transporter density in the striatum increases from PD 25 through PD50, and then decreases continuously until old age (Moll et al. 2000). Developmental changes in the central nervous system may partially contribute to ontogeny-dependent differences in the behavioral effects of psychostimulants. For instance, adolescent rodents and rhesus monkeys are less sensitive to the acute effects of amphetamine and cocaine (Bolanos et al. 1998; Paule et al. 1998). Others have shown that adolescent rats, unlike their adult counterparts, did not sensitize to cocaine-induced hyperlocomotion (Collins and Izenwasser 2002). In other studies adolescent rats and mice developed locomotor sensitization to cocaine and amphetamine, but sensitization to stereotyped behavior was not observed (Laviola et al. 1999; Laviola et al. 1995). Interestingly, young adolescent rats appear to be more sensitive than adults to some of the behavioral alterations induced by a single high dose of cocaine (Caster et al.
2007). Also, cocaine administered in binge patterns resulted in an exaggerated behavioral response in adolescent rats (Caster et al. 2005). Some of the inconsistencies in the literature on adolescent response to psychostimulants could be due to differences in animal species, strains, schedule of drug administration, and behavioral endpoints measured.

The long-term consequences of adolescent exposure to psychostimulants on vulnerability to drugs in adulthood were not reported in the above studies. Hence, the lasting effects of adolescent drug exposure remain to be investigated. The aims of the present study were to investigate a) the short- and long-term consequences of adolescent exposure to cocaine on the development of behavioral sensitization and b) the role of the nNOS gene in the development of behavioral sensitization in adolescent and adult mice. We report that a) adolescent exposure to cocaine resulted in long-term sensitization to cocaine into adulthood, b) the nNOS gene is essential for the development of behavioral sensitization in adulthood, and c) increased expression of striatal nNOS may be associated with behavioral sensitization to cocaine in adulthood but not in adolescence.

**Materials and Methods**

**Animals**

Mice purchased from Jackson Laboratories (Bar Harbor, Maine) were bred in our facilities at the University of Miami, Miller School of Medicine, Miami, FL as we described previously (Balda et al. 2006). Both genotypes, WT and nNOS KO, were generated on a mixed B6;129S genetic background (Huang et al. 1993). Animals were housed in a temperature- (22±0.5°C) and humidity- (50%) controlled room and maintained on a 12-h light/dark schedule with free access to food and water. Animal care
was in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, National Academy Press, 1996) and approved by the University of Miami Animal Care and Use Committee.

**Schedule of cocaine administration**

Cocaine-HCl (Sigma, St. Louis, MO) was dissolved in 0.9% NaCl. All injections were given intraperitoneally (IP) in a volume of 0.1ml/10g weight. Animals’ weights were monitored daily, and behavioral experiments were carried out between 10:00 and 16:00 hours.

**Experiment 1. Cocaine behavioral sensitization in adolescent WT and nNOS KO mice**

Behavioral testing of adolescent mice began on PD28. “Adolescence” represents the periadolescent period in mice and rats, which commences 7-10 days preceding the onset of puberty (PD38-40) and ends a few days thereafter (Spear and Brake 1983). Adolescent male mice (PD28) were divided into 12 groups: 6 WT and 6 nNOS KO groups. For each genotype Group 1 (n=10) received cocaine (20mg/kg) for 5 days and a single cocaine challenge (20mg/kg) on day 15 after a 10-day drug free period (PD42). Group 2 (n=10) received cocaine for 5 days and a single cocaine challenge on day 35 after a 30-day drug free period (PD62). Group 3 (n=10) received cocaine for 5 days and a single cocaine challenge on day 95 after a 90-day drug free period (PD122). Groups 4-6 represent control groups for groups 1-3, respectively. Groups 4, 5 and 6 (n=9-10) received saline for 5 days and a single cocaine challenge (20mg/kg) either on day 15 (PD42), 35 (PD62) or 95 (PD122).
**Experiment 2. Cocaine behavioral sensitization in adult WT and nNOS KO mice**

Behavioral testing of adult WT and KO mice began on PD80. Adult male mice (PD80) were divided into 12 groups: 6 WT and 6 nNOS KO groups. For each genotype Group 1 (n=7) received cocaine (20mg/kg) for 5 days and a single cocaine challenge (20mg/kg) on day 15 after a 10-day drug free period (PD94). Group 2 (n=8) received cocaine for 5 days and a single cocaine challenge on day 35 after a 30-day drug free period (PD114). Group 3 (n=10) received cocaine for 5 days and a single cocaine challenge on day 95 after a 90-day drug free period (PD174). Groups 4-6 represent control groups for groups 1-3, respectively. Group 4, 5 and 6 (n=8-10) received saline for 5 days and a single cocaine challenge (20mg/kg) either on day 15 (PD94), 35 (PD114) or 95 (PD174).

Routinely mice were habituated to the test cage for 30 min, then saline and cocaine injections were administered and locomotor activity was recorded for 1h. The comparison between locomotor activity on day 1 and day 5 after cocaine administration determined the induction phase of behavioral sensitization. The response to cocaine challenge 10 days after discontinuation of saline/cocaine administration (day 15) was compared to a) the response to cocaine on day 5, and b) the response of saline controls that received cocaine challenge for the first time on day 15. This allowed us to determine the influence of a short (10 day) drug-free period on the expression of sensitization. The response to cocaine challenge after a 30 and 90 day drug-free period (day 35 and 95) was determined in different groups in order to avoid multiple challenge injections. This allowed us to determine the influence of extended drug-free periods (30 or 90 days) on the persistence of behavioral sensitization.
**Experiment 3: Context-dependent hyperlocomotion in adolescent and adult WT and nNOS KO mice**

Context-dependent hyperlocomotion was determined by measuring the intensity of locomotion induced by a saline injection given to saline- and cocaine- pretreated mice in the cage that had previously paired with saline or cocaine (Itzhak 1997). WT and KO adolescent (PD 28) and adult (PD 80) mice (n=8-10) were treated with saline and cocaine as described in experiments 1 and 2, and after 3 days received a saline injection and locomotor activity was recorded for 60 min.

**Measurement of locomotor activity**

The locomotor activity cages are standard transparent rectangular rodent cages (42 x 24 x 20 cm high), and locomotion is monitored by an activity meter (Opto-Varimex-Mini Model B; Columbus Instruments, Columbus, OH). The activity meter consists of an array of 15 infrared emitter/detector pairs, spaced at 2.65 cm intervals, measuring activity along a single axis of motion. Emitters and detectors are mounted alongside the length of the cage (42 cm). Both the total counts and the ambulatory counts are recorded and transferred by a counter interface to a computer. The Opto-Varimix-Mini Model B separates counts (beam interruptions) of total activity from counts that correspond to ambulatory (horizontal) activity. This is accomplished by memorizing the location of the last broken beam and blocking additional counts from being scored on the front panel counter until a different beam is broken. Subtraction of the ambulatory counts from the total counts provides an index of vertical activity. Based on our previous observations (Itzhak 1997) it appears that the fraction of nonambulatory counts (25-30% of total counts) increase or decrease in parallel to corresponding changes in ambulatory counts. Because of this relationship, only ambulatory counts are reported. Counts were registered
every 10 min for a total of 60 min, and results are presented as mean ± SEM cumulative horizontal counts recorded.

**Immunohistochemistry of nNOS**

Adult (PD80) and adolescent (PD28) WT male mice (n=3-4/group) were administered saline and cocaine (20mg/kg) for 5 days. Although the final purpose of these experiments was immunohistochemical analysis, locomotor activity was measured as previously described and results were pooled with those from experiments 1 and 2. The acute effect of cocaine on nNOS-ir neurons was investigated in groups that showed changes in nNOS-ir neurons following repeated cocaine administration. Twenty four hours or 10 days after the last saline or cocaine injection, mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg). After loss of the foot-pinch response, mice were perfused transcardially via the left ventricle with sodium phosphate-buffered saline (PBS) followed by p-formaldehyde (4%) in PBS. The brains were removed and post-fixed overnight in the same fixative at 4°C. Serial coronal sections (50 μm) were cut with a Vibratome 1000 (TPI Inc., St. Louis, MO), collected in PBS and blocked for 1h at room temperature with normal goat serum (10%) in PBS containing Triton X-100 (0.3%). Sections were then incubated (72h; 4°C) with a rabbit polyclonal antibody to nNOS (1:3000, Santa Cruz Biotechnology, CA) diluted in a vehicle of 2% normal goat serum with 0.3% Triton X-100 in PBS. After 3x10min washes the sections were then incubated for 1h at room temperature in biotinylated goat anti-rabbit IgG diluted 1:100 in vehicle. The signal was then amplified using the Vectastain Elite ABC immunoperoxidase kit (Vector Laboratories, Burlingame, CA) and visualized with 0.05% DAB and 0.02% H₂O₂ in 50mM Tris Buffer. Sections were then rinsed 2x5min in PBS,
mounted onto slides, and coverslipped. Negative control sections were treated in the same way as described, except that the primary antibody was omitted from the staining procedure.

**Two-dimensional cell counting**

In initial studies counts of nNOS -ir cells were made using Image-Pro Plus image analysis software with the aid of a cooled monochrome camera (Retiga 2000R) attached to a compound light microscope (Olympus BX51; C. Squared Co., USA). Sections were viewed at 20× magnification and the number of nNOS labeled cells was quantified in the dorsal striatum (dST). Counts of nNOS -ir neurons were made by an observer blind to the treatment. Counts were obtained from 6 sections of each brain. Cells were included only when nuclear and cellular profiles in the plane of section were clearly distinguished to avoid differences that could arise from sectioning at different points of the cell.

**Stereological analysis of nNOS**

To quantify the total number of nNOS -ir neurons in the dST and NAC, the optical fractionator method, a stereological technique, was used. The term “stereology” refers to an analysis that provides a three-dimensional interpretation of structures, based on the observations obtained from two-dimensional sections (Sterio 1984; Weibel 1981) by which the biases and inaccuracies associated with non-stereological methods are avoided (West and Gundersen 1990). The limitations associated with two-dimensional images are 1.) the appearance probability of objects in an image is related to their size, shape, and orientation and 2.) tissue distortions such as shrinkage are not accounted for. Thus, the optical fractionator method was used because the cell number estimate is not
affected by the volume of reference (dST and NAC) or the size of counted elements (neurons).

For cell counts, sections were stained with DAB as the chromogen. Ten coronal sections (50 μm) were selected, taken at equally spaced intervals (100μm) through the full rostrocaudal extent of the striatum and analyzed using a Zeiss Axiophot microscope equipped with software that includes an optical fractionator probe (StereoInvestigator, Microbrightfield). To perform cell number estimation in the structure volume, the optical fractionator method and optical disector probe were used. Dimensions of the optical disector were designed based upon the cell distribution in the section, and optical fractionator grid size was determined based upon the results of preliminary counts of the naive brain sample to allow 100-200 counts per region of the striatum. Thus, the standard counting method used was a counting frame (dissector; 65μm x 65μm) and fractionator (150μm x 150μm) to meet the high standards of systematic random sampling. The appropriateness of the sampling scheme chosen was evaluated by calculating the precision of the estimates in each animal, expressed as the coefficient of error (West and Gundersen 1990). In all cases, the coefficient of error was less than 0.10, suggesting that a minimal amount of variance in the counts can be attributed to the technique.

**Western blotting experiments of nNOS**

Striatum and frontal cortex were microdissected and homogenized twice in 100 volumes of ice cold Buffer 1 (50mM Tris-HCl pH7.5, 5mM EDTA, 150 mM NaCl, 0.5% P-40 supplemented with 1mM PMSF, 1ug/ml pepstatin, 1ug/ml aprotinin, and 2ug/ml leupeptin) and incubated for 15 minutes. The homogenate was centrifuged and the supernatants collected. Protein concentrations were determined using the Lowry protein
assay with bovine serum albumin as a standard. Proteins were separated using 7.5% SDS-PAGE and transferred to PDVF membranes. Membranes were incubated in blocking buffer (5% nonfat dry milk in Tris buffered saline (TBS) [20mM Tris-HCl (pH 7.4), and 150mM NaCl]) containing 0.1% Tween-20 (TBS-T) for 1h at RT. Membranes were incubated with a rabbit polyclonal antibody to nNOS (1:250, Santa Cruz Biotechnology, CA) and a mouse monoclonal to β-tubulin (1:15,000, Upstate, NY) diluted in TBS-Tween. Membranes were then washed 30 minutes with 3 intermediate changes of TBS-T. The blots were then incubated (1hr at RT) with secondary antibodies, horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse at dilutions of 1:2000 and washed with TBS 3x10minutes. Visualization of the signal was enhanced by chemiluminescence using a Phototope-HRP Detection Kit (Cell Signaling). Quantification of bands corresponding to changes in protein levels was made using UN-SCAN-IT gel quantifying software (Silk Scientific Inc, Utah). Band densities were then analyzed by a Student t-test.

Data Analysis

Statistical analysis was performed with SPSS 16 software. Locomotor activity from the acute effect of saline and cocaine and the context-dependent sensitization studies were analyzed using a three-way ANOVA (treatment × age × genotype). Two-way ANOVA was used to analyze results of cocaine-induced behavioral sensitization studies (treatment x time) and the cell counts from the stereology experiments (treatment x age). Post hoc analysis using Bonferroni correction was used when warranted to determine differences between multiple groups. nNOS protein levels in saline- and cocaine-treated mice from Western blotting experiments were compared using unpaired,
two-tailed Student’s $t$ test. $P$ values less than 0.05 were considered significant for all
tests. Data are expressed as ±SEM.

**Results**

Results in Fig. 3.1 show the acute effect of saline and cocaine (20mg/kg) on
locomotor activity in WT and KO adolescent (PD28) and adult (PD80) mice. Three-way
ANOVA (treatment × genotype × age) revealed a significant treatment effect $F(1,145)=
212.38; p<0.0001$, a significant genotype effect $F(1,145)= 26.50; p<0.0001$ and a non-
significant age effect $F(1,145)= 0.67; p=0.4140$. Significant interactions were detected
between treatment and genotype $F(1,145)= 24.74; p <0.0001$ and between treatment,
genotype, and age $F(1,145)= 4.96; p=0.0280$. Post hoc analysis using Bonferroni
correction showed that KO adolescent mice were significantly more active than WT
adolescent mice when acutely administered cocaine (p<0.05), but not saline. Also, KO
adult mice were significantly more active than WT adult mice when acutely administered
cocaine (p<0.0001), but not saline. These results suggest that nNOS contributes to the
acute psychomotor stimulating effect of cocaine. The cocaine-induced hyperactivity
observed in the KO animals cannot be attributed to differences in basal locomotor
activity, as no differences were observed after saline administration.

**Experiment 1. Cocaine behavioral sensitization in adolescent WT and nNOS KO mice**

A two-way ANOVA (treatment × time) of the results of repeated saline and
cocaine administration to adolescent WT mice revealed a significant treatment effect $F[1,
180]=100.33; p<0.0001$ and a significant time effect $F[4, 180]=31.53; p<0.0001$. Post hoc
analysis using Bonferroni correction showed a significant difference between the
response to cocaine on days 1 and 5 and days 5 and 15 (p<0.001) suggesting the
development of sensitization during the initial five days and heightened sensitization after a 10 day drug-free period (Fig. 3.2A). In addition, on day 15, a comparison between saline and cocaine pretreated mice yielded a significant difference (p<0.01), further suggesting the development of sensitization (Fig. 3.2A). Additional groups that were treated with saline or cocaine in adolescence received a challenge cocaine injection after a 1 or 3 month drug free period (day 35 or 95); thus cocaine challenge was given at two time points in adulthood. Cocaine-induced locomotion in mice that had previously been treated with cocaine in adolescence was significantly higher than in saline pre-treated mice after drug free periods of 30 and 90 days (p<0.01 and p<0.001, respectively ) ( Fig. 3.2A). Therefore, WT mice treated with cocaine during adolescence remain sensitized to cocaine through adulthood.

The results of adolescent nNOS KO mice are shown in Figure 3.2B. A two-way ANOVA (treatment x time) yielded a significant treatment effect: $F[1,158]=109.92; p<0.0001$ and a significant time effect: $F[4,158]=28.41; p<0.0001$. Post-hoc Bonferroni correction showed significant differences between days 5 and 15 (p<0.001) but not between days 1 and 5 (Fig. 3.2B), suggesting a delayed development of sensitization compared to WT counterparts (Fig. 3.2A). Comparison between the responses of saline and cocaine pretreated mice to cocaine challenge on day 15 (p<0.01), day 35 (p<0.05), or day 95 (p<0.01) showed significant differences (Fig. 3.2B). Results suggest a) sensitization that developed during adolescence persists through early and late adulthood, and b) sensitization in adolescent nNOS KO mice, unlike in WT counterparts, is expressed only following a drug-free period.
Experiment 2. Cocaine behavioral sensitization in adult WT and nNOS KO mice

Figure 3.3A depicts cocaine behavioral sensitization in adult (PD 80) WT mice. A two-way ANOVA (treatment × time) revealed a significant treatment effect $F[1, 118]=49.42; p<0.0001$ and a significant time effect $F[4, 118]=13.78; p<0.0001$. Post hoc analysis using Bonferroni correction showed a significant difference between the response to cocaine on days 1 and 5 and days 5 and 15 (p<0.01 and p<0.05, respectively), suggesting the development of sensitization during the initial five days and heightened sensitization after a 10 day drug-free period. These results are similar to those from WT adolescent mice. On day 15, a comparison between the responses of saline and cocaine pretreated mice to cocaine challenge yielded a significant difference (p<0.05). Likewise, challenge cocaine given to four other saline and cocaine pretreated groups, after a one or three month drug-free period (day 35 and 95), resulted in a sensitized response in the cocaine pretreated groups compared to the saline groups (p<0.05).

Figure 3.3B depicts the results of repeated saline and cocaine administration to adult (PD 80) nNOS KO mice. A two-way ANOVA (treatment × time) revealed a significant treatment effect $F[1, 134]=50.04; p<0.0001$, a significant time effect $F[4, 134]=5.58; p<0.01$, and a significant interaction $F[4, 134]=6.53; p<0.0001$. However, Bonferroni correction showed non-significant differences between the response to cocaine on days 1 and 5 and on days 5 and 15, suggesting resistance to cocaine behavioral sensitization (Fig. 3.3B). Comparison between the results of cocaine challenge to saline and cocaine pretreated groups on days 15, 35, and 95 showed non-significant differences (Fig. 3.3B). These findings suggest that, unlike adult WT mice or adolescent nNOS KO mice, adult nNOS KO mice did not develop sensitization to cocaine. Similar
findings have previously been observed following administration of a lower dose of cocaine (15mg/kg) to adult nNOS KO mice (Itzhak et al. 1998a).

**Experiment 3: Context-dependent hyperlocomotion in adolescent and adult WT and nNOS KO mice**

Context-dependent hyperlocomotion was investigated by saline injection, given three days (day 8) after the termination of repeated saline and cocaine administration, in the same context that saline and cocaine pretreatment had been given (Fig. 3.4). Three-way ANOVA (treatment x age x genotype) revealed a significant treatment effect F(1,65)= 29.90; p<0.0001, a significant age effect F(1,65)= 12.20; p=0<0.0001, and a non-significant genotype effect F(1,65)= 0.03; p=0.8760. A significant interaction was detected between treatment and age F(1,65)= 9.58; p=0.0030. Post hoc analysis using Bonferroni correction showed that both WT and KO adult mice that received cocaine for 5 days were significantly more hyperactive than controls when challenged with saline, suggesting the development of conditioned locomotion (p<0.001 and p<0.01, respectively). However, there were no significant differences between saline and cocaine pretreated WT or KO adolescent mice when challenged with saline, suggesting the absence of conditioned hyperlocomotion.

**Immunohistochemistry of nNOS -ir neurons**

Initial studies analyzed the expression of nNOS-ir neurons in the dST by profile counting. Twenty four hours after repeated administration of cocaine (20mg/kg x 5 days) to adult WT mice, an increase of 65% was observed in the expression of striatal nNOS-ir neurons compared to saline control group (Fig. 3.5). WT adolescents administered cocaine (20mg/kg x 5 days), however, failed to show this increase in nNOS-ir neurons compared to controls.
Stereological estimation of the total number of nNOS-ir neurons

To quantify the changes in the number of nNOS-ir neurons in the dST and NAC, DAB stained sections were counted using stereology. The effects of repeated saline administration were compared to the effect of repeated cocaine administration (20mg/kg x 5 days) in adolescent and adult WT mice. Fig. 3.6A depicts the number of nNOS-ir neurons in the dST of adolescent and adult WT mice 24h after repeated (5 day) administration of either saline or cocaine (20mg/kg). A two-way ANOVA (age x treatment) resulted in a significant interaction between age and treatment $F[1, 8]=10.53; p=0.0118$, but non-significant main effects of age $F[1, 8]=1.50; p=0.2556$ or treatment $F[1, 8]=3.06; p=0.1186$. Post hoc analysis using Bonferroni correction revealed a significant difference between saline and cocaine treated adult animals ($p<0.05$). An increase of 96% in dST nNOS-ir neurons was observed in the cocaine-treated adult mice compared to saline controls. No significant difference was observed between adolescent WT mice treated repeatedly with either saline or cocaine (Fig. 3.6A). Given that differences in nNOS-ir neurons were detected after repeated cocaine administration only in the dST of adult WT mice, the acute effect of cocaine was further investigated in adult dST. A paired Student’s t-test analysis of nNOS-ir neurons in the dST of adult WT mice 24h after acute administration of saline or cocaine (20mg/kg) showed no significant changes in the number of nNOS-ir neurons (saline: $3010\pm227$; cocaine: $2480\pm450$). This finding suggests that repeated but not acute administration of cocaine resulted in enhanced expression of nNOS-ir neurons in the dST of adult WT mice. Fig. 3.6B depicts the number of nNOS-ir neurons in the NAC of adolescent and adult WT mice 24h after repeated (5 day) administration of saline or cocaine (20mg/kg). A two-way ANOVA (age
x treatment) resulted in a non-significant age effect $F[1, 13]=3.10; p=0.1.16$, a non-significant treatment effect $F[3, 13]=0.79; p=0.3901$, and non-significant interactions $F[1, 13]=1.57; p=0.2323$. These findings suggest that the increase of nNOS expression in the dST following cocaine administration was substrate-specific.

**Western blot analysis of nNOS**

In order to confirm the adult-specific increase in nNOS-ir neurons using stereology, we performed western blotting experiments of nNOS protein levels in the dST of adolescent and adult mice (Fig. 3.7). No significant difference in the amount of nNOS in the dST of saline and cocaine treated WT adolescent mice after a drug free period of 24hr was detected ($t=0.2873, p=0.7822$) (Fig. 3.7A). Fig. 3.7B shows the effect of cocaine on nNOS protein levels in the dST of adult mice. Student’s t-test (unpaired, two tail) showed that repeated cocaine administration induced a significant increase in striatal nNOS protein levels in adult WT mice when compared to saline-treated controls ($t=2.51, p<0.05$). These findings corroborate results from stereological experiments and lend support to the view that different neural adaptations, consequent to cocaine exposure, develop during adolescence and adulthood.

The dopamine projection most often linked with behavioral sensitization is the mesoaccumbens projection from the VTA to the NAC (Le Moal and Simon 1991). However, dopamine projections from the VTA to the PFC have also been implicated (Pierce and Kalivas 1997). To determine whether the cocaine-induced upregulation of striatal nNOS extends to other projections important in cocaine behavioral sensitization we investigated the changes in nNOS-ir in the PFC of cocaine and saline treated adult WT mice. An immunoblot analysis (Figure 3.8) showed no significant difference in the
amount nNOS protein levels in the PFC of saline and cocaine treated WT adult mice after a drug free period of 24 hr (t=0.1130, p=0.9137). These findings suggest that the increase of nNOS expression in the dST following cocaine administration was substrate specific as it was not observed in the PFC or NAC.

To determine if nNOS overexpression underlies the persistence of sensitization studies were carried out to verify if increased nNOS expression persists 10 days following cocaine administration. Figure 3.9 shows the cocaine-induced changes in nNOS-ir neurons and nNOS protein levels in the dST after a drug free period of 10 days to parallel the behavioral studies. Stereological analysis (Fig. 3.9A) showed that there was no significant difference in the number of nNOS-ir neurons in the dST of WT adult mice treated with saline and cocaine after 10 days of withdrawal (t=1.033, p=0.3415). Immunoblot analysis (Fig. 3.9B) also showed no significant difference in the amount nNOS protein levels in the dST of saline and cocaine treated WT adult mice after a drug free period of 10 days (t=0.3400, p=0.743). These results suggest that cocaine-induced upregulation in nNOS observed after a 24 hr drug free is transient in nature and return to control levels after 10 days.

Discussion

The present study investigated a) the roles of nNOS and ontogeny on the induction and persistence of short- and long-term behavioral sensitization to cocaine, and b) nNOS-dependent neural plasticity that may underlie cocaine sensitization.

Acute administration of cocaine, but not saline, to adolescent and adult nNOS KO mice resulted in heightened locomotor activity compared to WT counterparts. In contrast, inhibition of nNOS by 7-nitroindazole blocked the acute response to cocaine (Itzhak
We speculate that the deletion of the nNOS gene and not pharmacological blockade of nNOS resulted in neural adaptations which exacerbated the acute response to cocaine compared to WT mice.

The first major finding of the present study is that repeated administration of cocaine to either WT or nNOS KO mice during adolescence resulted in robust sensitization to cocaine in adulthood. Though several studies have investigated the development of sensitization to psychostimulants during the periadolescent period, the long-term consequences into adulthood are less known. Within the periadolescent period, some differences between WT and nNOS KO mice in the expression of sensitization to cocaine were observed. WT adolescent showed two phases of sensitization, first upon repeated cocaine administration (day 5 vs. day 1; Fig 3.2A.) and second following a 10-day drug free period. nNOS KO adolescent mice did not show the “first phase” of sensitization (on day 5) but showed the “second phase” after a 10-day drug free period. These findings suggest that the first phase in the development of sensitization to cocaine during adolescence is nNOS-dependent, while the second late phase may be nNOS-independent.

The second major finding of the present study is the noticeable difference between the response of adult WT and nNOS KO mice to repeated cocaine administration compared to the response of their adolescent counterparts. Adult WT mice developed short- and long-term sensitization to cocaine while adult nNOS KO mice did not develop sensitization to cocaine either after a 10-, 30-, or 90-day drug-free period. The latter finding is in agreement with our previous studies in adult WT and nNOS KO mice (Itzhak et al. 1998a). Although one may assume that a “ceiling effect” of cocaine in
adult KO mice may have obscured the expression of sensitization, there are two findings that do not support this assumption. First, in our previous studies the daily dose of cocaine administered to nNOS KO mice (of the same genetic background as in the current study) was 15 instead of 20mg/kg. The former dose resulted in lower ambulatory activity in KO mice than the latter dose, yet following administration of 15mg/kg cocaine sensitization did not develop (Itzhak et al. 1998a). Also dose-response studies indicated that 15mg/kg cocaine resulted in less locomotor activity than 30mg/kg. Hence, the dose of 15 mg/kg cocaine did not produce a ceiling effect, nevertheless sensitization did not develop (Itzhak et al. 1998a). In the same study we reported that the differences between the response of WT and nNOS KO mice to cocaine were not due to different pharmacokinetics (Itzhak et al. 1998a). A second observation that argues against a “ceiling effect” of cocaine in the KO mice appears from the adolescent study. Adolescent (like adult) KO mice demonstrated higher locomotor activity in response to acute cocaine administration compared to adolescent WT counterparts (Fig. 3.1) and yet adolescent but not adult KO mice developed sensitization to cocaine (Fig. 3.2B). We therefore propose that the nNOS gene has a major role in the development of sensitization to cocaine in adulthood, but that its role in sensitization during adolescence is less conspicuous. This hypothesis is supported by two findings. First, in adult subjects the presence of nNOS is needed for the development of sensitization. Second, repeated but not acute cocaine administration to WT mice resulted in significant increase in the expression of dorsostriatal nNOS-ir neurons in adults but not in adolescents.

The most important brain-reward circuits involve dopamine-containing neurons in the ventral tegmental area of the midbrain and their target areas in the limbic forebrain,
the nucleus accumbens and dorsal striatum, and frontal regions of cerebral cortex, the prefrontal cortex. In the dST, medium spiny neurons have connectivity to the substantia nigra pars reticulata and internal segment of the globus pallidus, which, in turn, control thalamocortical neurons and thus motor activity (Borgkvist and Fisone 2007). It is thought that the ventral striatum primarily controls the reinforcing effect of psychostimulants while the dST controls the psychomotor effects. Thus, the overexpression of nNOS-ir neurons following repeated cocaine administration was site-specific and occurred in the dorsal but not ventral striatum or the prefrontal cortex. The significance of this nNOS overexpression is not clear. However, given the role of NO/cGMP/PKG/CREB pathway in synaptic plasticity and long term potentiation (LTP) (Arancio et al. 2001; Lu et al. 1999; Puzzo et al. 2006) it is likely that the age-specific increase in nNOS expression in substrates involved in the psychomotor effect of cocaine contribute at least partly to the induction of sensitization. Although nNOS has traditionally been considered a constitutively expressed enzyme, the current study and others (Carretero et al. 2003; Sasaki et al. 2000; Wang and Lau 2001) provide evidence that nNOS expression is susceptible to regulation by various physiological or pathological conditions, including psychostimulant exposure. The overexpression of nNOS does not seem to underlie the persistence of sensitization since nNOS expression returned to control levels after a drug-free period of 10 days. Nevertheless, although the increased expression of nNOS following cocaine administration was transitory, modulation of downstream NO-signaling molecules (cGMP/ERK/CREB) may contribute to persistent sensitization.
Notably, the NO signaling system plays more of a role in sensitization during adulthood than in adolescence. During adolescence, developmental changes in the dopaminergic system may obscure the contribution of NO signaling molecules in the sensitization process. For instance, there is a constant increase in DA receptor densities throughout development which peaks at about PD28–30 (Murrin and Zeng 1990). Other studies have shown that striatal DA receptors are overexpressed before the onset of puberty, peak at around PD40 and then decrease to adult levels (Teicher et al. 1995). One possible consequence of an overdeveloped, immature dopaminergic system, characteristic of adolescence, is that nitrergic influences may be obscured during cocaine administration. As a result, adolescent mice may express sensitized response to cocaine independently of nNOS. These findings suggest that the adolescent period may be more vulnerable to cocaine sensitization than adulthood. Thus, blockade of the NO signaling pathway may prevent cocaine sensitization in adulthood but not in adolescence.

Further evidence for the differential response of adolescent and adult mice to cocaine-induced plasticity stems from the results of conditioned locomotion. Results showed significant development of context-dependent hyperlocomotion, 3 days following repeated cocaine administration, in adult WT and nNOS KO mice but not in their adolescent counterparts (Fig. 3.4). Other studies have also shown that conditioned hyperlocomotion is ontogeny-dependent in rats (Tirelli et al. 2003), suggesting the ability to form drug-context associations matures with time. Results also suggest a dichotomy between the magnitude of sensitization to the drug (unconditioned stimulus) and the context (conditioned stimulus). In other words, adolescent WT and KO mice that exhibited behavioral sensitization under the influence of cocaine did not show context-
dependent hyperlocomotion. In contrast, adult KO mice that did not show a sensitized response under the influence of cocaine exhibited robust context-dependent hyperlocomotion. These findings may be in general agreement with several studies that reported a lack of correlation between the magnitude of behavioral sensitization under the influence of cocaine and conditioned locomotion induced by saline in rats (Hotsenpiller and Wolf 2002) and mice (Tirelli et al. 2003; Tirelli et al. 2005). However, adult nNOS KO mice pretreated for 5 days with 15mg/kg cocaine (instead of 20mg/kg as in the present study) did not show context-dependent hyperlocomotion (Itzhak et al. 1998a), suggesting that the dose of cocaine influences conditioned locomotion in nNOS KO mice.

In a recent study we have shown that both adolescent and adult nNOS KO mice developed conditioned place preference to cocaine-associated context, but deficits in the persistence and reinstatement of conditioned response to cocaine were observed in both adolescent and adult nNOS KO mice compared to WT counterparts (Balda et al. 2006). In contrast, the findings of the present study have shown differential response of adolescent and adult KO mice to cocaine sensitization. Thus, it appears that NO-signaling pathway may contribute differently to cocaine behavioral sensitization and the learning of cocaine place preference, particularly in adolescent subjects. Given the prominent role of learning and memory in classical Pavlovian conditioning and the role of NO signaling in synaptic plasticity, it is likely that absence of the nNOS gene during adolescence has a more pronounced influence on learning and memory processes (conditioned place preference) than on the plasticity associated with behavioral sensitization.
In summary, the present study demonstrates that cocaine behavioral sensitization acquired in adolescence persists through adulthood. The nNOS gene is essential for the induction but not the persistence of long-term behavioral sensitization to cocaine during adulthood. The overexpression of nNOS -ir neurons in the dST may in part underlie the induction of behavioral sensitization in adulthood. In adolescence, however, cocaine-induced behavioral sensitization appears to be less dependent on the nNOS gene. Together these findings support the view that different neural adaptations, consequent to cocaine exposure, develop during adolescence and adulthood.
Figure 3.1. Effect of the nNOS gene and age on saline- and cocaine-induced locomotion in male mice. Adolescent (PD28) and adult (PD 80) mice received IP injections of saline and cocaine (20mg/kg) and locomotor activity was recorded for 60 minutes. The magnitude of locomotor activity in adolescent nNOS KO mice was significantly higher compared to WT counterparts following cocaine (*p<0.05) but not saline administration. Likewise, the intensity of locomotion in nNOS KO adults was significantly higher than in WT adults after cocaine (*p<0.0001) but not saline administration. (Taken from Balda et al. 2008).
Figure 3.2. Effect of the nNOS gene on cocaine behavioral sensitization in adolescent male mice. Mice received IP injections of saline or cocaine (20mg/kg) for 5 consecutive days (PD28-32). Locomotor activity was recorded for 60 minutes on days 1 through 5. Mice were challenged with cocaine (20mg/kg) after 10-, 30-, or 90-day drug-free period (day 15, day 35 or day 95, respectively). A) Comparison between the magnitude of cocaine-induced locomotor activity in adolescent WT mice on days 1 and 5 resulted in significant differences (a, p<0.001). Upon cocaine challenge, locomotor activity on day 15 was significantly higher than on day 5 (b, p<0.001). Likewise on day 15, locomotor activity in the cocaine group was significantly higher than in the control group (c, p<0.01). Cocaine challenge on day 35 and 95 resulted in higher locomotor activity in the cocaine groups than in controls (d and e, p<0.01 and p<0.001). B) Comparison between the magnitude of cocaine-induced locomotor activity in adolescent nNOS KO mice on days 1 and 5 resulted in non-significant differences. However,
cocaine challenge on day 15 resulted in higher locomotor activity than on day 5 (a, p<0.001) and also higher locomotor activity than in control mice that received cocaine challenge on day 15 (b, p<0.01). Cocaine challenge on day 35 and day 95 to mice that received cocaine during adolescence resulted in higher locomotor activity than in the control group that received cocaine challenge at corresponding time points (c and d, p<0.05 and p<0.01). (Modified from Balda et al. 2008).
Figure 3.3. Effect of the nNOS gene on cocaine behavioral sensitization in adult male mice. Mice received IP injections of saline or cocaine (20mg/kg) for 5 consecutive days (PD80-84). Locomotor activity was recorded for 60 minutes on days 1 through 5. Mice were challenged with cocaine (20mg/kg) after a 10-, 30-, or 90-day drug-free period (day 15, day 35, or day 95, respectively). A) Comparison between the magnitude of cocaine-induced locomotor activity in adult WT mice on days 1 and 5 resulted in significant differences (a, p<0.01). Upon cocaine challenge, locomotor activity on day 15 was significantly higher than on day 5 (b, p<0.05). Likewise on day 15, locomotor activity in the cocaine group was significantly higher than in the control group (c, p<0.05). Cocaine challenge on day 35 and day 95 resulted in higher locomotor activity in the cocaine groups than in controls (d and e, p<0.05). B) Cocaine administration to adult nNOS KO mice resulted in similar magnitude of locomotion on days 1 and 5. Challenge
cocaine given to cocaine pretreated groups after a 10-, 30-, and 90-day drug free period resulted in similar magnitude of locomotion as on days 1 and 5, suggesting the absence of a sensitized response. Likewise the responses of cocaine and saline pretreated groups to cocaine challenge on days 15, 35, and 95 resulted in non-significant differences in the magnitude of locomotor activity. (Modified from Balda et al. 2008).
Figure 3.4. Effect of saline injection on locomotor activity: Context-dependent hyperlocomotion in male mice. Three days following termination of the repeated drug administration (day 8), mice received a saline injection and locomotor activity was recorded for 60 min. Both WT and KO adult mice that received cocaine for 5 days were significantly more hyperactive than controls when challenged with saline (*p<0.001 and p<0.01, respectively), suggesting the development of conditioned hyperlocomotion. There were no significant differences between cocaine and saline pretreated adolescent mice when challenged with saline indicating the absence of conditioned hyperlocomotion. (Taken from Balda et al. 2008).
Figure 3.5. Effect of cocaine on nNOS-ir neurons in the dorsal striatum (dST) of adolescent and adult WT mice. WT adolescents that received cocaine (20mg/kg x 5 days; panel B) showed no significant difference in the number nNOS-ir neurons compared to saline treated age-matched controls (panel A). WT adults that received cocaine (20mg/kg x 5 days) showed a 65% increase in nNOS-ir neurons (panel D) compared to age-matched controls (panel C). Scale bar=100μm. Magnification=20x. (Taken from Balda et al. 2008).
Figure 3.6. Stereological analysis of nNOS expression in the dorsal striatum (dST) and the nucleus accumbens (NAC) of adolescent and adult mice after saline and cocaine administration. Adolescent (PD28) and adult (PD80) WT mice received saline or cocaine (20mg/kg) for 5 consecutive days. After 24 hr animals were perfused and brain tissue was prepared for staining of nNOS -ir neurons, as described in Materials and Methods. A. In the dST of adolescent mice there was no statistical difference in the number of nNOS-ir neurons in saline or cocaine treated mice. In contrast, in the dST of adult mice treated with cocaine for 5 days there was a significant increase in the number of nNOS-ir neurons compared to saline treated mice,*p<0.05. B. In the NAC, there were no statistical differences between saline or cocaine treated mice in the number of nNOS-ir neurons, either in adolescence or adulthood. (Taken from Balda et al. 2008).
Figure 3.7. Western blot analysis of nNOS in the dST of adolescent and adult mice 24hrs after saline and cocaine administration. Representative immunoblots demonstrating the effect of repeated cocaine on the levels of nNOS in adolescent (A) and adult (B) mice. dST lysates were immunoblotted with an antibody against nNOS. β-Tubulin was used as control for protein loading. A. In the dST of adolescent mice there was no statistical difference in the levels of nNOS in saline or cocaine treated mice. B. Conversely, cocaine administration significantly increases levels of striatal nNOS in WT adult mice when compared to saline treated controls (*<0.05). (Unpublished data).

Figure 3.8. Western blot analysis of nNOS in the PFC of adult mice 24hrs after saline and cocaine administration. Representative immunoblots demonstrating the effect of repeated cocaine on the levels of PFC nNOS in adult mice. PFC lysates were immunoblotted with an antibody against nNOS. β-Tubulin was used as control for protein loading. In the PFC of adult mice there was no statistical difference in the levels of nNOS between saline and cocaine treated mice. (Unpublished data).
Figure 3.9. Stereological and western blot analysis of nNOS in the dST of adult WT mice 10 days after saline and cocaine administration. A. After a drug-free period of 10 days, there was no statistical difference in the number of nNOS-ir neurons in the dST of adult mice treated with saline or cocaine. B. Similarly, in the PFC of adult mice there was no statistical difference in the levels of nNOS between saline and cocaine treated mice following a drug-free period of 10 days. (Unpublished data).
Chapter 4

Ontogeny-dependent contribution of the neuronal nitric oxide synthase (nNOS) gene to the regulation of tyrosine hydroxylase (TH) by cocaine (Balda et al. 2009b).

Summary

Recently, we demonstrated that intact NO signaling is essential for the development of cocaine behavioral sensitization in adulthood, but not adolescence (Balda et al. 2008). Given the requirement of dopamine transmission in cocaine behavioral sensitization and the interactions between NO and DA systems, the present study investigated the ontogeny-dependent role of the nNOS gene and the effect of cocaine on the expression of TH-ir neurons. Adolescent and adult WT and nNOS KO male mice received saline or a sensitizing regimen of cocaine (20mg/kg) for 5 days. After 24h, TH immunoreactivity was assessed in the ventral tegmental area and the dorsal striatum using stereology and western blotting, respectively. We report that a) adolescent and adult nNOS KO mice express lower levels of TH-ir neurons in the VTA compared to WT counterparts, b) cocaine administration to WT mice significantly increased striatal TH expression in adult but not adolescent mice, and c) the same cocaine administration to nNOS KO mice significantly decreased striatal TH expression in both adolescent and adult mice. Thus, the nitrergic system appears to contribute to behavioral sensitization by regulating dopaminergic systems in an ontogeny-dependent manner.

Background

In the central nervous system, NO is thought to be a highly reactive neuronal signaling molecule (Dawson and Snyder 1994). NO is produced from arginine via nNOS in response to calcium influx caused by stimulation of N-methyl-D-aspartate (NMDA) subtype of glutamate receptors (Garthwaite 1991; Snyder 1994). Compelling evidence
from neuroanatomical studies suggests the existence of interaction between dopaminergic and nitric ergic systems. In nearly all mesolimbic, corticostriatal, and nigrostriatal regions such as the ventral tegmental area, nucleus accumbens, dorsal striatum, substantia nigra, and frontal cortex, populations of TH-ir and NOS-ir neurons interact with each other as manifested by the presence of NOS-ir endings on TH-ir neurons and *vice versa* (Benavides-Piccione and DeFelipe 2003; Fujiyama and Masuko 1996; Hidaka and Totterdell 2001; Klejbor et al. 2004; Matthews et al. 1997). The close appositions provide the locus at which NO can affect the release and uptake of dopamine. NO exerts facilitatory influence on both tonic extracellular DA levels and phasic DA neuron spike activity (Grace 1991; West et al. 2002; West and Grace 2000). Striatal NO transmission is facilitated via nigrostriatal DA and frontal cortical glutamate afferents (Sammut et al. 2007a; Sammut et al. 2006; Sammut et al. 2007b). Among other mechanisms, NO has been shown to inhibit the function of DA transporters (Kiss 2000; Kiss et al. 2004; Lonart and Johnson 1994; Pogun et al. 1994; Volz and Schenk 2004) thereby facilitating dopaminergic transmission. The effect of NO on monoamine transporters is believed to represent a new form of interneuronal communication, that is, a nonsynaptic interaction that does not involve classical receptors (Kiss and Vizi 2001).

The interactions between DA, glutamate, and NO in mesolimbic and corticostriatal circuits (Carlsson and Carlsson 1990; Kiss 2000) suggest that NO may contribute to the effects of cocaine. Acute systemic administration of cocaine significantly increased NO efflux in the medial prefrontal cortex in a time-dependent manner (Sammut and West 2008). Furthermore, cocaine administration has been shown to regulate nNOS. Chronic cocaine administration followed by 1h of withdrawal
increases NOS activity in the cerebral cortex, cerebellum, midbrain, hypothalamus, hippocampus, amygdala, and spinal cord (Bhargava and Kumar 1997). A significant cocaine-induced up-regulation of nNOS expression was observed at 24h but not 72h or 14 days of withdrawal in the frontal and parietal cortices (Loftis and Janowsky 2000).

Recently we reported that repeated, but not acute, cocaine administration resulted in a significant increase in the expression of nNOS-ir neurons in the dST 24h (Balda et al. 2008) but not 10 days (unpublished data) after cocaine administration was discontinued. These results were observed in adult but not in adolescent B6;129S (WT) males. Also, while adult WT males developed long-lasting sensitization to cocaine, nNOS KO counterparts did not. On the other hand, adolescent WT and nNOS KO mice developed similar sensitization to cocaine. Together, these findings suggest that the nNOS gene has an ontogeny-dependent role in the development of behavioral sensitization to cocaine (Balda et al. 2008).

During adolescence, developmental changes in the dopaminergic system may obscure the contribution of NO signaling molecules in the sensitization process. For instance, there is a steady increase in DA receptor densities throughout development, which peaks at about PD28–30 (Murrin and Zeng 1990). Other studies have shown that striatal DA receptors are overexpressed before the onset of puberty, peak at around PD40 and then decrease to adult levels (Teicher et al. 1995). One possible consequence of an overdeveloped, immature dopaminergic system, characteristic of adolescence, is that nitrergic influences may be obscured during cocaine administration. Moreover, a study of the ontogeny of nNOS- and TH-ir neurons revealed that although these neurons appeared within the same striatal patches, the time course of their expression did not
coincide (Murata and Masuko 2003). Thus, a distinctive time course of the expression of TH- and nNOS-ir neurons within the same structure during development might differentially influence DA/NO interactions.

The present study investigated the ontogeny-dependent role of the nNOS gene in the expression of TH-ir neurons, and the effect of cocaine on TH-ir neurons. We report that a) adolescent and adult nNOS KO mice express lower levels of TH-ir neurons in the VTA compared to WT counterparts, b) cocaine administration to WT mice significantly increased striatal TH expression in adult but not adolescent mice, and c) the same cocaine administration to nNOS KO mice significantly decreased striatal TH expression in both adolescent and adult mice.

Materials and Methods

Animals

Mice purchased from Jackson Laboratories (Bar Harbor, Maine) were bred in our facilities at the University of Miami, Miller School of Medicine, Miami, FL as we described previously (Balda et al. 2006). Both genotypes, WT and nNOS KO, were generated on a mixed B6;129S genetic background (Huang et al. 1993). Animals were housed in a temperature- (22±0.5°C) and humidity- (50%) controlled room and maintained on a 12-h light/dark schedule with free access to food and water. Animal care was in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, National Academy Press, 1996) and approved by the University of Miami Animal Care and Use Committee.
Schedule of cocaine administration

Cocaine-HCl (Sigma, St. Louis, MO) was dissolved in 0.9% NaCl. All injections were given intraperitoneally (IP) in a volume of 0.1ml/10g weight.

Immunohistochemistry of TH

“Adolescence” represents the periadolescent period in mice and rats, which commences 7-10 days preceding the onset of puberty (PD38-40) and ends a few days thereafter (Spear and Brake 1983). Adolescent (PD28) and adult (PD80) WT male mice (n=4-5/group) were administered saline and cocaine (20mg/kg) for 5 days. The purpose of this schedule was to replicate the cocaine sensitizing-regimen we used previously in behavioral experiments (Balda et al. 2008). Twenty four hours after the last saline or cocaine injection, mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg). After loss of the foot-pinch response, mice were perfused transcardially via the left ventricle with sodium phosphate-buffered saline (PBS) followed by p-formaldehyde (4%) in PBS. The brains were removed and post-fixed overnight in the same fixative at 4°C. Serial coronal sections (50 μm) were cut with a Vibratome 1000 (TPI Inc., St. Louis, MO), collected in PBS and blocked for 1h at room temperature with normal goat serum (10%) in PBS containing Triton X-100 (0.3%). Sections were then incubated (72h; 4°C) with a rabbit polyclonal antibody to TH (1:3000, Chemicon, Temecula, CA) diluted in a vehicle of 2% normal goat serum with 0.3% Triton X-100 in PBS. After 3x10min washes the sections were then incubated for 1h at room temperature in biotinylated goat anti-rabbit IgG diluted 1:100 in vehicle. The signal was then amplified using the Vectastain Elite ABC immunoperoxidase kit (Vector Laboratories, Burlingame, CA) and visualized with 0.05% DAB and 0.02% H2O2 in
50mM Tris Buffer. Sections were then rinsed 2x5min in PBS, mounted onto slides, and coverslipped. Negative control sections were treated in the same way as described, except that the primary antibody was omitted from the staining procedure.

**Stereological analysis of TH**

To quantify the total number of TH-ir neurons in the VTA, the optical fractionator method, a stereological technique, was used as we described previously (Balda et al. 2008). For cell counts, sections were stained with DAB as the chromogen. Eight coronal sections (50 μm) were selected, taken at equally spaced intervals (100μm) through the full rostrocaudal extent of the midbrain and analyzed using a Zeiss Axiophot microscope equipped with software that includes an optical fractionator probe (StereoInvestigator, Microbrightfield). To perform cell number estimation in the structure volume, the optical fractionator method and optical disector probe were used. Dimensions of the optical disector were designed based upon the cell distribution in the section, and optical fractionator grid size was determined based upon the results of preliminary counts of the naive brain sample to allow 100-200 counts per region of the VTA. Thus, the standard counting method used was a counting frame (dissector; 65μm x 65 μm) and fractionator (150μm x 150μm) to meet the high standards of systematic random sampling. The appropriateness of the sampling scheme chosen was evaluated by calculating the precision of the estimates in each animal, expressed as the coefficient of error (West and Gundersen 1990). In all cases, the coefficient of error was less than 0.10, suggesting that a minimal amount of variance in the counts can be attributed to the technique.
Western blotting experiments of TH

Adolescent (PD28) and adult (PD80) WT male mice (n=4-5/group) were administered saline or cocaine (20mg/kg) for 5 days. Twenty four hours after the last saline or cocaine injection, striatum were microdissected and homogenized twice in 100 volumes of ice cold RIPA Buffer (50mM Tris-HCl pH7.5, 5mM EDTA, 150 mM NaCl, 0.5% NP-40 supplemented with 1mM PMSF, 1ug/ml pepstatin, 1ug/ml aprotinin, and 2ug/ml leupeptin) and incubated for 15 minutes. The homogenate was centrifuged and the supernatants collected. Protein concentrations were determined using the Lowry protein assay with bovine serum albumin as a standard. Proteins were separated using 7.5% SDS-PAGE and transferred to PDVF membranes. Membranes were incubated in blocking buffer (5% nonfat dry milk in Tris buffered saline (TBS) [20mM Tris-HCl (pH 7.4), and 150mM NaCl]) containing 0.1% Tween-20 (TBS-T) for 1h at RT. Membranes were incubated with a rabbit polyclonal antibody to TH (1:4500) (Santa Cruz Biotechnology, CA). Membranes were then washed 30 minutes with 3 intermediate changes of TBS-T. The blots were then incubated (1hr at RT) with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody at a dilution of 1:2000 and washed with TBS-T 3x10minutes. Visualization of the signal was enhanced by chemiluminescence using a Phototope-HRP Detection Kit (Pierce, Rockford, IL). To control for protein loading, immunoblots were stripped with Restore Western blot stripping buffer (Pierce, Rockford, IL), and blotted for a mouse monoclonal to β-tubulin (1:15,000, Upstate, NY). Quantification of band density was performed using UNSCAN-IT gel quantifying software (Silk Scientific Inc, Utah) and data were normalized to β-tubulin.
**Data Analysis**

Statistical analysis was performed with SPSS 16 software. The numbers of VTA TH-ir neurons were analyzed using a three-way ANOVA (age x treatment x genotype) followed by post hoc analysis using Bonferroni correction to determine differences between multiple groups. Striatal TH protein levels in saline- and cocaine-treated mice were compared using unpaired, two-tailed Student’s *t* test. *P* values less than 0.05 were considered significant for all tests. Data are expressed as ±SEM.

**Results**

**Stereological estimation of the total number of TH-ir neurons in the VTA**

To quantify the changes in the number of TH-ir neurons in the VTA, DAB stained sections were counted using stereology. Fig. 4.1 shows the effect of repeated saline administration compared to the effect of repeated cocaine administration (20 mg/kg×5 days) in WT and nNOS KO adolescent (PD28) and adult (PD80) mice. A three-way ANOVA (age x treatment x genotype) revealed a significant genotype effect \(F(1, 24)=84.33; p<0.0001\) and a significant age effect \(F(1,24)=51.77; p<0.0001\). Post hoc analysis using Bonferroni correction revealed a significant reduction in the number of TH-ir neurons in the VTA of adolescent nNOS KO animals when compared to their WT counterparts \((p<0.01)\). Similarly, adult nNOS KO animals also showed a decrease in TH-ir neurons when compared to WT counterparts after repeated saline and cocaine administration \((p<0.01)\). These findings suggest that the absence of nNOS leads to decreased expression of TH in the VTA. Interestingly, both control adult WT and nNOS KO animals showed significant increases in the number of TH-ir neurons when compared to their adolescent counterparts \((p<0.01 \text{ and } p<0.05, \text{ respectively})\) suggesting that there is
an age-dependent maturation of dopaminergic midbrain neurons. Notably, however, cocaine administration had no effect on VTA TH expression in either age group or genotype.

**Western blot analysis of TH-ir terminals in the dST**

To quantify the changes in dopaminergic (TH-ir) nerve terminals in the dST we performed western blot analysis. Fig. 4.2 shows the effect of age and genotype on TH protein levels in control (saline-treated) mice. Student’s t-tests (unpaired, two-tail) revealed that there was no significant difference in TH protein levels between adolescents and adults in either WT or nNOS KO mice (Fig. 4.2A and 4.2B). Also, there was no significant effect of genotype in either adolescent or adult mice (Fig. 4.2C and 4.2D). These findings suggest that the genotype- and age-dependent changes in the dopaminergic cell bodies in VTA observed under control conditions are region specific and do not extend to nerve terminals in the dST.

Fig. 4.3 shows the effect of age and genotype on cocaine mediated alterations in striatal TH. Student’s t-test (unpaired, two tail) showed that repeated cocaine administration induced a significant increase in striatal TH protein levels in adult WT mice when compared to saline-treated controls ($t=2.91$, $p<0.05$) (Fig. 4.3B). However, no difference was detected between adolescent WT mice treated with saline or cocaine (Fig. 4.3A) suggesting that cocaine modulates TH in an age-dependent manner. Conversely, in both adolescent and adult nNOS KO mice (Fig. 4.3C and 4.3D), repeated cocaine administration resulted in a reduction in striatal TH protein levels when compared to saline treated controls ($t=3.171$, $p<0.05$; $t=3.370$, $p<0.01$) suggesting that, in the absence of nNOS, TH is down-regulated after cocaine exposure.
Discussion

We have recently shown that administration of cocaine (20mg/kg) for 5 days to adolescent and adult WT and nNOS KO mice resulted in: 1) long-lasting sensitization of adolescent WT and nNOS KO mice, 2) long-lasting sensitization of adult WT mice but not adult nNOS KO mice, and 3) ontogeny-dependent increase in expression of nNOS-ir neurons in the dST of WT mice (Balda et al. 2008). Given the interactions between nitrergic and dopaminergic transmission (Background), the present study investigated the ontogeny-dependent role of the nNOS gene in the expression of TH-ir neurons, and the effect of cocaine on TH-ir neurons. The major findings are: 1) adolescent and adult nNOS KO mice express reduced levels of TH-ir neurons in the VTA compared to WT counterparts, 2) a sensitizing regimen of cocaine significantly increased striatal TH expression in adult but not adolescent WT mice, and 3) the same regimen of cocaine significantly decreased striatal TH expression in both adolescent and adult nNOS KO mice.

VTA TH-ir neurons: Effects of genotype, ontogeny, and cocaine

Cocaine-induced behavioral sensitization is largely a DA-dependent phenomenon. It has been shown that DA transmission in the VTA is necessary for the induction of cocaine sensitization (Vanderschuren and Kalivas 2000). Repeated microinjection of cocaine in the VTA (DA cell body region) and not into the NAC or dST (DA terminal fields) produces behavioral sensitization to subsequent systemic drug challenge, while microinjection of a DA antagonist directly into the VTA region prevents the development of behavioral sensitization to peripherally administered drugs (Vanderschuren and Kalivas 2000). These findings suggest that DA transmission in the VTA is necessary for
the induction of cocaine sensitization (Vanderschuren and Kalivas 2000). Thus, the
diminished expression of TH-ir neurons in the VTA of nNOS KO mice compared to WT
counterparts (Fig. 4.1) may contribute, in part, to the resistance of adult nNOS KO mice
to cocaine-induced behavioral sensitization (Balda et al. 2008; Itzhak et al. 1998a). We
conclude that psychomotor sensitization relies on an intact nitrergic system in adulthood
possibly due to modulation of dopaminergic neurons. The reduced expression of VTA
TH-ir neurons we observed in nNOS KO mice is in agreement with the reduced TH
protein levels observed in the adrenal glands and hypothalamus of these mice (Orlando et
al. 2008; Yamova et al. 2007).

Our results show, however, that even though adolescent nNOS KO mice show
reduced expression of VTA TH-ir neurons (Fig. 4.1), these mice developed cocaine-
induced behavioral sensitization (Balda et al. 2008). However, we reported that cocaine-
induced sensitization in adolescent nNOS KO mice was significantly delayed compared
to WT counterparts (Balda et al. 2008). Hence, in the case of adolescent nNOS KO mice,
reduced DA transmission in the VTA may delay, but not preclude, the development of
sensitization to cocaine. Studies have shown that the overproduction of DA receptors and
increased PFC DA activity may render adolescent animals more sensitive to
These neurobiological age-dependent differences may explain why in adolescence
cocaine psychomotor sensitization is, in part, independent of nNOS (Balda et al. 2008)
and subsequent modulation of TH-ir neurons (Fig. 4.2).

A noticeable age-dependent difference is that adult mice (both WT and nNOS
KO) have more TH-ir neurons in the VTA than their adolescent counterparts, suggesting
an ontogeny-dependent maturation of dopaminergic midbrain neurons. Previous studies have focused on other components of dopaminergic circuitry. For instance, studies in monkey PFC revealed a 6-fold increase in TH-ir neurons compared to younger ages; the density of PFC TH-ir neurons decline thereafter to adult levels (Rosenberg and Lewis 1995). DA receptors are overproduced prior to puberty (PD40) and are pruned back to adult levels thereafter. The density of DA D1 and D2 receptor families in male rat striatum (Andersen et al. 1997; Teicher et al. 1995) and PFC (Andersen and Teicher 2000; Leslie et al. 1991), but not the NAC, increases markedly between PD25-40, declines by PD60 and remains stable through adulthood. To the best of our knowledge, this is the first account of an ontogeny dependent difference in VTA TH-ir neurons. However, the finding that adolescent and adult WT mice developed similar pattern and magnitude of behavioral sensitization to cocaine (Balda et al. 2008) suggests that reduced VTA TH-ir neurons in adolescence does not influence cocaine-induced behavioral sensitization. In contrast, reduced VTA TH-ir neurons in the absence of the nNOS gene (Fig. 4.1) delayed or precluded the development of cocaine sensitization in adolescent and adult nNOS KO mice, respectively (Balda et al. 2008). Hence, the age-dependent differences in VTA TH-ir neurons influence cocaine-induced sensitization in the absence, but not in the presence, of the nNOS gene.

Finally, the number of TH-ir neurons in VTA were unaffected by repeated cocaine treatment, regardless of age or genotype (Fig. 4.1). These results are in agreement with previous reports demonstrating that TH protein and mRNA levels in the VTA were also unaffected by repeated cocaine administration (Hope et al. 2005; Sorg et al. 1993), but may be at odds with other reports showing increases in TH protein and
activity (Beitner-Johnson and Nestler 1991; Masserano et al. 1996). However, thus far, the direction of cocaine-induced modulation of VTA dopaminergic transmission remains controversial most likely due to differences in species, technical assessment (Western Blot vs in situ hybridization), cocaine regimen, and the time elapsed after cocaine treatment and assaying.

**Striatal TH-ir terminals: Effects of genotype, ontogeny, and cocaine**

DA projections most often linked with psychostimulant-induced behavioral sensitization are the mesoaccumbens projections from the VTA to the NAC (Le Moal and Simon 1991). However, DA projections from the substantia nigra pars compacta (SNc) to the dST have also been implicated (Pierce and Kalivas 1997). In the dST, medium spiny neurons have connectivity to the substantia nigra pars reticulata and internal segment of the globus pallidus, which, in turn, control thalamocortical neurons and thus motor activity (Borgkvist and Fisone 2007). It is thought that the ventral striatum primarily controls the reinforcing effect of psychostimulants while the dST controls the psychomotor effects and stimulus–response (S–R) habit formation (Mishkin et al. 1984; White 1989). A sensitized release of DA in the dST consistently contributes to the expression of behavioral sensitization to cocaine. This neurochemical sensitization in the dST is thought to be involved in habitual cocaine seeking behavior (Ito et al. 2002; Vanderschuren et al. 2005). Thus, both the mesolimbic and the nigrostriatal pathways have been implicated in behavioral sensitization.

Under basal conditions no major genotype or age dependent differences were detected in the TH-ir fibers of the dST (Fig. 4.2) suggesting that the corresponding changes observed in the VTA (Fig. 4.1) were region specific. It is important to bear in
mind that region specificity for ontogeny-dependent differences in neuroanatomical markers is not uncommon. For instance, striatal and cortical, but not accumbal, DA receptors are overproduced prior to puberty (PD40) and are pruned back to adult levels thereafter (Andersen et al. 1997; Andersen and Teicher 2000). In the present study, major ontogeny-dependent differences in the modulation of striatal TH levels were detected after cocaine administration. Our cocaine-sensitizing regimen resulted in a significant increase in TH protein levels in the dST of adult but not adolescent WT mice (Fig. 4.3). This cocaine-induced ontogeny-dependent overexpression of dST TH is reminiscent of the cocaine-induced ontogeny-dependent overexpression of dST nNOS-ir neurons we observed previously (Balda et al. 2008), suggesting mutual regulation of dST nitrergic/dopaminergic transmission by cocaine in adulthood. Yet the finding that WT mice from both age groups developed sensitization to cocaine (Balda et al. 2008), suggests that this behavioral plasticity in adolescence develops independently of TH (VTA or striatal) and nNOS. These findings support the view that different neural adaptations, consequent to cocaine exposure, develop during adolescence and adulthood.

While no genotypic differences in striatal TH-ir fibers were observed under basal conditions (Fig. 4.2), significant differences were detected after repeated cocaine administration (Fig. 4.3). Cocaine induced a decrease in dST TH-ir fibers in adolescent and adult nNOS KO mice. The findings that cocaine treatment leads to 1) a concomitant increase in both nNOS and TH in the dST of adult WT mice that develop sensitization, and 2) a reduction in TH in adult nNOS KO animals that are resistant to sensitization, suggests that nNOS may contribute to the cocaine-mediated increase in TH. This is in agreement with earlier reports of NO influence on TH regulation in which NO donors led
to phosphorylation of TH (Rodriguez-Pascual et al. 1999). Other have found that in addition to this short-term modification of existing TH protein, NO causes long-term upregulation of TH via induction of gene expression (Kim et al. 2003). Thus, it appears that in adulthood overexpression of dST nNOS and TH is associated with cocaine behavioral sensitization. The finding that adolescent nNOS KO mice, which developed cocaine-induced sensitization (Balda et al. 2008), showed reduced dST TH levels after cocaine administration (Fig. 4.3C), suggests once more, that dST TH levels in adolescence do not correlate with cocaine-induced sensitization.

In summary, results of the present study suggest that the nNOS gene, the level of TH expression in VTA, and the regulation of TH in the dST are associated with a sensitizing-regimen of cocaine in adulthood but not in adolescence. Hence, neuroadaptations necessary for the development of behavioral sensitization to cocaine in adolescence and adulthood may be different.
Figure 4.1. Stereological analysis of TH expression in the VTA of adolescent and adult WT and nNOS KO mice after saline and cocaine administration. Adolescent (PD28) and adult (PD80) WT and nNOS KO mice received saline or cocaine (20mg/kg) for 5 consecutive days. After 24 hr animals were perfused and brain tissue was prepared for staining of TH-ir neurons, as described in Materials and Methods. A significant genotype-dependent effect was observed (*p<0.01) as the number of TH-ir neurons in the VTA of nNOS KO mice was lower than that in their WT counterparts, regardless of age and drug treatment. Also, a significant age-dependent effect was observed as adult WT and nNOS KO mice showed significant increases in the number of TH-ir neurons when compared to their adolescent counterparts (#p<0.01 and p<0.05, respectively). Cocaine treatment had no significant effect on the number of VTA TH-ir neurons (Modified from Balda et al. 2009b).
Figure 4.2. Effects of ontogeny and genotype on striatal TH-ir terminals in control mice. Representative immunoblot analysis of dST lysates of control adolescent and adult mice showing no significant differences in the levels of TH in WT (A) and nNOS KO (B) mice. Control WT and nNOS KO mice also do not differ in the levels of TH in adolescent (C) and adult (D) mice. dST lysates were immunoblotted with an antibody against TH. β-Tubulin was used as control for protein loading. (A:adolescent; A’:adult) (Modified from Balda et al. 2009b).
Figure 4.3. Effects of ontogeny and genotype on cocaine-induced changes in striatal TH-ir terminals. Representative immunoblots demonstrating that cocaine administration significantly increased levels of dST TH in WT adult mice (B) (* p<0.05) but not WT adolescent mice (A) when compared to controls. Conversely, cocaine administration significantly decreases levels of striatal TH in adolescent (C) and adult (D) nNOS KO mice when compared to saline treated controls (*p<0.05 and p<0.01, respectively). dST lysates were immunoblotted with an antibody against TH. β-Tubulin was used as control for protein loading. (Modified from Balda et al. 2009b).
Chapter 5

Development and persistence of long-lasting behavioral sensitization to cocaine in female mice: role of the nNOS gene (Balda et al. 2009a).

Summary

Our recent studies have shown that the nNOS gene is required for the development and persistence of psychomotor sensitization to cocaine in adult but not adolescent male mice (Balda et al. 2008). The aim of the present study was to investigate the contribution of the nNOS gene to cocaine-induced behavioral sensitization in adolescent and adult female mice. Adolescent and adult WT and nNOS KO female mice received saline or cocaine (20mg/kg) for 5 days and then were challenged with cocaine (20mg/kg) after a drug-free period of either 10, 30, or 90 days. Context-dependent sensitization was determined by measuring saline-induced locomotor activity in the previously cocaine-paired environment. Results show that adolescent females of both genotypes, like their adult counterparts, developed long-lasting behavioral sensitization to cocaine (a three month period), suggesting high vulnerability of females to cocaine regardless of age. An effect of genotype was observed in the initiation of sensitization, e.g., delayed onset in the absence of the nNOS gene. The only age-dependent difference observed was that adult, but not adolescent mice developed context-dependent sensitization. The present study suggests that long-term expression of cocaine-induced behavioral sensitization in females (adolescent and adult) is nNOS-independent, unlike our previous findings in adult males.

Background

Initiation of drug use, particularly by vulnerable individuals, can progress through phases of escalated drug intake until addiction develops. Clinical and pre-clinical studies
have revealed that certain populations, specifically females and adolescents, may display unique vulnerabilities to the effects of psychostimulants (Roth and Carroll 2004a; Spear 2000; Spear and Brake 1983). Although the rates of cocaine abuse are currently lower in women than in men, women tend to escalate their rate of consumption of cocaine more rapidly (Lynch et al. 2002) and have more severe cocaine use compared to men (Kosten et al. 1993). Numerous studies suggest that gonadal hormones modulate behavioral responsivity to cocaine. In women, the subjective effects of stimulants such as euphoria, desire, and increased energy are potentiated during the follicular phase when estradiol levels are rising and progesterone levels are low (Justice and de Wit 2000). Moreover, administration of estradiol during the follicular phase further increases the subjective effects of psychostimulants (Justice and de Wit 2000). Once addicted, women report greater substance-dependence associated symptoms (Chen and Kandel 2002) and higher levels of craving following exposure to cocaine-related cues than do men (Robbins et al. 1999). Analogous sex differences have been observed using animal models of addiction. Adult female rats show enhanced cocaine-induced sensitization, self-administration, and conditioned place preference (Carroll et al. 2002; Russo et al. 2003; van Haaren and Meyer 1991). In addition, both adolescent and adult females show greater sensitization to cocaine than males (Laviola et al. 1995). Adult female rats consume more drug when estradiol levels are high, either during the estrus cycle or following estradiol replacement treatment in ovariectomized females (Lynch et al. 2001). In addition, sex differences in dopaminergic function have been identified and likely contribute to these effects (Walker et al. 2006). These findings suggest that gonadal hormones modulate the neural systems that mediate drug taking behaviors.
Clinical and animal studies have both shown that adolescents may be highly susceptible to the addictive properties of cocaine and other drugs. The rate of cocaine abuse and subsequent progression to dependence is greater in adolescence than in adulthood, particularly among females (SAMHSA 2007). Furthermore, the onset of drug use during adolescence is associated with an increased probability of developing life-long drug dependence (Barnes and Welte 1983; Grant and Dawson 1997; Hawkins et al. 1997). The heightened vulnerability characteristic of adolescence is thought to be a result of the reorganization of the dopaminergic systems in the brain during this period. Dopamine content, fiber density, and DA receptors reach peak levels during adolescence and undergo pruning in brain regions linked to drug abuse (Hohn and Wuttke 1979; Kalsbeek et al. 1988; Lidow et al. 1991; Tarazi et al. 1999; Teicher et al. 1995). Glutamatergic input to the prefrontal cortex also undergoes massive pruning during adolescence in humans, monkeys, and rats (Huttenlocher 1984; Insel et al. 1990).

Glutamatergic stimulation of the N-methyl-D-aspartate (NMDA) receptors opens ion channels, facilitates the influx of calcium, and activates the enzyme neuronal nNOS, which catalyzes the formation of NO from L-arginine and, subsequently, increases the synthesis of cyclic GMP (Garthwaite 1991; Snyder 1994). NO is a free radical gas with a major role in non-synaptic communication between glutamatergic and monoaminergic neurons (Kiss and Vizi 2001). NO exerts facilitatory influence on both tonic extracellular DA levels and phasic DA neuron spike activity (Grace 1991; West et al. 2002; West and Grace 2000). Striatal NO transmission is facilitated via nigrostriatal DA and frontal cortical glutamate afferents (Sammut et al. 2007a; Sammut et al. 2006; Sammut et al. 2007b). Multiple interactions between DA, glutamate, and nNOS in mesolimbic and
corticostriatal circuits (Carlsson and Carlsson 1990; Kiss 2000) suggest that NO plays a critical role in cocaine-induced behavioral plasticity. Indeed, acute systemic administration of cocaine significantly increased NO efflux in the medial prefrontal cortex in a time-dependent manner (Sammut and West 2008). Behavioral studies in adult males suggest that functional integrity of NO signaling is essential for the expression of cocaine-induced behavior including conditioned place preference (Balda et al. 2006; Kim and Park 1995), psychomotor sensitization (Balda et al. 2008; Itzhak 1997; Itzhak et al. 1998a), and cocaine self-administration (Pulvirenti et al. 1996).

Given that dopaminergic function and thus NO/DA/Glu interactions may vary in adolescents and females, we have been investigating the contribution of the nNOS gene to sex- and age-dependent effects of cocaine. Cocaine CPP studies revealed that the nNOS gene is essential during adolescence in both sexes for the maintenance and reinstatement of CPP (Balda et al. 2006). However, a prominent sexual dimorphism in response to cocaine CPP emerges in adulthood. While adult males require the nNOS gene for the maintenance and reinstatement of CPP, adult females do not, suggesting that the persisting effects of cocaine in females occur independently of nNOS (Balda et al. 2006). In a more recent study we found that the nNOS gene is essential for the induction of behavioral sensitization to cocaine in adult but not adolescent males (Balda et al. 2008). Together, this line of investigation has revealed that the NO-signaling pathway has a) a sex-dependent role in the neuroplasticity underlying cocaine CPP and b) an ontogeny-dependent influence on cocaine-induced psychomotor sensitization in males. The contribution of the nNOS gene to cocaine psychomotor sensitization in female mice remains unclear. The aim of the present study was to investigate the role of the nNOS
gene in the development of behavioral sensitization in adolescent and adult females. Although the long-term consequences of adolescent exposure to psychostimulants on vulnerability to drugs in adulthood in male rodents have been studied (Marin et al. 2008; McPherson and Lawrence 2006), little comparable information exists for female cohorts. Hence, the lasting effect of adolescent drug exposure in females was another critical aim of the present study. We report that (a) adolescent and adult exposure of females to cocaine results in long-term sensitization to cocaine into late adulthood, and (b) the persistence of long-term behavioral sensitization in females is independent of the nNOS gene. The latter finding, coupled with the results of our previous studies in adult males (Balda et al. 2008), further supports the hypothesis that the nNOS gene contributes to cocaine-induced behavioral plasticity in a sexually dimorphic manner in adulthood.

Materials and Methods

Animals

Mice purchased from Jackson Laboratories (Bar Harbor, Maine) were bred in our facilities at the University of Miami, Miller School of Medicine, Miami, FL as we described previously (Balda et al. 2006). Both genotypes, WT and nNOS KO, were generated on a mixed B6;129 genetic background (Huang et al. 1993). Animals were housed in a temperature- (22±0.5°C) and humidity- (50%) controlled room and maintained on a 12-h light/dark schedule with free access to food and water. Animal care was in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, National Academy Press, 1996) and approved by the University of Miami Animal Care and Use Committee.
Schedule of cocaine administration

Cocaine-HCl (20mg/kg) (Sigma, St. Louis, MO) was dissolved in 0.9% NaCl. All injections were given intraperitoneally (IP) in a volume of 0.1ml/10g weight. Animals’ weights were monitored daily, and behavioral experiments were carried out between 10:00 and 16:00 hours.

Experiment 1. Cocaine behavioral sensitization in adolescent WT and nNOS KO mice

Behavioral testing of adolescent mice began on PD28. “ Adolescence” represents the periadolescent period in mice and rats, which commences 7-10 days preceding the onset of puberty (PD38-40) and ends a few days thereafter (Spear and Brake 1983). Adolescent female mice (PD28) were divided into 12 groups: 6 WT and 6 nNOS KO groups. For each genotype Group 1 (n=9-10) received cocaine (20mg/kg) for 5 days and a single cocaine challenge (20mg/kg) on day 15 after a 10-day drug free period (PD42). Group 2 (n=10) received cocaine for 5 days and a single cocaine challenge on day 35 after a 30-day drug free period (PD62). Group 3 (n=8) received cocaine for 5 days and a single cocaine challenge on day 95 after a 90-day drug free period (PD122). Groups 4-6 represent control groups for groups 1-3, respectively. Groups 4, 5 and 6 (n=8-10) received saline for 5 days and a single cocaine challenge (20mg/kg) either on day 15 (PD42), 35 (PD62) or 95 (PD122).

Experiment 2. Cocaine behavioral sensitization in adult WT and nNOS KO mice

Behavioral testing of adult WT and KO mice began on PD80. Adult female mice (PD80) were divided into 12 groups: 6 WT and 6 nNOS KO groups. For each genotype Group 1 (n=7) received cocaine (20mg/kg) for 5 days and a single cocaine challenge (20mg/kg) on day 15 after a 10-day drug free period (PD94). Group 2 (n=8) received
cocaine for 5 days and a single cocaine challenge on day 35 after a 30-day drug free period (PD114). Group 3 (n=8) received cocaine for 5 days and a single cocaine challenge on day 95 after a 90-day drug free period (PD174). Groups 4-6 represent control groups for groups 1-3, respectively. Group 4, 5 and 6 (n=8-10) received saline for 5 days and a single cocaine challenge (20mg/kg) either on day 15 (PD94), 35 (PD114) or 95 (PD174).

Mice were routinely habituated to the test cage for 30 min, then saline and cocaine injections were administered and locomotor activity was recorded for 1h. The comparison between locomotor activity on day 1 and day 5 after cocaine administration determined the induction phase of behavioral sensitization. The response to cocaine challenge 10 days after discontinuation of saline/cocaine administration (day 15) was compared to a) the response to cocaine on day 5, and b) the response of saline controls that received cocaine challenge for the first time on day 15. This allowed us to determine the influence of a short (10 day) drug-free period on the expression of sensitization. The response to cocaine challenge after a 30 or 90 day drug-free period (day 35 or day 95) was determined in different groups in order to avoid multiple challenge injections. This allowed us to determine the influence of extended drug-free periods on the persistence of behavioral sensitization.

**Experiment 3: Context-dependent hyperlocomotion in adolescent and adult WT and nNOS KO mice**

Context-dependent hyperlocomotion was determined by measuring the intensity of locomotion induced by a saline injection given to saline- and cocaine- pretreated mice in the cage that had previously been paired with saline or cocaine (Itzhak 1997). WT and KO adolescent (PD 28) and adult (PD 80) mice (n=8-10) were treated with saline and
cocaine as described in experiments 1 and 2, and after 3 days received a saline injection, after which locomotor activity was recorded for 60 min.

**Measurement of locomotor activity**

The locomotor activity cages are standard transparent rectangular rodent cages (42 x 24 x 20 cm high), and locomotion is monitored by an activity meter (Opto-Varimex-Mini Model B; Columbus Instruments, Columbus, OH). The activity meter consists of an array of 15 infrared emitter/detector pairs, spaced at 2.65 cm intervals, measuring activity along a single axis of motion. Emitters and detectors are mounted alongside the length of the cage (42 cm). Both the total counts and the ambulatory counts are recorded and transferred by counter interface to a computer. The Opto-Varimex-Mini Model B separates counts (beam interruptions) of total activity from counts that correspond to ambulatory (horizontal) activity. This is accomplished by memorizing the location of the last broken beam and blocking additional counts from being scored on the front panel counter until a different beam is broken. Subtraction of the ambulatory counts from the total counts provides an index of vertical activity. Based on our previous observations (Itzhak 1997) it appears that the fraction of nonambulatory counts (25-30% of total counts) increase or decrease in parallel to corresponding changes in ambulatory counts. Because of this relationship, only ambulatory counts are reported. Counts were registered every 10 min for a total of 60 min, and results are presented as mean ± SEM cumulative horizontal counts.

**Data Analysis**

Statistical analysis was performed with SPSS 16 software. Locomotor activity due to the acute effect of saline and cocaine (Fig. 5.1) and the context-dependent hyperlocomotion
(Fig. 5.4) were analyzed by a three-way ANOVA (treatment × age × genotype). Two-way ANOVA was used to analyze results of cocaine-induced behavioral sensitization studies (treatment x time). Post hoc analysis using Bonferroni correction was used when warranted to determine differences between multiple groups. $P$ values less than 0.05 were considered significant for all tests.

**Results**

Results in Fig. 5.1 show the acute effect of saline and cocaine (20mg/kg) on locomotor activity in WT and KO adolescent (PD28) and adult (PD80) mice. Three-way ANOVA (treatment × genotype × age) revealed a significant treatment effect $F(1,219)=255.11; p<0.0001$, a significant genotype effect $F(1,219)=22.84; p<0.0001$, and a significant age effect $F(1,219)=25.42; p<0.0001$. Significant interactions were detected between treatment and genotype $F(1,219)=14.89; p<0.0001$ and between treatment and age $F(1,219)=8.17; p<0.01$. Post hoc analysis using Bonferroni correction showed that adolescent KO mice were significantly more active than adolescent WT mice when acutely administered cocaine ($p<0.01$), but not saline. Also, adult KO mice were significantly more active than adult WT mice when acutely administered cocaine ($p<0.0001$), but not saline. Interestingly, adolescent WT and KO mice were significantly less hyperactive than their adult counterparts when administered cocaine ($p<0.01$ and $p<0.001$ respectively), but not saline. Together, these results suggest that nNOS contributes to the acute psychomotor stimulating effect of cocaine and that adolescent mice are hyposensitive. However, the genotypic and ontogeny-dependent differences observed in response to cocaine cannot be attributed to differences in basal locomotor activity, as no differences were observed after saline administration.
Experiment 1: Cocaine behavioral sensitization in adolescent WT and nNOS KO mice

A two-way ANOVA (treatment × time) of the results of repeated saline and cocaine administration to adolescent WT mice revealed a significant treatment effect $F[1, 167]=98.11; p<0.0001$ and a significant time effect $F[4, 167]=30.24; p<0.0001$. Post hoc analysis using Bonferroni correction showed a significant difference between the response to cocaine on days 1 and 5 and days 5 and 15 ($p<0.001$) suggesting the development of sensitization during the initial five days and heightened sensitization after a 10 day drug-free period (Fig. 5.2A). In addition, on day 15, a comparison between saline and cocaine pre-treated mice yielded a significant difference ($p<0.001$) further suggesting the development of sensitization (Fig. 5.2A). Additional groups that had been treated with saline or cocaine in adolescence received a challenge cocaine injection after either a 1 month or a 3 month drug free period (days 35 or 95); thus cocaine challenge was given at two time points in adulthood. Cocaine-induced locomotion in mice that had previously been treated with cocaine in adolescence was significantly higher than in saline pre-treated mice after drug free periods of 30 and 90 days ($p<0.001$ and $p<0.01$, respectively ). The latter findings demonstrate long-lasting sensitization to the psychomotor stimulating effect of cocaine. Hence, WT female mice treated with cocaine during adolescence remain sensitized for an extended period of time into late adulthood.

The results of adolescent nNOS KO mice are shown in Figure 5.2B. A two-way ANOVA (treatment x time) revealed a significant treatment effect: $F[1,131]=91.95; p<0.0001$ and a significant time effect: $F[4,131]=23.15; p<0.0001$. No significant difference was detected between the response to cocaine on days 1 and 5, i.e., no
behavioral sensitization was detected during the first five days. However, post hoc analysis using Bonferroni correction showed a significant difference between the response to cocaine on days 5 and 15 (p<0.001), suggesting that a drug free period was necessary for the expression of sensitization. Comparison between the responses of saline and cocaine pre-treated mice to cocaine challenge on either day 15 (p<0.01) or day 35 (p<0.01) showed significant differences (Fig. 5.2B). On day 95, although the response of cocaine pre-treated mice appeared to be higher than controls, statistical significance was not reached. These results suggest, first, that sensitization in adolescent nNOS KO mice, unlike adolescent WT counterparts, is expressed only following a drug free period, and second, the development of sensitization during adolescence is maintained through adulthood similar to WT mice.

**Experiment 2: Cocaine behavioral sensitization in adult WT and nNOS KO mice**

Figure 5.3A depicts cocaine psychomotor sensitization in adult WT mice. A two-way ANOVA (treatment × time) revealed a significant treatment effect \( F[1, 163]=109.87; p<0.0001 \) and a significant time effect \( F[4, 163]=10.09; p<0.0001 \). Post hoc analysis using Bonferroni correction showed a significant difference between the response to cocaine on days 1 and 5 and days 5 and 15 (p<0.01 and p<0.05, respectively) suggesting the development of sensitization during the initial five days and heightened sensitization after a 10 day drug free period. These results are similar to those from adolescent WT mice. On day 15, a comparison between the responses of saline and cocaine pre-treated mice to cocaine challenge yielded a significant difference, (p<0.001). Likewise, in different groups of mice in which challenge cocaine injection was given after a 1 or 3 month drug free period, the result was a sensitized response to
cocaine (p<0.05 and p<0.001). These findings suggests that sensitization to the locomotor stimulating effects of cocaine persist for 3 months in adult WT mice, much like their adolescent counterparts.

The results of adult nNOS KO mice are shown in Figure 5.3B. A two-way ANOVA (treatment x time) revealed a significant treatment effect: $F[1,179]=102.06; p<0.0001$ and a significant time effect: $F[4,179]=10.66; p<0.0001$. Unlike adult WT mice, no significant difference was detected between the response to cocaine on days 1 and 5. Yet, Bonferroni post hoc test showed a significant difference between the response to cocaine on days 5 and 15 (p<0.001), suggesting that a drug free period was necessary for the expression of sensitization. These results are similar to those from adolescent KO mice. Comparison between the responses of saline and cocaine pre-treated mice to cocaine challenge on day 15 (p<0.05), day 35 (p<0.01), or day 95(p<0.05) showed significant differences (Fig. 5.3B) suggesting that locomotor stimulating effects of cocaine persist for 3 months in adult KO females.

Experiment 3: Context-dependent hyperlocomotion in adolescent and adult WT and nNOS KO mice

Context-dependent hyperlocomotion was investigated by saline injection, given three days (day 8) after the termination of repeated saline or cocaine administration, in the same context that saline or cocaine pretreatment had been given (Fig. 5.4). Three-way ANOVA (treatment x age x genotype) revealed a significant treatment effect $F(1,67)=55.47; p<0.0001$, a significant age effect $F(1,67)= 19.72; p<0.0001$, and a significant genotype effect $F(1,67)= 6.45; p<0.05$. A significant interaction was detected between genotype and age $F(1,67)=4.75; p<0.05$ and between age and treatment $F(1,67)=20.54; p<0.0001$. Post hoc analysis using Bonferroni correction showed that both adult WT and
KO mice that received cocaine for 5 days were significantly more hyperactive than controls when challenged with saline (p<0.0001), suggesting the development of conditioned locomotion. However, there were no significant differences between saline and cocaine pretreated adolescent WT or KO mice when challenged with saline, suggesting the absence of conditioned hyperlocomotion.

**Discussion**

The present study investigated the roles of nNOS and ontogeny in the induction and persistence of short- and long-term behavioral sensitization to cocaine in female mice. Acute administration of cocaine, but not saline, to adolescent and adult nNOS KO female mice resulted in heightened locomotor activity compared to WT counterparts (Fig 5.1). These results are reminiscent of our previous findings in males, in which adolescent and adult nNOS KO mice exhibited augmented hyperactivity in response to acute cocaine when compared to their WT counterparts (Balda et al. 2008). Together, these results suggest that nNOS contributes to the acute psychomotor stimulating effect of cocaine. Interestingly, adult WT and KO females were significantly more hyperactive than their adolescent counterparts suggesting that adolescent females are less sensitive to the acute effects of cocaine. This finding is in agreement with a number of studies pointing to adolescent hyposensitivity to acute cocaine, amphetamine, and apomorphine (Adriani et al. 1998; Bolanos et al. 1998; Laviola et al. 1999; Laviola et al. 1995; Shalaby and Spear 1980; Spear and Brake 1983).

The first finding of the present study is that repeated administration of cocaine to WT female mice during adolescence (PD28-32) resulted in robust and long-lasting sensitization to cocaine in adulthood (PD122). Though several studies have investigated
the development of sensitization to psychostimulants during the periadolescent or the adult period separately (Laviola et al. 1999; Laviola et al. 1995), the long-term consequences of adolescent exposure into adulthood are less known particularly in female subjects. The present results also indicate that a similar sensitization profile applies to WT adult females, i.e. sensitization persist for 3 months. Thus, no age effects were observed with regards to the sensitizing effect of cocaine.

The second finding of the present study is the genotypic difference between WT and nNOS KO mice in the initiation of sensitization to cocaine. Adolescent and adult WT showed two phases of sensitization, first upon repeated cocaine administration (day 5 vs. day 1; Fig 5.2A and Fig 5.3A) and second following a 10-day drug free period. Adolescent and adult nNOS KO mice did not show the “first phase” of sensitization (on day 5) but showed the “second phase” after a 10-day drug free period. Previously, we discovered this same pattern of sensitization in adolescent nNOS KO male mice (Balda et al. 2008). These findings suggest that the first phase in the development of sensitization to cocaine is nNOS-dependent, while the second later phase may be nNOS-independent. Given the prominent role of NO signaling in synaptic plasticity, primarily via cGMP/ERK/CREB signaling molecules, it is likely that absence of the nNOS gene may underlie the delayed development of behavioral sensitization. Importantly, in the present study repeated administration of cocaine to either adolescent or adult WT and nNOS KO females resulted in robust and long-lasting sensitization suggesting that the persistence of cocaine psychomotor sensitization is nNOS-independent in females. This is similar to the behavioral phenotype of adolescent males, but in contrast to that of adult males in
which the nNOS gene is essential for the development and persistence of behavioral sensitization to cocaine (Balda et al. 2008).

The results of the present study on cocaine sensitization in adult nNOS KO females differ from our previous study in adult nNOS KO females (Itzhak et al. 1998a). In the earlier study we reported that adult nNOS KO females did not develop cocaine sensitization. The cocaine dose used in the present study was 20mg/kg whereas in the previous study it was 15mg/kg; the current higher dose may have overcome any resistance to sensitization seen at the lower dose. Thus, it appears that the cocaine dose used may have significant effect on the development of sensitization in nNOS KO females.

Sexually dimorphic differences in adulthood have been previously reported in regards to other cocaine-related behaviors. For instance, we found that the maintenance and reinstatement of cocaine CPP is impaired in adult KO males when compared to adult WT males, while adult KO and WT females respond similarly to cocaine (Balda et al. 2006). These differences are likely due to post-pubertal hormonal changes because sexual dimorphism observed in CPP and psychomotor sensitization emerges only in adulthood but not adolescence. There is evidence that the gonadal hormones, estrogen and progesterone, stimulate DA release in the striatum and nucleus accumbens via a non-genomic mechanism (Becker and Rudick 1999) which may serve to enhance behavioral effects of cocaine in adult females. Furthermore, the estrous cycle was found to increase basal and amphetamine-stimulated DA in the striatum and nucleus accumbens of rats (Becker and Rudick 1999). These processes in females may contribute in part to long-term neuroadaptations underlying responsiveness to cocaine,
independently of nNOS function. Accordingly, nNOS contribution to long-term plasticity associated with the psychomotor stimulating and rewarding effect of cocaine appears to be sexually dimorphic in adulthood.

The distribution of nNOS coincides with that of gonadal hormone receptors in several regions of the limbic–hypothalamic system including the bed nucleus of the stria terminalis (BST), the amygdala, the preoptic region, the mediobasal hypothalamus, and the magnocellular nuclei (Merchenthaler et al. 2004; Shughrue and Merchenthaler 2001; Simerly et al. 1990) suggesting that steroid hormones may modulate nNOS expression. In the BST, ventromedial nucleus, and medial preoptic area there exists a significant sexual dimorphism (i.e. a greater number of nNOS-ir neurons in males than in estrus females) (Martini et al. 2008). Furthermore, male rats with a non-functional mutation of androgen receptors (Tfm males) exhibit a decrease of nNOS positive neurons suggesting that testosterone, through the action of androgen receptors, may play a significant role in the organization and modulation of the nNOS limbic-hypothalamic system (Martini et al. 2008). Furthermore, estrogen receptor α and androgen receptor interactions have been shown to regulate nNOS expression in medial amygdala, BST, MPA, and paraventricular nucleus (PVN) of male and female brain in a site-specific manner (Scordalakes et al. 2002). We propose that the sexual dimorphism in the nitrergic system, particularly that of the amygdala and BST, i.e. regions implicated in effects of cocaine (Shaham and Hope 2005; Stewart 2000), may partly contribute to the sexual dimorphism in the behavioral response to cocaine observed in nNOS KO mice. Future investigation of the potential sex-dependent differences in the expression of nNOS containing neurons in mesolimbic
structures after cocaine treatment may provide additional insight into sexual dimorphism in cocaine psychomotor sensitization and CPP.

Evidence for the differential response of adolescent and adult mice to cocaine-induced plasticity stems from the results of conditioned locomotion. Results showed significant development of context-dependent hyperlocomotion, 3 days following repeated cocaine administration, in adult WT and nNOS KO females but not in their adolescent counterparts (Fig. 5.4). Likewise, adolescent males failed to show conditioned hyperlocomotion, while adult males developed conditioned locomotion, again suggesting that context dependent hyperlocomotion is an ontogeny dependent phenomenon (Balda et al. 2008). Other studies have also shown that conditioned hyperlocomotion is ontogeny-dependent in rats (Tirelli et al. 2003), suggesting that the ability to form drug-context associations matures with time. Results also suggest a dichotomy between the magnitude of sensitization to the drug (unconditioned stimulus) and the context (conditioned stimulus). Adult WT and KO mice developed marked cocaine-induced sensitization and exhibited robust context-dependent hyperlocomotion. However, adolescent WT and KO mice exhibited behavioral sensitization under the influence of cocaine but failed to show context-dependent hyperlocomotion. These findings may be in general agreement with several studies that reported a lack of correlation between the magnitude of behavioral sensitization under the influence of cocaine and conditioned locomotion induced by saline in rats (Hotsenpiller and Wolf 2002) and mice (Tirelli et al. 2003; Tirelli et al. 2005).

In summary, behavioral sensitization to cocaine develops at a slower rate in the absence of the nNOS gene. Independent of age and genotype, cocaine behavioral
sensitization acquired in adolescence and adulthood persists for a remarkably long time in female mice. In contrast to adult males (Balda et al. 2008), the neuroadaptations underlying long-term behavioral sensitization to cocaine seem to be independent of the NO-signaling pathway in adult and adolescent female mice. These findings suggest that females and adolescents may be more vulnerable to cocaine sensitization than adult males. Thus, blockade of the NO-signaling pathway may prevent cocaine sensitization in adult males but not in females or adolescents.
Figure 5.1. Effect of the nNOS gene and age on saline- and cocaine- induced-locomotion in female mice. Adolescent (PD28) and adult (PD80) mice received IP injections of saline and cocaine (20mg/kg) and locomotor activity was recorded for 60 minutes. The magnitude of locomotor activity in adolescent nNOS KO mice was significantly higher compared to WT counterparts following cocaine (*p<0.01) but not saline administration. Likewise, the intensity of locomotion in adult nNOS KO mice was significantly higher than in adult WT mice after cocaine (*p<0.0001) but not saline administration. Both adolescent WT and KO mice were significantly less hyperactive than their adult counterparts when administered cocaine (#p<0.01 and #p<0.001, respectively), but not saline. (Taken from Balda et al. 2009a)
Figure 5.2. Effect of the nNOS gene on cocaine behavioral sensitization in adolescent female mice. Mice received IP injections of saline or cocaine (20mg/kg) for 5 consecutive days (PD28-32). Locomotor activity was recorded for 60 minutes on days 1 through 5. Mice were challenged with cocaine (20mg/kg) after a 10, 30, or 90 day drug-free period (day 15, 35, or 95 respectively). A) Comparison between the magnitude of cocaine-induced locomotor activity in adolescent WT mice on days 1 and 5 resulted in significant differences (a, p<0.001). Upon cocaine challenge, locomotor activity on day 15 was significantly higher than on day 5 (b, p<0.001). Likewise on day 15, locomotor activity in the cocaine group was significantly higher than in the control group (c, p<0.001). Cocaine challenges on day 35 and 95 resulted in higher locomotor activity in the cocaine group than in controls (d and e, p<0.001 and p<0.01, respectively). B) Comparison between the magnitude of cocaine-induced locomotor activity in adolescent
nNOS KO mice on days 1 and 5 resulted in non-significant differences. However, cocaine challenge on day 15 resulted in higher locomotor activity than on day 5 (a, \( p < 0.001 \)) and also higher locomotor activity than in control mice that received cocaine challenge on day 15 (b, \( p < 0.01 \)). Cocaine challenge on day 35 to mice that received cocaine during adolescence resulted in higher locomotor activity than in the control group that received cocaine challenge on day 35 (c, \( p < 0.01 \)). On day 95, although the response of cocaine pre-treated mice appeared to be higher than controls, statistical significance was not reached. (Taken from Balda et al. 2009a)
Figure 5.3. Effect of the nNOS gene on cocaine behavioral sensitization in adult female mice. Mice received IP injections of saline or cocaine (20mg/kg) for 5 consecutive days (PD80-84). Locomotor activity was recorded for 60 minutes on days 1 through 5. Mice were challenged with cocaine (20mg/kg) after a 10- or 30-day drug-free period (day 15 or day 35, respectively). A) Comparison between the magnitude of cocaine-induced locomotor activity in adult WT mice on days 1 and 5 resulted in significant differences (a, p<0.01). Upon cocaine challenge, locomotor activity on day 15 was significantly higher than on day 5 (b, p<0.05). Likewise on day 15, locomotor activity in the cocaine group was significantly higher than in the control group (c, p<0.001). Cocaine challenge on day 35 and day 95 resulted in higher locomotor activity in the cocaine group than in controls (d and e, p<0.05 and p<0.001). B) Cocaine
administration to adult nNOS KO mice resulted in similar magnitude of locomotion on days 1 and 5. However, cocaine challenge on day 15 resulted in higher locomotor activity than on day 5 (a, p<0.001). Comparison between the responses of saline and cocaine pre-treated mice to cocaine challenge on day 15 (b, p<0.05), day 35 (c, p<0.01), or day 95(d, p<0.05) showed significant differences. ( Taken from Balda et al. 2009a)
Figure 5.4. Effect of saline injection on locomotor activity: Context-dependent hyperlocomotion in female mice. Three days following termination of the repeated drug administration (day 8), mice received a saline injection and locomotor activity was recorded for 60 min. Both adult WT and KO mice that received cocaine for 5 days were significantly more hyperactive than controls when challenged with saline (*p<0.0001), suggesting the development of conditioned hyperlocomotion. There were no significant differences between cocaine and saline pretreated adolescent mice when challenged with saline indicating the absence of conditioned hyperlocomotion. (Taken from Balda et al. 2009a)
Chapter 6
Discussion

Enduring behavioral effects of cocaine from adolescence to adulthood

My studies have been instrumental in identifying the long-term susceptibility to drug-seeking behavior and psychomotor stimulation in adulthood following exposure to cocaine in adolescence. In chapter 2 it was demonstrated that cocaine administration to both WT male and female mice during adolescence renders the organism sensitive to reinstatement of cocaine CPP following a low-dose priming injection of cocaine in adulthood (PD70). Given that the paradigm of CPP reinstatement is relevant to drug relapse (Mueller and Stewart 2000), the results suggest that exposure to cocaine during adolescence facilitates susceptibility to future drug relapse in adulthood. This finding is particularly relevant considering the lack of investigations on the persistence of CPP acquired in adolescence into adulthood. In fact, the majority of studies have investigated CPP within the adolescent and adult periods separately and the general consensus is that adolescent rodents display greater CPP at lower drug doses (Badanich et al. 2006; Zakharova et al. 2009) while no age-dependent differences are detected at higher doses (Campbell et al. 2000; Laviola et al. 1992; Schramm-Sapyta et al. 2004).

Similarly, the long-term consequences of adolescent exposure to cocaine behavioral sensitization into adulthood have been largely overlooked. My findings in Chapters 3 (males) and 5 (females) revealed that repeated cocaine administration during adolescence (PD28-32) resulted in robust and long-lasting sensitization to cocaine in adulthood (PD122) indicating the remarkable persistence of this cocaine-induced behavioral plasticity. Only one other study (Marin et al. 2008) has shown that cocaine
administration in adolescence induced behavioral sensitization that lasted until early adulthood (PND 64), but not late adulthood (PD94). This discrepancy is most likely due to the dose of cocaine as the aforementioned study used 10mg/kg compared to the higher dose of 20mg/kg cocaine used in the current studies. Also, to my knowledge, this is the first report to describe long lasting sensitization from adolescence through adulthood in female mice (Balda et al. 2009a). Taken together these findings imply the development of long-lasting neural adaptations from adolescence through adulthood that promote high vulnerability to psychomotor stimulation and drug reward.

**Sexually dimorphic contribution of nNOS to the behavioral effects of cocaine**

Human and animal studies have revealed that females tend to display unique vulnerabilities to the effects of psychostimulants (Roth and Carroll 2004a). This enhanced susceptibility may be due to sex differences in dopaminergic function that are, in part, modulated by gonadal hormones (Walker et al. 2006). In Chapter 2 sex differences using the CPP paradigm were reported. Specifically, the magnitude of cocaine CPP in adult WT females was significantly higher than in their male counterparts suggesting that the rewarding effects of cocaine are more intense in females.

In Chapters 2, 3, and 5 ample evidence is provided that nNOS contributes to both the rewarding effects and psychomotor stimulating effects of cocaine in a sexually dimorphic manner. Adult nNOS KO male mice show major deficiencies in all measured cocaine-induced behaviors when compared to their WT counterparts. They fail to maintain and reinstate cocaine CPP and are completely resistant to cocaine-induced behavioral sensitization. Conversely, adult nNOS KO females did not differ from their WT counterparts. In adult nNOS KO females CPP was maintained for 3 weeks
posttraining and successfully reinstated following a priming cocaine injection. Furthermore, adult nNOS KO females remain fully sensitized to cocaine for up to 3 months. Collectively, these findings suggest that intact NO signaling is necessary for long-term neural plasticity associated with responsiveness to the rewarding and psychomotor stimulating effects of cocaine in adult male, but not adult female, mice.

Both behavioral sensitization and CPP are thought to be mediated, in part, by enhanced dopaminergic neurotransmission after repeated cocaine administration. The nitrergic system may contribute to these effects by amplifying DA signaling either by increasing vesicular DA release and/or by inhibiting the dopamine transporter. In adult males, these events appear to have profound effects on the response to cocaine as genetically deleting the nNOS gene attenuates most of the cocaine related behaviors analyzed. In adult females, however, enhanced basal and psychostimulant-induced striatal DA release and uptake most likely contribute to the augmented behavioral responsiveness, independently of nNOS function. Further evidence suggest that sexual dimorphism in striatal DA function may be a direct result of gonadal hormones, particularly estrogen (Becker and Rudick 1999). Although adult females in the current studies were administered cocaine without monitoring of the estrous cycle, the influence of estradiol on the strengthening of cocaine-induced behaviors must be considered. The present studies were conducted without monitoring the estrous cycle for two major reasons: 1) repeated administration of psychostimulants has been shown to alter the estrous cycle (King et al. 1993; Raap et al. 2000; Walker et al. 2001) and 2) vaginal lavage, the method commonly used to monitor the estrus cycle, interferes with the effect of cocaine (Walker et al. 2002). Moreover, it has been shown that estrogen and
progesterone may stimulate hippocampal eNOS which is unaffected in the nNOS KO females. The resulting enhanced production of NO may facilitate learning processes and thus cocaine-conditioned behavior (Grohe et al. 2004) in adult females compared to adult males.

Finally, the pronounced behavioral differences between adult KO males and females may also stem from the sexual dimorphism of the limbic–hypothalamic nNOS system. The distribution of nNOS overlaps with that of gonadal hormone receptors in the bed nucleus of the stria terminalis (BST), the amygdala, the preoptic region, the mediobasal hypothalamus, and the magnocellular nuclei (Merchenthaler et al. 2004; Shughrue and Merchenthaler 2001; Simerly et al. 1990). Evidence indicates that there is a greater quantity of nNOS-ir neurons in the BST, ventromedial nucleus, and medial preoptic area of the male rodents when compared to female rodents (Martini et al. 2008). Furthermore, male rats with a non-functional mutation of androgen receptors (Tfm males) exhibit a decrease of nNOS positive neurons suggesting that testosterone, through the action of androgen receptors, may play a significant role in the organization and modulation of the nNOS limbic-hypothalamic system (Martini et al. 2008). The sexual dimorphism in the nitricergic system, particularly that of the amygdala and BST, is especially pertinent since these regions have been implicated in drug reward (Shaham and Hope 2005; Stewart 2000). Thus, the sexual dimorphism in the nNOS limbic neurons may render adult males more sensitive to the influence of the nitricergic system on the effects of cocaine.
**Contribution of nNOS to the behavioral effects of cocaine during adolescence**

In chapter 2 evidence is provided that the nitrergic system is critically involved in the development of persistent drug seeking behavior from adolescence through adulthood. Similar to adult KO males, adolescent KO males and females failed to maintain cocaine CPP, while their WT counterparts retained the cocaine associations for at least two weeks. Additionally, a low-dose cocaine priming injection which effectively reinstated cocaine CPP in adolescent WT male and female mice failed to do so in their adolescent KO counterparts. Results suggest that the nNOS gene is essential during adolescence in both sexes for the development of long-term neural plasticity underlying responsiveness to the incentive value of cocaine reward.

In chapters 3 and 5 results demonstrate that the role of the nNOS gene in cocaine behavioral sensitization during adolescence is less prominent. Unlike adult KO males, repeated administration of cocaine to either WT or nNOS KO mice during adolescence (both sexes) resulted in robust and long-lasting sensitization to cocaine in adulthood. Subtle genotypic differences were detected in the initiation of sensitization to cocaine. Adolescent WT showed two phases of sensitization, first upon repeated cocaine administration (day 5 vs. day 1) and second following a 10-day drug free period. Adolescent nNOS KO mice did not show the “first phase” of sensitization (on day 5) but showed the “second phase” after a 10-day drug free period. Given the prominent role of NO signaling in synaptic plasticity, it is likely that absence of the nNOS gene may underlie the delayed development of behavioral sensitization.

Clearly, the NO signaling system plays more of a role in sensitization during adulthood than in adolescence. As mentioned above, behavioral sensitization is absent in
adult nNOS KO male mice, but nearly normal in nNOS KO adolescent mice. During adolescence, developmental changes in the striatal dopaminergic system may obscure the contribution of NO signaling molecules in the sensitization process which is dependent on the striatum. During adolescence, mesolimbic and striatal dopamine receptors and dopamine transporters are overproduced and subsequently pruned back to adulthood levels (Lidow et al. 1991; Tarazi et al. 1998a; b; 1999; Teicher et al. 1995). Age related differences in dopamine degradation were reported including an adult specific cocaine-induced dopamine transporter upregulation that was not expressed in adolescents (Collins and Izenwasser 2002). Also, age differences in enzymatic degradation of dopamine in the striatum (Nakano and Mizuno 1996) and the nucleus accumbens were observed (Philpot and Kirstein 2004). One possible consequence of the immature dopaminergic system, characteristic of adolescence, is that nitrergic influences may be obscured during cocaine administration. As a result, adolescent mice express a sensitized response to cocaine independently of nNOS.

My data suggest that in adolescent subjects the NO-signaling pathway contributes more to the learning of cocaine place preference than to cocaine behavioral sensitization. Although both behavioral sensitization and place conditioning paradigms are valid measures of drug responsiveness, it is thought that these two behaviors are associated with the activity of two distinct pathways, the nigrostriatal and mesolimbic dopamine systems, respectively. Although psychostimulants act on both of these pathways, drug-induced behavior is associated with different dopaminergic responses in the terminal areas of these two pathways (Cadoni and Di Chiara 1999). Further, drug-induced sensitization does not necessarily predict expression of place conditioning (Hemby et al.
1992; Martin-Iverson and Reimer 1996). Therefore differences between behavioral sensitization and CPP are not entirely unexpected. Given the prominent role of learning and memory in classical Pavlovian conditioning and the role of NO signaling in synaptic plasticity, it is likely that absence of the nNOS gene during adolescence has a more pronounced influence on learning and memory processes (conditioned place preference) than on the plasticity associated with behavioral sensitization. This may explain the deficits exhibited by adolescent KO mice in cocaine CPP that do not occur in behavioral sensitization studies.

My findings on the roles of nNOS, ontogeny, and gender on the behavioral effects of cocaine are summarized in Table 1. First, in WT mice, there were no major ontogeny- or sex-dependent differences in either cocaine sensitization or CPP. All WT subjects showed 1) development and maintenance of behavioral sensitization and 2) acquisition, maintenance, and reinstatement of cocaine CPP. The only major divergence was that adult WT females showed significantly greater cocaine CPP than male counterparts, a finding that is in agreement with the current literature supporting a trend of enhanced vulnerability to psychostimulant in females. Second, major sex-dependent differences were observed in adult KO animals. While adult KO males showed deficits in sensitization and CPP, adult KO females behaved like adult WT females in both paradigms. These findings strongly suggest that NO signaling contributes to the sensitizing and rewarding effect of cocaine in a sexually dimorphic manner. Furthermore, no sex differences were observed in adolescent KO animals suggesting that the sexual dimorphism in adulthood is most likely due to post-pubertal hormonal changes. Finally, major age-dependent differences were observed with regards to the
sensitizing effects of cocaine in KO animals. While both adolescent KO males and females developed long-lasting sensitization to cocaine, adult KO male mice did not, indicating that the nitrergic contribution to the sensitizing effects of cocaine is ontogeny-dependent. Conversely, no age dependent differences were observed in the CPP paradigm. Similar to adult KO males, adolescent KO mice failed to maintain and reinstate CPP, providing evidence that NO signaling is essential for the rewarding effects of cocaine in adolescents (both sexes), as well as in adult males. Together, this line of investigation has revealed that the NO-signaling pathway has a) a sex-dependent role in the neuroplasticity underlying cocaine CPP and b) an ontogeny- and sex-dependent influence on cocaine-induced behavioral sensitization.

**Neural correlates of behavioral sensitization to cocaine in adulthood: the role of nNOS and TH**

I have demonstrated that intact NO signaling is necessary for the behavioral plasticity associated with the sensitizing effects of cocaine in adult male mice. In Chapter 3, it was also shown that the same sensitizing regimen of cocaine resulted in a significant increase in the expression of nNOS-ir neurons (stereology) and nNOS protein levels (western blotting) in the dorsal striatum of adult male mice after a 24h drug free period. Therefore, this cocaine-induced upregulation of nNOS may underlie the induction of behavioral sensitization, since nNOS KO adult males fail to sensitize to cocaine. The neuroadaptation in nNOS is transient as the number of nNOS-ir neurons return to control levels after 10 days suggesting that nNOS does not underlie the persistence of behavioral sensitization. Instead, the modulation of downstream NO-signaling molecules (cGMP/ERK/CREB) may contribute to persistent sensitization. In addition to the temporal specificity, the increase in nNOS is also restricted to a specific brain region, the
dorsal striatum. Cocaine did not affect the number of nNOS-ir neurons in the NAC or PFC, two areas often linked with behavioral sensitization (Le Moal and Simon 1991; Pierce and Kalivas 1997).

In chapter 4 further evidence is provided for the importance of the nNOS system in behavioral sensitization. In the absence of nNOS there is a considerable reduction in dopaminergic neurons of the VTA and dopaminergic terminals of the dST. This result is consistent with reports of reduced TH protein levels observed in the adrenal glands and hypothalamus of these mice (Orlando et al. 2008; Yamova et al. 2007). Considering that DA transmission in the VTA is necessary for the induction of cocaine sensitization (Vanderschuren and Kalivas 2000), the decrease in dopaminergic neurons could explain the lack of sensitization in adult KO males. The significance of decreased TH in KO adolescence is less clear. However, the delayed induction of sensitization in the younger animals could be a consequence of reduced VTA TH-ir neurons. Nevertheless, nNOS seems to have a significant role in modulating the levels of TH in the VTA.

Interestingly, the same cocaine-sensitizing regimen also resulted in a significant increase in TH protein levels in the dST of adult WT mice after a 24h drug free period. This cocaine-induced overexpression of dST TH parallells the cocaine-induced overexpression of dST nNOS-ir neurons suggesting regulation of dST nitrergic/dopaminergic transmission by cocaine. Conversely, in the nNOS KO mice cocaine induced a decrease in dST TH-ir fibers. Together, these findings suggest that a constitutive lack of nNOS causes an imbalance in the dopaminergic system under resting conditions and after cocaine administration. Although no direct data is provided, the following lines of evidence suggests that nNOS contributes to cocaine-mediated increase
in TH observed in adult mice: 1) In nearly all mesolimbic, corticostriatal, and nigrostriatal regions populations of TH-ir and NOS-ir neurons interact with each other as manifested by the presence of NOS-ir endings on TH-ir neurons and *vice versa* (Benavides-Piccione and DeFelipe 2003; Fujiyama and Masuko 1996; Hidaka and Totterdell 2001; Klejbor et al. 2004; Matthews et al. 1997), 2) a constitutive lack of nNOS leads to reduction in TH levels in the adrenal gland, hypothalamus, and VTA in nNOS KO mice (Orlando et al. 2008; Yamova et al. 2007; Balda et al. 2009b), 3) while adult WT mice repeatedly administered cocaine experience an increase in dST TH, the opposite effect is seen in adult nNOS KO mice and 4) a recent study demonstrated that the NO generators, sodium nitroprusside (SNP) and S-nitroso-\(N\)-acetyl-D,L-penicillamine, upregulated transcript levels of tyrosine hydroxylase, dopamine \(\beta\)-hydroxylase, and phenylethanolamine \(N\)-methyltransferase, accompanied by long-term increases in their enzyme activities and the intracellular catecholamine levels (Kim et al. 2003). Thus, it appears that overexpression of dST nNOS and subsequent increase in TH are associated with sensitization in adulthood.

**Neural correlates of behavioral sensitization to cocaine in adolescence: the roles of nNOS and TH**

The contribution of nNOS to the sensitizing effects of cocaine depends largely on the age of the subject. While adult KO male mice were incapable of sensitizing to cocaine, adolescent KO males developed long-lasting cocaine-induced sensitization, suggesting sensitization occurs independently of nNOS during adolescence. This finding is most likely related to the fact that cocaine modulation of the nitrergic system occurs in an age-specific manner. Specifically, repeated cocaine treatment had no effect on the expression of striatal nNOS-ir neurons or protein levels in adolescent WT male mice, as
it did in adult WT mice. Thus, the increased expression of striatal nNOS seems to be associated with behavioral sensitization to cocaine in adulthood, but not in adolescence.

In parallel with the striatal nitrergic system, cocaine modulation of dopaminergic terminals in the dST also occurs in an ontogeny-dependent manner. While a sensitizing regimen of cocaine increased striatal TH-ir terminals in adult WT males, it had no effect on striatal TH-ir terminals in adolescent WT mice. Similarly, others have found that TH levels were not altered in the NAC when rats received cocaine during adolescence (Marin et al. 2008). Thus, the inability of cocaine to alter TH is probably related to its inability to upregulate nNOS in adolescence. Yet WT mice from both age groups developed sensitization to cocaine suggesting that this behavioral plasticity in adolescence develops independently of nNOS and TH. Adolescent specific developmental CNS changes most likely obscure the contribution of these enzymes to the sensitization process.

Figure 6.1 summarizes my findings on the cocaine-induced molecular changes in nNOS and TH thought to underlie cocaine-induced behavioral sensitization in WT mice. **Acutely**, cocaine binds and inhibits the DAT causing an accumulation of DA in the synaptic cleft (1). In addition, cocaine has the ability to elevate extracellular glutamate levels (2) which in turn stimulates NMDA receptors causing an influx of Ca\(^{2+}\) responsible for the activation of nNOS and formation of NO (3). The increase in EC DA (4) may be further potentiated by NO-mediated inhibition of the DAT and/or cGMP-mediated vesicular release. Up to this point, the molecular events are similar in the brains of adolescent and adult mice. However, **repeated** administration of cocaine for 5 days causes an increase in the number of nNOS-ir neurons (5) and protein levels of TH (6) exclusively in the dST of WT adult males. Presumably these enzymatic changes translate
into to an increase in bioactive NO from nNOS as well as DA from TH. Whether the increase in nNOS triggers the increase in TH remains unclear. Nevertheless, the ensuing increase in DA neurotransmission is thought to underlie the development of behavioral sensitization in WT adult mice. In adolescent WT mice, repeated cocaine administration is not associated with an upregulation of either nNOS or TH (events 5 and 6 are missing on the left side of the panel). Regardless, long-lasting behavioral sensitization occurs in adolescent WT suggesting this behavioral plasticity during this time period develops independently of nNOS and TH.

Figure 6.2 summarizes my findings on behavioral sensitization and molecular changes in TH induced by cocaine in KO mice. As in WT mice, cocaine is thought to (1) increase synaptic DA by inhibiting the DAT and (2) elevate EC glutamate levels. However, the glutamate mediated formation of NO is not possible due to the deficiency in nNOS (3). nNOS KO mice are also unable to undergo the nNOS upregulation seen in adult WT mice (5). Furthermore, instead of the cocaine-induced upregulation in TH-ir terminals observed in adult WT mice, cocaine causes the reverse effect in the absence of nNOS (6). Similarly, nNOS deficient mice display reduced levels of dopaminergic neurons in the VTA when compared to their WT counterparts (7). Combined, these differences most likely result in an overall reduction in DA (4) and a lack of cocaine-induced behavioral sensitization in adult KO male mice. In adolescent KO mice these differences have very little effect on the development and long-term persistence of behavioral sensitization. The finding that nNOS adolescent mice develop sensitization despite a downregulation in TH (VTA and dST), together with the lack of nitrergic and dopaminergic cocaine-induced neuroadaptations in adolescent WT mice, support my
claim that dST TH and nNOS levels in adolescence do not correlate with cocaine-induced sensitization.

In conclusion my studies have contributed to our understanding of the roles of nNOS, ontogeny, and gender in the rewarding and psychomotor stimulating effects of cocaine. My studies revealed that the NO-signaling pathway has a) a sex-dependent role in the neuroplasticity underlying cocaine CPP and b) an ontogeny- and sex-dependent influence on cocaine-induced behavioral sensitization. Furthermore, cocaine upregulates nitrergic and dopaminergic neurons in adult, but not adolescent mice, suggesting different neural adaptations, consequent to cocaine exposure, develop during adolescence and adulthood. Thus, results from my studies indicate that clinical treatment of drug craving and drug-seeking should be tailored to the gender and age of the patient population.

**Future directions**

My findings have opened up several avenues of additional research. First, since all of my studies identifying a role for nNOS in behavioral sensitization and cocaine CPP were carried out in nNOS KO mice, confirmatory pharmacological studies using nNOS inhibitors or NO donors would be of great value. Second, given that one dose of cocaine -20mg/kg- was utilized in all experiments, dose response studies could be conducted to determine the relationship between different doses of cocaine and the accompanying changes in physiology and behavior. Third, my investigations could be extended to probe the neural substrates underlying cocaine CPP by examining cocaine-induced nitrergic neuroadaptations in relevant brain regions such as the hippocampus and amygdala. Fourth, the significant role of sex and possibly gonadal hormones in the effects of nNOS on cocaine-induced behaviors should be investigated 1.) by performing
ovariectomy studies in adult nNOS KO females and 2.) studying potential gender-dependent neuroadaptations in nitrergic neurons as potential reasons for the sexually dimorphic contribution of nNOS to the behavioral effects of cocaine. Fifth, the molecular mechanisms responsible for the cocaine-induced upregulation of nNOS should be thoroughly addressed. Finally, the relationship between the cocaine-mediated up-regulations of nNOS and TH should be elucidated.

a) **Pharmacological studies to supplement current findings**

Major deficits in the maintenance and reinstatement of cocaine CPP were observed in adult KO male mice and adolescent KO mice of both sexes. This data was partially corroborated by Itzhak and Anderson (2007) who demonstrated that in adult male mice pharmacological inhibition of nNOS with 7-nitroindazole (7-NI) prevented maintenance and reinstatement of cocaine CPP, while augmentation of NO signaling with molsidomine rescued such deficits. Analogous testing in adolescent mice would be advantageous. With regards to behavioral sensitization, it was shown that adolescent mice, but not adult males, sensitized to cocaine independently of nNOS. This is in agreement with an earlier study which showed that 7-NI attenuated behavioral sensitization in adult male Swiss Webster mice (Itzhak 1997). Future efforts should be directed at determining whether 7-NI would be ineffective in hindering behavioral sensitization in adolescent mice. Additionally, the ability of molsidomine to reverse the resistance of adult KO male mice to cocaine sensitization would be significant. Collectively, these studies would support my hypothesis on the critical role of nNOS in cocaine CPP and behavioral sensitization. Furthermore, pharmacological manipulation
of this pathway offers a potential application in the clinical management of sensitization to drug craving and drug-seeking behavior.

**b) Dose response studies**

The inclusion of dose response studies would greatly broaden my discoveries. In this way, an association between varying doses of cocaine and the accompanying changes in sensitization and CPP as well neural changes would be possible. This is of particular importance given that adolescent mice display greater cocaine sensitization and cocaine CPP at lower doses of 5-10 mg/kg (Badanich et al. 2006; Laviola et al. 1995; Niculescu et al. 2005; Ujike et al. 1995; Zakharova et al. 2009), but not at higher doses (Bolanos et al. 1996; Campbell et al. 2000; Collins and Izenwasser 2002; Guerriero et al. 2006; Laviola et al. 1992; Schramm-Sapyta et al. 2004), suggesting that adolescents are more sensitive to the addictive properties of low dose cocaine. In this thesis, age-dependent behavioral differences in WT mice were undetectable most likely because a relatively high dose of cocaine was used. In the gender studies, it was discovered that when compared to WT adult males, WT adult female rodents developed higher cocaine CPP, but not higher behavioral sensitization. Perhaps, sex-dependent differences in behavioral sensitization would have been revealed at lower doses. Furthermore, the initial dose of cocaine could potentially have an influence on the long-term effects of drug exposure, such as long-term sensitization and persistence and reinstatement of CPP. Finally, it would also be interesting to determine if the changes in nNOS and TH associated with repeated cocaine administration would be dose dependent.
c) Neural substrates underlying cocaine CPP-the role of nNOS

The molecular studies in this thesis were designed primarily to probe the role of nNOS in the neural plasticity underlying behavioral sensitization. However, a major influence of nNOS in the rewarding effects of cocaine using the CPP paradigm was also identified. Various studies have implicated the amygdala and hippocampus as key players in the Pavlovian learning needed for cocaine CPP. For example, lesions of various nuclei of the amygdala and hippocampus interfere with both acquisition and retrieval of cocaine CPP (Fuchs et al. 2003; Meyers et al. 2003). Furthermore, numerous studies have established that glutamatergic signaling is necessary for appetitive Pavlovian conditioning (Di Ciano et al. 2001; Mead and Stephens 2003). Similarly, major deficits in cocaine CPP maintenance and reinstatement in mice lacking nNOS were discovered (Chapter 2). Thus, a comprehensive investigation into the expression of nNOS in hippocampal and amygdalar nuclei after cocaine CPP training may provide insight into the genotypic differences in cocaine CPP.

d) The role of sex and gonadal hormones in the effects of nNOS on cocaine-induced behaviors

The nNOS gene contributes to the behavioral effects of cocaine in a sexually dimorphic manner using two independent measures, cocaine behavioral sensitization and cocaine CPP. Specifically, it was discovered that adult females develop and maintain both behaviors regardless of the presence of nNOS. I speculate that the nNOS-independent augmented behavioral responses to cocaine may be due to the effects of gonadal hormones. Thus, an important future experiment would be to determine if ovariectomies in adult nNOS KO females would switch their behavioral phenotype to resemble their KO male counterparts (i.e., attenuation of cocaine-induced sensitization
and CPP maintenance and reinstatement). In turn, treating ovariectomized adult KO female with estrogen should reinstate long-term cocaine sensitization and CPP when compared to ovariectomized females treated with vehicle.

The influence of sex on cocaine-induced nitricergic neuroadaptations should also be addressed. Just as age-specific differences in the modulation of nNOS and TH by cocaine were found, comparable studies should be extended to include female subjects. nNOS and TH immunoreactivity before and after cocaine administration should be assessed in dST, NAC, VTA, amygdala and hippocampus in adult females and compared to the changes in male subjects. The existence of basal sexual dimorphism in the nNOS system has already been reported in the amygdala and BST (Martini et al. 2008; Scordalakes et al. 2002). These and undiscovered sex differences in cocaine-induced neuroadaptations may provide insight into sexual dimorphism in the behavioral response to cocaine.

e.) Molecular mechanisms underlying the cocaine-induced upregulation of nNOS

Others, including myself, have shown that repeated psychostimulant administration is associated with increases in NO formation (Sammut and West 2008), nNOS activity (Bhargava and Kumar 1997; Lin et al. 1999), striatal nNOS mRNA expression (Wang and Lau 2001), number of nNOS-ir neurons and protein levels (Balda et al. 2008). The mechanism by which cocaine causes an upregulation of the nNOS system is not known and studies addressing this question are warranted. One such possible mechanism is the upregulation of cAMP/CREB pathway which is one of the earliest and well studied cocaine-induced neuroadaptions (Carlezon et al. 1998; Miserendino and Nestler 1995; Nestler et al. 1990; Pliakas et al. 2001; Self 1998; Striplin...
and Kalivas 1992; Terwilliger et al. 1991). Several studies indicate that cAMP regulates nNOS (promoter activity and/or mRNA/protein) (Bachir et al. 2003; Boissel et al. 2003). Furthermore, the classical cAMP-PKA-CREB pathway mediates a significant portion of the cAMP-induced upregulation of nNOS via gene transcription (Boissel et al. 2004). Thus, cocaine-mediated increases in cAMP-PKA-CREB pathway may partially contribute to the increases in nNOS reported in this thesis. Therefore, a potential line of investigation could include studying cocaine-induced changes in nNOS following manipulation cAMP and PKA levels.

f.) Investigation of the relationship between cocaine-mediated increases in nNOS and TH

Repeated cocaine treatment leads to increases in both dST nNOS and TH in adult WT males. There is evidence suggesting that nNOS may trigger the upregulation of TH. For instance, amplification of NO signaling results in long-term increases in TH transcript levels, enzymatic activity, and intracellular levels (Kim et al. 2003). Conversely, a lack of constitutive nNOS leads to a downregulation of TH levels in the adrenal gland, hypothalamus, and VTA (Orlando et al. 2008; Yamova et al. 2007; Balda et al. 2009b). Lastly, cocaine treatment which causes an increase in TH in the dST of WT adult males, has the reverse effect on TH levels in nNOS KO adult mice (Balda et al. 2009b). Therefore, future research investigating the link between nNOS and TH changes is needed. One potential strategy includes tissue-specific and temporal inactivation of the nNOS gene in conditional knock out mice. In this way, it could be determined if controlled inactivation of the nNOS gene causes impairments in both cocaine-induced behavioral sensitization and TH alterations. More importantly, this method would allow us to determine if activating the gene reverses the behavioral and molecular phenotype.
Table 6.1. Summary of the roles of nNOS, ontogeny, and gender on the behavioral effects of cocaine. In WT mice, there were no major ontogeny- or sex-dependent differences in either cocaine sensitization or CPP. All WT subjects showed 1) development and maintenance of behavioral sensitization and 2) acquisition, maintenance, and reinstatement of cocaine CPP. The only major divergence was that adult WT females showed significantly greater cocaine CPP (+++) than male counterparts, a finding that is in agreement with the current literature supporting a trend of enhanced vulnerability to psychostimulant in females. In KO mice, major sex-dependent differences were observed in adulthood. While adult KO males showed deficits in sensitization and CPP, adult KO females behaved like adult WT females in both paradigms. These findings strongly suggest that NO signaling contributes to the sensitizing and rewarding effect of cocaine in a sexually dimorphic manner. In KO mice, major age-dependent differences were observed with regards to the sensitizing effects of cocaine in KO animals. While both adolescent KO males and females developed long-lasting sensitization to cocaine, adult KO male mice did not, indicating that the nitrergic contribution to the sensitizing effects of cocaine is ontogeny-dependent. Conversely, no age dependent differences were observed in the CPP paradigm. Similar to adult KO males, adolescent KO mice failed to maintain and reinstate CPP, providing evidence that NO signaling is essential for the rewarding effects of cocaine in adolescents (both sexes), as well as in adult males. Together, this line of investigation has revealed that the NO-signaling pathway has a) a sex-dependent role in the neuroplasticity underlying cocaine CPP and b) an ontogeny- and sex-dependent influence on cocaine-induced behavioral sensitization.

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Figure 6.1. Proposed model of the relationship between cocaine-induced behavioral and neural plasticity in WT mice. Acutely, cocaine inhibits the DAT causing an accumulation of DA in the synaptic cleft (1). In addition, cocaine has the ability to elevate extracellular glutamate levels (2) which in turn stimulates NMDA receptors causing an influx of Ca^{2+} responsible for the activation of nNOS and formation of NO (3). The increase in EC DA (4) may be further potentiated by NO-mediated inhibition of the DAT and/or cGMP-mediated vesicular release. Up to this point, the molecular events 1-4 are thought to be similar in the adolescent and adult dST. However, repeated administration of cocaine for 5 days causes an increase in the number of nNOS-ir neurons (5) and protein levels of TH (6) exclusively in the dST of WT adult males. The ensuing increase in DA neurotransmission is thought to underlie the development of behavioral sensitization in WT adult mice. In adolescent WT mice, repeated cocaine administration is not associated with an upregulation of either nNOS or TH (events 5 and 6 are missing on the left side of the panel). Regardless, long-lasting behavioral sensitization occurs in adolescent WT suggesting this behavioral plasticity during this time period develops independently of nNOS and TH. Abbreviations: D1R, D1 dopamine receptor; D2R, D2 dopamine receptor; DA, dopamine; DAT, dopamine reuptake transporter; DDC, dopamine decarboxylase; Glu, glutamate; L-DOPA, levodopa; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; TH, tyrosine hydroxylase.
Figure 6.2. Proposed model of the relationship between cocaine-induced behavioral and neural plasticity in nNOS KO mice. Acutely, cocaine is thought to (1) increase synaptic DA by inhibiting the DAT and (2) elevate EC glutamate levels. However, the glutamate mediated formation of NO is not possible due to the deficiency in nNOS (3). nNOS KO mice are also unable to undergo the nNOS upregulation seen in adult WT mice (5). Furthermore, instead of the cocaine-induced upregulation in TH-ir terminals observed in adult WT mice, cocaine causes the reverse effect in the absence of nNOS (6). Similarly, nNOS deficient mice display reduced levels of dopaminergic neurons in the VTA when compared to their WT counterparts (7). Combined, these differences most likely result in an overall reduction in DA (4) and a lack of cocaine-induced behavioral sensitization in adult KO male mice. In adolescent KO mice these differences have very little effect on the development and long-term persistence of behavioral sensitization.

The finding that nNOS adolescent mice develop sensitization despite a downregulation in TH (VTA and dST) support the claim that dST TH and nNOS levels in adolescence do not correlate with cocaine-induced sensitization. Abbreviations: D1R, D1 dopamine receptor; D2R, D2 dopamine receptor; DA, dopamine; DAT, dopamine reuptake transporter; DDC, dopamine decarboxylase; Glu, glutamate; L-DOPA, levodopa; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; TH, tyrosine hydroxylase; VTA, ventral tegmental area.
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