INDIVIDUAL SENSITIVITY TO NOVELTY AND (+)-3,4-METHYLENEDIOXYMETHAMPHETAMINE: ROLES FOR SEROTONIN AND GABA NEUROTRANSMISSION

by

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To my friends and family, who have supported and loved me from the beginning.

And to my beautiful mother, who taught me that there is no more powerful force in the world than education.

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Drug addiction continues to be a problem in our society, and better understanding of the neuroanatomical and neurochemical alterations that delineate the switch between causal drug use and compulsive drug addiction is needed. Characterizing what makes one individual more vulnerable to the development of compulsive drug-taking behaviors may hold the key to this complex phenomenon. Because individual differences in humans exist to the subjective effects of 3,4-methylenedioxymethamphetamine (MDMA) and these differences are rooted, in part, in individual sensitivity to the drug effects, we utilized two animal models of increased sensitivity in the current studies. First, in a sensitization animal model we examined the mechanisms of increased sensitivity to (+)-MDMA and found a critical role for serotonin (5-HT) neurotransmission, in particular the 5-HT_{2A} receptor (5-HT_{2A}R) in the nucleus accumbens (NAc) and prefrontal cortex (PFC). We then carried this finding into a model of individual difference in which animals are separated based on their differential locomotor response to a novel environment into high responder rats (HR) and low responder rats (LR). In addition to an increased sensitivity to (+)-MDMA, we uncovered basal differences in the 5-HT system between HR and LR rats, an increased level of expression of the 5-HT_{2A}R in the NAc of HR rats in particular. Additionally, we examined the brain structures activated secondary to novelty in HR vs. LR rats and the phenotype-specific behavioral changes after repeated exposure to the environment. Our findings revealed a strong influence of GABA neurotransmission that may underlie the differences between HR vs. LR behavioral phenotypes. These findings lend support to the idea that the neural systems underlying drug-induced and stress-induced behaviors overlap and may help to understand how individual sensitivity to both (+)-MDMA and novelty may confer an increased vulnerability to the development of compulsive drug-taking behavior.

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LIST OF ABBREVIATIONS

5-HT	Serotonin
5-HT R	Serotonin receptor
5-HT_{1A}R	Serotonin 1A receptor
5-HT_{1B}R	Serotonin 1B receptor
5-HT_{2A}R	Serotonin 2A receptor
5-HT_{2C}R	Serotonin 2C receptor
5,7-DHT	5,7-dihydroxytrypamine
6-OHDA	6-hydroxydopamine
ACTH	Adrenocorticotropin releasing hormone
ANOVA	Analysis of variance
AON	Anterior olfactory nucleus, dorsolateral
AOM	Anterior olfactory nucleus, medial
BLA	Basolateral nucleus of amygdala
BNST	Bed nucleus of the stria terminalis
CA1	CA1 of hippocampus

CeA	Central nucleus of amygdala
Cing	Cingulate cortex
CL	Centrolateral thalamic nucleus
CLi	Caudal linear nucleus of raphe
CM	Central medial thalamic nucleus
CPu	Caudate putamen
CRF	Corticotrophin-releasing factor
DA	Dopamine
DAB	3,3'-diaminobenzidine
DOI	2,5-dimethoxy-4-iodoamphetamine
DRN	Dorsal raphe nucleus
GABA	?-aminobutyric acid
GAD-67	Glutamate decarboxylase-67
Hab	Habenular complex
HPA	Hypothalamic-pituitary-adrenal

HR	High responder rats
IF	Interfascicular nucleus of the ventral tegmental area
IL	Infralimbic cortex
IMD	Intermediodorsal thalamic nucleus
IP	Intraperitoneal
LC	Locus coeruleus
LOrbC	Lateral orbital cortex
LR	Low responder rats
LSI	Lateral septal nucleus, intermediate portion
M100907	R-(+)-(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenylethyl)]-4-piperidine-methanol
MD	Mediodorsal thalamic nucleus
MDMA	3,4-methylenedioxymethamphetamine
MK 212	6-chloro-2-(1-piperazinyl)pyrazine

MRN	Median raphe nucleus
NAc	Nucleus accumbens
NAcC	Nucleus accumbens core
NAcS	Nucleus accumbens shell
PB	Parabrachial pigmented nucleus of the ventral tegmental area
PBS	Phosphate-buffered saline
PBS-T	Phosphate-buffered saline containing 0.4% Triton-X
PFC	Prefrontal cortex
PN	Paranigral nucleus of the ventral tegmental area
PTSD	Post-traumatic stress disorder
PrL	Prelimbic cortex
PVA	Paraventricular thalamic nucleus
PVN	Paraventricular hypothalamic nucleus
RLi	Rostral linear nucleus of raphe

Sal	Saline
SC	Subcutaneous
SD	Standard deviation
SEM	Standard error of the mean
SN	Substantia nigra
SNc	Substantia nigra pars compacta
TH	Tyrosine hydroxylase
VP	Ventral pallidum
VTA	Ventral tegmental area

CHAPTER 1:

INTRODUCTION

The evolution of casual drug use to compulsive drug addiction is a process that has defied understanding. Drug addiction is defined as continued drug use and compulsive drug-seeking despite a desire to stop and at the expense of other priorities (Edwards et al., 1981). There are individual differences in the number of drug exposures and duration of drug use that precede a diagnosis of dependence. While many people experiment, few users actually become addicted. Of the 60% of Americans who use illicit drugs at some point in their lives (Johnston et al., 2001), only a small subset will go on to develop addiction. For example, of those who consider themselves regular users of cocaine, only 16% will develop dependence (Wagner and Anthony, 2002). Conversely, some individuals in treatment for opiate dependence report that they felt the full throes of addiction after only one exposure to the drug (O'Brien et al., 1986). There are also reported studies of individual differences in the discrimination of the subjective effects of drugs of abuse in humans (De Wit et al., 1986; O'Brien et al., 1986). Subjects report a wide range of subjective effects to the initial dose of amphetamine (De Wit et al., 1986), and this initial sensitivity can be correlated with how many drug exposures are required to meet the criteria for drug addition (O'Brien et al., 1986).

Model of differential vulnerability to drug addiction

Individual differences have also been documented in animal models of drug-seeking behavior. In one animal model, rats are separated based on their locomotor activity in response to the mild stress of a novel environment. Rats who exhibit higher levels of locomotor activity, or high responder rats (HR), are more sensitive to the locomotor-stimulating and reinforcing properties of drugs of abuse than those who exhibit lower levels of activity, or low responder rats (LR) (Piazza et al., 1989). HR rats have been hypothesized to have an underlying increased sensitivity of the dopamine (DA) mesolimbic pathway (Dellu et al., 1996) consisting of DAergic projections from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) (Swanson, 1982). The DA mesolimbic circuit in HR rats appears to be more reactive to a variety of different stimuli, with differential responses to novelty exposure (Cain et al., 2004; Piazza et al., 1989), drugs of abuse [amphetamine (Piazza et al., 1989), cocaine (Hooks et al., 1991b) and morphine (Deroche et al., 1992)] and food reinforcement (Hooks et al., 1994). HR rats also have a greater susceptibility to developing drug-taking behaviors in the self-administration paradigm (Piazza et al., 1989; Piazza et al., 2000), as well as an increased sensitivity to the acute locomotor-stimulating (Hooks et al., 1991a) and sensitizing effects of drugs (Hooks et al., 1992b; Hooks et al., 1991a). Because of these underlying differences in both overt drug-induced behaviors and in the reinforcing properties of drugs of abuse, the HR vs. LR animal model provides an excellent opportunity to examine the factors that contribute to individual differences in the development of compulsive drug-taking behaviors.

History of MDMA

Individual differences in the use patterns have been demonstrated for the club drug 3,4-methylenedioxymethamphetamine (MDMA, 'ecstasy') (Gerra et al., 1998; Laviola et al., 1999). MDMA is a substituted amphetamine with psychostimulant properties similar to amphetamine and mescaline (Shulgin et al., 1991; Steele et al., 1994). MDMA was first synthesized by Merck pharmaceuticals in 1912 and patented in 1914 for its anorectic properties. MDMA fell into obscurity due to lack of clinical utility until the drug resurfaced as a psychotherapeutic adjunct (Greer and Tolbert, 1998). MDMA is classified as Schedule I narcotic by the Drug Enforcement Agency, and therefore is defined as a drug which has a high potential for abuse and no currently accepted medical use in the United States (i.e. LSD, heroin, marijuana). Use of MDMA has steadily increased, especially in younger age groups (Adlaf et al., 1996), but despite its abuse potential has several viable therapeutic possibilities. MDMA was briefly employed as an adjunct to psychotherapy (Greer and Tolbert, 1998). Also, clinical trials of its efficacy in treating anxiety in terminally ill cancer patients (Multidisciplinary Association for Psychedelic Studies, 2004) and in patients with post-traumatic stress disorder (PTSD) (Braff et al., 2001; Doblin, 2002; Multidisciplinary Association for Psychedelic Studies, 2004) are ongoing.

Behavioral and Physiological Effects of MDMA

The psychotropic effects of MDMA in humans include increased self-confidence, energy, mood and decreased inhibitions (Liester et al., 1992). The time course of onset is approximately 20 minutes after oral administration with maximal subjective effects between 90 and 120 minutes (Morgan, 2000). MDMA induces feelings of empathy, love and connectedness with other people along with an increased ability for self-exploration (Liester et al., 1992). Some users report flashes of light or objects in their visual field, but there have been no reports of florid hallucinations (Cami et al., 2000; Shulgin et al., 1991; Steele et al., 1994). Negative side effects of MDMA include insomnia, difficulty concentrating, anxiety, depression and memory impairment, the extent of which correlates to degree of self-reported MDMA exposure (Bolla et al., 1998). Physiological changes include increased blood pressure and heart rate and hyperthermia which can be life-threatening (Cami et al., 2000; McCann et al., 1996), as well as head and muscle aches which present anywhere from 24 to 48 after drug exposure (Morgan, 2000). A survey of human MDMA users revealed that tolerance to the positive subjective effects of MDMA develops quickly, while there is a sensitization to the negative effects (Peroutka et al., 1988).

While the racemic mixture of isomers is the form of MDMA used by humans on the street [(±)-MDMA], studies in animals have also been conducted using the more [(+)-MDMA] and less potent [(-)-MDMA] forms of the drug. (+)-, (-)- and (±)-MDMA in animal models induces hypermotility, particularly in the periphery of the activity chamber (Gold et al., 1988; Herin et al., 2004; Paulus and Geyer, 1992). In addition, rats

are able to discriminate between the subjective effects of (±)-, (+)- or (-)-MDMA and saline (Baker et al., 1997; Bubar et al., 2004; Schechter, 1986), and to a certain extent both isomers as well as racemic MDMA support drug-seeking in a self-administration paradigm (Fantegrossi et al., 2002; Ratzenboeck et al., 2001; Schenk et al., 2003). Also, (+)- and (±)-MDMA have been shown to induce behavioral sensitization, or an enduring enhancement in the behavioral effects of psychomotor stimulants with repeated, intermittent exposure (Bubar and Cunningham, 2005; Kalivas et al., 1998).

Inidividual Sensitivity to Novelty and MDMA

Drug addiction continues to be a problem in our society, and better understanding of the neuroanatomical and neurochemical alterations that delineate the switch between causal drug use and compulsive drug addiction is needed. Characterizing what makes one individual more vulnerable to the development of compulsive drug-taking behaviors may hold the key to this complex phenomenon. Because human individual differences in the initial reinforcing effects of MDMA are thought to be rooted in individual sensitivity to the drug, we utilized two animal models of increased sensitivity in the current studies. First, in a sensitization animal model we studied the role of serotonin (5-HT) neurotransmission in the behavioral effects evoked by acute administration of (+)-MDMA. These findings led us to hypothesize a role for 5-HT neurotransmission in other models of increased sensitivity to psychostimulants, so we examined the sensitivity of HR vs. LR rats to (+)-MDMA, alterations in 5-HT neurotransmission and phenotype-specific behavioral changes after repeated exposure to the environment. Finally, we

examined the brain structures activated secondary to novelty in HR vs. LR rats using c-Fos expression. A member of the AP-1 family of transcription factors, expression of the immediate-early gene protein c-Fos (as well as *c-fos* mRNA) is often used as a marker of neuronal activity (Dragunow and Faull, 1989; Sagar et al., 1988). Increases in c-Fos expression are induced rapidly in the brain in response a variety of stimuli and control the transcription of a number of other genes (Kouzarides and Ziff, 1988). In this way, we can map the anatomical circuits differentially engaged by HR vs. LR rats in response to a novel environment. The goal of the current studies is to identify potential behavioral and anatomic differences between vulnerable rats (sensitized rats and HR rats) and their less sensitive counterparts that may reveal differences in neurotransmitter systems and their underlying anatomic substrates.

Even in the face of the recent progress made in the understanding of the mechanisms of developing drug addiction and neurobiology of craving (Robinson and Berridge, 1993), pharmacotherapies in the treatment of drug dependence are extremely limited (Kaplan and Sadock, 1998). The current standard of care is individual, family and group psychotherapy in an inpatient setting with complete isolation from social situations in which patients obtained or used drugs (Higgins et al., 1993; Kang et al., 1991; Kaplan and Sadock, 1998). With these studies, we hope to better understand how increased sensitivity to drugs of abuse may increase individual vulnerability to developing compulsive drug-taking behaviors. We hope to find common patterns between the two animal models that lend insight into factors that may predispose one individual to higher sensitivity to the effects of drugs of abuse.

CHAPTER 2:

CHRONIC TREATMENT WITH THE SEROTONIN$_2$ RECEPTOR (5-HT$_2$R) AGONIST DOI MODULATES THE BEHAVIORAL AND CELLULAR RESPONSE TO (+)-3,4-METHYLENEDIOXYMETHAMPHETAMINE [(+)-MDMA]

Background and Significance

3,4-Methylenedioxymethamphetamine (MDMA or 'ecstasy') is a substituted amphetamine with both stimulant and hallucinogenic properties. MDMA has a complex mechanism of action: it binds to and reverses monoamine transporters (Rudnick and Wall, 1992) resulting in the release of serotonin (5-HT), dopamine (DA) and norepinephrine into the synapse (Fitzgerald and Reid, 1990; White et al., 1996). Of note, MDMA elicits a relatively large efflux of 5-HT in the brain compared to other psychostimulants (Crespi et al., 1997) due to its high affinity for the 5-HT transporter (Battaglia et al., 1988). MDMA also has modest affinity for several neurotransmitter receptor subtypes, including α_2-adrenergic, 5-HT$_2$, M$_1$ muscarinic and H$_1$ histamine receptors (Battaglia et al., 1988).

The manner in which these neural mechanisms contribute to the observed behavioral effects of MDMA has been the subject of recent intense interest. The

behavioral effects of MDMA in animals include hypermotility, particularly in the periphery of the activity chamber (Gold et al., 1988; Herin et al., 2004; Paulus and Geyer, 1992) and the 5-HT syndrome (e.g., flat body posture, forepaw treading and headweaving) (Spanos and Yamamoto, 1989). In addition, rats have been trained to discriminate between (±)-, (+)- or (-)-MDMA and saline (Baker et al., 1997; Bubar et al., 2004; Schechter, 1986), and to a certain extent both isomers as well as racemic MDMA support drug-seeking in a self-administration paradigm (Fantegrossi et al., 2002; Ratzenboeck et al., 2001; Schenk et al., 2003). Recent pharmacological studies have demonstrated an important role for 5-HT in mediating the behavioral effects of (±)-MDMA, the form used on the street, and the more potent (+)-isomer of MDMA. For example, antagonists of the 5-HT_{1B}R effectively attenuated hyperactivity induced by a low dose of (+)-MDMA (McCreary et al., 1999) or (±)-MDMA (Fletcher et al., 2002), although a 5-HT_{1A}R antagonist was ineffective (McCreary et al., 1999). The 5-HT_{2A}R and the 5-HT_{2C}R appear to play oppositional roles in the generation of hyperactivity induced by (+)-MDMA and (±)-MDMA. For example, antagonists with varying selectivity for the 5-HT_{2A}R attenuate horizontal hyperactivity and rearing produced by (+)-MDMA (Bankson and Cunningham, 2002; Herin et al., 2004) and (±)-MDMA (Kehne et al., 1996), and are most reliable at suppressing hyperactivity induced by the highest doses of (±)-MDMA (Kehne et al., 1996) and (+)-MDMA (Bankson and Cunningham, 2002; Herin et al., 2004). In contrast, selective 5-HT_{2C}R antagonists have been shown to greatly increase hyperactivity induced by low doses of (+)-MDMA (Bankson and Cunningham, 2002; Herin and Cunningham, 2001) and (±)-MDMA

(Fletcher et al., 2002). These data suggest that activation of the 5-HT_{2A}R is involved in the generation of MDMA-evoked hyperactivity while activation of 5-HT_{2C}R serves to limit hyperactivity produced by the substituted amphetamine. Thus, the locomotor activation elicited by MDMA is, in part, a culmination of the balancing influences of the 5-HT_{1B}R, 5-HT_{2A}R and 5-HT_{2C}R.

These 5-HT receptors are distributed within the mesocorticoaccumbens and nigrostriatal DA circuits which control expression of behaviors evoked by psychostimulants, such as amphetamine and cocaine (Pettit et al., 1984; Delfs et al., 1990; Callahan et al., 1997; Schmidt et al., 1992; Chesselet and Delfs, 1996; Tanji and Hoshi, 2001; Tzschentke, 2001; Nambu et al., 2002). Serotonin neurons of the dorsal raphe nucleus (DR) provide dense innervation to the nodes of these key pathways, and moderate to high levels of the 5-HT_{1B}R, 5-HT_{2A}R and 5-HT_{2C}R have been localized to the mesocorticoaccumbens DA circuit, including the ventral tegmental area (VTA) where the cell bodies of DA neurons are located (Di, V et al., 2000; Doherty and Pickel, 2000; Yan and Yan, 2001), and their projections to the nucleus accumbens (NAc; Mengod et al., 1990; Pazos and Palacios, 1985) and prefrontal cortex (PFC; Bruinvels et al., 1993; Mengod et al., 1990; Pazos and Palacios, 1985) as well as the cell bodies of the nigrostriatal DA system in the substantia nigra (SN; Ikemoto et al., 2000; Mengod et al., 1990; Pazos and Palacios, 1985) and their terminal regions in the caudate putamen (CPu; Mengod et al., 1990; Pazos and Palacios, 1985; Pazos et al., 1985). The possible importance of the mesocorticolimbic and nigrostriatal systems in the control of the behavioral effects of MDMA is further suggested by the findings that (+)-, (-)- and (±)-

MDMA enhanced DA efflux in the CPu (Johnson et al., 1986; Stone et al., 1986), an effect that was blocked by the 5-HT_{2A}R antagonist M100907 (Schmidt et al., 1992). Furthermore, systemic administration of DA D_1R and D_2R antagonists (Bubar et al., 2004) and 6-hydroxydopamine (6-OHDA) lesions of the NAc (Gold et al., 1989) attenuated (±)-MDMA-induced hyperactivity, while 5,7-dihydroxytrypamine (5,7-DHT) lesions of the NAc were also effective in this regard (Bankson and Cuningham, unpublished observation). The ability of MDMA to recruit these same brain circuits has also been validated in studies of expression of the immediate-early gene c-Fos as a marker of neuronal activation (Sagar et al., 1988). For example, acute administration of (+)-MDMA increased c-Fos expression in nodes of both the mesocorticoaccumbens (VTA, NAc, PFC) and nigrostriatal (CPu) circuit as well as the DRN (Stephenson et al., 1999).

Stimulation of the 5-HT_{2A}R by low doses of a preferential 5-HT_{2A}R agonist, such as 2,5-dimethoxy-4-iodoamphetamine (DOI), evokes hyperactivity (Bankson and Cunningham, unpublished observations; Bishop et al., 2004; Hillegaart et al., 1996) while stimulation of the 5-HT_{2C}R by preferential agonists, such as MK 212, generate hypomotility (Filip et al., 2004a; Halford et al., 1997; Lucki et al., 1989). Long term exposure to a preferential 5-HT_{2A}R or 5-HT_{2C}R agonist results in a down-regulation of receptors as assessed by decreased receptor binding (Bmax; (Gray et al., 2003; Van Oekelen et al., 2003)) and tolerance to their behavioral effects (Bubar and Cunningham, 2003; Darmani et al., 1990). Given the sensitivity of these receptors to down-regulation following repeated stimulation, receptor regulation in the 5-HT_2R family may play an

important role in the response to repeated exposure to MDMA (Bubar and Cunningham, 2005; Scheffel et al., 1992) and may contribute to the development of locomotor sensitization to MDMA (enhanced behavioral effects of repeated expression) or other sequelae seen following repeated exposure (McCreary et al., 1999; Bull et al., 2004).

In the present experiments, we examined the interrelationship between down-regulation of 5-HT_2R expression and the behavioral effects evoked by acute administration of (+)-MDMA. We utilized a chronic regimen of DOI previously shown to selectively decrease 5-HT_{2A}R binding and induce tolerance to the discriminative stimulus properties of DOI with no significant alteration in 5-HT_{2C}R binding (Smith et al., 1999). We hypothesized that chronic DOI treatment would decrease 5-HT_{2A}R expression to remove this stimulatory component underlying (+)-MDMA-induced locomotor activity. Therefore, this down-regulation of 5-HT_{2A}R would reduce the degree of behavioral and cellular activation by (+)-MDMA, decreasing (+)-MDMA-induced locomotor activity and c-Fos expression. Twenty-four hours after the last DOI injection, we assessed locomotor sensitivity to (+)-MDMA and the pattern of (+)-MDMA-induced c-Fos expression. Then, in order to assess any functional changes that might have been induced by chronic DOI in 5-HT_{2A}R and 5-HT_{2C}R, we measured both 5-HT_{2A}R-mediated head shakes across the chronic regimen of DOI administration as well as suppression of locomotor activity secondary to administration of the 5-HT_{2C}R agonist MK 212 during DOI withdrawal. Tissues were collected for Western blotting and immunocytochemistry to determine changes in expression of 5-HT_{2A}R and 5-HT_{2C}R protein.

Materials and Methods

Subjects

Adult male Sprague-Dawley rats (virus antibody-free; Harlan Sprague-Dawley Inc., Indianapolis IN) weighing 250-300 g were used for all studies. Rats were allowed to acclimate for 7 days in a colony room at a constant temperature (21-23°C) and humidity (45-50%) on a 12 hr light-dark cycle (light 0700-1900 hr). Rats were housed four per cage with food and water ad libitum. All experiments were carried out in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996) and with approval of the Institutional Animal Care and Use Committee.

Drugs

(-)-2,5-Dimethoxy-4-iodoamphetamine hydrochloride [DOI; Sigma, St. Louis MO], (+)-3,4-methylenedioxymethamphetamine HCl [(+)-MDMA; National Institute on Drug Abuse, Research Triangle, NC] and 6-chloro-2-(1-piperazinyl)pyrazine hydrochloride [MK 212; Tocris, Ellisville MO] were dissolved in sterile saline (0.9% NaCl) and injected in a volume of 1 ml/kg. The doses of all drugs refer to the weight of the salt and were chosen based on previous studies (Bankson and Cunningham, 2002; Bubar and Cunningham, 2003; Herin et al., 2004; Smith et al., 1999).

Behavioral Protocols

Apparatus. To assess locomotor activity, modified open-field activity monitors were used under low-light conditions (San Diego Instruments, San Diego CA). Each monitor was housed in a sound-attenuating cabinet and consisted of a Plexiglas cube (40 x 40 x 40 cm) with two sets of photobeams: one positioned 4 cm above the cage floor to measure horizontal locomotor activity and one 16 cm from the cage floor to measure rearing. Interruptions in the photobeams were recorded as activity counts. Separate counts of horizontal activity and rearing were made by the control software (Photobeam Activity Software; San Diego Instruments) and stored for subsequent statistical analysis. Video cameras located above the chambers were used to monitor activity continuously without disruption of behavior.

Chronic DOI treatment: Effects on (+)-MDMA Challenge. After 3 days of habituation to handling, rats (N=64) were removed from the home cage, weighed and injected with either saline (1 ml/kg, SC) or DOI (1 mg/kg, SC) once a day for 8 days (Smith et al., 1999). All injections were given between 0800 and 1100 hours. Four hours after injection on the last two days of chronic saline or DOI treatment, rats were habituated to the activity monitors by exposure to the activity monitors (3 hr/day for 2 days). Twenty-four hours following the last injection, rats were weighed and placed in the activity monitors for 90 min. Animals were then briefly removed from the apparatus and injected with either saline (Sal; 1 ml/kg, SC) or (+)-MDMA (3, 6 or 12 mg/kg, SC). Recording of activity in 5-min epochs began immediately following replacement into the activity monitors and continued for 90 min. Following completion of behavioral testing,

rats treated with Sal-Sal, Sal-(+)-MDMA (12 mg/kg), DOI-Sal and DOI-(+)-MDMA (12 mg/kg) were removed from the locomotor activity monitors and brain tissue was collected for c-Fos and 5-HT_{2A}R immunocytochemistry.

Chronic DOI treatment: Effects on Headshake Behavior and MK 212 challenge. After 3 days of habituation to handling, a second cohort of rats (N=64) was treated with either saline (1 ml/kg, SC) or DOI (1 mg/kg, SC) once a day for 8 days similar to the above protocol. On days 1, 3, 5 and 7, the number of 5-HT_{2A}R-mediated head shakes was observed in the home cage for 15 min immediately post-injection. Twenty-four hours after the last DOI injection, rats were challenged with saline (1 ml/kg, SC) or the 5-HT_{2C}R agonist MK 212 (2 mg/kg, SC) and immediately placed into the activity monitors. Animals were not habituated to the activity monitors in this experiment as MK 212-induced locomotor suppression is most robust upon initial exposure to a novel environment (Bubar and Cunningham, 2002; Halford et al., 1997; Lucki et al., 1989). Recording of activity in 5-min epochs began immediately following placement into the activity monitors and continued for 20 min.

Data analysis. Behaviors measured in activity monitors included horizontal activity [mean counts (± SEM)] and rearing [mean counts (± SEM)]. Total counts were summed for each animal throughout the monitoring session: 90 min for (+)-MDMA challenge and 20 min for MK 212 challenge. Head shakes [mean number observed (± SEM)] were measured in the home cage for 15 min immediately following DOI treatment. Initial analyses for all behavioral data were conducted with two-way analysis

of variance (ANOVA; SAS for Windows, Version 8.2, SAS Institute, Cary, NC). A two-way ANOVA for independent groups was used to analyze the effects of DOI treatment (factor 1) and either (+)-MDMA or MK 212 challenge (factor 2) on horizontal activity and rearing measures. A two-way ANOVA was also used to analyze the effects of DOI treatment (factor 1) and day of treatment (factor 2) on expression of head shakes with repeated measures for day of treatment. Pre-planned comparisons were then made with the Bonferroni procedure (experimentwise $\alpha = 0.05$; SAS for Windows, V. 8.2). This approach to statistical analysis is supported by a number of statisticians (Keppel, 1973; Kirk, 1995; Sheskin, 2000).

Protein Expression Protocols

Immunocytochemistry. Immediately following (+)-MDMA challenge and behavioral monitoring, animals were deeply anesthetized with pentobarbital (Sigma; 100 mg/kg; IP) and transcardially perfused with phosphate-buffered saline (PBS) followed by 3%-buffered paraformaldehyde. Brains were removed, blocked at the mid-pons and post-fixed for 2 hr at room temperature. Brains were then transferred into a 30% sucrose solution at 4°C for 48 hr, rapidly frozen on crushed dry ice and stored at -80°C until use.

Fifty-micrometer sections were prepared with a cryostat (Leica CM1850) at -20°C and processed using immunodetection procedures previously described (Paris et al., 1991; Frankel and Cunningham, 2002). Briefly, sections were washed extensively with PBS and blocked with 1.5% normal goat serum (Vectastain Elite kit; Vector Laboratories, Burlingame CA) in PBS containing 0.4% Triton-X (PBS-T; Sigma).

Sections were then incubated in PBS-T for 2 days at 4°C with primary antibody for c-Fos (polyclonal; Ab-5, Oncogene Research, San Diego CA; diluted 1:50000) or 5-HT_{2A}R [(monoclonal; 556326, BD PharMingen, San Diego CA; diluted 1:5000) or (polyclonal; courtesy of Dr. Bryan L. Roth, Case Western University, Cleveland OH; diluted 1:1000)]. The sections were then washed in PBS and incubated with either biotinylated goat anti-rabbit or anti-mouse IgG (Vector; diluted 1:400) in PBS-T for 1 hr at 25°C. Following additional washes, sections were incubated in an avidin/biotin-horseradish peroxidase complex (Vectastain Elite Kit, Vector) for 1 hr at 25°C to amplify the signal. The pH of the sections was brought to 7.6 with 3 washes in Tris buffer, and the sections were developed in 3,3'-diaminobenzidine (0.5 mg/ml; Sigma) with 0.005% H_2O_2. Additional control sections were developed in parallel in which either no primary antibody or no secondary antibody was added. The sections were rinsed in PBS to terminate the chromagen reaction, mounted onto gelatin chrom alum-coated slides and coverslipped with Permount (Fisher Scientific, Houston TX).

Digital images were captured from brain sections using an Olympus Vanox-T AH2 microscope and a Pixera Professional camera (model VCS10132; Sherwood Dallas Co., Dallas TX) interfaced to a personal computer and analyzed using Scion Image software (v. Beta 4.0.2; Scion Image Corp., Frederick MD). A 2X objective was used to capture all photomicrographs for a final magnification of 12.5X. Sections from each brain were captured from anatomically appropriate rostral-caudal levels (**Figure 4**): Bregma +1.7 mm for prefrontal cortex (PFC), nucleus accumbens core (NAcC), nucleus

accumbens shell (NAcS) and caudate-putamen (CPu); Bregma -0.3 mm for ventral pallidum (VP) and Bregma -6.04 for ventral tegmental area (VTA) (Paxinos and Watson, 1998). A fixed-size rectangle was used to represent each brain area. Images were corrected for uneven background staining using digital subtraction of an unfocused image (Frankel and Cunningham, 2002), and cells immunopositive for c-Fos or 5-HT_{2A}R were counted automatically by Scion Image after applying the density slice function to the corrected image.

Western Blotting. After 3 days of habituation to handling, a third cohort of rats (N=16) was treated with either saline (1 ml/kg, SC) or DOI (1 mg/kg, SC) once a day for 8 days (see Behavioral Protocols). This cohort was not challenged or assessed behaviorally. Twenty-four hours after the last injection, the rats were anesthetized with chloral hydrate (Sigma; 800 mg/kg, IP) and decapitated. Brains were removed rapidly using a rodent brain matrix (Harvard Apparatus, Warren MI), and brain areas corresponding to the PFC, NAc, CPu and VTA were isolated on a cool tray (4°C) by gross dissection (Heffner et al., 1980). Samples were suspended in PBS containing 1% NP-40, 0.1% SDS, 12 mM sodium deoxycholate and protease inhibitor cocktail (Sigma; diluted 1:100) and immediately homogenized. The samples were incubated at 4°C for 30 min then centrifuged at 10000 x g for 15 min, and the supernatant fraction was collected for Western analysis. Protein concentration of the supernatant was determined using the BCA protein determination kit (Pierce, Rockford IL). Total protein (7.5 µg/lane) from individual animals was boiled in Laemmli sample buffer (5 min) and run on a 10% Tris-

glycine using SDS-PAGE. The gel was blotted onto a PVDF membrane using a semi-dry electroblotter (Alltech, Deerfield IL; 18 V, 10 min). After brief staining with Ponceau S (Sigma; 500 mg/ml in 1% acetic acid) to ensure transfer, blots were blocked with 5% nonfat dry milk in Tris-buffered saline (TBS, pH 7.4; 1.5 hr) and incubated with primary antibody diluted in TBS (1 hr). Primary antibodies included 5-HT_{2A}R (monoclonal; 556326, BD PharMingen, San Diego CA; diluted 1:5000), 5-HT_{2C}R (monoclonal; 556335, BD Pharmingen; diluted 1:5000) and actin (monoclonal; MAB1501, Chemicon, Temecula CA; diluted 1:5000). After extensive washing (3 X 20 min in TBS), sheep anti-mouse IgG horseradish peroxidase-conjugated secondary antibody (AP300P, Chemicon; diluted 1:10000) was added (1 hr). The membranes were again washed before development with the ECL plus Western Blotting Detection System (Amersham Biosciences, Piscataway NJ) and exposed to Kodak Biomax MR film (Eastman Kodak, Rochester NY). Digital images obtained from the films were analyzed with Scion Image to obtain band densities. Actin content was determined in each lane to control for loading variability. Comparisons were only made between groups run on the same gel.

Data analysis. Measures of protein expression included number of c-Fos immunoreactive cells [mean number (± SEM)] and number of 5-HT_{2A}R immunoreactive cells [mean number (± SEM)] measured with immunocytochemistry as well as relative amount of 5-HT_{2A}R and 5-HT_{2C}R expression [mean band density normalized to actin (± SEM)] measured with Western blotting. A two-way ANOVA (SAS for Windows, V. 8.2) for independent groups was used to analyze the effects of DOI treatment (factor 1)

and (+)-MDMA challenge (factor 2) on number of c-Fos immunopositive cells in each brain area analyzed. Planned pairwise comparisons were then made with the Bonferroni procedure (experimentwise $\alpha = 0.05$; SAS for Windows, Version 8.2). Two-tailed Student's *t*-tests (experimentwise $\alpha = 0.05$) were used to analyze the effects of DOI treatment on both the number of 5-HT_{2A}R immunopositive cells as measured by immunocytochemistry and on 5-HT_{2A}R and 5-HT_{2C}R band density as measured by Western blotting.

Results

Effect of repeated DOI on (+)-MDMA-induced locomotor activity

Rats were treated for 8 days (once/day) with saline (1 ml/kg, SC) or DOI (1 mg/kg, SC). On day 9, animals from each treatment group were challenged with either saline (1 ml/kg, SC) or (+)-MDMA (3, 6 or 12 mg/kg, SC), and locomotor activity was monitored for 90 min (**Figure 1**). There was a main effect of DOI treatment on both horizontal activity ($F_{1,79}$=5.99, p=0.0168) and rearing ($F_{1,75}$=17.00, p<0.001), and (+)-MDMA challenge resulted in significant increases in both horizontal activity ($F_{3,79}$=60.42, p<0.001) and rearing ($F_{3,75}$=23.43, p<0.001). However, the treatment x challenge interaction was not significant for either horizontal activity ($F_{3,79}$=1.29, p=0.2859) or rearing ($F_{3,75}$=1.31, p=0.2779) suggesting that the dose-effect curves for (+)-MDMA after saline and DOI treatment are parallel. Pre-planned comparisons using the Bonferroni procedure indicated that (+)-MDMA challenge enhanced both horizontal activity and rearing at all doses tested (p<0.05) and that DOI treatment significantly enhanced (+)-MDMA-induced horizontal activity (**Figure 1A**) and rearing (**Figure 1B**) at the 6 mg/kg dose of (+)-MDMA compared to saline-treated controls (p<0.05).

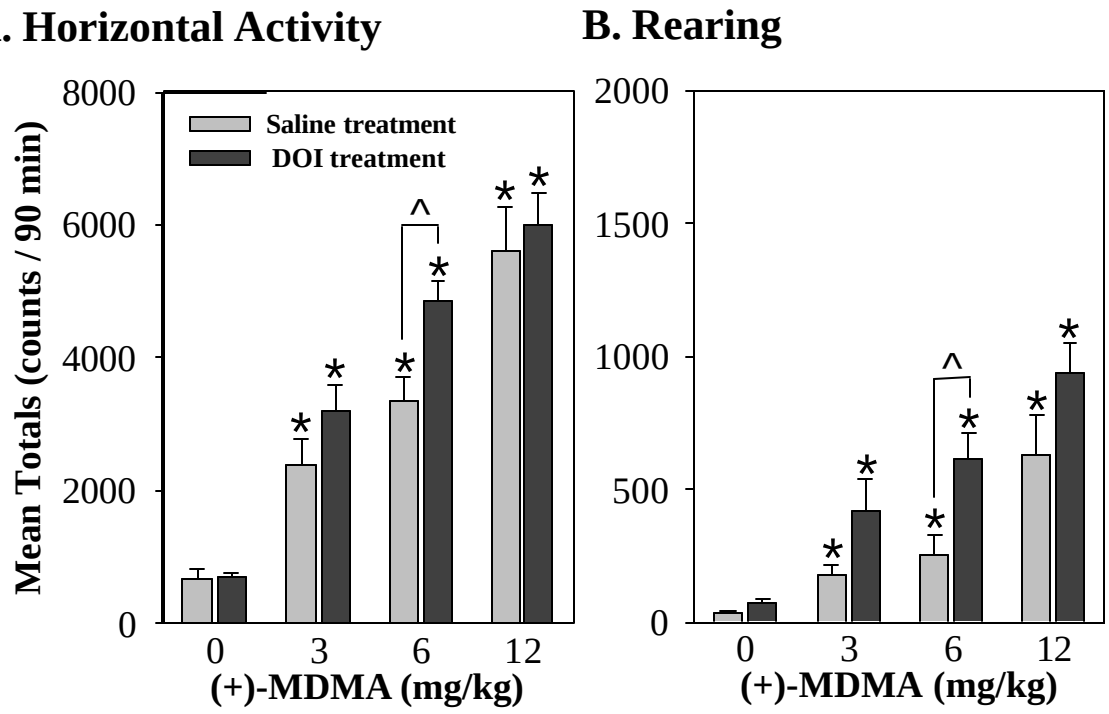


Figure 1. Effect of repeated DOI on (+)-MDMA-induced locomotor activity. Animals were treated for 8 days (once/day) with saline (1 ml/kg, SC) or DOI (1 mg/kg, SC) and challenged on day 9 with either saline (1 ml/kg; SC) or (+)-MDMA (3, 6 or 12 mg/kg, SC). **A.** Horizontal activity and **B.** rearing are represented as mean total number of counts per 90 min (± SEM). [*p<0.05 vs. Saline-MDMA (0 mg/kg); ^p<0.05 vs. Saline treatment at a given (+)-MDMA dose based on *a priori* comparisons with Bonferroni procedure.]

Effect of repeated DOI on head shakes and MK 212-induced hypomotility

In a separate group, animals were injected with either saline (1 ml/kg, SC) or DOI (1 mg/kg, SC) daily for 8 days. On days 1, 3, 5 and 7 of DOI treatment, the number of 5-HT_{2A}R-induced head shakes was observed in the home cage for 15 min immediately following injection (**Figure 2**). There was a main effect of DOI treatment ($F_{1,127}$=123.14, p<0.001), day of treatment ($F_{3,127}$=3.65, p=0.0155) and a treatment x day interaction ($F_{3,127}$=6.26, p=0.0007). Pre-planned comparisons indicated that on each day tested, rats receiving DOI exhibited a greater number of head shakes compared to saline controls (p<0.05). Although there was a trend toward attenuation of DOI-induced head shakes on day 3 as compared to day 1 (p<0.10), the head shake response to DOI had recovered to acute levels by day 5 suggesting that some aspects of the functional tolerance of 5-HT_{2A}R that had developed after the first DOI treatments had abated by day 5 of the treatment regimen.

On day 9 (24 hours after the last DOI injection), these animals were then challenged with either saline (1 ml/kg, SC) or the 5-HT_{2C}R agonist MK 212 (2 mg/kg, SC) and placed in the activity monitors for 20 min (**Figure 3**). While there was no main effect of DOI treatment on horizontal activity ($F_{1,31}$=0.65, p=0.4286), there was a main effect of DOI treatment on rearing ($F_{1,31}$=176.38, p<0.0001), and MK 212 challenge resulted in significant decreases in both horizontal activity ($F_{1,31}$=179.31, p<0.0001) and rearing ($F_{1,31}$=68.23, p<0.001). However, there was no significant treatment x challenge interaction for horizontal activity ($F_{1,31}$=0.35, p=0.5572) or rearing ($F_{1,31}$=1.06, p=0.3115) suggesting that MK 212 suppressed locomotor activity equally in saline- and DOI-treated

animals. Pre-planned comparisons using the Bonferroni procedure indicated that MK 212 significantly decreased both horizontal activity (**Figure 3A**) and rearing (**Figure 3B**) in both saline- and DOI-treatment groups ($p<0.05$), and that DOI treatment significantly attenuated rearing in response to the novel chamber ($p<0.05$). While the DOI regimen did not appear to affect the functional sensitivity of 5-HT_{2C}R as assessed by MK 212 challenge, there is an effect of DOI treatment on investigatory behavior in a novel environment.

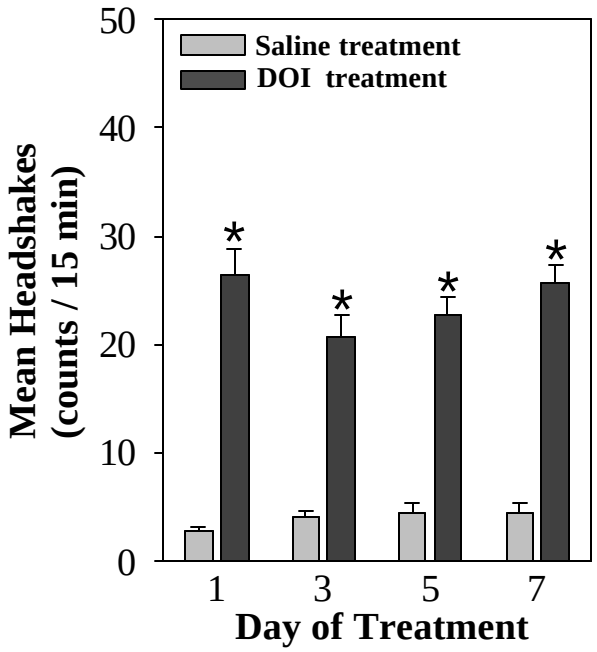


Figure 2. Effect of repeated DOI on head-shake behavior. Animals were treated for 8 days (once/day) with saline (1 ml/kg, SC) or DOI (1 mg/kg, SC) and the number of head shakes was counted for the 15-min period immediately post-injection on days 1, 3, 5 and 7. The data are represented as mean number of head shakes per 15 min (± SEM) per day. [*p<0.05 vs. Saline-Day 1 based on *a priori* comparisons with Bonferroni procedure.]

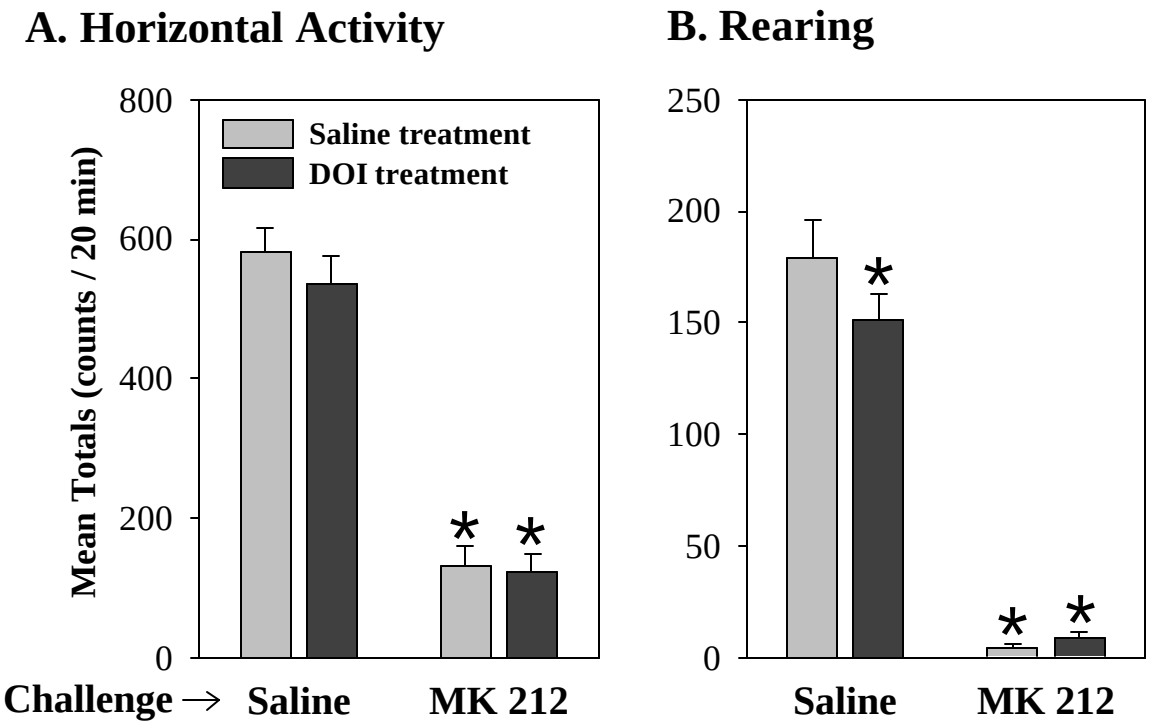
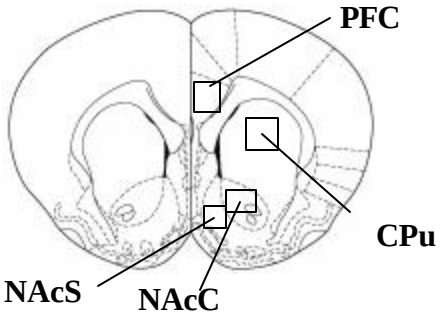


Figure 3. Effect of repeated DOI on MK 212-induced hypomotility. Animals were treated for 8 days (once/day) with saline (1 ml/kg, SC) or DOI (1 mg/kg, SC) and challenged on day 9 with either saline (1 ml/kg; SC) or MK 212 (2 mg/kg, SC). **A.** Horizontal activity and **B.** rearing are represented as mean total number of counts per 20 min (± SEM). [*p<0.05 vs. Saline-Saline based on *a priori* comparisons with Bonferroni procedure.]

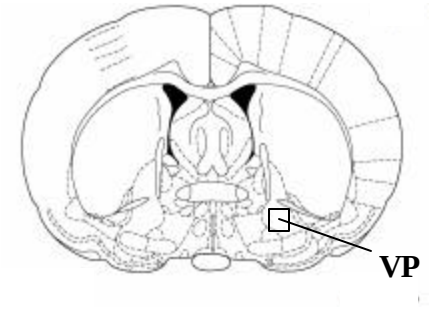
Effect of repeated DOI on (+)-MDMA-induced c-Fos

Because DOI treatment potentiated (+)-MDMA-induced locomotor activity, we analyzed individual components of the DA reward circuit for expression of the immediate-early gene product c-Fos (**Figure 4**). Immediately following (+)-MDMA challenge and behavioral monitoring, animals were perfused for c-Fos immunocytochemistry to determine the number of c-Fos immunopositive cells (**Figure 5**). While no main effect of DOI treatment on c-Fos expression was observed in any of the regions examined, (+)-MDMA administration increased c-Fos expression in the PFC ($F_{1,22}$=7.30, p=0.0141), NAcS ($F_{1,22}$=38.49, p<0.0001), NAcC ($F_{1,22}$=22.73, p<0.0001), CPu ($F_{1,22}$=53.25, p<0.0001), VP ($F_{1,22}$=20.66, p=0.0002) and VTA ($F_{1,15}$=29.90, p<0.0001). There was a significant treatment x challenge interaction in the CPu ($F_{1,22}$=8.09, p=0.0104), but no other significant interactions. Pre-planned comparisons indicated that (+)-MDMA challenge significantly increased c-Fos expression in both saline- and DOI-treatment groups in the NAcS, NAcC, VP and VTA (p<0.05; **Figure 5**). This pattern also holds in the CPu (p<0.05), although DOI treatment significantly attenuated the number of c-Fos positive cells (p<0.05) seen after injection of (+)-MDMA. A trend toward a similar pattern was observed in the PFC: (+)-MDMA significantly increased c-Fos expression in the saline-treated group (p<0.05), but not in the DOI-treated group (**Figure 5**). Control sections developed with either no primary antibody or no secondary antibody showed no c-Fos staining (data not shown).

A. Bregma +1.7 mm



B. Bregma -0.3 mm



C. Bregma -6.04 mm

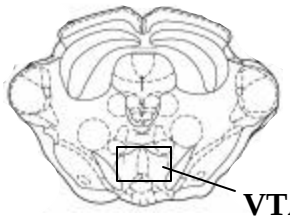


Figure 4. Schematic drawings of brain areas analyzed for DOI effects on (+)-MDMA-induced c-Fos. Adapted from The Rat Brain in Stereotaxic Coordinates (Paxinos and Watson, 1998). For anatomical reference, structures are labeled as follows: **A.** Bregma +1.7 mm: prefrontal cortex (PFC); nucleus accumbens core (NAcC); nucleus accumbens shell (NAcS); caudate-putamen (CPu). **B.** Bregma -0.3 mm: ventral pallidum (VP). **C.** Bregma -6.04 mm: ventral tegmental area (VTA).

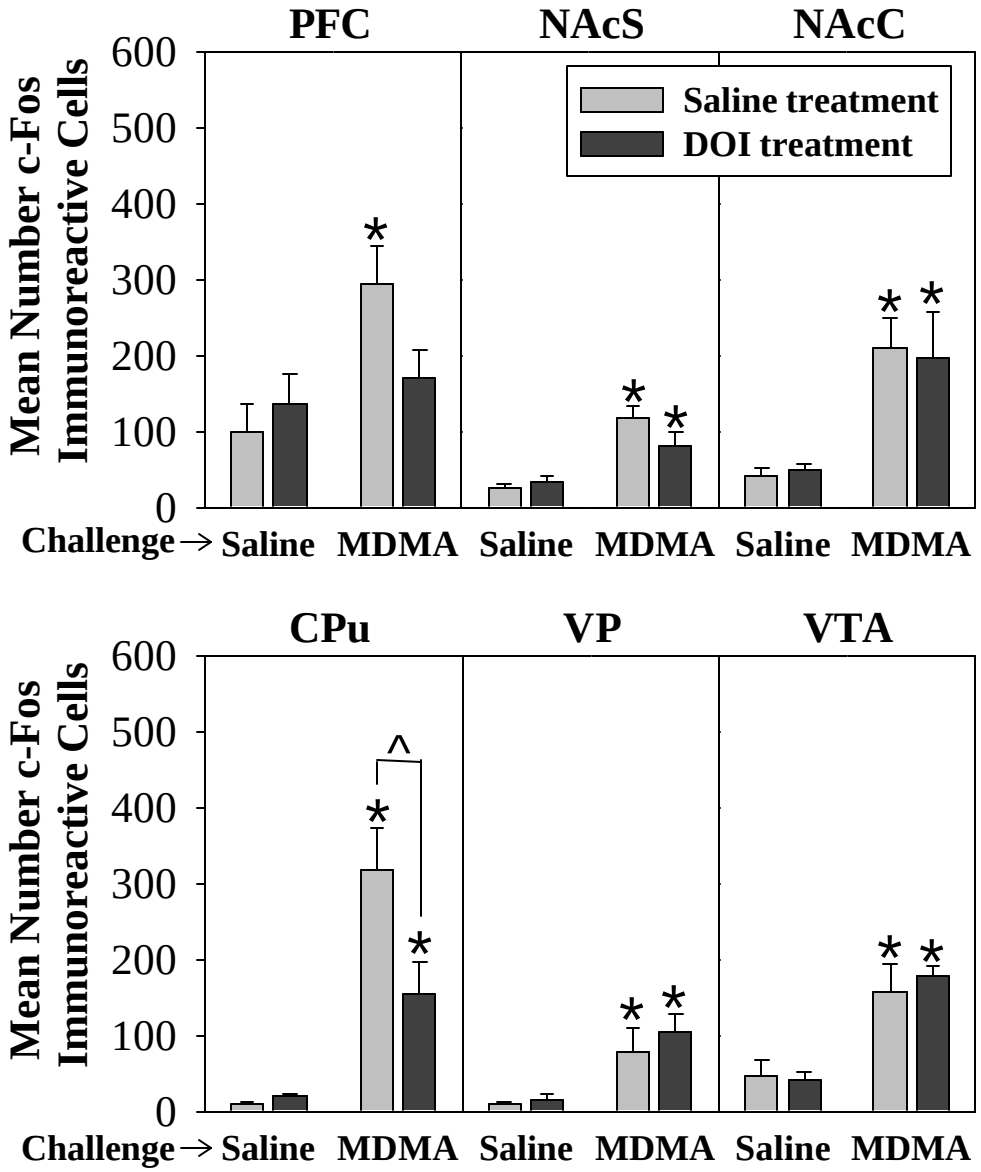


Figure 5. Effect of repeated DOI on (+)-MDMA-induced c-Fos. Mean numbers of c-Fos immunopositive cells (± SEM) in the prefrontal cortex (PFC), nucleus accumbens shell (NAcS), nucleus accumbens core (NAcC), caudate-putamen (CPu), ventral pallidum (VP) and ventral tegmental area (VTA). Animals were treated for 8 days (once/day) with saline (1 ml/kg, SC) or DOI (1 mg/kg, SC) and challenged on day 9 with either saline (1 ml/kg; SC) or (+)-MDMA (12 mg/kg, SC). [*p<0.05 vs. Saline-Saline; ^p<0.05 vs. Saline treatment at given (+)-MDMA dose based on *a priori* comparisons with Bonferroni procedure.]

Effect of repeated DOI on expression of 5-HT_{2A}R and 5-HT_{2C}R as measured by Western blot

We examined protein expression patterns for the 5-HT_{2A}R and 5-HT_{2C}R on day 9 following the chronic DOI regimen in the third groups of DOI-treated rats (not challenged or behaviorally monitored) using immunoblot analysis (**Figure 6**). No differences in expression of the 5-HT_{2A}R or 5-HT_{2C}R were observed in the PFC, CPu, NAc or VTA by immunoblotting ($p>0.05$) in pairwise *t*-tests indicating that there was no effect of DOI treatment on expression of 5-HT_{2A}R or 5-HT_{2C}R protein.

Effect of repeated DOI on expression of 5-HT_{2A}R as measured by immunocytochemistry

Small differences in protein levels might have been obscured in a homogenized tissue sample used for immunoblotting. Therefore, we analyzed the expression of the 5-HT_{2A}R in tissue sections using immunocytochemistry; brain tissue from the c-Fos immunocytochemistry study (**Figure 4**) were analyzed in parallel for 5-HT_{2A}R immunoreactivity (**Figure 7**). Saline- and DOI-treated groups exhibited significant differences in expression of 5-HT_{2A}R in PFC and NAcS ($p<0.05$), with no significant differences in NAcC, CPu, VP or VTA ($p>0.05$) in pairwise *t*-tests. Control sections developed with either no primary antibody or no secondary antibody showed no 5-HT_{2A}R staining (data not shown). The pattern of 5-HT_{2C}R immunocytochemical staining was neither discrete nor distinct enough to enable accurate quantification of 5-HT_{2C}R protein expression in tissue sections (data not shown).

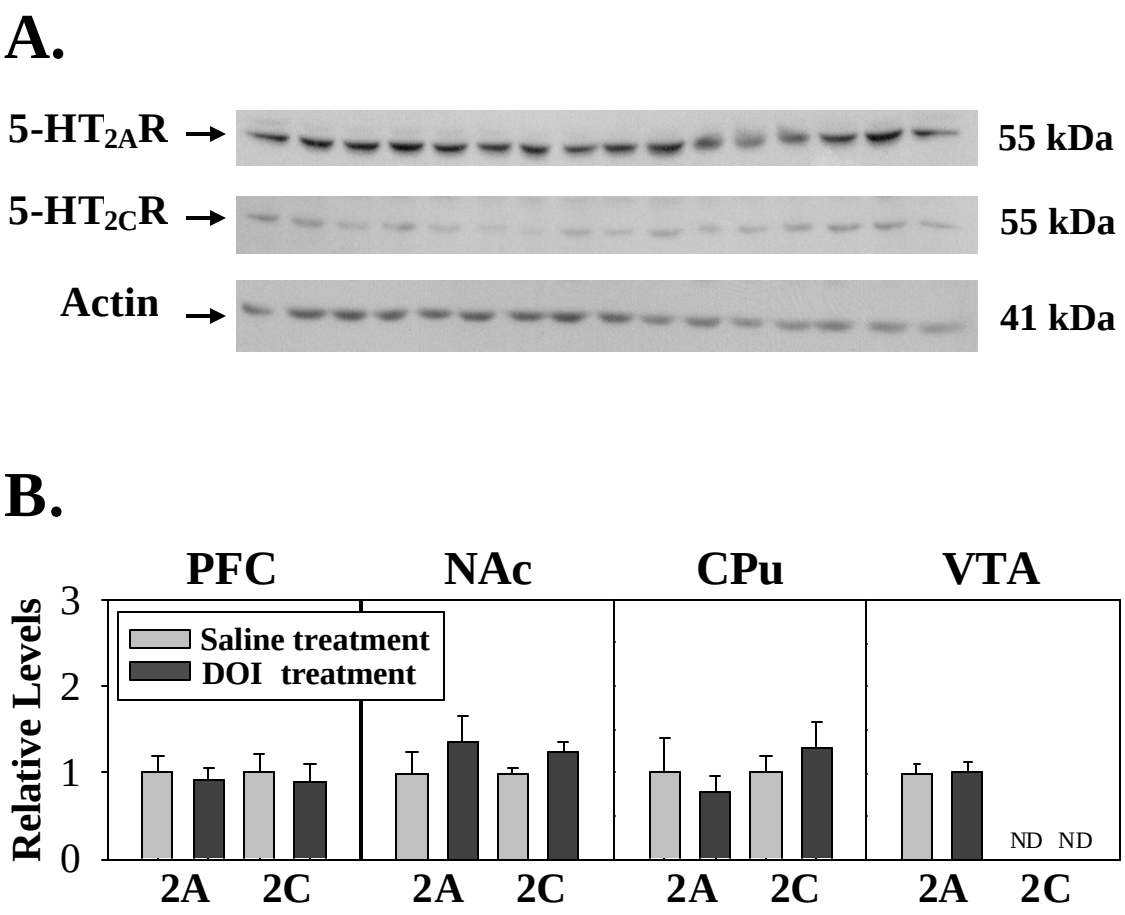


Figure 6. Effect of repeated DOI on expression of 5-HT_{2A}R and 5-HT_{2C}R as measured by Western blot. Animals were treated for 8 days (once/day) with saline (1 ml/kg, SC) or DOI (1 mg/kg, SC), and tissue was isolated for immunoblotting. **A.** Representative immunoblot from prefrontal cortex illustrating bands corresponding to the 5-HT_{2A}R (55 kDa), 5-HT_{2C}R (55 kDa), and actin (41 kDa). Each band represents an individual animal, and samples were run in alternating lanes (Sal, DOI, Sal…). **B.** Relative density of staining normalized to actin (± SEM) in the CPu, NAc, PFC and VTA. No 5-HT_{2C}R signal was detectable (ND) in the VTA. [No significant differences were observed (pairwise *t*-test).]

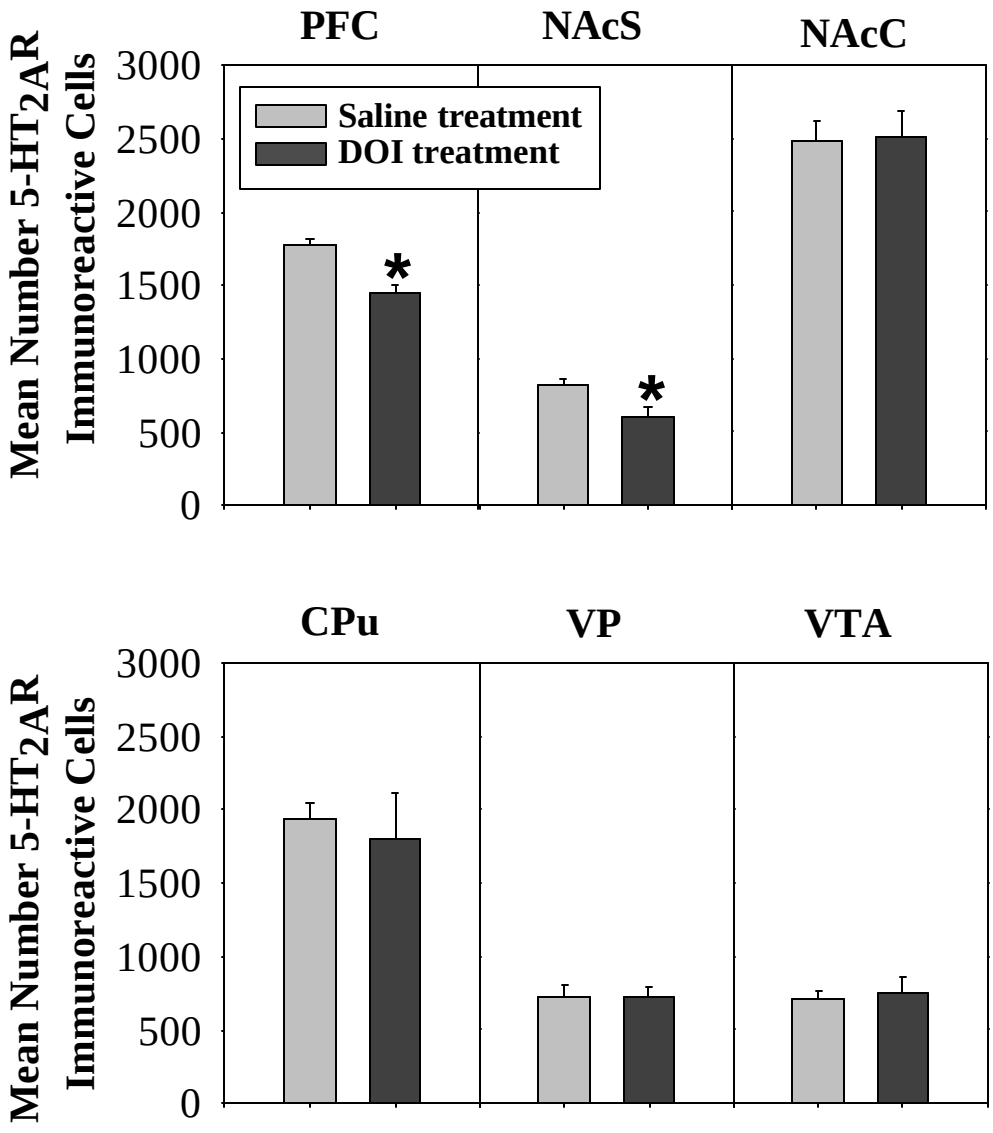


Figure 7. Effect of repeated DOI on expression of 5-HT_{2A}R as measured by immunocytochemistry. Mean numbers of 5-HT_{2A}R immunopositive cells (± SEM) in the prefrontal cortex (PFC), nucleus accumbens shell (NAcS), nucleus accumbens core (NAcC), caudate-putamen (CPu), ventral pallidum (VP) and ventral tegmental area (VTA). Animals were treated for 8 days (once/day) with saline (1 ml/kg, SC) or DOI (1 mg/kg, SC) and challenged on day 9 with saline (1 ml/kg; SC) prior to perfusion. [*p < 0.05 vs. Saline (pairwise t-test).]

Discussion

The present study indicates that withdrawal from repeated DOI treatment is associated with enhanced (+)-MDMA-induced hyperactivity in the absence of changes in 5-HT_{2A}R- or 5-HT_{2C}R-mediated behaviors or the expression of 5-HT_{2A}R and 5-HT_{2C}R as measured by Western blotting. However, DOI treatment decreased both (+)-MDMA-induced c-Fos expression and 5-HT_{2A}R expression in cortical and striatal areas as measured by immunocytochemistry. These data suggest that repeated DOI results in anatomically-specific changes in the cellular response to (+)-MDMA that may underlie the enhanced behavioral sensitivity to (+)-MDMA.

We originally hypothesized that repeated DOI treatment would result in the down-regulation of 5-HT_{2A}R expression (Smith et al., 1999) and a concomitant attenuation of (+)-MDMA-induced hyperactivity (Bankson and Cunningham, 2002; Herin et al., 2004). Surprisingly, however, we observed an *enhancement* of (+)-MDMA-induced locomotor activity at the intermediate 6 mg/kg dose, despite confirmation of selective 5-HT_{2A}R down-regulation in select cortical and striatal areas, namely the PFC and NAcS. Interestingly, however, DOI treatment did not significantly enhance locomotor activity evoked by (+)-MDMA at the 3 or 12 mg/kg doses. The relative contributions of the different 5-HT receptors in (+)-MDMA-induced locomotor activity shifts across doses of (+)-MDMA (Bankson and Cunningham, 2002; Herin et al., 2004; McCreary et al., 1999). At the low, 3 mg/kg dose of (+)-MDMA, a limited role for the 5-HT_{2A}R in (+)-MDMA-induced locomotor activity is indicated (Bankson and Cunningham, 2002; Herin et al., 2004), while there is a greater role for the 5-HT_{1B}R

(McCreary et al., 1999). At the high, or 12 mg/kg dose of (+)-MDMA, however, the relative role of the 5-HT_{2A}R in the generation of (+)-MDMA-induced locomotor activity is far more influential: the 5-HT_{2A}R antagonist M100907 eliminated locomotor activity produced by 12 mg/kg of (+)-MDMA (Herin et al., 2004). Perhaps a balance of multiple mechanisms that drive (+)-MDMA-induced locomotor activity at different doses is critical in the manifestation of the DOI-induced enhancement of the locomotor response to (+)-MDMA, requiring an intermediate dose of (+)-MDMA that employs the signaling system to an adequate degree to result in gross alterations in behavior but does not overwhelm the modifications induced by DOI.

Since DOI has roughly equal affinity for the 5-HT_{2A}R and 5-HT_{2C}R (Roth et al., 2000), the possibility exists that the expression of the 5-HT_{2C}R may have been modulated by chronic DOI treatment. If repeated DOI administration down-regulated the 5-HT_{2C}R, then the normal inhibitory effect of 5-HT_{2C}R stimulation on (+)-MDMA-induced locomotor activity (Bankson and Cunningham, 2002) would be lost and might offset the down-regulation of the stimulatory 5-HT_{2A}R (Bankson and Cunningham, 2002) and account for the increase in locomotor activity upon (+)-MDMA challenge (Bubar and Cunningham, 2005) after DOI treatment. Thus, a greater net effect of 5-HT_{2C}R down-regulation could have presented a relatively simple explanation for the increase in (+)-MDMA-induced locomotor activity observed in the face of lesser amounts of 5-HT_{2A}R protein expression. However, the functional sensitivity of both 5-HT_{2A}R and 5-HT_{2C}R appears to be intact after repeated DOI treatment. While slightly reduced on day 3 of treatment, the expression of DOI-evoked head shakes recovered to control levels for the

remainder of the regimen in a pattern consistent with that seen in the literature (Darmani et al., 1990). Additionally, the ability of the 5-HT_{2C}R agonist to evoke hypomotility was unchanged during withdrawal from the chronic DOI regimen. Therefore, at the timepoint at which an enhanced effect of (+)-MDMA was observed after DOI administration (24 hours after the last DOI injection), the ability of stimulation of either 5-HT_{2A}R (Bedard and Pycock, 1977; Pranzatelli, 1990; Yap and Taylor, 1983) or 5-HT_{2C}R (Filip et al., 2004a; Halford et al., 1997; Lucki et al., 1989) to result in their expected behavioral effects was normal. These data do not support a simple decrease in function of one or both receptors as the etiology of the enhanced (+)-MDMA-induced locomotor activity in DOI-treated animals but imply more complex downstream changes.

Our results also demonstrate that during withdrawal from DOI treatment, there is a microanatomical down-regulation of 5-HT_{2A}R in the PFC and NAcS as measured by immunocytochemistry. These DOI-induced changes in protein expression detected using immunocytochemistry were not borne out in the Western blot assays. It appears that, for the 5-HT_{2A}R, Western blotting techniques in homogenized tissue samples obscured subtle differences in protein expression that could be detected in intact tissue sections. Unlike Western blot, immunocytochemical techniques grant a level of anatomical specificity showing exactly where in the tissue the staining occurred, and perhaps small changes in 5-HT_{2A}R expression that were detectable in individual cells with immunocytochemistry were washed out.

To a certain extent, the relative roles of the 5-HT_{2A}R resident in the PFC and NAcS have been characterized following acute microinjection of 5-HT_{2A}R ligands. The

5-HT_{2A}R in the PFC, but not the NAcS (McMahon et al., 2001), appears to play a stimulatory role in cocaine-induced locomotor activity (Filip et al., 2004b). Thus, *acute* reduction of the PFC 5-HT_{2A}R component of locomotor drive might attenuate (not enhance) (+)-MDMA-induced hyperactivity. Other evidence, however, suggests that chronic down-regulation might produce the opposite effect. In a study in which animals were administered a chronic regimen of (±)-MDMA and were subsequently challenged with the same drug, an increased sensitivity to (±)-MDMA was associated with a decreased expression of the 5-HT_{2A}R in the frontal cortex and NAc (Scheffel et al., 1992). Consequently, while acute blockade of 5-HT_{2A}R in PFC (but not in NAc) attenuated psychostimulant-induced locomotor activity (Filip et al., 2004b; McMahon et al., 2001), the chronic effect of 5-HT_{2A}R down-regulation in one or both of these areas may contribute to the increased (+)-MDMA-induced locomotor activity observed during DOI withdrawal.

Repeated, intermittent exposure to psychostimulants generally results in an enduring enhancement in the behavioral effects of psychomotor stimulants ("behavioral sensitization") (Kalivas et al., 1998) which is accompanied by a variety of lasting neurochemical changes (White and Kalivas, 1998). Cross-sensitization between different drugs occurs in which repeated treatment with one drug can make an animal more sensitive to subsequent challenge with another. For example, chronic (±)-MDMA treatment enhances the subsequent response to cocaine administration by increasing the amount of DA efflux in the NAc after cocaine challenge in animals that received chronic (±)-MDMA (Morgan et al., 1997) and vice versa (White et al., 1995). Similar

neurochemical effects are seen in the interaction between cocaine and DOI (Yan et al., 2000). We propose that the ability of repeated DOI treatment to result in enhanced (+)-MDMA behavioral activation may be related to a cross-sensitizing effect between DOI and (+)-MDMA. In other words, the repeated exposure to DOI induces downstream changes that mimic (+)-MDMA sensitization, therefore, increasing subsequent locomotor sensitivity to (+)-MDMA challenge. While classified as a hallucinogen (Arnt, 1989; Shannon et al., 1984), DOI has some similar actions in the DA mesocorticolimbic pathway as MDMA. For example, systemic DOI administration increases both locomotor activity (Chapter 3; Bankson and Cunningham, unpublished observations; Bishop et al., 2004; Hillegaart et al., 1996) and DA release in the NAc (Yan, 2000), effects previously shown to occur with psychostimulants (Bradberry and Roth, 1989; Fitzgerald and Reid, 1990) Additionally, the effects of psychostimulants are enhanced by DOI: systemic pretreatment with DOI enhanced both cocaine-induced hyperactivity (Filip et al., 2004a) and amphetamine-induced DA release in CPu and NAc (Ichikawa and Meltzer, 1995; Kuroki et al., 2003). Repeated treatment with cocaine enhanced the ability of DOI microinjected into the NAc to elicit DA release (Yan et al., 2000). Thus, we hypothesize that the chronic DOI regimen used here evoked similar downstream changes to those observed following sensitizing regimens of psychostimulants resulting in an increase in sensitivity to (+)-MDMA.

Studies of c-Fos expression support for this hypothesis. The classic pattern of c-Fos activation after acute MDMA challenge is a dose-dependent increase in expression in the PFC, CPu, NAc and VTA that parallels increases in MDMA-induced hyperactivity

(Stephenson et al., 1999). Consistent with these findings, we observed that acute (+)-MDMA injection in animals chronically treated with saline increased c-Fos expression in these same areas. We observed an enhancement of (+)-MDMA-induced locomotor activity during DOI withdrawal that tracked with a decrease in (+)-MDMA-evoked c-Fos expression in the CPu. This pattern was similar to the decrements noted in stimulant-evoked c-Fos expression upon repeated, intermittent exposure to such psychostimulants as amphetamine and cocaine that result in sensitization (Asin et al., 1995; Curran et al., 1996; Jaber et al., 1995). The fact that (+)-MDMA-induced behavioral and cellular activation after chronic DOI treatment mirrors the pattern seen after a sensitizing regimen of psychostimulants suggests a possible early role for activation of 5-HT_{2A}R and/or 5-HT_{2C}R in the processes of neural adaptations that lead to increased sensitivity to (+)-MDMA.

In summary, results from the present study suggest a prominent role of the 5-HT_{2A}R in the PFC and NAc in the sensitivity to the locomotor stimulating effects (+)-MDMA. For the first time, we demonstrate that chronic DOI treatment results in an enhanced behavioral response to (+)-MDMA and in a pattern of neuronal activation that resembles psychostimulant sensitization. Further understanding of the downstream neuronal changes underlying DOI-induced increases in sensitivity to psychostimulants may help to better elucidate the potential general mechanisms involved in the sensitization process, i.e., how these drug-induced alterations generalize within the DA mesolimbic system to result in exaggerated responses to other psychostimulants.

CHAPTER 3:

INDIVIDUAL DIFFERENCES IN RESPONSE TO A NOVEL ENVIRONMENT: STUDIES OF HABITUATION, THE EFFECTS OF (+)-3,4-METHYLENEDIOXYMETHAMPHETAMINE [(+)-MDMA; ECSTASY] AND THE ROLE OF THE SEROTONIN$_2$ RECEPTOR (5-HT$_2$R)

Background and Significance

Individual human drug users report a wide range of reactions to the acute subjective effects of drugs of abuse (De Wit et al., 1986; O'Brien et al., 1986). The sensitivity of an individual to the initial positive effects of drug administration correlates to how many drug exposures will be required to meet the criteria for drug addiction and the likelihood that drug use will escalate into drug dependence (O'Brien et al., 1986). Attempts to model individual sensitivity to drugs of abuse and to predict whether an individual will engage in drug-seeking behaviors have utilized an animal model of individual differences studied best by Piazza and colleagues. In this model, an outbred rat population is separated into high responder (HR) and low responder rats (LR) based on the degree of individual locomotor activation observed upon exposure to a novel environment (Piazza et al., 1989). Those animals exhibiting high levels of novelty-

induced locomotor activity have an increased sensitivity to psychostimulants (Hooks et al., 1991b; Piazza et al., 1989) as compared to those with lower levels of novelty-induced locomotor activity.

In parallel to humans who exhibit high sensitivity to the positive subjective effects of stimulants and appear to develop addictive behaviors readily (O'Brien et al., 1986), HR rats acquire patterns of self-administration for amphetamine while LR rats do not (Piazza et al., 1989). The self-administration assay is a paradigm in which animals are given intravenous access to a drug that is contingent upon the accurate performance of an operant behavior (e.g., lever pressing). It is an accurate method to study drug-taking behavior in rats and has face validity to model the problem of human drug-taking behavior. When given access to a low dose of cocaine in the self-administration paradigm, only HR rats acquired drug-seeking behaviors (Piazza et al., 2000). This increased sensitivity in HR rats is not limited to low doses of drug and is present across the entire dose-response curve (Piazza et al., 2000), a vertical shift implying that HR rats are generally more responsive to the reinforcing effects of psychostimulants than LR rats. Thus, the HR vs. LR rat model is an important tool to elucidate the role of individual differences in sensitivity to drugs of abuse in animals and its possible extension to the process by which occasional drug taking in humans can evolve into compulsive drug addiction.

The behavioral phenotypes seen in HR vs. LR rats are based at least in part on a differential reactivity of the dopamine (DA) mesolimbic circuit (Dellu et al., 1996). The DA mesolimbic circuit consists of DAergic projections from the midbrain ventral

tegmental area (VTA) to various forebrain structures, including the nucleus accumbens (NAc); this pathway has been termed the 'reward circuit' (Swanson, 1982). The DA mesolimbic circuit in HR rats appears to be more reactive to a variety of different stimuli, with differential responses to novelty exposure (Cain et al., 2004; Piazza et al., 1989), drugs of abuse [amphetamine (Piazza et al., 1989), cocaine (Hooks et al., 1991b) and morphine (Deroche et al., 1992)] and food reinforcement (Hooks et al., 1994). Each of these stimuli increases DA efflux in the NAc and is perceived as reinforcing in outbred populations of rats (Bedford et al., 1980; Bevins and Bardo, 1999; Dellu et al., 1996; Smith et al., 1976; Yokel and Pickens, 1973), but the exaggerated response in HR rats suggests an underlying increased sensitivity of the DA mesolimbic circuit relative to LR rats (Dellu et al., 1996).

The HR phenotype has been compared to the human sensation-seeking trait (Dellu et al., 1996). Sensation-seeking is defined as a need for emotional stimulation, variety, and novelty and a willingness to take risks in pursuit of such experiences (Zuckerman, 1979). Human sensation seekers are more likely to abuse drugs (Wagner, 2001; Zuckerman, 1979), and one drug that has been positively associated with this trait is the club drug 3,4-methylenedioxymethamphetamine (MDMA, 'ecstasy') (Gerra et al., 1998; Laviola et al., 1999). MDMA is a substituted amphetamine with prominent subjective effects including elevated mood, increased feelings of self-confidence, self-acceptance and closeness toward others (Vollenweider et al., 1998). Interestingly, noted individual differences in the subjective response to MDMA have been observed in human psychopharmacology studies (Gatchalian et al., 2002). People who sense the effects of

MDMA at lower doses are also more likely to report 'liking' the experience and feeling 'high' than those for whom higher doses are required (Gatchalian et al., 2002).

A prominent component of the mechanism of action of MDMA involves the reversal of the serotonin (5-HT) transporter (Rudnick and Wall, 1992) and subsequent enhancement of synaptic 5-HT levels (Fitzgerald and Reid, 1990; White et al., 1996). Of the 16 available 5-HT receptors (Hoyer et al., 2002), roles for the 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A} and 5-HT_{2C} receptors in modulating the hyperactivity induced by (+)-MDMA have been observed (Bankson and Cunningham, 2002; Callaway et al., 1990; Callaway et al., 1992; Fletcher et al., 2002; Herin et al., 2004; McCreary et al., 1999). Of particular interest is the 5-HT_{2A}R, which plays a stimulatory role in (+)-MDMA-induced hyperactivity; the selective 5-HT_{2A}R antagonist M100907 attenuated hyperactivity in response to (+)-MDMA treatment (Bankson and Cunningham, 2002; Herin et al., 2004). Direct stimulation of the 5-HT_{2A}R with the agonist DOI also evokes hyperactivity (Bankson and Cunningham, unpublished observations)(Bishop et al., 2004; Hillegaart et al., 1996), and while DOI also has affinity for both the 5-HT_{2B}R and 5-HT_{2C}R (Roth et al., 2000), antagonist studies indicated that the behavioral effects of DOI are mediated by the 5-HT_{2A}R (Bedard and Pycock, 1977; Schreiber et al., 1995). This makes DOI a useful tool in studying the function of 5-HT_{2A}R behaviorally.

The first objective of this study was to characterize the behavioral phenotype of HR rats as compared to LR rats. After separating the animals into HR and LR groups, we assessed the stability of the phenotype to repeated exposure to the test environment and to challenge in a second distinct environment. The challenge of the HR and LR rats in a

secondary novel environment after the initial separation day is undertaken to establish if the presentation of a second novel environment is an adequate stimulus to differentially activate HR vs. LR rats. The second objective was to test whether HR and LR rats exhibit a differential reaction to (+)-MDMA after habituation. Because we hypothesize that HR rats will exhibit a greater locomotor response to (+)-MDMA, and 5-HT_{2A}R plays a stimulatory role in (+)-MDMA-induced hyperactivity, we examined the function and expression of this receptor in HR vs. LR rats. We also examined the sensitivity of HR vs. LR rats to 5-HT_{2A}R-induced head shakes, assessed the role of the 5-HT_{2A}R in novelty-induced locomotor activity and quantified the expression of 5-HT_{2A}R in several brain regions in HR vs. LR rats. These studies were conducted in order to better understand the HR vs. LR animal model in terms of the stability of the trait and the role of the 5-HT_{2A}R in response to novelty and locomotor activity.

Materials and Methods

Subjects

Adult male Sprague-Dawley rats (virus antibody-free; Harlan Sprague-Dawley Inc., Indianapolis IN) weighing 250-300 g were used for all studies. Rats were allowed to acclimate for 7 days in a colony room at a constant temperature (21-23°C) and humidity (45-50%) on a 12 hr light-dark cycle (light 0700-1900 hr). Rats were housed four per cage with food and water *ad libitum*. All experiments were carried out in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996) and with approval of the Institutional Animal Care and Use Committee.

Drugs

(+)-3,4-methylenedioxymethamphetamine HCl [(+)-MDMA; National Institute on Drug Abuse, Research Triangle, NC], (-)-2,5-Dimethoxy- 4-iodoamphetamine hydrochloride [DOI; Sigma, St. Louis MO] and *R*-(+)-(2,3-dimethoxyphenyl)- 1-[2-(4-fluorophenylethyl)]- 4-piperidine-methanol [M100907; synthesized by Thomas Ullrich and Kenner Rice; National Institutes of Health, Bethesda MD] were dissolved in sterile saline (0.9% NaCl) and injected in a volume of 1 ml/kg. The doses of all drugs refer to the weight of the salt and were chosen based on previous studies (Bankson and Cunningham, 2002; Bubar and Cunningham, 2003; Herin et al., 2004; Smith et al., 1999). ^{125}I-MPPI [4-(29-methoxyphenyl)- 1-[29-[*N*-(20-pyridinyl-)-iodo-benzamido] ethyl] piperazine] and ^{125}I-DOI was purchased from NEN Life Science Products (Boston MA),

and 8-[5-(2,4-dimethoxy-5-(4-trifluoromethyl phenylsulfonamido) phenyl-5-oxopentyl]-1,3,8-triazaspiro[4,5]decane-2,4-dione (RS 102221) was purchased from Tocris (Ballwin MO).

Activity Screening to Separate HR and LR Rats

To assess locomotor activity, modified open-field activity monitors were used under low-light conditions (San Diego Instruments, San Diego CA). Each monitor was housed in a sound-attenuating cabinet and consisted of a Plexiglas cube (40 x 40 x 40 cm) with a set of photobeams positioned 4 cm above the cage floor to measure horizontal locomotor activity. Video cameras located above the chambers were used to monitor activity continuously without disruption of behavior. Counts of horizontal activity were made by the control software (Photobeam Activity Software; San Diego Instruments) and stored for subsequent statistical analysis.

In order to separate HR and LR rats, naïve male Sprague-Dawley rats (N=56 rats per cohort) were removed from the home cage and placed the activity monitors. Recording of activity in 5-min epochs began immediately and continued for 30 min. Rats were separated into HR and LR groups such that individuals with horizontal activity in the top 15% were designated HR rats (n=8) and those in the bottom 15% were designated LR rats (n=8). HR and LR rats were weighed following behavioral screening. All separations were run between 0800 and 1300 during the light phase. In each of the seven cohorts of rats separated into HR and LR groups (n=56 rats per phenotype) for the current study, horizontal activity counts were evaluated as a time course in 5-min bins and summed for individual animals throughout the 30-min monitoring session to evaluated

group means. Differences in locomotor activity and weights between HR and LR groups were assessed using a two-tailed Student's *t*-test and were considered statistically significant when $p < 0.05$ (Keppel, 1973).

Habituation and novelty challenge in HR vs. LR rats

Two cohorts of HR and LR rats (n=16 rats per phenotype) were separated and tested for the effects of habituation, subsequent novelty and (+)-MDMA challenge. After separation, HR and LR rats were habituated to the locomotor activity monitors by repeated exposure to the environment (3 hr/day for 4 days), and the first 30 min of each session was monitored. After four days of habituation, the animals were challenged with a distinct environment: HR and LR rats were placed into a novel three-chambered conditioned place-preference apparatus (Med Associates, Georgia VT) that consisted of one black chamber and one white chamber (21 x 21 x 28 cm) separated by a small gray corridor (21 x 21 x 12 cm). The chambers are separated from one another by guillotine doors which were in the open position during the test. Horizontal activity was monitored for 30 min by photobeams that line the apparatus and stored for subsequent statistical analysis.

Horizontal activity counts were summed for each animal throughout each 30-min session. The changes in locomotor activity observed across days of repeated exposure to the original activity monitors were initially analyzed using a two-way analysis of variance (ANOVA; SAS for Windows, Version 8.2, SAS Institute, Cary NC). For this habituation data, the effects of behavioral phenotype (factor 1) and day of habituation (factor 2; with repeated measures) were analyzed and included data from the initial

separation for a total of five different exposures to the activity monitors across five days. Pre-planned comparisons were then made with the Bonferroni procedure (experimentwise $\alpha = 0.05$; SAS for Windows, V. 8.2). This approach to statistical analysis is supported by a number of statisticians (Keppel, 1973; Kirk, 1995; Sheskin, 2000). Two-tailed Student's *t*-tests were used to compare activity levels of HR and LR rats seen upon challenge in the novel place preference chambers.

(+)-MDMA challenge in HR vs. LR rats

On the following day, animals from the same two cohorts of HR and LR rats were tested for (+)-MDMA-induced hyperactivity after an additional 90-min acclimation period in the test chambers to ensure that the effect of differential novelty-induced increases in locomotor activity were minimal. Rats were briefly removed from the apparatus and injected with either saline (1 ml/kg, SC) or (+)-MDMA (3 mg/kg, SC). (+)-MDMA was administered at a relatively low dose that has been shown to evoke hyperactivity (Bankson and Cunningham, 2001). Recording of activity in 5-min epochs began immediately following placement into the activity monitors and continued for 90 min. Horizontal activity counts were summed for each animal throughout the 90-min monitoring session, and a two-way ANOVA was used to analyze the effects of behavioral phenotype (factor 1) and challenge (factor 2) on horizontal activity. Time course data were broken down into 5-min time bins, and a three-way ANOVA was used to detect group x treatment x time interactions. In the case that a significant interaction was found, differences between groups were determined at each 5-min time point using a one-way

ANOVA. Pre-planned comparisons were then made with the Bonferroni procedure (experimentwise $\alpha = 0.05$).

Effect of the 5-HT_{2A}R agonist DOI on head shakes and novelty-induced activity in HR vs. LR rats

Another two cohorts of HR and LR rats (n=16 rats per phenotype) were separated and tested for DOI-induced (1) head shakes and (2) locomotor activity. (1) Following the separation procedure in the novel activity monitors, rats were returned to the home cage and left undisturbed for four days. Over the next 3 days, the animals were handled briefly and weighed in order to minimize the effects of handling stress. Animals were treated with either saline (1 ml/kg, SC) or DOI (1 mg/kg, SC), and the number of 5-HT_{2A}R-mediated head shakes was observed in the home cage for 15 min immediately following DOI treatment. (2) The animals were subsequently left in the home cage undisturbed for an additional seven days (with handling and weighing as above). HR and LR rats were then treated with either saline (1 ml/kg, SC) or DOI (0.25 mg/kg, SC) and immediately placed into the activity monitors. DOI at this dose has been shown to cause an increase in locomotor activity in unhabituated animals (Bankson and Cunningham, unpublished observations; Bishop et al., 2004; Hillegaart et al., 1996). Recording of activity in 5-min epochs for 30 min began immediately following replacement into the activity monitors. In order to test for differences between HR and LR rats in number of head shakes, the effects of behavioral phenotype (factor 1) and treatment (factor 2) were analyzed in a two-way ANOVA for independent groups. For DOI-induced hyperactivity, horizontal activity counts were summed for each animal throughout the 30-min

monitoring session, and the effects of behavioral phenotype (factor 1) and treatment (factor 2) were analyzed in another two-way ANOVA for independent groups. Pre-planned comparisons were then made with the Bonferroni procedure (experimentwise $\alpha = 0.05$). To determine if DOI induced differential effects on activity of HR vs. LR rats, activity counts were expressed as a percentage of baseline activity and compared between phenotypes using a Student's *t*-test.

Effect of 5-HT_{2A}R antagonist M100907 on novelty-induced activity in HR vs. LR rats

Two additional cohorts of HR and LR rats (n=16 rats per phenotype) were separated and tested for M100907-induced suppression of locomotor activity in response to a novel environment. Following the separation, rats were returned to the home cage and left undisturbed for four days. For the next 3 days, the animals were handled briefly and weighed in order to eliminate the effects of handling stress. Seven days after the initial novelty separation, HR and LR rats were challenged with either saline (1 ml/kg, IP) or the 5-HT_{2A}R antagonist M100907 (0.5 mg/kg, IP) and immediately placed into the activity monitors. This dose of M100907 was chosen based on previous studies in our laboratory (Herin et al., 2004) and has been shown to decrease locomotor activity in response to a novel environment (Frankel and Cunningham, unpublished observation). Recording of activity in 5-min epochs began immediately following placement into the activity monitors and continued for 30 min. Horizontal activity counts were summed for each animal throughout the 30-min monitoring session. Data were analyzed for behavioral phenotype (factor 1) and treatment (factor 2) effects using a two-way ANOVA

for independent groups, and pre-planned comparisons were then made with the Bonferroni procedure (experimentwise $\alpha = 0.05$). To establish if M100907 induced differential affects on activity in HR vs. LR rats, activity counts were expressed as a percentage of baseline activity and compared between phenotypes using a Student's *t*-test.

5-HT$_{2A}$R autoradiography

One final cohort of HR and LR rats (n=8 rats per phenotype) was separated and 5-HT$_{2A}$R-ligand binding was assessed in brain sections. Seven days after separation, HR and LR rats were anesthetized with chloral hydrate (Sigma; 800 mg/kg; IP) and decapitated. Brains were removed rapidly and immediately frozen in dry ice-cooled isopentyl alcohol for 15 sec. Brains were then placed on crushed dry ice for an additional 10 min until completely frozen and stored at -80°C until use. Fifteen-micrometer sections were prepared with a cryostat (Leica CM1850) at -20°C, were thaw-mounted onto gelatin chrom alum-coated glass slides and stored at -80°C. Each slide contained brain sections from three rats (alternating HR and LR rats) to limit variation between slides.

Slides were processed using autoradiographic procedures previously described for 5-HT$_{2A}$R and 5-HT$_{2C}$R radioligand binding (Li et al., 2003). Brain sections were preincubated for 30 min at room temperature in assay buffer (50 mM Tris-HCl, pH 7.4, containing 0.5 mM EDTA, 10 mM $MgSO_4$, 0.1% ascorbic acid, 0.1% bovine serum albumin and 10 μM pargyline). The slides were incubated for 1 h at room temperature

with ^{125}I-DOI (0.2 nM) in the presence of RS 102221 (100 nM, a 5-HT_{2C}R antagonist) for determination of the density of 5-HT_{2A}R binding sites. Non-specific binding was defined in the presence of 10 µM M100907. The slides were then washed twice with assay buffer at 4°C for 10 min and rinsed with cold distilled H_2O. After air blow-drying, the slides were exposed for 7 days to ^{3}H-Hyperfilm® (Amersham).

Brain images were captured and analyzed with MCID Basic 7.0 (Imaging Research, Inc., St. Catharines, Ontario). A set of ^{125}I microscales (Amersham) was exposed with the slides to calibrate the gray scale density readings into nCi / mg tissue. Adjacent brain sections were used for all three autoradiographic studies, and data for each brain region in each rat represent the mean of four adjacent brain sections. Specific binding was determined by subtracting the non-specific labeling from the total binding for each region. Sections for analysis were taken from five different rostral-caudal levels (Paxinos and Watson, 1998): Bregma +3.20 mm for anterior olfactory nucleus, medial (AOM), infralimbic cortex (IL), lateral orbital cortex (LOrbC) and prelimbic cortex (PrL); Bregma +1.70 mm for cingulate cortex (Cing), caudate-putamen (CPu), lateral septal nucleus, intermediate portion (LSI), nucleus accumbens, core (NAcC) and nucleus accumbens, shell (NAcS); Bregma -3.30 mm for basolateral nucleus of amygdala (BLA), CA1 of hippocampus (CA1), central nucleus of amygdala (CeA), central medial thalamic nucleus (CM), habenular complex (Hab), hypothalamus, dorsal portion (DHyp), hypothalamus, ventral portion (VHyp), mediodorsal thalamic nucleus (MD) and paraventricular hypothalamic nucleus (PVN); and Bregma -5.20 mm for interfascicular nucleus (IF), rostral linear nucleus of raphe (RLi), substantia nigra pars compacta (SNc)

and ventral tegmental area (VTA). Measures of protein expression included relative density of 5-HT_{2A}R labeling in HR vs. LR rats (**Figure 16; Table 1**). Two-tailed Student's *t*-tests (experimentwise $\alpha = 0.05$) were used to analyze differences in ligand binding between HR and LR groups.

Results

Separation of behavioral phenotypes

In total, seven separate cohorts of 56 rats were separated into HR and LR groups (n=56 rats per phenotype). A typical 30-min separation for a single cohort is shown (**Figure 8 and 9**). The activity level of this group of HR rats was significantly higher than that of the LR rats ($p<0.05$) over the initial 30-min separation period (**Figure 8A**). Likewise, HR rats exhibited higher levels of locomotor activity at each time point ($p<0.05$) throughout the 30 min separation period as compared to LR rats (**Figure 8B**). Population dynamics of this cohort of animals are represented as the number of total horizontal counts per 30 min plotted in ascending order for all 56 individual animals (**Figure 9A**). The locomotor activity level of rats around the mean is represented well by a straight line, but linear regression demonstrated that the slope of the line changes at the top and bottom of the activity range ($F_{2, 50}=64.26$; $p<0.0001$). This shift roughly translates to the top and bottom 15% of animals tested, so HR and LR groups are defined using this stringent definition. The distribution of locomotor activity exhibited a normal Gaussian distribution (**Figure 9B**) and illustrates that all HR and LR rats fall outside one standard deviation of the mean in terms of total horizontal activity with this separation criterion. Additionally, weights did not differ ($p=0.58$) in this group of LR rats (232.25 ± 3.63 g) and HR rats (229.50 ± 3.14 g).

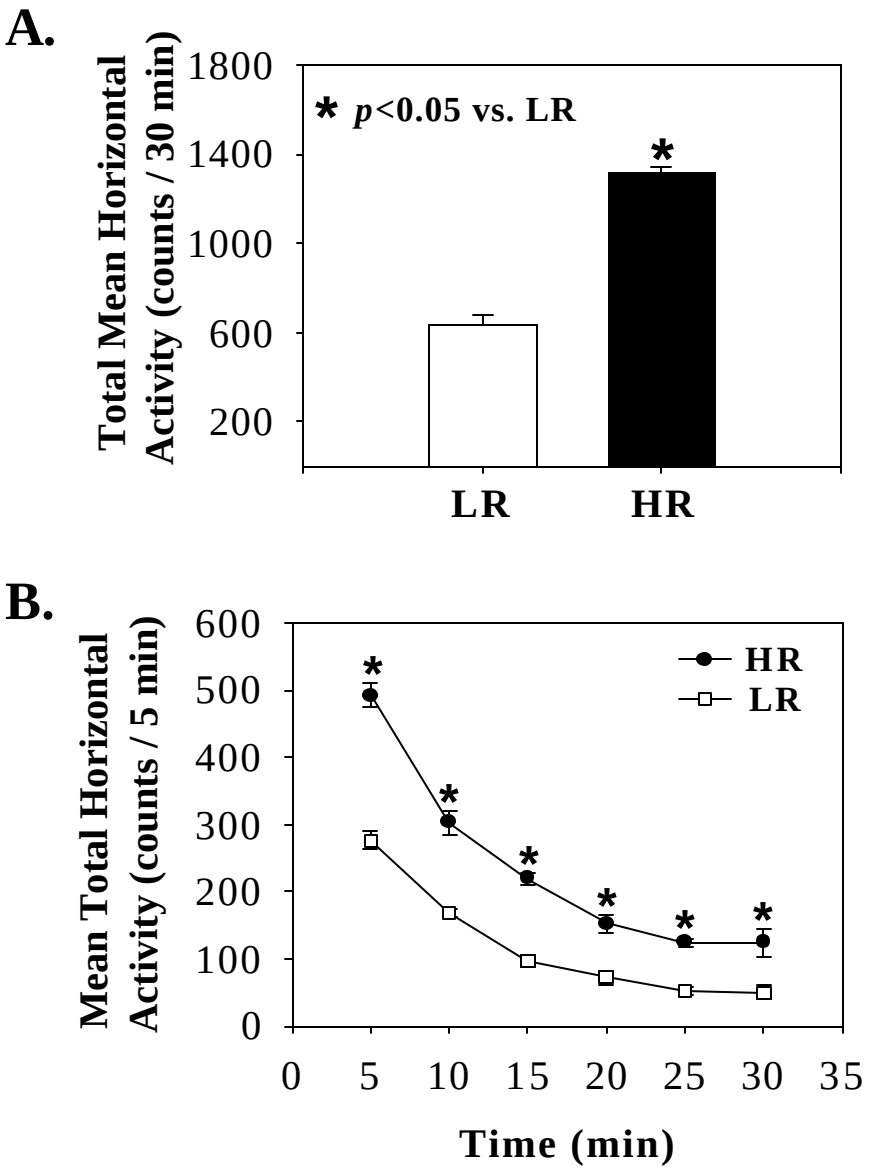


Figure 8. Separation of HR and LR rats based on novelty-induced locomotor activity. Naïve male Sprague-Dawley rats were placed into activity monitors, and spontaneous locomotor activity was recorded for 30 min. A. Locomotor activity of HR and LR rats (n=8 per phenotype) is represented as total mean horizontal activity in counts / 30 min (± SEM). B. Time course of locomotor activity is represented as total mean horizontal activity in counts / 5 min (± SEM). [* $p < 0.05$ vs. LR based on Student's *t*-test.]

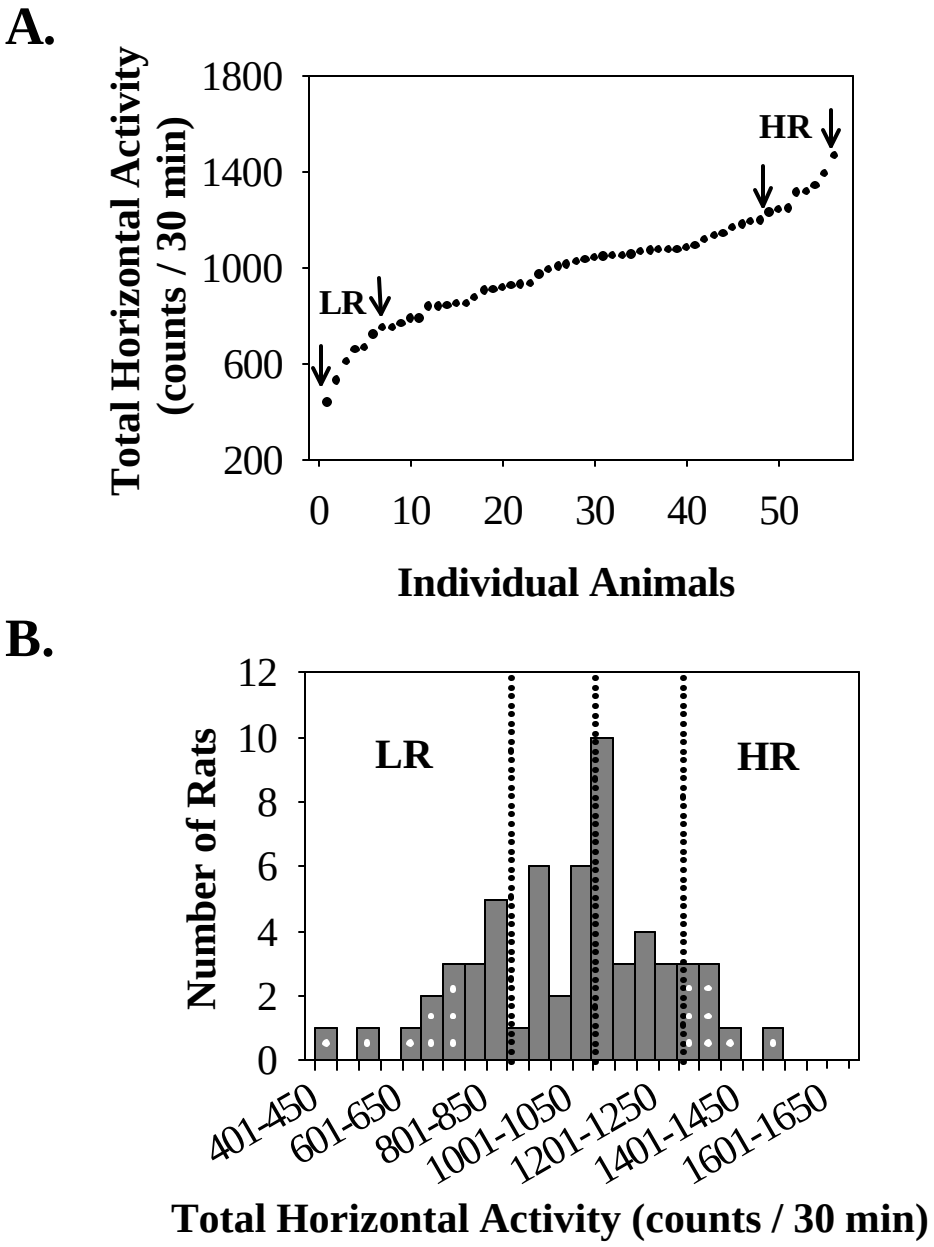


Figure 9. Population characteristics of HR and LR rats at separation. A. Range of locomotor activity of all rats in this separation is represented, plotted in ascending order of total horizontal activity (counts / 30 min). HR and LR ranges are indicated with arrows. **B.** The histogram depicting the frequencies of occurrence of total horizontal activity (counts / 30 min) is broken down into 50-count bins. Circles represent HR and LR rats, and dashed lines indicate ± 1 SD from the mean.

Expression of behavioral phenotype during habituation

Two cohorts (n=16 rats per phenotype) were separated for this experiment. The mean total horizontal activity level of all 56 animals in the first cohort was 1064 ± 215 counts, with a significant difference between mean total horizontal activities of LR rats (714 ± 52 counts) and HR rats (1413 ± 48 counts) ($p<0.05$; **Figure 8 and 9**). The mean total horizontal activity level of all animals in the second cohort was 991 ± 216 counts, with a significant difference between mean total horizontal activities of LR rats (637 ± 111 counts) and HR rats (1318 ± 83 counts) ($p<0.05$; data not shown). After separation, HR and LR rats were placed into the locomotor activity monitors on four consecutive days (3 hr/day), and the first 30 min of each session were recorded (**Figure 10**); the rats were exposed to the activity monitors a total a five times (one separation day plus four days of habituation). There was a main effect of behavioral phenotype ($F_{1, 79}=27.78$; $p=0.0001$), day of habituation ($F_{4, 79}=12.98$; $p<0.0001$) and a phenotype x day interaction ($F_{4, 79}=12.40$; $p<0.0001$). While LR rats showed no change in total activity counts across the separation and the four habituation sessions, locomotor activity levels in HR rats steadily declined with each subsequent exposure to the apparatus. Pre-planned comparisons indicated that, on the separation day as well as the first three days of habituation, HR rats had higher levels of horizontal activity than LR rats; however, on the fourth day of habituation, locomotor activity was no longer significantly different between HR and LR rats. These data demonstrate that after the initial separation screening, repeated exposure to the monitors reduced the degree of hyperactivity expressed by HR rats such that HR and LR rats ultimately exhibited similar degrees of

activity in response to the test environment.

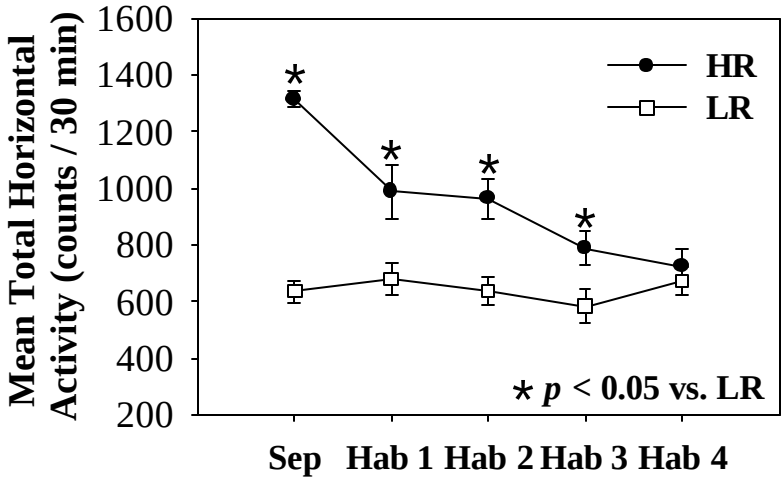


Figure 10. Expression of behavioral phenotype during habituation. HR and LR rats (n=16 rats per phenotype) were placed into locomotor activity monitors for four consecutive days (3 hr / day), and the first 30 min of spontaneous locomotor activity was recorded. Locomotor activity is represented as total mean horizontal activity in counts / 30 min (± SEM). [***** $p < 0.05$ vs. LR based on *a priori* comparisons with Bonferroni procedure.]

Expression of behavioral phenotype upon subsequent novelty challenge

After the separation and four-day habituation protocol, the same HR and LR rats were placed into monitors configured for the study of place preference to which all rats were naïve, and activity was monitored for 30 min (**Figure 11**). The mean horizontal activity levels of HR rats were not significantly different from that of LR rats ($p<0.05$) over the 30-min testing period. Thus, exposure to a completely distinct environment failed to allow re-separation of HR vs. LR rats.

Effects of (+)-MDMA challenge in HR vs. LR rats

Twenty-four hours after the novelty challenge, the same two cohorts of HR and LR rats were placed once again into the locomotor activity monitors and allowed to acclimate for 90 min. The first 30 min of activity was recorded, and activity levels were similar to that seen on day four of habituation (data not shown). At the termination of this 90-min acclimation period, activity was recorded for an additional 90 min after injection with either saline (1 mg/kg, SC) or (+)-MDMA (3 mg/kg, SC; **Figure 12A**). While there was no main effect of behavioral phenotype ($F_{1, 31}=2.67$; $p=0.1133$) or a phenotype x day interaction ($F_{1, 31}=2.02$; $p=0.1660$), there was a main effect of saline or (+)-MDMA challenge ($F_{1, 31}=136.94$; $p<0.0001$). Pre-planned comparisons indicated that (+)-MDMA treatment increased locomotor activity in both HR and LR rats compared to saline controls, and HR rats treated with (+)-MDMA exhibited greater locomotor activity than LR rats treated with (+)-MDMA. HR and LR rats exhibited similar locomotor activity after a saline injection. Analysis of the time course of (+)-MDMA-induced

locomotor activity in HR vs. LR rats (**Figure 12B**) was undertaken using a three-way ANOVA and revealed a group x treatment x time interaction ($F_{1, 575}$=2.41; p=0.0279). Subsequent one-way ANOVA at each time point followed by pre-planned comparisons indicated that at the 5-, 15-, 25-, 30- and 35-min time points, HR rats exhibited significantly greater hyperactivity than LR rats upon challenge with (+)-MDMA. While exposure to novelty secondary to habituation failed to differentially activate locomotor activity in HR vs. LR rats, (+)-MDMA treatment reinstated the HR vs. LR phenotype.

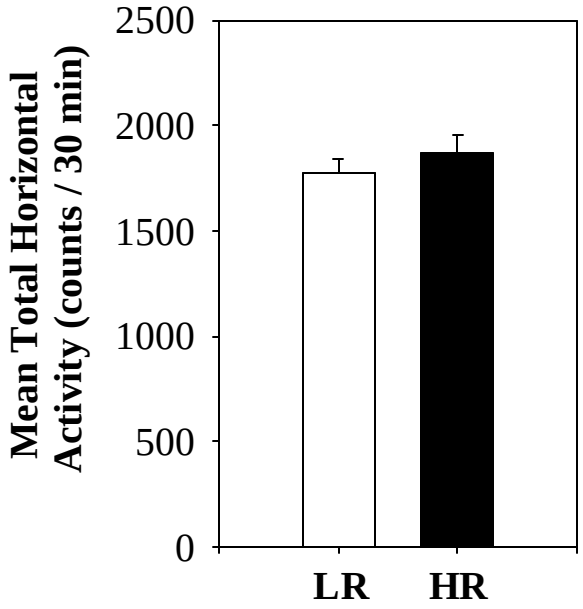


Figure 11. Expression of behavioral phenotype upon subsequent novelty challenge. After habituation, HR and LR rats (n=16 rats per phenotype) were placed into novel place-preference chambers (one white and one black chamber, separated by a small gray corridor). Locomotor activity is represented as total mean horizontal activity in counts / 30 min (± SEM). The difference between HR and LR rats was not statistically significant. [$p > 0.05$ vs. LR based on Student's *t*-test.]

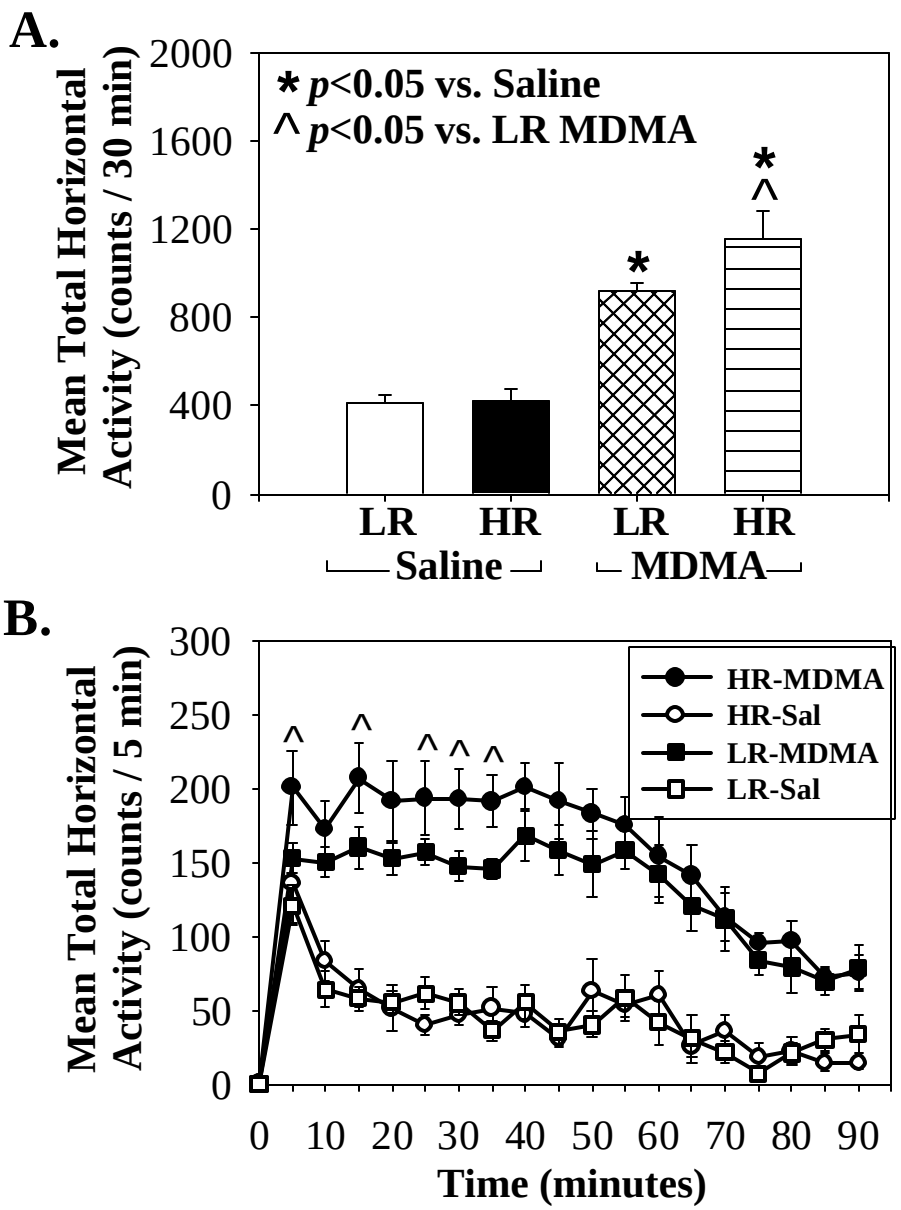


Figure 12. Effects of (+)-MDMA challenge in HR vs. LR rats. HR and LR rats (n=8 rats per phenotype-treatment) were placed into locomotor activity monitors and allowed to acclimate for 90 min. Animals were then administered either (+)-MDMA (3 mg/kg, SC) or saline (1 ml/kg, SC). **A.** Locomotor activity is represented as total mean horizontal activity in counts / 30 min (± SEM). **B.** The time course of locomotor activation is represented as total horizontal activity in counts / 5 min (± SEM). [***** $p < 0.05$ vs. Saline (within group), ^ $p < 0.05$ vs. LR MDMA based on *a priori* comparisons with Bonferroni procedure.]

Effects 5-HT_{2A}R agonist on HR vs. LR rats

Another two cohorts (n=16 rats per phenotype) were separated for this experiment. The mean total horizontal activity level of all 56 animals in the first cohort was 855 ± 115 counts, with a significant difference between mean total horizontal activities of LR rats (678 ± 60 counts) and HR rats (1102 ± 28 counts) ($p<0.05$; data not shown). The mean total horizontal activity level of all animals in the second cohort was 923 ± 111 counts, with a significant difference between mean total horizontal activities of LR rats (722 ± 53 counts) and HR rats (1201 ± 32 counts) ($p<0.05$; data not shown). Seven days after separation, HR vs. LR rats were injected with either saline (1 ml/kg, SC) or DOI (1 mg/kg, SC), and the number of 5-HT_{2A}R-induced head shakes was observed in the home cage for 15 min immediately following injection (**Figure 13A**). There was a main effect of behavioral phenotype ($F_{1, 31}=14.55$; $p=0.0007$), treatment ($F_{1, 31}=625.90$; $p<0.0001$) and a phenotype x treatment interaction ($F_{1, 31}=22.27$; $p<0.0001$). Pre-planned comparisons indicated that DOI treatment evoked a greater number of head shakes in both HR and LR rats compared to saline controls ($p<0.05$) and that HR rats exhibited significantly fewer DOI-induced head shakes compared to LR rats ($p<0.05$). This suggests that HR rats are less sensitive to DOI-induced head shake behavior than LR rats.

Seven days after the first DOI test, the same HR and LR rats were injected with either saline (1 ml/kg, SC) or DOI (0.25 mg/kg, SC) and placed into the into the activity monitors. We examined the locomotor-activating effects of DOI in HR and LR rats during a 30-min exposure to the novel apparatus (**Figure 13B**). There was a main effect of behavioral phenotype ($F_{1, 31}=8.51$; $p=0.0113$) and treatment ($F_{1, 31}=58.30$; $p<0.0001$),

but no phenotype x treatment interaction ($F_{1, 31}$=1.56; p=0.2317). While pre-planned comparisons indicated that DOI treatment induced hyperactivity in both behavioral phenotypes (p<0.05) and the absolute level of DOI-induced locomotor activity in HR rats was greater that observed in LR rats (p<0.05), DOI treatment did not differentially activate HR vs. LR rats. When DOI-induced activity was compared to novelty-induced activity in each behavioral phenotype (i.e., in saline-treated controls), the increase in activity above baseline in LR rats (251%) was not significantly different from the increase in HR rats (226%; p=0.937). These data imply that while HR rats are less sensitive to DOI-induced head shakes compared to LR rats, no differential effect on novelty-induced locomotor activity was observed after DOI treatment in HR vs. LR rats.

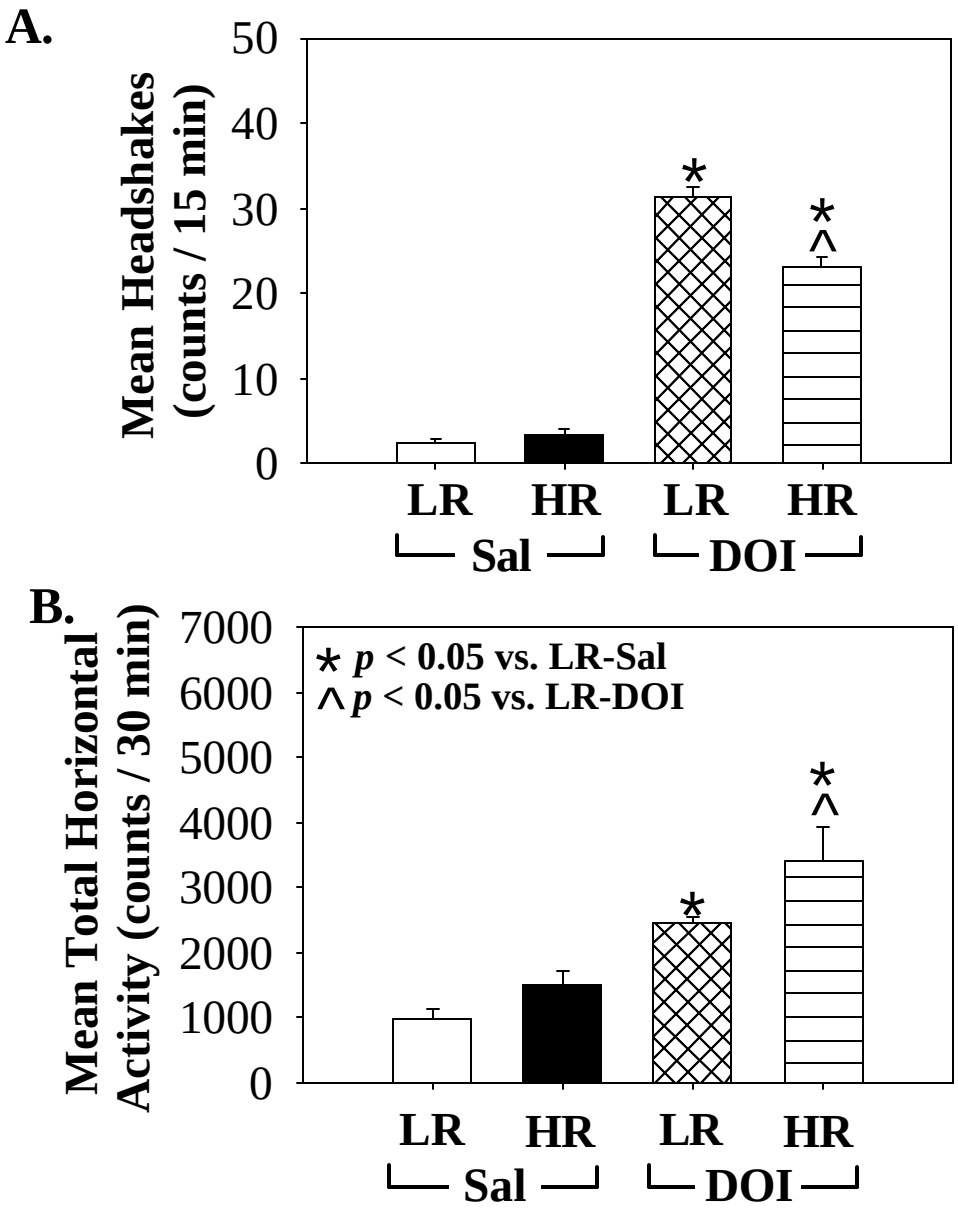


Figure 13. Effects 5-HT_{2A}R agonist on HR vs. LR rats. A. Rats were separated into HR and LR groups (n=8 rats per phenotype-treatment) and challenged seven days later in the home cage with saline (1 ml/kg; SC) or DOI (1 mg/kg; SC). Number of head shakes was scored for 15 min immediately after injection. Data are represented as mean number of head shakes (± SEM). **B.** Seven days later, animals were administered either saline (1 ml/kg, SC) or DOI (0.25 mg/kg, SC), and locomotor activity was monitored for 30 min. Data are represented as mean horizontal activity in counts / 30 min (± SEM). [*****$p < 0.05$ vs. LR Sal; ^$p < 0.05$ vs. LR DOI based on *a priori* comparisons with Bonferroni procedure.]

Effects of the 5-HT_{2A}R antagonist M100907 on novelty-induced locomotor activity in HR vs. LR rats

Two more cohorts (n=16 rats per phenotype) were separated for this experiment. The mean total horizontal activity level of all 56 animals in the first cohort was 912 ± 142 counts, with a significant difference between mean total horizontal activities of LR rats (677 ± 43 counts) and HR rats (1132 ± 48 counts) ($p<0.05$; data not shown). The mean total horizontal activity level of all animals in the second cohort was 880.05 ± 126.10 counts, with a significant difference between mean total horizontal activities of LR rats (698 ± 51 counts) and HR rats (1091 ± 87 counts) ($p<0.05$; data not shown). Seven days after separation, HR vs. LR rats were injected with either saline (1 ml/kg, IP) or M100907 (0.5 mg/kg, IP), and placed into the activity monitors. We examined the locomotor-suppressing effects of M100907 in HR and LR rats during a 30-min exposure to a novel apparatus (**Figure 14**). While there was a main effect of behavioral phenotype ($F_{1, 31}=36.93$; $p<0.0001$), there was no treatment effect ($F_{1, 31}=1.91$; $p=0.1783$) or phenotype x treatment interaction ($F_{1, 31}=1.10$; $p=0.3024$). Pre-planned comparisons indicated that HR rats exhibited a greater amount of locomotor activity compared to LR rats upon second exposure to the novel environment ($p<0.05$) and M100907 treatment had no effect on locomotor activity within the first 30 min in either behavioral phenotype ($p>0.05$).

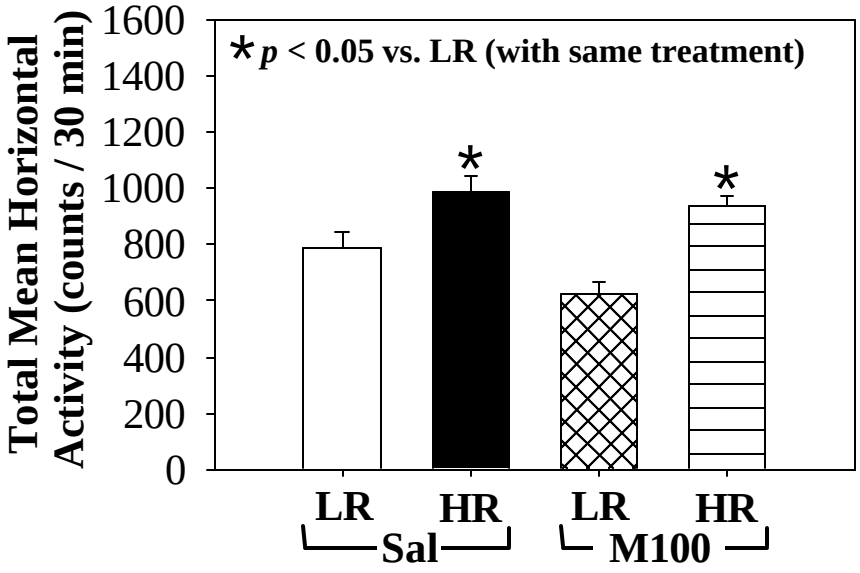


Figure 14. Effects of the 5-HT_{2A}R antagonist M100907 on novelty-induced locomotor activity in HR vs. LR rats. Rats were separated into HR and LR groups (n=8 rats per phenotype-treatment) and allowed to remain in the home cage for seven days. HR and LR rats were then challenged with either saline (1 ml/kg; IP) or M100907 (0.5 mg/kg; IP), and locomotor activity was monitored. Data are represented as mean total horizontal activity counts / 30 min (± SEM). [*****p < 0.05 vs. LR (with the same treatment) based on *a priori* comparisons with Bonferroni procedure.]

5-HT_{2A}R radioligand binding in HR vs. LR rats

One cohort (n=8 rats per phenotype) was separated for this experiment. The mean total horizontal activity level of all 56 animals was 1019 ± 149 counts, with a significant difference between mean total horizontal activities of HR rats (1264 ± 47 counts) and LR rats (791 ± 61 counts) ($p<0.05$; data not shown). HR and LR rats were allowed to remain in the home cages for seven days and sacrificed for 5-HT_{2A}R autoradiography. While expression of the 5-HT_{2A}R (**Figure 15**; **Table 1**) did not differ in the majority of the brain areas examined ($p>0.05$; AOM, LOrbC, PrL, Cing, CPu, LSI, NAcS, BLA, CA1, CeA, CM, Hab, DHyp, VHyp, MD, PVN, IF, RLi, SNc, VTA), there was significantly more radioligand binding to the NAcC of HR rats compared to LR rats ($p<0.05$).

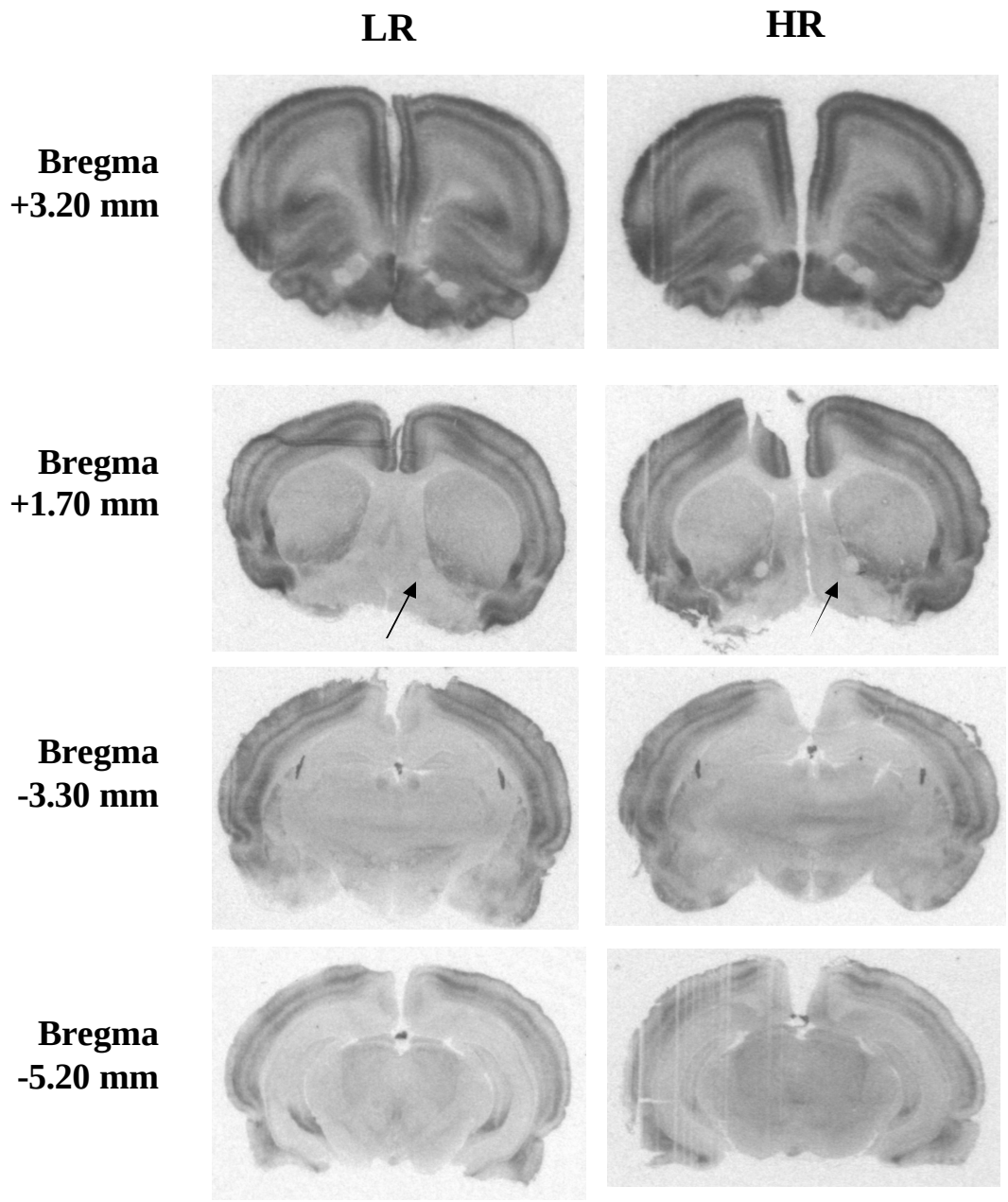


Figure 15. 5-HT_{2A}R radioligand binding in HR vs. LR rats. Left panel: LR rats; right panel: HR rats (n=8 rats per phenotype). Binding assay for 5-HT_{2A}R using ^{125}I-DOI (0.2 nM) in the presence of RS 102221 (100 nM, a 5-HT_{2C}R antagonist) for 1 hr at room temperature. Sections are from Bregma +3.20 mm, Bregma +1.70 mm, Bregma -3.30 mm and Bregma -5.20 mm. Arrows indicate the NAcC in HR (right) and LR (left) rats. The slides were exposed to film for 7 days.

Brain Area	Abbr.	Density (nCi/mg tissue) LR	vs.	Density (nCi/mg tissue) HR	*p*
Ant. olfactory n. (Medial)	AOM	2.873 ± 0.3746	vs.	2.936 ± 0.8817	0.9485
Infralimbic cortex	IL	5.343 ± 0.2636	vs.	5.385 ± 0.4725	0.9393
Lateral orbital cortex	LOrbC	6.002 ± 0.2115	vs.	5.631 ± 0.5414	0.5373
Prelimbic cortex	PrL	2.426 ± 0.1664	vs.	2.569 ± 0.1711	0.5623
Cingulate cortex	Cing	2.258± 0.1727	vs.	2.565 ± 0.2319	0.3129
Caudate-putamen	CPu	0.237 ± 0.1566	vs.	0.310 ± 0.0643	0.6753
Lateral septal n., int.	LSI	0.192 ± 0.1095	vs.	0.390 ± 0.0692	0.1581
Nucleus accumbens, core	**NAcC**	**0.562 ± 0.1498**	**vs.**	**1.077 ± 0.1688**	**0.0457**
Nucleus accumbens, shell	NAcS	0.455 ± 0.0289	vs.	0.570 ± 0.0595	0.1118
Basolateral n. of amygdala	BLA	0.943 ± 0.0703	vs.	1.178 ± 0.1972	0.2879
CA1 of hippocampus	CA1	-0.034 ± 0.0471	vs.	0.151 ± 0.2619	0.5030
Central n. of amygdala	CeA	0.726 ± 0.0576	vs.	0.986 ± 0.1718	0.1821
Thalamus	CM	0.020 ± 0.0787	vs.	0.204 ± 0.3468	0.6168
Habenular complex	Hab	0.308 ± 0.0443	vs.	0.316 ± 0.2681	0.9776
Hypothalamus, dorsal portion	DHyp	0.276 ± 0.1116	vs.	0.368 ± 0.0884	0.5330
Hypothalamus, ventral portion	VHyp	0.602 ± 0.1675	vs.	0.754 ± 0.1877	0.5592
Thalamus	MD	-0.011 ± 0.0664	vs.	0.227 ± 0.3323	0.4991
Paraventricular thalamic n.	PVN	0.016 ± 0.0647	vs.	0.087 ± 0.3357	0.8389
Interfascicular n.	IF	0.320 ± 0.1141	vs.	0.316 ± 0.1166	0.9802
Rostral linear n. of raphe	RLi	0.309 ± 0.1065	vs.	0.467 ± 0.0771	0.2577
Substantia nigra pars compacta	SNc	0.124 ± 0.0502	vs.	0.261 ± 0.0574	0.1020
Ventral tegmental area	VTA	0.230 ± 0.0605	vs.	0.367 ± 0.1016	0.2726

Table 1. 5-HT_{2A}R radioligand binding in HR vs. LR rats. Binding assay for 5-HT_{2A}R using ^{125}I-DOI (0.2 nM) in the presence of RS 102221 (100 nM, a 5-HT_{2C}R antagonist) for 1 hr at room temperature. Data for HR and LR rats (n=8 rats per phenotype) are represented as mean density (nCi / mg tissue; ± SEM). Sections are from Bregma +3.20 mm, Bregma +1.70 mm, Bregma -3.30 mm and Bregma -5.20 mm. [Significance defined as $p > 0.05$; HR vs. LR rats based on Student's *t*-test (bold).]

Discussion

After separation of HR and LR rats based upon levels of activity in a novel environment, repeated exposure to the same activity monitors eliminated the differential locomotor activation originally observed between HR and LR rats. While an attempt to reinstate the phenotypic difference by exposure to a second novel environment was not successful, subsequent challenge with (+)-MDMA resulted in higher levels of hyperactivity in HR vs. LR rats. Thus, the initial novelty and (+)-MDMA challenge served as strong stimuli to elicit differential locomotor activity in HR vs. LR rats while the secondary novelty exposure was not. HR rats displayed a diminished sensitivity to 5-HT_{2A}R-induced head shakes and increased levels of 5-HT_{2A}R binding in the NAc compared to LR rats, although neither 5-HT_{2A}R stimulation nor blockade differentially modulated HR vs. LR responses to a novel environment. These data suggest that the HR vs. LR phenotype is stable and may involve signaling through the 5-HT_{2A}R.

Repeated daily exposure to the activity monitors eliminated the differential responding of HR vs. LR rats to these originally-novel enclosures. Consistent with these data, other studies have similarly demonstrated that differences in novelty-induced locomotor activity between HR and LR rats disappeared after repeated exposure to the once-novel environment (Piazza et al., 1990; Thiel et al., 1999). This phenomenon enables the testing of other factors (e.g., drugs) in the HR and LR rats without the complication of differential locomotor responding to the environment in which they are tested.

The elimination of the differences in activity between HR and LR rats may be related to the diminished novelty and/or stress response of the HR rats to the inescapable experience of the novel chamber. The HR vs. LR animal model has often been linked to differential novelty-seeking behaviors in humans (Dellu et al., 1996; Kabbaj and Akil, 2001), however, it is unclear whether this parallel is appropriate. When allowed to roam freely, HR rats spend more time in a novel vs. familiar environment (Dellu et al., 1996), but they do not interact more with a novel object as compared to LR rats (Cain et al., 2004). Additionally, phenotypic patterns in novelty-induced place preference tests are unclear. HR rats have been shown to have both a greater preference for novelty (Bevins et al., 1997) as well as no difference in preference in a novel-object induced place preference paradigm (Cain et al., 2004) as compared to LR rats. In addition, animals separated based on differential free-choice novelty-seeking behaviors do not mimic HR vs. LR phenotypic patterns (Bevins and Peterson, 2004; Cain et al., 2004; Klebaur et al., 2001). In this task, instead of using an inescapable novel environment to separate groups as in the present study, high and low novelty-seeking rats are separated based upon latency of free-choice entrance into a novel environment (Bevins and Peterson, 2004), time spent interacting with a novel object (Bevins and Peterson, 2004; Klebaur et al., 2001), or degree of novel object-induced place preference (Cain et al., 2004). Patterns of differential sensitivity to the reinforcing and locomotor-stimulating effects of psychostimulants observed in HR vs. LR rats, however, were not borne out in these models. High and low novelty-seeking rats did not display differential acquisition of amphetamine self-administration behaviors (Klebaur et al., 2001) as seen in HR and LR rats (Piazza et al., 1989), nor do they exhibit a differential response to acute administration of methamphetamine (present results; Bevins and Peterson, 2004), as

would have been predicted in HR vs. LR rats (Hooks et al., 1991b; Piazza et al., 1989). This suggests that high novelty-seeking rats separated based on a free-choice novelty are not the same phenotypically as the rats who exhibit hyperactivity secondary to the mild stress of an inescapable novel environment as studied here.

Novelty may serve to be a mild stressor in the present studies (Dellu et al., 1996). Upon exposure to the mild stress of an inescapable novel environment, HR rats exhibited a longer duration of elevated corticosterone levels than LR rats (Piazza et al., 1991a). Also, exposure to a tail-pinch stressor HR rats elicited a greater increase in DA efflux in the NAc as compared to LR rats (Rouge-Pont et al., 1993), and this differential DA response is dependent on stress-induced increases in blood corticosterone levels (Rouge-Pont et al., 1998). Moreover, HR and LR rats exhibit a differential pattern of neural activation in response to stress with greater induction of *c-fos* mRNA in limbic and reward-related areas and with less induction in the hippocampus in HR rats as compared to LR rats (Kabbaj and Akil, 2001).

If the differential locomotor activation that separates the HR and LR phenotypes is a purely a reaction to any novel environment independent of the newness of the procedural experience itself (i.e., the combination of handling, removal from the home cage, transport and first exposure to an inescapable novel environment), HR rats should exhibit greater activity than LR rats not only on the initial separation day but upon exposure to a second novel environment as well. In order to address this question, we challenged previously-separated HR and LR rats with exposure to a second novel environment (place preference chambers) after a four-day habituation to the activity monitors. Place preference chambers have previously been employed to separate HR and LR rats (Bevins et al., 1997), however, in the present experiment HR and LR rats

responded with identical activity levels upon exposure. While the possibility exists that the rats did not perceive the place preference chambers as distinct from the activity monitors, in reality a wide variety of "novel" environments have been successfully utilized in separation procedures (Bevins et al., 1997; Marinelli and White, 2000; Piazza et al., 1989). Although some of the enclosures used to separate HR and LR rats are vastly different from any environment that the animals have previously experienced (Piazza et al., 1989), other separation enclosures are identical to the home cage environment (Marinelli and White, 2000). Thus, separation of HR and LR rats is not contingent upon a physical dissimilarity from environments with which rats have had previous experience but on the distinctiveness of the separation experience itself.

There seems to be some indispensable quality in the *initial* experience of the inescapable novel environment that renders the ability to differentially activate HR vs. LR rats. First, exposure to an inescapable novel environment is considered a stressor. In addition to its ability to elicit increases in blood corticosterone (Piazza et al., 1991a), multiple parallels have been demonstrated between the pattern of c-Fos expression secondary to an inescapable novel environment and that observed following a variety of other stressors (Cullinan et al., 1995; Emmert and Herman, 1999; Grahn et al., 1999; Handa et al., 1993). Second, HR rats exhibit exaggerated responses to stressful stimuli, including the mild stress of exposure to an inescapable novel environment (Piazza et al., 1991a), tail-pinch (Rouge-Pont et al., 1993), social (Touyarot et al., 2004) and free-choice stressors (Kabbaj et al., 2000). The ability of the stress axis to modulate the function of the DA mesolimbic circuit is well known (Le Moal and Simon, 1991). Intravenous corticosterone administration facilitates the firing of DA cells in the VTA, increasing the probability of burst firing (Overton et al., 1996), and both tail-pinch and

social interaction stressors increase DA transmission in the NAc (Louilot et al., 1986). As differences in DA release in NAc are directly correlated with differential responding in a novel environment (Bradberry et al., 1991), the exaggerated responses to stressful stimuli in HR rats, therefore, may culminate in the differential functioning of the DA mesolimbic circuit seen in HR vs. LR rats.

Although the locomotor reaction to a novel environment diminishes with repeated exposure for HR rats, the behavioral difference between HR and LR rats re-emerges upon administration of the psychostimulant (+)-MDMA. These data indicate that the HR vs. LR phenotype cannot be eliminated through habituation to the separation environment and reinforce the idea that separation into the HR or LR phenotype is a stable characterization. Consistent with this hypothesis, differences between HR and LR groups in novelty-induced locomotor activity were stable when tested with 6 days (Rosario and Abercrombie, 1999), 21 days (Cordero et al., 2003) and one month (Piazza et al., 1990) between exposures to the novel environment, and habituated HR rats have been consistently shown to be more sensitive to the locomotor-stimulating effects of amphetamine (Hooks et al., 1992b), morphine (Deroche et al., 1993) and cocaine (Hooks et al., 1993) compared to LR rats. These data suggest that specific, stable neurobiological differences between HR and LR rats underlie the differential behavioral phenotype observed in the two groups.

As HR rats are more sensitive to the locomotor-stimulating effects of (+)-MDMA (present results) and given that the 5-HT_{2A}R plays stimulatory role in (+)-MDMA-induced hyperactivity (Bankson and Cunningham, 2002; Herin et al., 2004), we expected that HR rats would exhibit a greater sensitivity to 5-HT_{2A}R-mediated behaviors compared to LR rats, coupled to increased 5-HT_{2A}R expression. While HR rats did exhibit greater

levels of 5-HT_{2A}R in the NAc compared to LR rats, the differential 5-HT_{2A}R expression did not translate into the expected behavioral differences between the behavioral phenotypes. HR rats exhibited less sensitivity to DOI-induced head shake behavior compared to LR rats and did not differ in DOI-induced hyperactivity or M100907-induced locomotor suppression. As DOI microinfused into the NAc results in a local increase in DA concentration (Yan, 2000), and the amount of DA released in the NAc directly correlates to the degree of locomotor activity in a novel environment (Bradberry et al., 1991), we expected the greater NAc 5-HT_{2A}R to translate into greater locomotor activity in HR vs. LR rats. Thus, it seems that the 5-HT_{2A}R in the NAc are not likely involved in the differential locomotor activity observed in HR vs. LR rats in response to a novel environment. While it is still possible that the greater 5-HT_{2A}R expression in HR vs. LR rats results in greater (+)-MDMA-induced hyperactivity in HR rats, the 5-HT_{2A}R resident in the NAc does not appear to play a role in cocaine-induced activity (McMahon et al., 2001). A more likely explanation for the differential locomotor response to (+)-MDMA in HR vs. LR rats lies in the ability of (+)-MDMA to differentially activate the DA mesolimbic system. (+)-MDMA administration results in efflux of DA in the NAc (Fitzgerald and Reid, 1990; White et al., 1996), and like cocaine (Hooks et al., 1991b), this effect may be more pronounced in HR vs. LR rats. Also, in the face a diminished sensitivity of HR rats to DOI-induced head shakes, no difference between HR and LR rats in 5-HT_{2A}R in the PFC was detected. It has been shown that DOI directly infused into the PFC induces head shake behavior (Willins and Meltzer, 1997), thus we would have expected decreased levels of 5-HT_{2A}R expression in the PFC of HR vs. LR rats. The fact that such a pattern was not uncovered implies that the PFC is not involved in the differential head shake behavior observed in HR vs. LR rats. As studies suggest that

DOI-induced head shakes might also be mediated by brainstem structures (Bedard and Pycock, 1977; Fone et al., 1989; Fone et al., 1991; Yap and Taylor, 1983), the differential sensitivity to 5-HT_{2A}R stimulation in other brain areas might underlie the differential sensitivity to DOI-induced head shakes observed in HR vs. LR rats.

In summary, results from the present study suggest that while the phenotypic difference between HR and LR rats cannot be reinstated by exposure to a second novel environment, the HR vs. LR phenotype is a stable phenomenon based on underlying neurobiological differences. For the first time, we demonstrate that HR rats are more sensitive than LR rats to the locomotor-stimulating effect of (+)-MDMA and that HR rats have increased expression of the 5-HT_{2A}R in the NAc. It is thought that the neural circuits underlying novelty-, stress- and drug-induced reactions overlap (Emmert and Herman, 1999; Handa et al., 1993; Kabbaj and Akil, 2001; Stephenson et al., 1999), and our findings indicate that stimuli activating the system to a greater degree [e.g., inescapable novelty stress and (+)-MDMA] can elicit differential activation in HR vs. LR rats with possible involvement of a 5-HT_{2A}R-mediated mechanism. These findings lead us to question what brain areas are involved in the generation of the HR vs. LR phenotype. Further understanding of the crossover between the stress system and the DA mesolimbic circuit might, in part, help to explain the role of stress in triggering drug-seeking behavior and relapse to abuse and point to a role of the stress circuit in the HR vs. LR animal model that might lead to potential therapeutic breakthroughs in the fight against drug addiction in humans.

CHAPTER 4:

EXPOSURE TO A NOVEL ENVIRONMENT INDUCES DIFFERENTIAL EXPRESSION OF C-FOS IMMUNOREACTIVITY IN HIGH AND LOW RESPONDING RATS: EVIDENCE FOR DIFFERENTIAL ACTIVATION OF GABA NEURONS

Background and Significance

Drug addiction is characterized by a unique set of behaviors including continued drug use despite a desire to stop and compulsive drug seeking at the expense of other priorities (Edwards et al., 1981). The process by which occasional drug-taking behavior evolves into drug addiction is a key issue to researchers in the field. There are widely reported individual differences in humans with regard to the course of developing drug addiction. The amount of drug exposure necessary before an individual makes the transition from occasional drug-taking behavior to drug addiction varies and depends in part on the reported sensitivity of the user to the subjective reinforcing effects of the drug (O'Brien et al., 1986). Individual differences have also been documented in animal models of drug-seeking behavior: rats who exhibit higher levels of locomotor activity in response to the mild stress of a novel environment are more sensitive to the locomotor-stimulating and reinforcing properties of drugs of abuse (Piazza et al., 1989). These high responder (HR) rats seem to have an underlying heightened sensitivity of the dopamine

(DA) mesolimbic circuit (Dellu et al., 1996) conferring an increased vulnerability to drug-taking behavior relative to low responder (LR) rats (Piazza et al., 1989; Piazza et al., 2000). Neurochemical differences have been uncovered between HR and LR rats in both the origin of the DA mesolimbic circuit in the ventral tegmental area (VTA) and the terminal region in the nucleus accumbens (NAc). Dopamine neurons recorded in the VTA of HR rats exhibit more bursts and higher basal firing rates than VTA DA cells of LR rats (Marinelli and White, 2000), and this high-frequency burst firing in the VTA results in higher levels of DA release in the NAc as compared to equivalent numbers of impulses at lower frequencies (Garris and Wightman, 1994; Gonon, 1988). Consistent with these findings, HR rats exhibit higher basal DA levels in NAc as compared to LR rats (Hooks et al., 1992a). Thus, the weight of evidence supporting the link between NAc DA and the locomotor-stimulating and reinforcing effects of psychostimulants (Deminiere et al., 1989; Robinson and Berridge, 1993) is supported by the HR vs. LR animal model.

In addition to the differential sensitivity of the DA mesolimbic circuit, HR and LR rats also exhibit a differential reactivity to a variety of different stressful stimuli (Piazza et al., 1991a; Rouge-Pont et al., 1993; Touyarot et al., 2004), and the phenotypic sensitivity of HR and LR rats to stressors could be based upon differential reactivity of the stress axis. The physiologic response to stress is mediated by the hypothalamic-pituitary-adrenal (HPA) axis which controls the secretion of glucocorticoid hormones (e.g., corticosterone) into the peripheral circulation by the adrenal glands and is under central control via corticotrophin-releasing factor (CRF) (Herman and Cullinan, 1997) as

well as afferent connections from a variety of different anatomic loci like the amygdala, bed nucleus of the stria terminalis, locus coeruleus, and dorsal raphe nucleus. It is well known that the stress axis modulates the function of the DA mesolimbic circuit (Le Moal and Simon, 1991). In outbred rats, intravenous corticosterone administration facilitates the firing of DA cells in the VTA, increasing the probability of burst firing (Overton et al., 1996), and both tail-pinch and social interaction stressors increase DA transmission in the NAc (Louilot et al., 1986).

Of note, there is abundant evidence that the HPA axis may contribute to the phenotypic distinction between HR and LR rats. For example, HR rats have greater stress-induced DA release in the NAc compared to LR in reaction to a 10-minute tail-pinch stressor (Rouge-Pont et al., 1993). In addition, HR rats exhibit a higher degree of disruption of spatial learning following chronic social stress than LR rats (Touyarot et al., 2004). HR rats exhibit a longer duration of increased blood corticosterone in response to novelty than LR rats (Piazza et al., 1991a). HR rats also exhibit a differential pattern of neural activation as measured by *c-fos* mRNA in response to the stress of placement in a novel dark-light box (Kabbaj and Akil, 2001). Together, these data suggest that the phenotypic differences between HR and LR rats may be driven by functional distinctions in the processing of both rewarding and stressful stimuli. Elucidating the nature of these differences may hold the key to understanding what factors underlie the enhanced vulnerability to developing drug-seeking behaviors in HR rats as compared to their LR counterparts.

The objective of the current study was to map the neuroanatomical circuits which exhibit differential activation in HR rats as compared to LR rats after a single exposure to a novel environment. In order to identify potential brain areas that drive the differential behavioral response of HR vs. LR rats to an initial novelty exposure, we utilized the immediate-early gene product, c-Fos, as a marker of neuronal activity. Because c-Fos acts as a widespread activator of transcription (Rylski and Kaczmarek, 2004), its pattern of expression is a convenient tool to examine anatomic elements activated in response to a given stimulus (Sagar et al., 1988). In addition to characterizing c-Fos expression in the nodes of the DA mesolimbic system, we sought out anatomical regions that might influence HR vs. LR behavior with a general survey of c-Fos expression in a collection of other brain areas. We also characterized the cell types in which differential c-Fos expression occurred using double-label immunocytochemistry for serotonin (5-HT), tyrosine hydroxylase (TH) and glutamic acid decarboxylase-67 (GAD-67) to label 5-HT, DA and ?-aminobutyric acid (GABA) cells, respectively. The hypothesis is that differential anatomical sites will be engaged in HR vs. LR rats secondary to the initial novelty stimulus, leading to a divergent pattern of neural activation that may point to anatomical sites (and systems) involved in the basic expression of the HR vs. LR phenotype.

Materials and Methods

Subjects

Adult male Sprague-Dawley rats (virus antibody-free; Harlan Sprague-Dawley Inc., Indianapolis IN) weighing 250-300 g were used for all studies. Rats were allowed to acclimate for 7 days in a colony room at a constant temperature (21-23°C) and humidity (45-50%) on a 12 hr light-dark cycle (light 0700-1900 hr). Rats were housed four per cage with food and water *ad libitum*. All experiments were carried out in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996) and with approval of the Institutional Animal Care and Use Committee.

Activity Screening to Separate HR and LR Rats

To assess locomotor activity, modified open-field activity monitors were used under low-light conditions (San Diego Instruments, San Diego CA). Each monitor was housed in a sound-attenuating cabinet and consisted of a Plexiglas cube (40 x 40 x 40 cm) with a set of photobeams positioned 4 cm above the cage floor to measure horizontal locomotor activity. Video cameras located above the chambers were used to monitor activity continuously without disruption of behavior. Counts of horizontal activity were made by the control software (Photobeam Activity Software; San Diego Instruments) and stored for subsequent statistical analysis.

In order to separate HR and LR rats, naïve male Sprague-Dawley rats (n=56 rats per cohort) were removed from the home cage and placed the activity monitors.

Recording of activity in 5-min epochs began immediately and continued for 30 min. Rats were separated into HR and LR groups such that individuals with horizontal activity in the top 15% were designated HR rats (n=8) and those in the bottom 15% were designated LR rats (n=8). HR and LR rats were weighed following behavioral screening. All separations were run between 0800 and 1300 during the light phase. In the two cohorts of rats separated into HR and LR groups (n=56 rats per cohort) for the current study, horizontal activity counts were summed for individual animals throughout the 15-min monitoring session. The separation protocol was shortened in the present study in order to facilitate the visualization of c-Fos protein within the window of peak expression after exposure to a novel environment (Papa et al., 1993). In previous 30-min separations, animals that were determined to be in HR and LR groups at 30 min would have been similarly defined at 15 min (Chapter 3). Thus, the validity of the shortened separation necessary in the immunocytochemistry separations was confirmed. Differences in locomotor activity between HR and LR groups were assessed using a two-tailed Student's *t*-test and were considered statistically significant when $p < 0.05$ (Keppel, 1973).

Immunocytochemistry

Two cohorts of HR and LR rats (n=16 rats per phenotype) were separated and returned to the home cage for three hours. Rats were then deeply anesthetized with pentobarbital (Sigma; 100 mg/kg; IP) and transcardially perfused with phosphate-buffered saline (PBS) followed by 3% buffered paraformaldehyde. Brains were removed, blocked at the mid-pons and post-fixed for 2 hr at room temperature. Brains

were then transferred into a 30% sucrose solution at 4°C for 48 hr, rapidly frozen on crushed dry ice and stored at -80°C until use.

Fifty-micrometer sections were prepared with a cryostat (Leica CM1850) at -20°C and processed using immunodetection procedures previously described (Paris et al., 1991; Frankel and Cunningham, 2002). Briefly, sections were washed extensively with PBS and blocked with 1.5% normal goat serum (Vectastain Elite kit; Vector Laboratories, Burlingame CA) in PBS containing 0.4% Triton-X (PBS-T; Sigma). Sections were then incubated in PBS-T for 2 days at 4°C with primary antibody for c-Fos (polyclonal; Ab-5, Oncogene Research, San Diego CA; diluted 1:50000). The sections were then washed in PBS and incubated with biotinylated goat anti-rabbit IgG (Vector; diluted 1:400) in PBS-T for 1 hr at 25°C. Following additional washes, sections were incubated in an avidin/biotin-horseradish peroxidase complex (Vectastain Elite Kit, Vector) for 1 hr at 25°C to amplify the signal. The pH of the sections was brought to 7.6 with 3 washes in Tris buffer, and the sections were developed in 3,3'-diaminobenzidine (DAB; 0.5 mg/ml; Sigma) with 0.005% H_2O_2. Additional control sections were developed in parallel in which either no primary antibody or no secondary antibody was added. For double labeling, initial c-Fos immunoreactivity was developed in DAB enhanced with nickel (black). This was followed by a second primary antibody incubation using either 5-HT (polyclonal; 20080; Immunostar, Inc., Hudson WI; diluted 1:5000), GAD-67 (polyclonal; MAB 5406; Chemicon International, Temecula CA; diluted 1:1000) or TH (polyclonal; AB 152; Chemicon; diluted 1:1000) that was

developed in DAB without nickel (brown). The sections were rinsed in PBS to terminate the chromagen reaction, mounted onto gelatin chrom alum-coated slides and coverslipped with Permount (Fisher Scientific, Houston TX).

Digital images were captured from brain sections using an Olympus Vanox-T AH2 microscope and a Pixera Professional camera (model VCS10132; Sherwood Dallas Co., Dallas TX) interfaced to a personal computer and analyzed using Scion Image software (v. Beta 4.0.2; Scion Image Corp., Frederick MD). A 2X objective was used to capture all photomicrographs for a final magnification of 12.5X. Sections from each brain were captured from anatomically appropriate rostral-caudal levels (**Figure 17 and 18**): Bregma +4.2 mm for anterior olfactory nucleus, dorsolateral (AON), anterior olfactory nucleus, medial (AOM), lateral orbital cortex (LOrbC); Bregma +2.7 mm for infralimbic cortex (IL), prelimbic cortex (PrL); Bregma +2.2 mm for caudate-putamen (CPu), nucleus accumbens, core (NAcC), nucleus accumbens, shell (NAcS); Bregma +1.7 mm for cingulate cortex (Cing), caudate-putamen (CPu), lateral septal nucleus, intermediate portion (LSI), nucleus accumbens, core (NAcC), nucleus accumbens, shell (NAcS); Bregma +1.0 mm for caudate-putamen (CPu), nucleus accumbens, core (NAcC), nucleus accumbens, shell (NAcS); Bregma -0.3 mm for bed nucleus of the stria terminalis (BNST), caudate-putamen (CPu), ventral pallidum (VP); Bregma -1.4 mm for paraventricular hypothalamic nucleus (PVN), paraventricular thalamic nucleus (PVA); Bregma -2.3 mm for central nucleus of amygdala (CeA), basolateral nucleus of amygdala (BLA), CA1 of hippocampus (CA1), centrolateral thalamic nucleus (CL), central medial thalamic nucleus (CM), intermediodorsal thalamic nucleus (IMD), mediodorsal thalamic

nucleus (MD); Bregma -4.16 mm for habenular complex (Hab); Bregma -5.8 mm for interfascicular nucleus (IF), parabrachial pigmented nucleus (PB), paranigral nucleus (PN), rostral linear nucleus of raphe (RLi), substantia nigra pars compacta (SNc); Bregma -6.0 mm for caudal linear nucleus of raphe (CLi); Bregma -7.3 mm for dorsal raphe nucleus (DRN), median raphe nucleus (MRN); Bregma -10.04 mm for locus coeruleus (LC). (Paxinos and Watson, 1998). A fixed-size rectangle was used to represent each brain area. Images were corrected for uneven background staining using digital subtraction of an unfocused image (Frankel and Cunningham, 2002), and cells immunopositive for c-Fos were counted automatically by Scion Image after applying the density slice function to the corrected image. Co-labeling of neurons (i.e., c-Fos with TH, 5-HT or GAD-67) was scored directly by an observer blind to experimental condition from sections in which staining for each antigen was distinct.

Data analysis

Measures of protein expression included number of c-Fos-, 5-HT-, TH- and GAD-immunoreactive cells [mean number (± SEM)] as well as number of cells co-labeled for c-Fos and 5-HT, c-Fos and TH and c-Fos and GAD [mean number (± SEM)]. Two-tailed Student's *t*-tests (experimentwise $\alpha = 0.05$) were used to analyze differences in numbers of immunopositive cells in HR and LR rats.

Results

HR and LR rats exhibited differential c-Fos protein expression in several brain areas

One cohort (n=8 rats per phenotype) was separated for this experiment, and the HR and LR rats were perfused for c-Fos immunocytochemistry. The mean total horizontal activity level of all 56 animals was 759 ± 136 counts, with a significant difference between mean total horizontal activities of HR rats (985 ± 65 counts) and LR rats (561 ± 45 counts) ($p<0.05$; data not shown). Of the rostral brain areas examined (**Figure 16**), HR rats exhibited greater numbers of c-Fos immunopositive nuclei in three cortical areas, the AON, LOrbC and Cing (for which a representative comparison is shown; **Figure 17**) compared to LR rats ($p<0.05$; **Table 2**); there were no differences in c-Fos expression in the other cortical areas examined (AOM, IL and PrL; $p>0.05$; **Table 2**). Neither the septum (LSI) nor the striatum at any level assessed (NAcC, NAcS, CPu; $p>0.05$; **Table 2**) exhibited differential c-Fos expression based upon behavioral phenotype. Of the caudal brain areas examined (**Figure 18**), HR rats had greater numbers of c-Fos immunopositive nuclei in two midbrain A10 DA cell-group subnuclei, the RLi and PB ($p<0.05$; **Table 3**), while the remaining DA subnuclei analyzed (IF, PN, SNc, CLi) showed no differences in c-Fos expression between HR and LR rats ($p>0.05$; **Table 3**). The ventral pallidum (VP), extended amygdala (BNST, CeA, BLA), thalamus (PVA, CL, CM, IMD, MD, Hab), PVN of the hypothalamus and CA1 of the hippocampus exhibited no differential c-Fos expression between HR and LR rats ($p>0.05$; **Table 3**). Interestingly, HR exhibited greater c-Fos expression in the DRN

when compared to LR rats (p<0.05; **Table 3**) but not in the MRN or LC (p>0.05; **Table 3**). Control sections developed with either no primary antibody or no secondary antibody showed no c-Fos staining (data not shown).

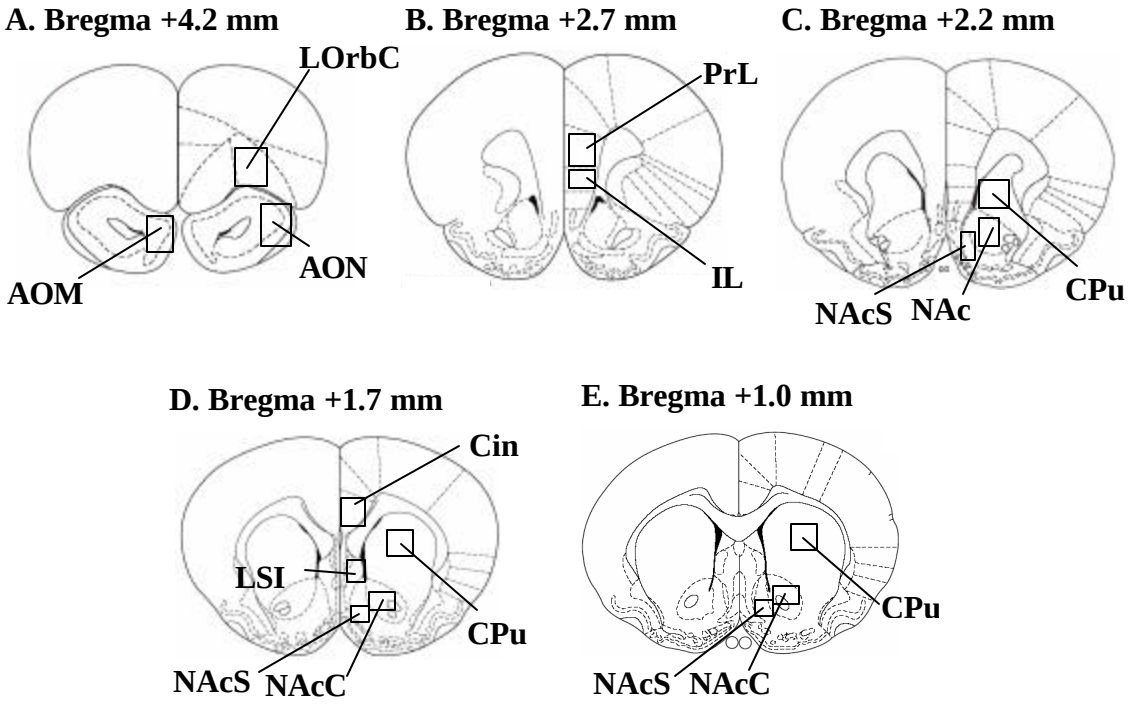


Figure 16. Schematic drawings of rostral brain areas analyzed for HR vs. LR c-Fos immunoreactivity. Adapted from The Rat Brain in Stereotaxic Coordinates (Paxinos and Watson, 1998). For anatomical reference, structures were labeled as follows: **A.** Bregma +4.2 mm: anterior olfactory nucleus, dorsolateral (AON); anterior olfactory nucleus, medial (AOM); lateral orbital cortex (LOrbC). **B.** Bregma +2.7 mm: infralimbic cortex (IL); prelimbic cortex (PrL). **C.** Bregma +2.2 mm: caudate-putamen (CPu); nucleus accumbens, core (NAcC); nucleus accumbens, shell (NAcS). **D.** Bregma +1.7 mm: cingulate cortex (Cing); caudate-putamen (CPu); lateral septal nucleus, intermediate portion (LSI); nucleus accumbens, core (NAcC); nucleus accumbens, shell (NAcS). **E.** Bregma +1.0 mm: caudate-putamen (CPu); nucleus accumbens, core (NAcC); nucleus accumbens, shell (NAcS).

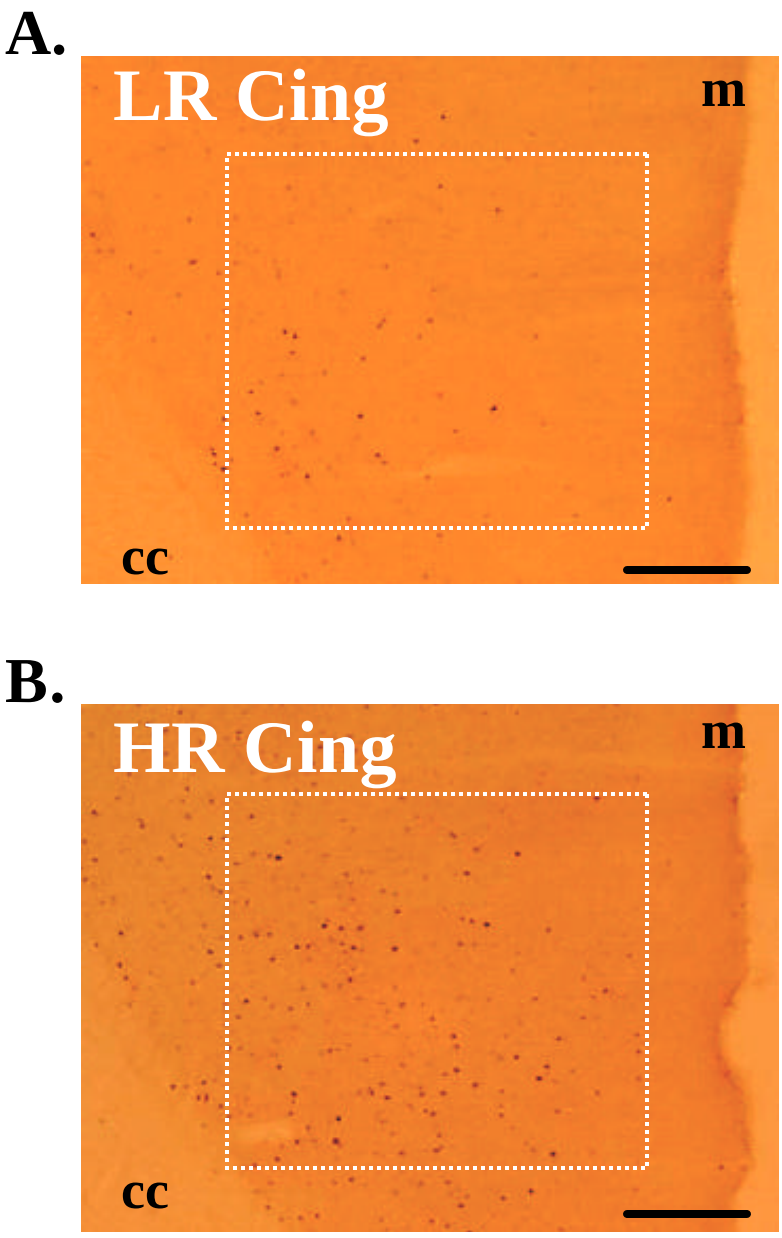


Figure 17. c-Fos expression in Cing in HR vs. LR rats after exposure to a novel environment. Digital images of c-Fos expression in **A.** a HR rat and **B.** a LR rat are shown. Abbreviations: cc, corpus callosum; m, midline. Scale bar = 250 µm.

Brain Area	**Abbr.**	**LR**	**vs.**	**HR**	***p***
Ant. olfactory n. (dorsolateral)*	**AON**	**102.0 ± 8.5**	**vs.**	**170.6 ± 27.8**	**0.0334**
Ant. olfactory n. (medial)	AOM	155.6 ± 10.0	vs.	161.0 ± 20.0	0.8137
Cingulate cortex*	**Cing**	**15.4 ± 5.2**	**vs.**	**30.8 ± 2.8**	**0.0201**
Infralimbic cortex	IL	76.4 ± 12.8	vs.	71.8 ± 17.2	0.8325
Lateral orbital cortex*	**LOrbC**	**62.5 ± 7.5**	**vs.**	**126.6 ± 21.2**	**0.0128**
Prelimbic cortex	PrL	133.6 ± 23.5	vs.	200.5 ± 45.7	0.2144
Caudate-putamen (Bregma +2.2 mm)	CPu	5.5 ± 1.0	vs.	4.6 ± 0.8	0.5102
Caudate-putamen (Bregma +1.7 mm)	CPu	11.0 ± 2.9	vs.	11.5 ± 3.0	0.9069
Caudate-putamen (Bregma +1.0 mm)	CPu	33.5 ± 7.3	vs.	34.0 ± 8.8	0.9659
Nucleus accumbens, core (Bregma +2.2 mm)	NAcC	13.3 ± 1.2	vs.	15.5 ± 5.5	0.6937
Nucleus accumbens, core (Bregma +1.7 mm)	NAcC	11.3 ± 2.1	vs.	10.5 ± 3.1	0.8446
Nucleus accumbens, core (Bregma +1.0 mm)	NAcC	15.0 ± 1.6	vs.	18.1 ± 4.0	0.4817
Nucleus accumbens, shell (Bregma +2.2 mm)	NAcS	6.8 ± 1.1	vs.	7.3 ± 2.7	0.8675
Nucleus accumbens, shell (Bregma +1.7 mm)	NAcS	9.4 ± 2.0	vs.	6.1 ± 1.6	0.2307
Nucleus accumbens, shell (Bregma +1.0 mm)	NAcS	10.9 ± 0.9	vs.	10.0 ± 2.6	0.7549
Lateral septal n., int.	LSI	31.0 ± 4.5	vs.	35.8 ± 5.1	0.4986

Table 2. c-Fos expression in rostral brain areas in HR vs. LR rats after exposure to a novel environment. Brain areas analyzed for c-Fos immunoreactivity rostral to Bregma. Data represented as mean number of c-Fos immunostained cells (± SEM; n=8 rats per phenotype). [* $p < 0.05$ vs. LR based on Student's *t*-test.]

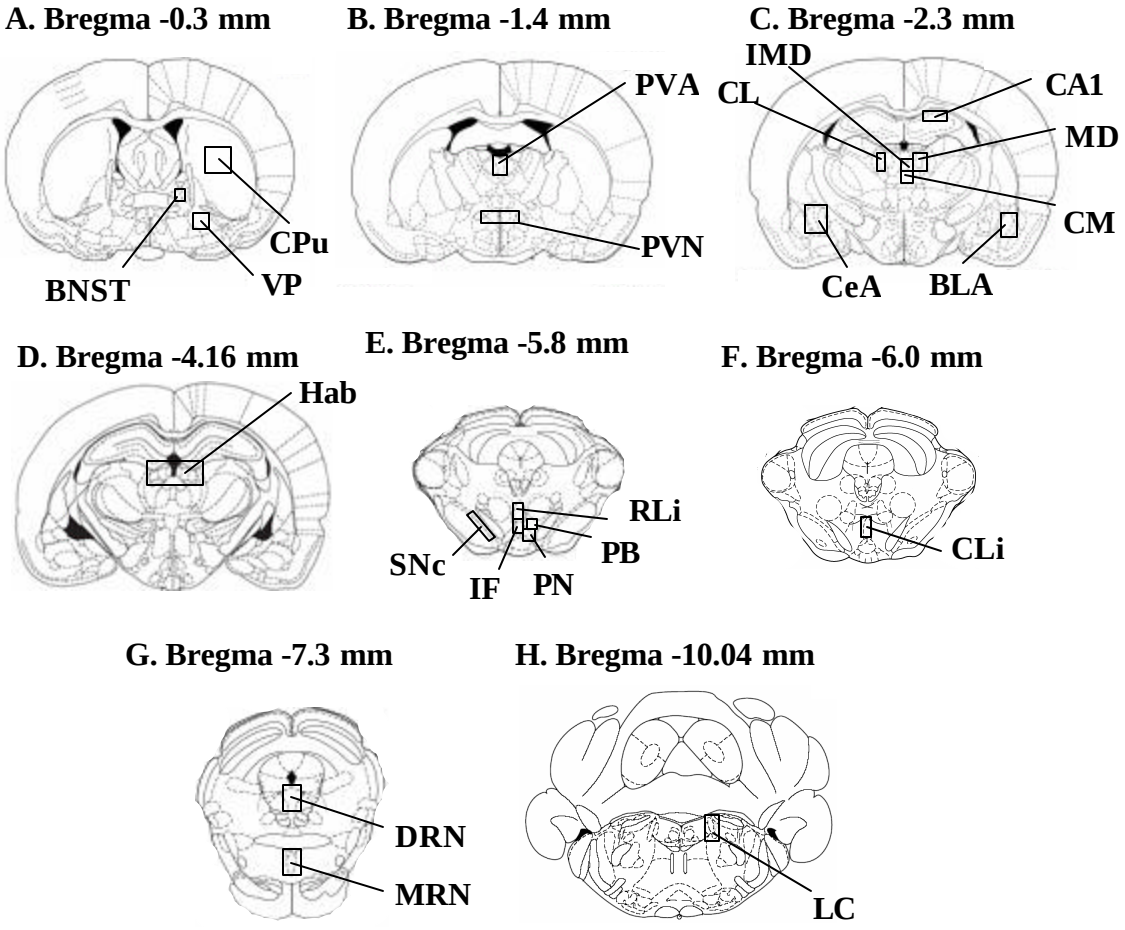


Figure 18. Schematic drawings of caudal brain areas analyzed for HR vs. LR c-Fos immunoreactivity. Adapted from The Rat Brain in Stereotaxic Coordinates (Paxinos and Watson, 1998). For anatomical reference, structures were labeled as follows: **A.** Bregma -0.3 mm: bed nucleus of the stria terminalis (BNST); caudate-putamen (CPu); ventral pallidum (VP). **B.** Bregma -1.4 mm: paraventricular hypothalamic nucleus (PVN); paraventricular thalamic nucleus (PVA). **C.** Bregma -2.3 mm: central nucleus of amygdala (CeA); basolateral nucleus of amygdala (BLA); CA1 of hippocampus (CA1); centrolateral thalamic nucleus (CL); central medial thalamic nucleus (CM); intermediodorsal thalamic nucleus (IMD); mediodorsal thalamic nucleus (MD). **D.** Bregma -4.16 mm: habenular complex (Hab). **E.** Bregma -5.8 mm: interfascicular nucleus (IF); parabrachial pigmented nucleus (PB); paranigral nucleus (PN); rostral linear nucleus of raphe (RLi); substantia nigra pars compacta (SNc). **F.** Bregma -6.0 mm: caudal linear nucleus of raphe (CLi). **G.** Bregma -7.3 mm: dorsal raphe nucleus (DRN); median raphe nucleus (MRN). H. Bregma -10.04 mm: locus coeruleus (LC).

Brain Area	**Abbr.**	**LR**	**vs.**	**HR**	***p***
Bed n. of the stria terminalis	BNST	58.5 ± 21.9	vs.	66.5 ± 24.6	0.8117
Caudate-putamen (Bregma -0.3 mm)	CPu	47.6 ± 6.0	vs.	52.8 ± 11.2	0.6939
Ventral pallidum	VP	50.4 ± 6.7	vs.	49.6 ± 11.4	0.9556
Hypothalamus, paraventricular n.	PVN	250.6 ± 38.4	vs.	264.5 ± 58.0	0.8448
Thalamus, paraventricular n.	PVA	216.4 ± 17.3	vs.	224.1 ± 37.1	0.8526
Amygdala, central n.	CeA	25.4 ± 4.6	vs.	40.6 ± 8.1	0.1235
Amygdala, basolateral n.	BLA	21.6 ± 5.1	vs.	25.1 ± 6.3	0.6740
CA1 of hippocampus	CA1	11.1 ± 2.3	vs.	14.8 ± 2.4	0.2892
Thalamus, centrolateral n.	CL	21.0 ± 4.1	vs.	18.0 ± 2.5	0.5444
Thalamus, central medial n.	CM	22.3 ± 6.7	vs.	19.4 ± 3.3	0.7055
Thalamus, intermediodorsal n.	IMD	32.0 ± 4.5	vs.	22.4 ± 4.0	0.1320
Thalamus, mediodorsal n.	MD	12.6 ± 1.8	vs.	10.0 ± 2.5	0.4052
Habenular complex	Hab	40.6 ± 5.8	vs.	47.3 ± 7.7	0.5023
Interfascicular n. (VTA)	IF	36.8 ± 5.2	vs.	34.4 ± 4.7	0.7386
Parabrachial pigmented n. (VTA) *	**PB**	**29.3 ± 2.5**	**vs.**	**46.3 ± 4.2**	**0.0035**
Paranigral n. (VTA)	PN	18.6 ± 2.3	vs.	24.3 ± 3.2	0.1813
Rostral linear n. of raphe*	**RLi**	**23.6 ± 3.7**	**vs.**	**51.5 ± 7.1**	**0.0037**
Substantia nigra pars compacta	SNc	24.5 ± 3.3	vs.	35.6 ± 5.0	0.0834
Caudal linear n. of raphe	CLi	22.5 ± 3.6	vs.	34.4 ± 6.1	0.1162
Dorsal raphe n.*	**DRN**	**25.6 ± 4.1**	**vs.**	**56.5 ± 8.3**	**0.0048**
Median raphe n.	MRN	55.8 ± 8.7	vs.	55.9 ± 7.3	0.9913
Locus coeruleus	LC	15.6 ± 2.0	vs.	11.9 ± 1.2	0.1337

Table 3. c-Fos expression in caudal brain areas in HR vs. LR rats after exposure to a novel environment. Brain areas analyzed for c-Fos immunoreactivity caudal to Bregma. Data represented as mean number of c-Fos immunostained cells (± SEM; n=8 rats per phenotype). [* $p < 0.05$ vs. LR based on Student's *t*-test.]

Differential expression of c-Fos immunoreactivity in GABA- and DA-containing cells in two subnuclei of the VTA

Another cohort (n=8 rats per phenotype) was separated for this experiment, and the RLi and PB subnuclei of the VTA were examined for c-Fos labeling in DA and GABA cells labeled for TH- and GAD-67-immunoreactivity. The mean total horizontal activity level of all 56 animals was 567 ± 78 counts, with a significant difference between mean total horizontal activities of HR rats (704 ± 34 counts) and LR rats (461 ± 14 counts) ($p<0.05$; data not shown). Photomicrographs from the PB depict a cell positive for GAD-67 alone and a cell double-labeled for both c-Fos and GAD-67 (**Figure 19A**) as well as a cell positive for TH alone and a cell double-labeled for both c-Fos and TH (**Figure 19B**). Data are represented as mean number of c-Fos-, TH-, and GAD-67-positive cells and mean number of cells co-labeled for both c-Fos-TH and c-Fos-GAD-67 (± SEM; **Table 4**). In the RLi, HR rats exhibited a greater number of c-Fos-positive cells as compared to LR rats ($p<0.05$; **Table 4A**) in keeping with earlier results, and both HR and LR rats exhibited similar numbers of TH- and GAD-67-positive cells in this region ($p>0.05$; **Table 4A**). HR and LR rats also exhibited similar mean numbers of cells co-labeled with both c-Fos and TH ($p>0.05$; **Table 4A**). However, a greater number of cells co-labeled with both c-Fos and GAD-67 were observed in HR rats compared to LR rats ($p<0.05$; **Table 4A**).

The pattern of c-Fos staining in the PB was similar to that seen in the RLi. Of note, HR rats again exhibited a greater number of c-Fos-positive cells as compared to LR rats and a greater number of cells co-labeled for both c-Fos and GAD-67 ($p<0.05$; **Table**

4B). The mean number of TH- and GAD-67-positive cells in the PB and the mean number of cells co-labeled with both c-Fos and TH did not differ between HR and LR rats ($p>0.05$; **Table 4B**). Thus after exposure to novelty, HR rats had a greater number of c-Fos immunoreactive cells in both RLi and PB, and it appears that the difference in activation of these two nuclei lies in the differential activation of VTA GABA cells between HR and LR rats. Control sections developed with either no primary antibody or no secondary antibody showed no c-Fos, TH or GAD-67 staining (data not shown).

Differential expression of c-Fos immunoreactivity in GABA- and 5-HT-containing cells in the DRN

In the same cohort of rats, the DRN was examined for c-Fos labeling in 5-HT- and GAD-67-immunoreactive cells. A photomicrograph from the DRN depicts a cell positive for 5-HT alone and a cell double-labeled for both c-Fos and 5-HT (**Figure 19C**). Data are represented as mean number of c-Fos-, 5-HT-, and GAD-67-positive cells and mean number of cells co-labeled for both c-Fos-5-HT and c-Fos-GAD-67 (± SEM; **Table 4**). In the DRN, HR rats exhibited a greater number of c-Fos-positive cells as compared to LR rats ($p<0.05$; **Table 4C**), while total mean number of 5-HT- and GAD-67-positive cells did not differ between HR and LR rats ($p>0.05$; **Table 4C**). The mean number of cells co-labeled with both c-Fos and 5-HT was not significantly different in HR rats compared to LR rats ($p>0.05$; **Table 4C**). However, HR rats expressed a greater number of cells co-labeled for both c-Fos and GAD-67 ($p<0.05$; **Table 4C**). After exposure to novelty, HR rats had a greater number of c-Fos immunoreactive cells in DRN, and in a

pattern similar to that observed in the VTA, it appears that the difference between HR and LR rats in activation of the DRN lies in the differential activation of GABA cells as well. Control sections developed with either no primary antibody or no secondary antibody showed no c-Fos, 5-HT or GAD-67 staining (data not shown).

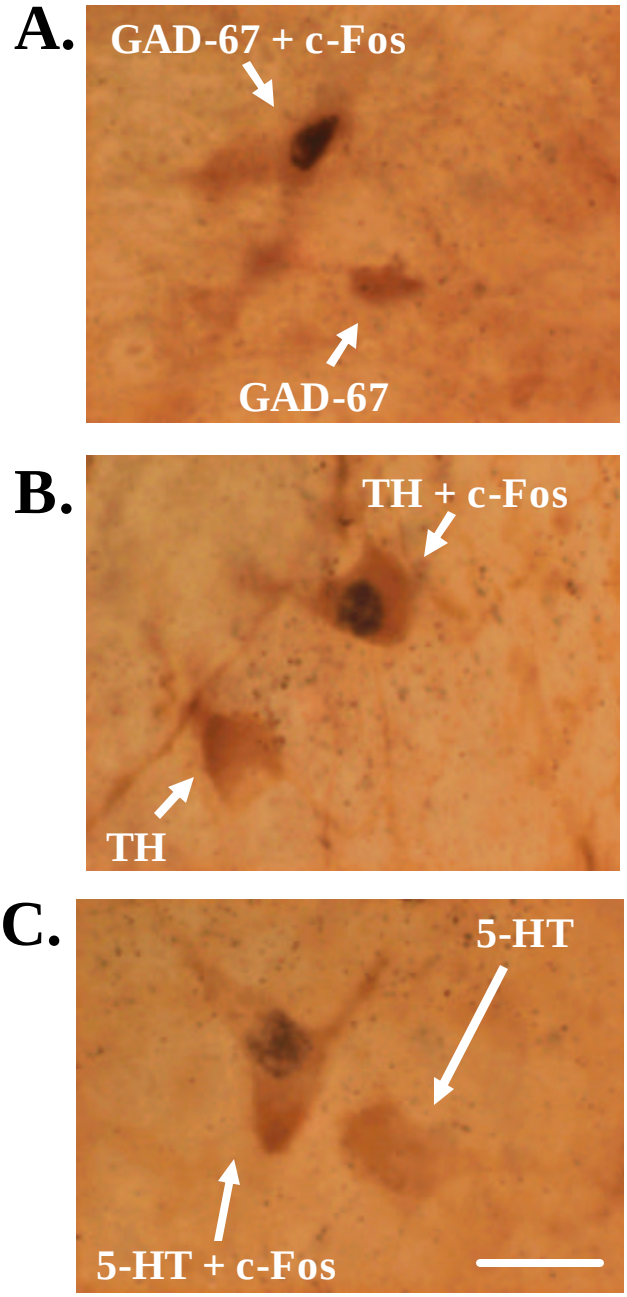


Figure 19. Photomicrographs depicting double-labeled sections in HR vs. LR rats. Digital images of c-Fos expression in three different cell types. **A.** Photomicrographs taken from PB of VTA depicting both GAD-67 labeling alone and GAD-67 + c-Fos co-labeling. **B.** Photomicrographs taken from PB of VTA depicting both TH labeling alone and TH + c-Fos co-labeling. **C.** Photomicrographs taken from DRN depicting both 5-HT labeling alone and 5-HT + c-Fos co-labeling. Scale bar represents 10 µm.

A. RLi

	LR	vs.	HR	*p*
c-Fos positive cells	8.13 ± 0.65	vs.	23.38 ± 2.24	<0.0001
TH positive cells	26.38 ± 2.96	vs.	24.88 ± 2.35	0.6976
Co-labeled	**1.38 ± 0.32**	**vs.**	**2.63 ± 0.65**	**0.1084**
GAD-67 positive cells	67.63 ± 4.23	vs.	65.25 ± 2.69	0.6427
Co-labeled	**1.38 ± 0.26**	**vs.**	**9.88 ± 1.30**	**<0.0001**

B. PB

	LR	vs.	HR	*p*
c-Fos positive cells	6.00 ± 1.13	vs.	17.00 ± 1.44	<0.0001
TH positive cells	55.75 ± 3.60	vs.	58.25 ± 3.44	0.6235
Co-labeled	**0.75 ± 0.25**	**vs.**	**1.00 ± 0.27**	**0.5057**
GAD-67 positive cells	97.50 ± 5.30	vs.	95.13 ± 6.86	0.7882
Co-labeled	**1.13 ± 0.30**	**vs.**	**9.00 ± 1.31**	**<0.0001**

C. DRN

	LR	vs.	HR	*p*
c-Fos positive cells	33.94 ± 5.59	vs.	49.81 ± 4.95	0.0046
5-HT positive cells	34.75 ± 3.66	vs.	35.00 ± 3.73	0.9625
Co-labeled	**10.36 ± 1.00**	**vs.**	**7.25 ± 1.79**	**0.1220**
GAD-67 positive cells	66.38 ± 5.61	vs.	57.50 ± 5.11	0.2617
Co-labeled	**12.00 ± 1.52**	**vs.**	**19.00 ± 2.55**	**0.0335**

Table 4. Characterization of c-Fos expressing cells in HR and LR rats after exposure to a novel environment. Data represented as mean number of immunostained cells (± SEM) for c-Fos, TH and GAD-67 labeling alone as well as c-Fos-TH and c-Fos-GAD-67 co-labeling in **A.** rostral linear nucleus of raphe (RLi) and **B.** parabrachial pigmented nucleus (PB) of VTA. Data represented as mean number of immunostained cells (± SEM) for c-Fos, 5-HT and GAD-67 labeling alone as well as c-Fos-5-HT and c-Fos-GAD-67 co-labeling in **C.** dorsal raphe nucleus (DRN). [* $p < 0.05$ vs. LR based on Student's *t*-test.]

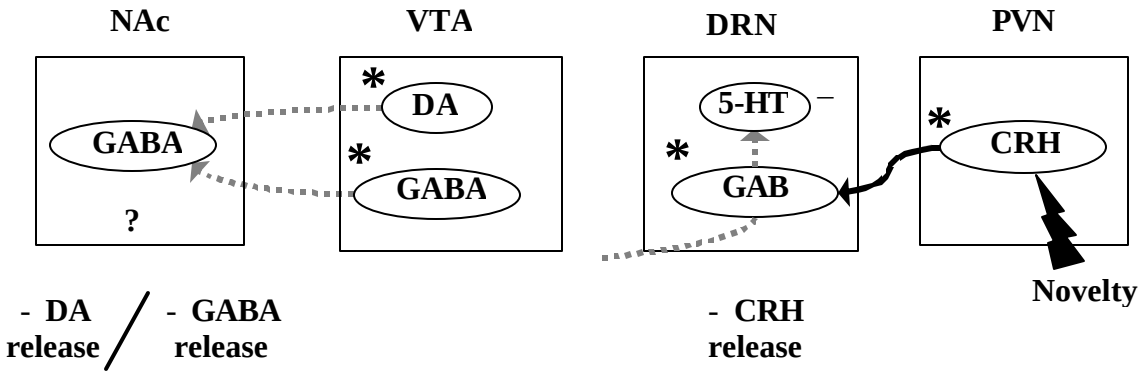


Figure 20. Brain areas involved in the generation of differential novelty-induced locomotor activity in HR vs. LR rats. The action of both DA and GABA in the NAc increases locomotor activity in rats. HR rats exhibit basal differences in DA firing and greater levels of DA in the NAc in response to a novel environment compared to LR rats. In addition, novelty exposure results in a greater activation of GABA cells in the VTA in HR vs. LR rats, which project to the NAc. No
velty has also been shown to increase activation of cells in the PVN, which by targeting GABA cells in the DRN cause greater GABA activation in the DRN in HR vs. LR rats. GABA in the DRN acts locally to decrease 5-HT release throughout the brain, and it is postulated that the GABA cells in the DRN project to the NAc. The increased activation of the GABA projections to the NAc from both the VTA and the DRN in HR vs. LR rats might underlie the increased locomotor activity in HR vs. LR rats in response to novelty. Solid black lines represent excitatory connections, and hatched gray lines represent inhibitory connections. Asterisks indicate steps where differences between HR and LR rats have been detected.

Discussion

Six neuroanatomical nuclei were differentially engaged in HR vs. LR rats after exposure to a novel environment. Greater numbers of c-Fos positive cells were observed in the AON, LOrbC, Cing, PB, RLi and DRN of HR rats compared to LR rats. Upon further analysis, we discovered that TH-positive VTA cells (PB and RLi) and 5-HT-positive DRN cells expressed c-Fos equally in HR and LR rats after the novelty stimulus. Strikingly, however, HR rats exhibited significantly greater numbers of c-Fos-positive cells that stained for GAD-67-immunoreactivity in both VTA subnuclei (PB and RLi) and in the DRN as compared to LR rats. Our findings are the first to reveal that the exposure to novelty results in expression of c-Fos predominately in GABA neurons of specific midbrain subnuclei of HR rat, but not LR rats. This differential activation of GABA subsystems of the VTA and DRN seen consequent to novelty challenge may reflect the important mechanistic distinctions that set the stage for the differential behaviors seen in HR and LR phenotypes.

When the current picture of neuronal activation in HR vs. LR rats in response to a novel environment is compared to that elicited by a challenge of the two groups in a light-dark box (Kabbaj and Akil, 2001), the patterns are similar but not identical. In both paradigms, HR rats exhibit greater c-Fos expression in the AON, LOrbC and Cing compared to LR rats; however, exposure to the light-dark box induced more *c-fos* mRNA in the PVN of HR rats as compared to LR rats (Kabbaj and Akil, 2001), an effect not present in the current study. Additionally, exposure to the novel environment (present results) produced differential expression of c-Fos in the VTA and DRN of HR and LR

rats, but no differential expression was localized to either of these brain areas after light-dark box exposure (Kabbaj and Akil, 2001). While some of the differences noted might be accounted for based on the fact that documented *c-fos* mRNA changes (Kabbaj and Akil, 2001) might not translate into differences in the expression of the c-Fos protein, we hypothesize that the two studies actually examined two different stages of the differential activation process in HR vs. LR rats. In the light-dark box experiment, animals were sacrificed at an early time point in the differential activation of HR vs. LR rat (either immediately or 15 minutes after the behavioral test) in order to assess *c-fos* mRNA changes (Kabbaj and Akil, 2001), while the current study examined a later time point in order to maximize the c-Fos protein expression observed after exposure to a novel environment (three hours post exposure; Papa et al., 1993). The combination of results from the two studies gives insight into the sequential nature of differential brain area activation in HR vs. LR rat after exposure to novelty.

HR rats exhibited greater c-Fos expression in GABA cells in both the VTA and DRN in response to a novel environment. In the following discussion, we will elaborate on how these early and late changes influence the proposed mechanisms by which greater GABA activation might result in the increased locomotor activity observed in HR vs. LR rats in response to novelty. To set the stage, it is known that GABA neurotransmission in the NAc results in the generation of locomotor activity. The infusion of the $GABA_A$ receptor antagonists picrotoxin (Morgenstern et al., 1984; Plaznik et al., 1990; Wong et al., 1991) and bicucullin (Wong et al., 1991) into the NAc decrease locomotor activity (Morgenstern et al., 1984; Plaznik et al., 1990; Wong et al., 1991), and the infusion of the

$GABA_A$ receptor agonist muscimol (Plaznik et al., 1990) and the $GABA_B$ receptor baclofen (Plaznik et al., 1990; Wong et al., 1991) into the NAc increase locomotor activity. Therefore, compounds which mimic the action of GABA in the NAc enhance locomotor activity, while those block the action of GABA attenuate locomotor activity.

We propose two possible mechanisms by which HR rats may have increased GABA neurotransmission in the NAc after exposure to a novel environment (**Figure 20**). The first involves the differential expression of c-Fos in GABA cells in the VTA. It is known that non-TH-staining (putatively GABAergic) efferent projections from the VTA terminate in the NAc (Swanson et al., 1981). We found that after novelty exposure, HR rats exhibited greater c-Fos expression in GABA cells in the VTA. If this greater GABA activation in HR rats translates into greater firing, it would result in increased GABA release in the NAc and differential locomotor activation in HR vs. LR rats. We propose, therefore, that the increase in c-Fos expression in GABA cells in the VTA of HR rats could result in greater GABA release in the NAc and greater subsequent novelty-induced locomotor activity in HR vs. LR rats (**Figure 20**).

The second mechanism by which HR rats might have increased GABA neurotransmission in the NAc involves stress-induced changes in the DRN. As the responsiveness of the stress circuit appears to be a key contributor to the behavioral distinctions between HR and LR rats, we propose that the increased c-Fos expression observed in the DRN of HR vs. LR rats is a downstream consequence of their increased stress reactivity (**Figure 20**). Differences in the reaction to stress (Rouge-Pont et al., 1993) and in the expression of certain stress-related molecules in the brain (Kabbaj et al.,

2000) have been reported in HR vs. LR rats. While the mechanisms are not well understood, the interaction of reward and stress signaling is hypothesized to result in differential activation of the DA mesolimbic system and result in higher novelty-induced levels of locomotor activity in HR vs. LR rats (Dellu et al., 1996).

The PVN is a hypothalamic nucleus that acts as an integrator of stress signals, responding to both stimulatory and inhibitory inputs (Sawchenko et al., 1993). It is the central regulator of the HPA axis and projects to a number of brain areas including the anterior pituitary (Vale et al., 1981). Stimulation of the PVN leads to the release of CRF in the pituitary (Vale et al., 1981), which then releases adrenocorticotropin releasing hormone (ACTH) into the bloodstream resulting in the synthesis and release of corticosterone by the adrenal glands (Herman et al., 1996). A promising early mediator of the HR vs. LR phenotypic difference might be CRF released from the PVN, as novelty exposure induces greater expression of *c-fos* mRNA in this structure in HR vs. LR rats at the early time point (Kabbaj and Akil, 2001) and causes an increase in corticosterone levels (Piazza et al., 1991a), a downstream effect of stress axis activation.

In addition to its effect on the HPA axis, CRF released from the PVN in response to stress acts as a neurotransmitter (Owens and Nemeroff, 1991). The projections from the PVN exhibit a widespread distribution, with mRNA for the CRF receptor present throughout the brain (Chalmers et al., 1995). The DRN receives afferent input from PVN (Valentino et al., 2001), and CRF receptors are found on both 5-HT and GABA cells (Waselus et al., 2005). Although the DRN is comprised mostly of 5-HT cells (Molliver, 1987; Jacobs and Azmitia, 1992), the preponderance of CRH synapses are on GABA

cells (Waselus et al., 2005). Interestingly, acute swim stress specifically targets stress-responsive GABA cells in DRN (Roche et al., 2003). By this mechanism we propose that the early increased activation of the PVN in HR vs. LR rats (Kabbaj and Akil, 2001) might trigger the later GABA-specific activation of the DRN in response to novelty (**Figure 20**).

The DRN contains projection neurons that terminate in the NAc (Imai et al., 1986; Vertes, 1991), but the nature of these projections is unknown. While they could be strictly 5-HT or GABA, it seems most likely that the projections are a mix of 5-HT and GABA fibers similar to the pattern observed in the VTA (Swanson et al., 1981). If the increased c-Fos expression in GABA cells in the DRN results in increases in GABA neurotransmission and some of these GABA cells project to the NAc, it is intriguing to speculate a second mechanism by which novelty exposure might result in greater GABA neurotransmission in the NAc of HR vs. LR rats. In addition to the putative GABA from the VTA, HR rats might have increased GABA release in the NAc compared to LR rats from DRN GABA projection neurons that are activated to a greater degree after exposure to a novel environment (**Figure 20**).

This hypothesis of stress-inducing changes in GABA transmission in the NAc is supported by evidence in the literature. First, our hypothesis that exposure to novelty might release more CRF in HR rats compared to LR rats is consistent with the fact that CRF administration has been shown to enhance novelty-induced behaviors (Britton et al., 1982; Sutton et al., 1982). In addition, increases in GABA neurotransmission within the DRN have been associated with behaviors reminiscent of the HR vs. LR animal model.

The application of the $GABA_B$ agonist baclofen in the DRN resulted in increased novelty-induced locomotor activity, increased appetitive behaviors and increase in DA turnover in the NAc (Wirtshafter et al., 1993). HR rats are similarly more active in a novel environment (present results)(Piazza et al., 1989), show greater motivational excitement for food reinforcement (Hooks et al., 1994) and have higher DA turnover in the NAc (Piazza et al., 1991b). Also, our proposed model differential GABA activation in the DRN would have consequences on 5-HT neurotransmission. The DRN contains numerous GABA interneurons (Gervasoni et al., 2000; Nanopoulos et al., 1982; Varga et al., 2003) whose tonic activity decreases the firing of nearby 5-HT cells (Innis and Aghajanian, 1987; Gallager, 1978; Gervasoni et al., 2000; Varga et al., 2003). Therefore, increased GABA cell activation in HR vs. LR rats would result in a greater inhibition of 5-HT cell firing and a decrease in 5-HT neurotransmission throughout the brain (**Figure 20**). This hypothesized effect is consistent with the fact that HR rats have decreased 5-HT release in various forebrain structures, including the PFC, NAc and CPu compared to LR rats (Piazza et al., 1991b). Thus, it seems that increased GABA neurotransmission in the DRN may indeed be involved in the generation of the HR vs. LR behavioral phenotype.

Surprisingly, in neither c-Fos study conducted were differences in expression detected in the DA cells of the VTA in HR and LR rats after novelty exposure (present results)(Kabbaj and Akil, 2001). It is known that the generation of novelty-induced locomotor activity in rats involves the function of the DA mesolimbic circuit: increases in DA in the NAc result in increases in locomotor activity (Wise and Bozarth, 1987), and

higher levels of DA have been correlated to higher levels of locomotor activation in HR vs. LR rats (Bradberry et al., 1991). Additionally, both DA depletion (Fink and Smith, 1979; Koob et al., 1981; Taghzouti et al., 1985) and the injection of DA receptor antagonists into the NAc (Bardo et al., 1990; Ossowska et al., 1990) attenuate novelty-induced locomotor activity with no effect on habituated animals. There is, however, conflicting evidence that implies that novelty-induced locomotor activity does not appear to be mediated by DA. In our model of novelty-induced activity, the stress response (signaling through CRF) plays a prominent role, and it has been shown that CRF-induced locomotor activity is independent of DA function. Neither systemic DA receptor blockade nor DA depletion in the NAc was able to attenuate CRF-induced locomotor activity while both of these manipulations block amphetamine-induced activity (Swerdlow et al., 1986). Thus, it is reasonable to assign a prominent role for GABA in the generation of novelty-induced activity that is independent of the DAergic projections from the VTA to the NAc.

In addition to the increased c-Fos expression in the VTA and DRN, HR rats exhibited greater novelty-induced c-Fos expression in three cortical areas, the AON, LOrbC and Cing, compared to LR rats (present results)(Kabbaj and Akil, 2001). The LOrbC and Cing are part of PFC (Kolb et al., 2004; Rose and Woolsey, 1948) and have been implicated stress responsivity (Diorio et al., 1993). Exposure to novelty, swim or restraint stress has been shown to induce c-Fos expression in the PFC of outbred rats (Cullinan et al., 1995; Emmert and Herman, 1999; Handa et al., 1993). The PFC appears to dampen both the behavioral (Brake et al., 2000) and hormonal (Herman et al., 2004)

response to stress, as rats with PFC lesions exhibited an exaggerated response to tail-pinch stress with a greater increase in corticosterone release compared to normal controls (Brake et al., 2000). Thus, it seems that the increased stress response in HR rats may induce greater levels of c-Fos in the PFC, and the increased activation in the PFC might then function to dampen the stress response in HR vs. LR rats in response to novelty.

HR rats also exhibited greater c-Fos induction in the AON, part of the olfactory bulb, a possible reaction to the greater degree of exploration in HR rats compared to their LR counterparts (Kabbaj and Akil, 2001). However, evidence points to a more important role for the olfactory bulb in the modulation of stress responsivity (Kelly et al., 1997). Rats with ablations of the olfactory bulb, or bulbectomized rats, share several traits in common with HR rats. Like HR rats (Piazza et al., 1989), bulbectomized rats are hyperactive in response to a novel environment compared to sham-operated controls (van Riezen et al., 1977). Also, HR rats have been shown to have greater response to stress compared to LR rats (Rouge-Pont et al., 1993), and bulbectomized rats have increased stress-induced corticosterone secretion compared to controls (Cairncross et al., 1977). Finally, HR rats exhibit decreased 5-HT in various forebrain structures (Piazza et al., 1991b) similar to bulbectomized rats (Lumia et al., 1992). As the olfactory bulb expresses CRF immunoreactivity (Imaki et al., 1989; Bassett et al., 1992), it reasonable to speculate that the increased stress signaling proposed in HR rats compared to LR rats might increase activation in this area of cortex and exhibit a similar dampening function as the PFC.

The fact that we observed a pattern of c-Fos induction in both HR and LR groups that represents an amalgam of results from both novelty- (Emmert and Herman, 1999; Handa et al., 1993; Papa et al., 1993) and stress-induced (Cullinan et al., 1995) c-Fos studies implies that our separation paradigm is the culmination of both novelty- and stress-induced reactions in HR and LR rats, consistent with earlier studies out of our laboratory (Chapter 3). Notably, no elements of the classic motor circuit (SNc, CPu, NAcC, NAcS) differed between HR and LR rats, although the two groups exhibited significant differences in locomotor activity in response to novelty. This suggests that increases in c-Fos expression in these brain areas are not merely a consequence of increases in locomotor activity. It is notable, however, that even though the 5-HT system modulates the stress axis (Lowry, 2002), no other components of the central stress circuit (CeA, BLA, BNST, Hab, Sep, PVN) were differentially activated in HR vs. LR rats. This suggests that the individual differences in stress reactivity seen at this time point in the HR vs. LR rat model may be due to the sensitivity of the DRN; however, increased activation of GABA cells in the DRN may have downstream sequelae that cascade to trigger additional pathways differentially in HR vs. LR rats.

In summary, results from the present study suggest that the differential reactivity in response to a novel environment observed in HR vs. LR rats is coupled to differential reward and stress reactivity. While confirming that HR rats exhibit greater activation in several cortical areas compared to LR rats after novelty, we demonstrated the GABA cells in both the VTA and the DRN are differentially activated in HR vs. LR rats, and for the first time we have uncovered a potential modulatory function for GABA in the HR vs.

LR animal model. Better understanding of the anatomical relationships that support the interaction between the stress system and the DA mesolimbic circuit in HR vs. LR rats might lend better understanding to the development of future therapeutics to pharmacologically reduce the increased risk of addiction in vulnerable populations.

DISCUSSION

Drug addiction continues to be a problem in our society, and the neuroanatomical and neurochemical basis for the switch between causal drug use and compulsive drug addiction is an important missing link in current knowledge. Characterizing what makes one individual more vulnerable to the development of compulsive drug-taking behaviors may hold the key to this complex phenomenon. Because human individual differences in the initial reinforcing effects of MDMA are thought to be rooted in individual sensitivity to the drug, we utilized two animal models of increased sensitivity in the current studies: the sensitization animal model and HR vs. LR rat.

Role of GABA neurotransmission in Sensitivity to Novelty and (+)-MDMA

For the first time, we have demonstrated that differences in GABA neurotransmission are evident in HR vs. LR rats: we uncovered differential activation of GABA cells in the DRN and VTA in HR vs. LR rats (**Figure 20**; Chapter 4). While c-Fos expression is not a direct measure of neuronal firing, there is evidence to support a link between the two measures. HR rats have a greater activation of the stress circuit after exposure to a novel environment as evidenced by an increased expression of *c-fos* mRNA in the PVN (Kabbaj and Akil, 2001) and greater corticosterone secretion (Piazza et al., 1991a) compared to LR rats after the same stimulus. As the activation of the PVN is necessary for the secretion of corticosterone, the expression of *c-fos* mRNA seems to

be linked to neuronal activity in this brain area. Therefore, we propose that the greater increases c-Fos expression in GABA cells in both the DRN and VTA of HR rats might result in increases in locomotor activity in HR vs. LR rats in response to novelty via increases in GABA neurotransmission.

The increased GABA neurotransmission in HR vs. LR rats in response to novelty may be mediated by two different mechanisms. First, GABA neurotransmission in the NAc results in the generation of locomotor activity: GABA agonists enhance locomotor activity while GABA antagonists attenuate locomotor activity (Morgenstern et al., 1984; Plaznik et al., 1990; Wong et al., 1991), and it is known that putatively GABAergic efferent projections from the VTA terminate in the NAc (Swanson et al., 1981). We found that after novelty exposure, HR rats exhibited greater c-Fos expression in GABA cells in the VTA which could result in increased GABA release in the NAc in HR vs. LR rats. This increased GABA release, might then contribute to the differential locomotor activation in HR vs. LR rats. We propose, therefore, that the increase in c-Fos expression in GABA cells in the VTA of HR rats could result in greater GABA release in the NAc and greater subsequent novelty-induced locomotor activity in HR vs. LR rats (**Figure 20**). Second, we propose that the increase in GABA cell activation in the DRN of HR rats is a direct result of the increased stress responsivity in HR vs. LR rats. Briefly, novelty induces greater *c-fos* mRNA in the PVN of HR vs., LR rats (Kabbaj and Akil, 2001). In response to stress, the PVN then specifically activates GABA cells in the DRN (Roche et al., 2003). These GABA cells might then project to the NAc where increased GABA tone functions to increase locomotor activity (**Figure 20**; Imai et al., 1986;

Vertes, 1991). Thus, GABA neurotransmission in the NAc seems to be an important point of divergence in terms of behavioral differences between HR and LR rats in response to novelty.

In addition to its role in individual differences in reaction to novelty, GABA neurotransmission has also been implicated in the phenomenon of sensitization. Sensitized animals have greater GABA release in NAc after sensitizing regimens of amphetamine (De Rover et al., 2004) and morphine (De Rover et al., 2005). Cross-sensitization between different drugs of abuse occurs in which repeated treatment with one drug can make an animal more sensitive to subsequent challenge with another, and this phenomenon is observed with cocaine, amphetamine and morphine (Bonate et al., 1997; Lett, 1989). The hypothesis is that drugs of abuse have in common their ability to sensitize the central reward system (i.e., induce similar changes in the DA mesolimbic system that increase the sensitivity to the reinforcing effect of all drugs of abuse). It is promising, therefore, to speculate that some of the changes in GABA neurotransmission in sensitized animals may be due to increased reward reactivity, or 'incentive sensitization', a process hypothesized to be common to all drugs of abuse (Robinson and Berridge, 1993; Robinson and Berridge, 2003). Thus, both HR rats and sensitized rats exhibit alterations in GABA neurotransmission, and these changes appear to be qualitatively similar. This parallel between the increased sensitivity seen in the two models underscores the potential importance of GABA neurotransmission in the individual sensitivity novelty and drugs of abuse.

Role of 5-HT neurotransmission in Sensitivity to Novelty and (+)-MDMA

In contrast to the pattern seen in GABA neurotransmission, the role of 5-HT neurotransmission in the sensitivity to drugs of abuse in the two models is not as clear. First, we demonstrated that HR rats exhibited a greater sensitivity to the locomotor-stimulating effects of (+)-MDMA compared to LR rats (Chapter 3), and because the 5-HT_{2A}R plays a stimulatory role in (+)-MDMA hyperactivity, we then investigated HR vs. LR rats for any functional and expression differenced in 5-HT_{2A}R. While HR rats displayed a diminished sensitivity to 5-HT_{2A}R-induced head shakes and increased levels of 5-HT_{2A}R binding in the NAc compared to LR rats, neither 5-HT_{2A}R stimulation nor blockade differentially modulated HR vs. LR responses to a novel environment (Chapter 3). When we examined the differential sensitivity to the locomotor-stimulating effects of (+)-MDMA in a sensitization model, we found changes of opposite magnitude compared to HR rats. We observed that enhanced (+)-MDMA-induced hyperactivity is associated with decreased 5-HT_{2A}R expression in NAc. Thus, in spite of the fact that both HR rats and DOI-treated rats exhibited a greater sensitivity to the locomotor-activating effects of (+)-MDMA, there were no obvious parallels in the observed alterations in the 5-HT_{2A}R between the two different models. The increase in sensitivity to (+)-MDMA was coupled in HR rats to increased levels of 5-HT_{2A}R expression in the NAc compared to LR rats and in sensitized rats to decreased levels of 5-HT_{2A}R expression in the NAc compared to saline-treated controls. These conflicting patterns in 5-HT_{2A}R expression imply that the 5-HT_{2A}R is not involved in the same way in both models and that there exists limited

crossover between the mechanisms underlying the increased sensitivity to (+)-MDMA in the two models.

Concluding Remarks

The question of what predisposing factors make an individual vulnerable to developing compulsive drug-taking behaviors is an important one. As this phenomenon in humans seems to be the critical component determining whether casual drug use will evolve into frank addiction (O'Brien et al., 1986), findings from the HR vs. LR animal model are particularly interesting. The present findings lend collective support to the hypothesis that the specialized functions of overlapping stress and reward circuits might underlie the expression of "vulnerability" to psychostimulants, and that GABA neurotransmission may feature prominently in future research. In particular, the DRN may serve as the integration point as we have identified a potentially important role for GABA neurotransmission in the DRN in the individual differences that confer increased vulnerability to developing drug-taking behaviors. To a certain extent, repeated administration of some psychoactive drugs (e.g., stimulants, DOI) can mimic the increased sensitivity observed in HR vs. LR rats: the alterations in GABA (but not 5-HT) neurotransmission are similar in the two models. These results beg further research into the role of the DRN and GABA neurotransmission in differential vulnerability to the development of drug taking-behaviors and in stress-induced relapse to drug-taking behaviors.

Additionally, the overlap between animal models predisposed to drug-taking behavior basally (HR vs. LR rats) and those whose increased sensitivity is derived from prior exposure to drugs of abuse (sensitization) may provide insight toward this goal. The HR vs. LR animal model has excellent face validity that parallels individual differences in human populations to developing drug addiction: initial sensitivity to the effects of drugs of abuse correlates closely with the likelihood of developing compulsive drug-seeking behaviors in both HR vs. LR rats (Piazza et al., 1989) and in humans (O'Brien et al., 1986). Additionally, sensitization in animals has been shown to increase drug-taking behaviors (Fletcher et al., 2001; Horger et al., 1992; Suto et al., 2002; Suto et al., 2003; de Vries et al., 1998) and has been tentatively linked to the development of compulsive drug-taking behaviors in humans in that it parallels the pattern development of drug craving (Robinson and Berridge, 1993; Robinson and Berridge, 2003). Thus, a better understanding of how increased sensitivity to drugs of abuse may increase individual vulnerability to developing compulsive drug-taking behaviors should be an important goal of future research.

REFERENCES

1. Adlaf EM, Ivis FJ, Smart RG, Walsh GW. (1996). Enduring resurgence or statistical blip? Recent trends from the Ontario Student Drug Use Survey. *Can J Public Health.* **87**: 189-192.

2. Arnt J. (1989). Characterization of the discriminative stimulus properties induced by 5-HT1 and 5-HT2 agonists in rats. *Pharmacol Toxicol*. **64**: 165-172.

3. Asin KE, Bednarz L, Nikkel A, Perner R. (1995). Rotation and striatal c-fos expression after repeated, daily treatment with selective dopamine receptor agonists and levodopa. *J Pharmacol Exp Ther*. **273**: 1483-1490.

4. Baker LE, Virden TB, Miller ME, Sullivan CL. (1997). Time course analysis of the discriminative stimulus effects of the optical isomers of 3,4-methylenedioxymethamphetamine (MDMA). *Pharmacol Biochem Behav*. **58**: 505-516.

5. Bankson MG, Cunningham KA. (2001). 3,4-Methylenedioxymethamphetamine (MDMA) as a unique model of serotonin receptor function and serotonin-dopamine interactions. *J Pharmacol Exp Ther*. **297**: 846-852.

6. Bankson MG, Cunningham KA. (2002). Pharmacological studies of the acute effects of (+)-3,4-methylenedioxymethamphetamine on locomotor activity: role of 5-HT(1B/1D) and 5-HT(2) receptors. *Neuropsychopharmacology*. **26**: 40-52.

7. Bardo MT, Bowling SL, Pierce RC. (1990). Changes in locomotion and dopamine neurotransmission following amphetamine, haloperidol, and exposure to novel environmental stimuli. *Psychopharmacology (Berl)*. **101**: 338-343.

8. Bassett JL, Shipley MT, Foote SL. (1992). Localization of corticotropin-releasing factor-like immunoreactivity in monkey olfactory bulb and secondary olfactory areas. *J Comp Neurol*. **316**: 348-362.

9. Battaglia G, Brooks BP, Kulsakdinun C, de Souza EB. (1988). Pharmacologic profile of MDMA (3,4-methylenedioxymethamphetamine) at various brain recognition sites. *Eur J Pharmacol*. **149**: 159-163.

10. Bedard P, Pycock CJ. (1977). "Wet-dog" shake behaviour in the rat: a possible quantitative model of central 5-hydroxytryptamine activity. *Neuropharmacology*. **16**: 663-670.

11. Bedford JA, Borne RF, Wilson MC. (1980). Comparative behavioral profile of cocaine and norcocaine in rats and monkeys. *Pharmacol Biochem Behav*. **13**: 69-75.

12. Bevins RA, Bardo MT. (1999). Conditioned increase in place preference by access to novel objects: antagonism by MK-801. *Behav Brain Res*. **99**: 53-60.

13. Bevins RA, Klebaur JE, Bardo MT. (1997). Individual differences in response to novelty, amphetamine-induced activity and drug discrimination in rats. *Behav Pharmacol*. **8**: 113-123.

14. Bevins RA, Peterson JL. (2004). Individual differences in rats' reactivity to novelty and the unconditioned and conditioned locomotor effects of methamphetamine. *Pharmacol Biochem Behav*. **79**: 65-74.

15. Bishop C, Tessmer JL, Ullrich T, Rice KC, Walker PD. (2004). Serotonin 5-HT2A Receptors Underlie Increased Motor Behaviors Induced in Dopamine-Depleted Rats by Intrastriatal 5-HT2A/2C Agonism. *J Pharmacol Exp Ther*. **310**: 687-694.

16. Bolla KI, McCann UD, Ricaurte GA. (1998). Memory impairment in abstinent MDMA ("Ecstasy") users. *Neurology*. **51**: 1532-1537.

17. Bonate PL, Swann A, Silverman PB. (1997). Context-dependent cross-sensitization between cocaine and amphetamine. *Life Sci*. **60**: L1-L7.

18. Bradberry CW, Gruen RJ, Berridge CW, Roth RH. (1991). Individual differences in behavioral measures: correlations with nucleus accumbens dopamine measured by microdialysis. *Pharmacol Biochem Behav*. **39**: 877-882.

19. Bradberry CW, Roth RH. (1989). Cocaine increases extracellular dopamine in rat nucleus accumbens and ventral tegmental area as shown by in vivo microdialysis. *Neurosci Lett*. **103**: 97-102.

20. Braff DL, Geyer MA, Swerdlow NR. (2001). Human studies of prepulse inhibition of startle: normal subjects, patient groups, and pharmacological studies. *Psychopharmacology (Berl)*. **156**: 234-258.

21. Brake WG, Flores G, Francis D, Meaney MJ, Srivastava LK, Gratton A. (2000). Enhanced nucleus accumbens dopamine and plasma corticosterone stress responses in adult rats with neonatal excitotoxic lesions to the medial prefrontal cortex. *Neuroscience*. **96**: 687-695.

22. Britton DR, Koob GF, Rivier J, Vale W. (1982). Intraventricular corticotropin-releasing factor enhances behavioral effects of novelty. *Life Sci*. **31**: 363-367.

23. Bruinvels AT, Palacios JM, Hoyer D. (1993). Autoradiographic characterisation and localisation of 5-HT1D compared to 5-HT1B binding sites in rat brain. *Naunyn Schmiedebergs Arch Pharmacol*. **347**: 569-582.

24. Bubar, M. J. and Cunningham, K. A. (2002). Persistent enhancement of (+)-MDMA ("Ecstacy")-evoked hyperactivity during withdrawal from 5-HT2C receptor down-regulation . *2002 Abstract Viewer/Itinerary Planner.* Society for Neuroscience: Online.

25. Bubar, M. J. and Cunningham, K. A. (2003). Repeated 5-HT2CR agonist induces persistent enhancement of (+)-MDMA-induced hyperactivity despite recovery of 5-HT2CR sensitivity. *Behavioural Pharmacology.* 14: S64.

26. Bubar, M. J. and Cunningham, K. A. Transient changes in 5-HT2CR sensitivity accompany short term sentitisation following repeated (+)-MDMA. *Neuropharmacology*. (In Press.)

27. Bubar MJ, Pack KM, Frankel PS, Cunningham KA. (2004). Effects of dopamine D1- or D2-like receptor antagonists on the hypermotive and discriminative stimulus effects of (+)-MDMA. *Psychopharmacology (Berl).* **173**: 326-336.

28. Bull EJ, Hutson PH, Fone KC. (2004). Decreased social behaviour following 3,4-methylenedioxymethamphetamine (MDMA) is accompanied by changes in 5-HT(2A) receptor responsivity. *Neuropharmacology*. **46**: 202-210.

29. Cain ME, Smith CM, Bardo MT. (2004). The effect of novelty on amphetamine self-administration in rats classified as high and low responders. *Psychopharmacology (Berl)*. **176**: 129-138.

30. Cairncross KD, Wren A, Cox B, Schnieden H. (1977). Effects of olfactory bulbectomy and domicile on stress-induced corticosterone release in the rat. *Physiol Behav*. **19**: 485-487.

31. Callahan PM, De La GR, Cunningham KA. (1997). Mediation of the discriminative stimulus properties of cocaine by mesocorticolimbic dopamine systems. *Pharmacol Biochem Behav*. **57**: 601-607.

32. Callaway CW, Rempel N, Peng RY, Geyer MA. (1992). Serotonin 5-HT1-like receptors mediate hyperactivity in rats induced by 3,4-methylenedioxymethamphetamine. *Neuropsychopharmacology*. **7**: 113-127.

33. Callaway CW, Wing LL, Geyer MA. (1990). Serotonin release contributes to the locomotor stimulant effects of 3,4-methylenedioxymethamphetamine in rats. *J Pharmacol Exp Ther*. **254**: 456-464.

34. Cami J, Farre M, Mas M, Roset PN, Poudevida S, Mas A, San L, De La TR. (2000). Human pharmacology of 3,4-methylenedioxymethamphetamine ("ecstasy"): psychomotor performance and subjective effects. *J Clin Psychopharmacol*. **20**: 455-466.

35. Chalmers DT, Lovenberg TW, de Souza EB. (1995). Localization of novel corticotropin-releasing factor receptor (CRF2) mRNA expression to specific subcortical nuclei in rat brain: comparison with CRF1 receptor mRNA expression. *J Neurosci*. **15**: 6340-6350.

36. Chesselet MF, Delfs JM. (1996). Basal ganglia and movement disorders: an update. *Trends Neurosci*. **19**: 417-422.

37. Cordero MI, Kruyt ND, Sandi C. (2003). Modulation of contextual fear conditioning by chronic stress in rats is related to individual differences in behavioral reactivity to novelty. *Brain Res*. **970**: 242-245.

38. Crespi D, Mennini T, Gobbi M. (1997). Carrier-dependent and Ca(2+)-dependent 5-HT and dopamine release induced by (+)-amphetamine, 3,4-methylendioxymethamphetamine, p-chloroamphetamine and (+)-fenfluramine. *Br J Pharmacol*. **121**: 1735-1743.

39. Cullinan WE, Herman JP, Battaglia DF, Akil H, Watson SJ. (1995). Pattern and time course of immediate early gene expression in rat brain following acute stress. *Neuroscience*. **64**: 477-505.

40. Curran EJ, Akil H, Watson SJ. (1996). Psychomotor stimulant- and opiate-induced c-fos mRNA expression patterns in the rat forebrain: comparisons between acute drug treatment and a drug challenge in sensitized animals. *Neurochem Res*. **21**: 1425-1435.

41. Darmani NA, Martin BR, Glennon RA. (1990). Withdrawal from chronic treatment with (+/-)-DOI causes super-sensitivity to 5-HT2 receptor-induced head-twitch behaviour in mice. *Eur J Pharmacol*. **186**: 115-118.

42. De Rover M, Lodder JC, Schoffelmeer AN, Brussaard AB. (2005). Intermittent morphine treatment induces a long-lasting increase in cholinergic modulation of GABAergic synapses in nucleus accumbens of adult rats. *Synapse*. **55**: 17-25.

43. De Rover M, Mansvelder HD, Lodder JC, Wardeh G, Schoffelmeer AN, Brussaard AB. (2004). Long-lasting nicotinic modulation of GABAergic synaptic transmission in the rat nucleus accumbens associated with behavioural sensitization to amphetamine. *Eur J Neurosci.* **19**: 2859-2870.

44. de Vries TJ, Schoffelmeer AN, Binnekade R, Mulder AH, Vanderschuren LJ. (1998). Drug-induced reinstatement of heroin- and cocaine-seeking behaviour following long-term extinction is associated with expression of behavioural sensitization. *Eur J Neurosci*. **10**: 3565-3571.

45. De Wit H, Uhlenhuth EH, Johanson CE. (1986). Individual differences in the reinforcing and subjective effects of amphetamine and diazepam. *Drug Alcohol Depend*. **16**: 341-360.

46. Delfs JM, Schreiber L, Kelley AE. (1990). Microinjection of cocaine into the nucleus accumbens elicits locomotor activation in the rat. *J Neurosci*. **10**: 303-310.

47. Dellu F, Piazza PV, Mayo W, Le Moal M, Simon H. (1996). Novelty-seeking in rats--biobehavioral characteristics and possible relationship with the sensation-seeking trait in man. *Neuropsychobiology*. **34**: 136-145.

48. Deminiere JM, Piazza PV, Le Moal M, Simon H. (1989). Experimental approach to individual vulnerability to psychostimulant addiction. *Neurosci Biobehav Rev*. **13**: 141-147.

49. Deroche V, Piazza PV, Casolini P, Maccari S, Le Moal M, Simon H. (1992). Stress-induced sensitization to amphetamine and morphine psychomotor effects depend on stress-induced corticosterone secretion. *Brain Res*. **598**: 343-348.

50. Deroche V, Piazza PV, Le Moal M, Simon H. (1993). Individual differences in the psychomotor effects of morphine are predicted by reactivity to novelty and influenced by corticosterone secretion. *Brain Res.* **623**: 341-344.

51. Di M, V, Di Giovanni G, Di Mascio M, Esposito E. (2000). Biochemical and electrophysiological evidence that RO 60-0175 inhibits mesolimbic dopaminergic function through serotonin(2C) receptors. *Brain Res*. **865**: 85-90.

52. Diorio D, Viau V, Meaney MJ. (1993). The role of the medial prefrontal cortex (cingulate gyrus) in the regulation of hypothalamic-pituitary-adrenal responses to stress. *J Neurosci*. **13**: 3839-3847.

53. Doblin R. (2002). A clinical plan for MDMA (Ecstasy) in the treatment of posttraumatic stress disorder (PTSD): partnering with the FDA. *J Psychoactive Drugs*. **34**: 185-194.

54. Doherty MD, Pickel VM. (2000). Ultrastructural localization of the serotonin 2A receptor in dopaminergic neurons in the ventral tegmental area. *Brain Res*. **864**: 176-185.

55. Dragunow M, Faull R. (1989). The use of c-fos as a metabolic marker in neuronal pathway tracing. *J Neurosci Methods*. **29**: 261-265.

56. Edwards G, Arif A, Hadgson R. (1981). Nomenclature and classification of drug- and alcohol-related problems: a WHO memorandum. *Bull World Health Organization*. **59**: 225-242.

57. Emmert MH, Herman JP. (1999). Differential forebrain c-fos mRNA induction by ether inhalation and novelty: evidence for distinctive stress pathways. *Brain Research*. **845**: 60-67.

58. Fantegrossi WE, Ullrich T, Rice KC, Woods JH, Winger G. (2002). 3,4-Methylenedioxymethamphetamine (MDMA, "ecstasy") and its stereoisomers as reinforcers in rhesus monkeys: serotonergic involvement. *Psychopharmacology (Berl)*. **161**: 356-364.

59. Filip M, Bubar MJ, Cunningham KA. (2004a). Contribution of serotonin (5-HT) 5-HT2 receptor subtypes to the hyperlocomotor effects of cocaine: Acute and chronic pharmacological analyses. *J Pharmacol Exp Ther*.

60. Filip, M., Przegalinski, E., and Cunningham, K. A. (2004b). Characterization of the roles for serotonin (5-HT) 5-HT2A receptors (5-HT2AR) in the ventral tegmental area and prefrontal cortex in spontaneous activity and cocaine-induced hyperactivity. *SOME CONFERENCE???*

61. Fink JS, Smith GP. (1979). Decreased locomotor and investigatory exploration after denervation of catecholamine terminal fields in the forebrain of rats. *J Comp Physiol Psychol.* **93**: 34-65.

62. Fitzgerald JL, Reid JJ. (1990). Effects of methylenedioxymethamphetamine on the release of monoamines from rat brain slices. *Eur J Pharmacol*. **191**: 217-220.

63. Fletcher PJ, Korth KM, Robinson SR, Baker GB. (2002). Multiple 5-HT receptors are involved in the effects of acute MDMA treatment: studies on locomotor activity and responding for conditioned reinforcement. *Psychopharmacology (Berl)*. **162**: 282-291.

64. Fletcher PJ, Robinson SR, Slippoy DL. (2001). Pre-exposure to (+/-)3,4-methylenedioxy-methamphetamine (MDMA) facilitates acquisition of intravenous cocaine self-administration in rats. *Neuropsychopharmacology*. **25**: 195-203.

65. Fone KC, Johnson JV, Bennett GW, Marsden CA. (1989). Involvement of 5-HT2 receptors in the behaviours produced by intrathecal administration of

selected 5-HT agonists and the TRH analogue (CG 3509) to rats. *Br J Pharmacol*. **96**: 599-608.

66. Fone KC, Robinson AJ, Marsden CA. (1991). Characterization of the 5-HT receptor subtypes involved in the motor behaviours produced by intrathecal administration of 5-HT agonists in rats. *Br J Pharmacol*. **103**: 1547-1555.

67. Frankel PS, Cunningham KA. (2002). The hallucinogen d-lysergic acid diethylamide (d-LSD) induces the immediate-early gene c-Fos in rat forebrain. *Brain Res.* **958**: 251-260.

68. Gallager DW. (1978). Benzodiazepines: potentiation of a GABA inhibitory response in the dorsal raphe nucleus. *Eur J Pharmacol*. **49**: 133-143.

69. Garris PA, Wightman RM. (1994). Different kinetics govern dopaminergic transmission in the amygdala, prefrontal cortex, and striatum: an in vivo voltammetric study. *J Neurosci*. **14**: 442-450.

70. Gatchalian, K, Tancer, M, Lockhart, N, and Johanson, C. (2002). Discriminative stimulus properties of 3,4-methylenedioxymethamphetamine (MDMA, 'Ecstacy') in recreational drug users. *Drug and Alcohol Dependence*. 66: S62.

71. Gerra G, Zaimovic A, Giucastro G, Maestri D, Monica C, Sartori R, Caccavari R, Delsignore R. (1998). Serotonergic function after (+/-)3,4-methylene-dioxymethamphetamine ('Ecstasy') in humans. *Int Clin Psychopharmacol*. **13**: 1-9.

72. Gervasoni D, Peyron C, Rampon C, Barbagli B, Chouvet G, Urbain N, Fort P, Luppi PH. (2000). Role and origin of the GABAergic innervation of dorsal raphe serotonergic neurons. *J Neurosci.* **20**: 4217-4225.

73. Gold LH, Hubner CB, Koob GF. (1989). A role for the mesolimbic dopamine system in the psychostimulant actions of MDMA. *Psychopharmacology (Berl).* **99**: 40-47.

74. Gold LH, Koob GF, Geyer MA. (1988). Stimulant and hallucinogenic behavioral profiles of 3,4-methylenedioxymethamphetamine and N-ethyl-3,4-methylenedioxyamphetamine in rats. *J Pharmacol Exp Ther*. **247**: 547-555.

75. Gonon FG. (1988). Nonlinear relationship between impulse flow and dopamine released by rat midbrain dopaminergic neurons as studied by in vivo electrochemistry. *Neuroscience*. **24**: 19-28.

76. Grahn RE, Will MJ, Hammack SE, Maswood S, McQueen MB, Watkins LR, Maier SF. (1999). Activation of serotonin-immunoreactive cells in the dorsal

raphe nucleus in rats exposed to an uncontrollable stressor. *Brain Res*. **826**: 35-43.

77. Gray JA, Compton-Toth BA, Roth BL. (2003). Identification of two serine residues essential for agonist-induced 5-HT2A receptor desensitization. *Biochemistry*. **42**: 10853-10862.

78. Greer GR, Tolbert R. (1998). A method of conducting therapeutic sessions with MDMA. *J Psychoactive Drugs*. **30**: 371-379.

79. Halford JC, Lawton CL, Blundell JE. (1997). The 5-HT2 receptor agonist MK-212 reduces food intake and increases resting but prevents the behavioural satiety sequence. *Pharmacol Biochem Behav*. **56**: 41-46.

80. Handa RJ, Nunley KM, Bollnow MR. (1993). Induction of c-fos mRNA in the brain and anterior pituitary gland by a novel environment. *Neuroreport*. **4**: 1079-1082.

81. Heffner TG, Hartman JA, Seiden LS. (1980). A rapid method for the regional dissection of the rat brain. *Pharmacol Biochem Behav*. **13**: 453-456.

82. Herin, D. V. and Cunningham, K. A. (2001). Potentiation of (+)-3,4-Methylenedioxymethamphetamine [(+)-MDMA]-induced hyperactivity by the selective 5-HT2C receptor (5-HT2CR) antagonist SB 242084. *2001 Abstract Viewer/Itinerary Planner*. Society for Neuroscience: Online.

83. Herin DV, Liu S, Ullrich T, Rice KC, Cunningham KA. (2004). Role of the serotonin 5-HT(2A) receptor in the hyperlocomotive and hyperthermic effects of (+)-3,4-methylenedioxymethamphetamine. *Psychopharmacology (Berl).*

84. Herman JP, Cullinan WE. (1997). Neurocircuitry of stress: central control of the hypothalamo-pituitary-adrenocortical axis. *Trends Neurosci*. **20**: 78-84.

85. Herman JP, Mueller NK, Figueiredo H. (2004). Role of GABA and glutamate circuitry in hypothalamo-pituitary-adrenocortical stress integration. *Ann N Y Acad Sci*. **1018**: 35-45.

86. Herman JP, Prewitt CM, Cullinan WE. (1996). Neuronal circuit regulation of the hypothalamo-pituitary-adrenocortical stress axis. *Crit Rev Neurobiol*. **10**: 371-394.

87. Higgins ST, Budney AJ, Bickel WK, Hughes JR, Foerg F, Badger G. (1993). Achieving cocaine abstinence with a behavioral approach. *Am J Psychiatry*. **150**: 763-769.

88. Hillegaart V, Estival A, Ahlenius S. (1996). Evidence for specific involvement of 5-HT1A and 5-HT2A/C receptors in the expression of patterns of spontaneous motor activity of the rat. *Eur J Pharmacol*. **295**: 155-161.

89. Hooks MS, Colvin AC, Juncos JL, Justice JB, Jr. (1992a). Individual differences in basal and cocaine-stimulated extracellular dopamine in the nucleus accumbens using quantitative microdialysis. *Brain Res*. **587**: 306-312.

90. Hooks MS, Jones GH, Hemby SE, Justice JB, Jr. (1993). Environmental and pharmacological sensitization: effects of repeated administration of systemic or intra-nucleus accumbens cocaine. *Psychopharmacology (Berl)*. **111**: 109-116.

91. Hooks MS, Jones GH, Juncos JL, Neill DB, Justice JB. (1994). Individual differences in schedule-induced and conditioned behaviors. *Behav Brain Res*. **60**: 199-209.

92. Hooks MS, Jones GH, Neill DB, Justice JB, Jr. (1992b). Individual differences in amphetamine sensitization: dose-dependent effects. *Pharmacol Biochem Behav*. **41**: 203-210.

93. Hooks MS, Jones GH, Smith AD, Neill DB, Justice JB, Jr. (1991a). Individual differences in locomotor activity and sensitization. *Pharmacol Biochem Behav*. **38**: 467-470.

94. Hooks MS, Jones GH, Smith AD, Neill DB, Justice JB, Jr. (1991b). Response to novelty predicts the locomotor and nucleus accumbens dopamine response to cocaine. *Synapse*. **9**: 121-128.

95. Horger BA, Giles MK, Schenk S. (1992). Preexposure to amphetamine and nicotine predisposes rats to self-administer a low dose of cocaine. *Psychopharmacology (Berl)*. **107**: 271-276.

96. Hoyer D, Hannon JP, Martin GR. (2002). Molecular, pharmacological and functional diversity of 5-HT receptors. *Pharmacol Biochem Behav*. **71**: 533-554.

97. Ichikawa J, Meltzer HY. (1995). DOI, a 5-HT2A/2C receptor agonist, potentiates amphetamine-induced dopamine release in rat striatum. *Brain Res*. **698**: 204-208.

98. Ikemoto K, Nishimura A, Okado N, Mikuni M, Nishi K, Nagatsu I. (2000). Human midbrain dopamine neurons express serotonin 2A receptor: an immunohistochemical demonstration. *Brain Res*. **853**: 377-380.

99. Imai H, Steindler DA, Kitai ST. (1986). The organization of divergent axonal projections from the midbrain raphe nuclei in the rat. *J Comp Neurol*. **243**: 363-380.

100. Imaki T, Nahon JL, Sawchenko PE, Vale W. (1989). Widespread expression of corticotropin-releasing factor messenger RNA and immunoreactivity in the rat olfactory bulb. *Brain Res*. **496**: 35-44.

101. Innis RB, Aghajanian GK. (1987). Pertussis toxin blocks 5-HT1A and GABAB receptor-mediated inhibition of serotonergic neurons. *Eur J Pharmacol*. **143**: 195-204.

102. Jaber M, Cador M, Dumartin B, Normand E, Stinus L, Bloch B. (1995). Acute and chronic amphetamine treatments differently regulate neuropeptide messenger RNA levels and Fos immunoreactivity in rat striatal neurons. *Neuroscience*. **65**: 1041-1050.

103. Jacobs BL, Azmitia EC. (1992). Structure and function of the brain serotonin system. *Physiol Rev*. **72**: 165-229.

104. Johnson MP, Hoffman AJ, Nichols DE. (1986). Effects of the enantiomers of MDA, MDMA and related analogues on [3H]serotonin and [3H]dopamine release from superfused rat brain slices. *Eur J Pharmacol*. **132**: 269-276.

105. Johnston LD, O'Malley PM, Bachman JG. (2001). Monitoring the Future National Survey Results on Drug Use, 1975-2000: College Students and Adults Ages 19-40. *Natl Inst Drug Abuse: NIH.* **Vol II**: Publ. No. 01-4935.

106. Kabbaj M, Akil H. (2001). Individual differences in novelty-seeking behavior in rats: a c-fos study. *Neuroscience*. **106**: 535-545.

107. Kabbaj M, Devine DP, Savage VR, Akil H. (2000). Neurobiological correlates of individual differences in novelty-seeking behavior in the rat: differential expression of stress-related molecules. *J Neurosci*. **20**: 6983-6988.

108. Kalivas PW, Duffy P, White SR. (1998). MDMA elicits behavioral and neurochemical sensitization in rats. *Neuropsychopharmacology*. **18**: 469-479.

109. Kang SY, Kleinman PH, Woody GE, Millman RB, Todd TC, Kemp J, Lipton DS. (1991). Outcomes for cocaine abusers after once-a-week psychosocial therapy. *Am J Psychiatry*. **148**: 630-635.

110. Kaplan HI, Sadock BJ. (1998). *Synopsis of Psychiatry*. Philadelphia: Lippincott WIlliams and Wilkins.

111. Kehne JH, Ketteler HJ, McCloskey TC, Sullivan CK, Dudley MW, Schmidt CJ. (1996). Effects of the selective 5-HT2A receptor antagonist MDL 100,907 on MDMA-induced locomotor stimulation in rats. *Neuropsychopharmacology*. **15**: 116-124.

112. Kelly JP, Wrynn AS, Leonard BE. (1997). The olfactory bulbectomized rat as a model of depression: an update. *Pharmacol Ther*. **74**: 299-316.

113. Keppel G. (1973). *Design and Analysis: A Researcher's Handbook*. Englewood Cliffs, N.J.: Prentis Hall Inc.

114. Kirk R. (1995). *Experimental Design: Procedures for the Behavioral Sciences*. Pacific Grove: Brooks/Cole Publishing Company.

115. Klebaur JE, Bevins RA, Segar TM, Bardo MT. (2001). Individual differences in behavioral responses to novelty and amphetamine self-administration in male and female rats. *Behav Pharmacol*. **12**: 267-275.

116. Kolb B, Pellis S, Robinson TE. (2004). Plasticity and functions of the orbital frontal cortex. *Brain Cogn*. **55**: 104-115.

117. Koob GF, Stinus L, Le Moal M. (1981). Hyperactivity and hypoactivity produced by lesions to the mesolimbic dopamine system. *Behav Brain Res*. **3**: 341-359.

118. Kouzarides T, Ziff E. (1988). The role of the leucine zipper in the fos-jun interaction. *Nature*. **336**: 646-651.

119. Kuroki T, Meltzer HY, Ichikawa J. (2003). 5-HT 2A receptor stimulation by DOI, a 5-HT 2A/2C receptor agonist, potentiates amphetamine-induced dopamine release in rat medial prefrontal cortex and nucleus accumbens. *Brain Res*. **972**: 216-221.

120. Laviola G, Adriani W, Terranova ML, Gerra G. (1999). Psychobiological risk factors for vulnerability to psychostimulants in human adolescents and animal models. *Neurosci Biobehav Rev*. **23**: 993-1010.

121. Le Moal M, Simon H. (1991). Mesocorticolimbic dopaminergic network: functional and regulatory roles. *Physiol Rev*. **71**: 155-234.

122. Lett BT. (1989). Repeated exposures intensify rather than diminish the rewarding effects of amphetamine, morphine, and cocaine. *Psychopharmacology (Berl)*. **98**: 357-362.

123. Li Q, Wichems CH, Ma L, Van de Kar LD, Garcia F, Murphy DL. (2003). Brain region-specific alterations of 5-HT2A and 5-HT2C receptors in serotonin transporter knockout mice. *J Neurochem*. **84**: 1256-1265.

124. Liester MB, Grob CS, Bravo GL, Walsh RN. (1992). Phenomenology and sequelae of 3,4-methylenedioxymethamphetamine use. *J Nerv Ment Dis*. **180**: 345-352.

125. Louilot A, Le Moal M, Simon H. (1986). Differential reactivity of dopaminergic neurons in the nucleus accumbens in response to different behavioral situations. An in vivo voltammetric study in free moving rats. *Brain Res*. **397**: 395-400.

126. Lowry CA. (2002). Functional subsets of serotonergic neurones: implications for control of the hypothalamic-pituitary-adrenal axis. *J Neuroendocrinol.* **14**: 911-923.

127. Lucki I, Ward HR, Frazer A. (1989). Effect of 1-(m-chlorophenyl)piperazine and 1-(m-trifluoromethylphenyl)piperazine on locomotor activity. *J Pharmacol Exp Ther*. **249**: 155-164.

128. Lumia AR, Teicher MH, Salchli F, Ayers E, Possidente B. (1992). Olfactory bulbectomy as a model for agitated hyposerotonergic depression. *Brain Res.* **587**: 181-185.

129. Marinelli M, White FJ. (2000). Enhanced vulnerability to cocaine self-administration is associated with elevated impulse activity of midbrain dopamine neurons. *J Neurosci.* **20**: 8876-8885.

130. McCann UD, Slate SO, Ricaurte GA. (1996). Adverse reactions with 3,4-methylenedioxymethamphetamine (MDMA; 'ecstasy'). *Drug Saf*. **15**: 107-115.

131. McCreary AC, Bankson MG, Cunningham KA. (1999). Pharmacological studies of the acute and chronic effects of (+)-3, 4-methylenedioxymethamphetamine on locomotor activity: role of 5-hydroxytryptamine(1A) and 5-hydroxytryptamine(1B/1D) receptors. *J Pharmacol Exp Ther*. **290**: 965-973.

132. McMahon LR, Filip M, Cunningham KA. (2001). Differential regulation of the mesoaccumbens circuit by serotonin 5-hydroxytryptamine (5-HT)2A and 5-HT2C receptors. *J Neurosci.* **21**: 7781-7787.

133. Mengod G, Pompeiano M, Martinez-Mir MI, Palacios JM. (1990). Localization of the mRNA for the 5-HT2 receptor by in situ hybridization histochemistry. Correlation with the distribution of receptor sites. *Brain Res.* **524**: 139-143.

134. Molliver ME. (1987). Serotonergic neuronal systems: what their anatomic organization tells us about function. *J Clin Psychopharmacol*. **7**: 3S-23S.

135. Morgan AE, Horan B, Dewey SL, Ashby CR, Jr. (1997). Repeated administration of 3,4-methylenedioxymethamphetamine augments cocaine's action on dopamine in the nucleus accumbens: a microdialysis study. *Eur J Pharmacol*. **331**: R1-R3.

136. Morgan MJ. (2000). Ecstasy (MDMA): a review of its possible persistent psychological effects. *Psychopharmacology (Berl)*. **152**: 230-248.

137. Morgenstern R, Mende T, Gold R, Lemme P, Oelssner W. (1984). Drug-induced modulation of locomotor hyperactivity induced by picrotoxin in nucleus accumbens. *Pharmacol Biochem Behav*. **21**: 501-506.

138. Multidisciplinary Association for Psychedelic Studies. (2004). MAPS News Update, 11/22/04. *http://www maps org/news/*.

139. Nambu A, Tokuno H, Takada M. (2002). Functional significance of the cortico-subthalamo-pallidal 'hyperdirect' pathway. *Neurosci Res*. **43**: 111-117.

140. Nanopoulos D, Belin MF, Maitre M, Vincendon G, Pujol JF. (1982). Immunocytochemical evidence for the existence of GABAergic neurons in the nucleus raphe dorsalis. Possible existence of neurons containing serotonin and GABA. *Brain Res*. **232**: 375-389.

141. National Research Council. (1996). *Guide for the care and use of laboratory animals*. Washington D.C.: National Academy Press.

142. O'Brien CP, Ehrman RN, Terns JN. (1986). Classical Conditioning in Human Opioid Dependence. In: *Behavioral Analysis of Drug Dependence*. (Goldberg SR, Stolerman IP, eds), pp 329-338. London: Academic Press.

143. Ossowska K, Karcz M, Wardas J, Wolfarth S. (1990). Striatal and nucleus accumbens D1/D2 dopamine receptors in neuroleptic catalepsy. *Eur J Pharmacol*. **182**: 327-334.

144. Overton PG, Tong ZY, Brain PF, Clark D. (1996). Preferential occupation of mineralocorticoid receptors by corticosterone enhances glutamate-induced burst firing in rat midbrain dopaminergic neurons. *Brain Res*. **737**: 146-154.

145. Owens MJ, Nemeroff CB. (1991). Physiology and pharmacology of corticotropin-releasing factor. *Pharmacol Rev*. **43**: 425-473.

146. Papa M, Pellicano MP, Welzl H, Sadile AG. (1993). Distributed changes in c-Fos and c-Jun immunoreactivity in the rat brain associated with arousal and habituation to novelty. *Brain Res Bull.* **32**: 509-515.

147. Paris JM, Callahan PM, Lee JM, Cunningham KA. (1991). Behavioral sensitization to cocaine is not associated with changes in serotonin (5-HT) fiber immunoreactivity in rat forebrain. *Brain Res Bull*. **27**: 843-847.

148. Paulus MP, Geyer MA. (1992). The effects of MDMA and other methylenedioxy-substituted phenylalkylamines on the structure of rat locomotor activity. *Neuropsychopharmacology*. **7**: 15-31.

149. Paxinos W, Watson C. (1998). *The Rat Brain in Stereotaxic Coordinates*. San Diego CA: Academic Press.

150. Pazos A, Cortes R, Palacios JM. (1985). Quantitative autoradiographic mapping of serotonin receptors in the rat brain. II. Serotonin-2 receptors. *Brain Res*. **346**: 231-249.

151. Pazos A, Palacios JM. (1985). Quantitative autoradiographic mapping of serotonin receptors in the rat brain. I. Serotonin-1 receptors. *Brain Res.* **346**: 205-230.

152. Peroutka SJ, Newman H, Harris H. (1988). Subjective effects of 3,4-methylenedioxymethamphetamine in recreational users. *Neuropsychopharmacology*. **1**: 273-277.

153. Pettit HO, Ettenberg A, Bloom FE, Koob GF. (1984). Destruction of dopamine in the nucleus accumbens selectively attenuates cocaine but not heroin self-administration in rats. *Psychopharmacology (Berl)*. **84**: 167-173.

154. Piazza PV, Deminiere JM, Le Moal M, Simon H. (1989). Factors that predict individual vulnerability to amphetamine self-administration. *Science*. **245**: 1511-1513.

155. Piazza PV, Deminiere JM, Maccari S, Mormede P, Le Moal M, Simon H. (1990). Individual reactivity to novelty predicts probability of amphetamine self-administration. *Behav Pharmacol*. **1**: 339-345.

156. Piazza PV, Deroche-Gamonent V, Rouge-Pont F, Le Moal M. (2000). Vertical shifts in self-administration dose-response functions predict a drug-vulnerable phenotype predisposed to addiction. *J Neurosci*. **20**: 4226-4232.

157. Piazza PV, Maccari S, Deminiere JM, Le Moal M, Mormede P, Simon H. (1991a). Corticosterone levels determine individual vulnerability to amphetamine self-administration. *Proc Natl Acad Sci U S A*. **88**: 2088-2092.

158. Piazza PV, Rouge-Pont F, Deminiere JM, Kharoubi M, Le Moal M, Simon H. (1991b). Dopaminergic activity is reduced in the prefrontal cortex and increased in the nucleus accumbens of rats predisposed to develop amphetamine self-administration. *Brain Res*. **567**: 169-174.

159. Plaznik A, Stefanski R, Kostowski W. (1990). GABAergic mechanisms in the nucleus accumbens septi regulating rat motor activity: the effect of chronic treatment with desipramine. *Pharmacol Biochem Behav*. **36**: 501-506.

160. Pranzatelli MR. (1990). Evidence for involvement of 5-HT2 and 5-HT1C receptors in the behavioral effects of the 5-HT agonist 1-(2,5-dimethoxy-4-iodophenyl aminopropane)-2 (DOI). *Neurosci Lett*. **115**: 74-80.

161. Ratzenboeck E, Saria A, Kriechbaum N, Zernig G. (2001). Reinforcing effects of MDMA ("ecstasy") in drug-naive and cocaine-trained rats. *Pharmacology*. **62**: 138-144.

162. Robinson TE, Berridge KC. (1993). The neural basis of drug craving: an incentive-sensitization theory of addiction. *Brain Res Brain Res Rev*. **18**: 247-291.

163. Robinson TE, Berridge KC. (2003). Addiction. *Annu Rev Psychol*. **54**: 25-53.

164. Roche M, Commons KG, Peoples A, Valentino RJ. (2003). Circuitry underlying regulation of the serotonergic system by swim stress. *J Neurosci.* **23**: 970-977.

165. Rosario LA, Abercrombie ED. (1999). Individual differences in behavioral reactivity: correlation with stress-induced norepinephrine efflux in the hippocampus of Sprague-Dawley rats. *Brain Res Bull.* **48**: 595-602.

166. Rose J, Woolsey CN. (1948). The orbitofrontal cortex and its connections with the mediodorsal nucleus in rabbit, sheep, and cat. *Research publications of the association of nervous and mental disorders*. **27**: 210-232.

167. Roth BL, Kroeze WK, Patel S, Lopez E. (2000). The Multiplicity of Serotonin Receptors: Uselessly diverse molecules or an embarrasment of riches? *The Neuroscientist*. **6**: 252-262.

168. Rouge-Pont F, Deroche V, Le Moal M, Piazza PV. (1998). Individual differences in stress-induced dopamine release in the nucleus accumbens are influenced by corticosterone. *Eur J Neurosci*. **10**: 3903-3907.

169. Rouge-Pont F, Piazza PV, Kharouby M, Le Moal M, Simon H. (1993). Higher and longer stress-induced increase in dopamine concentrations in the nucleus accumbens of animals predisposed to amphetamine self-administration. A microdialysis study. *Brain Res.* **602**: 169-174.

170. Rudnick G, Wall SC. (1992). The molecular mechanism of "ecstasy" [3,4-methylenedioxy-methamphetamine (MDMA)]: serotonin transporters are targets for MDMA-induced serotonin release. *Proc Natl Acad Sci U S A.* **89**: 1817-1821.

171. Rylski M, Kaczmarek L. (2004). Ap-1 targets in the brain. *Front Biosci*. **9**: 8-23.

172. Sagar SM, Sharp FR, Curran T. (1988). Expression of c-fos protein in brain: metabolic mapping at the cellular level. *Science*. **240**: 1328-1331.

173. Sawchenko PE, Imaki T, Potter E, Kovacs K, Imaki J, Vale W. (1993). The functional neuroanatomy of corticotropin-releasing factor. *Ciba Found Symp*. **172**: 5-21.

174. Schechter MD. (1986). Discriminative profile of MDMA. *Pharmacol Biochem Behav*. **24**: 1533-1537.

175. Scheffel U, Lever JR, Stathis M, Ricaurte GA. (1992). Repeated administration of MDMA causes transient down-regulation of serotonin 5-HT2 receptors. *Neuropharmacology*. **31**: 881-893.

176. Schenk S, Gittings D, Johnstone M, Daniela E. (2003). Development, maintenance and temporal pattern of self-administration maintained by ecstasy (MDMA) in rats. *Psychopharmacology (Berl).* **169**: 21-27.

177. Schmidt CJ, Fadayel GM, Sullivan CK, Taylor VL. (1992). 5-HT2 receptors exert a state-dependent regulation of dopaminergic function: studies with MDL 100,907 and the amphetamine analogue, 3,4-methylenedioxymethamphetamine. *Eur J Pharmacol*. **223**: 65-74.

178. Schreiber R, Brocco M, Audinot V, Gobert A, Veiga S, Millan MJ. (1995). (1-(2,5-dimethoxy-4 iodophenyl)-2-aminopropane)-induced head-twitches in the rat are mediated by 5-hydroxytryptamine (5-HT) 2A receptors: modulation by novel 5-HT2A/2C antagonists, D1 antagonists and 5-HT1A agonists. *J Pharmacol Exp Ther*. **273**: 101-112.

179. Shannon M, Battaglia G, Glennon RA, Titeler M. (1984). 5-HT1 and 5-HT2 binding properties of derivatives of the hallucinogen 1-(2,5-dimethoxyphenyl)-2-aminopropane (2,5-DMA). *Eur J Pharmacol*. **102**: 23-29.

180. Sheskin D. (2000). *Handbook of parametric and nonparametric statistical procedures*. Boca Raton: Chapman & Hall/CRC.

181. Shulgin AT, Shulgin A, Jacob P, III. (1991). Central nervous system (CNS) activity of two new psychoactive compounds. *J Psychoactive Drugs*. **23**: 95-96.

182. Smith RL, Barrett RJ, Sanders-Bush E. (1999). Mechanism of tolerance development to 2,5-dimethoxy-4-iodoamphetamine in rats: down-regulation of the 5-HT2A, but not 5-HT2C, receptor. *Psychopharmacology (Berl)*. **144**: 248-254.

183. Smith SG, Werner TE, Davis WM. (1976). Effect of unit dose and route of administration on self-administration of morphine. *Psychopharmacology (Berl)*. **50**: 103-105.

184. Spanos LJ, Yamamoto BK. (1989). Acute and subchronic effects of methylenedioxymethamphetamine [(+/-)MDMA] on locomotion and serotonin syndrome behavior in the rat. *Pharmacol Biochem Behav*. **32**: 835-840.

185. Steele TD, McCann UD, Ricaurte GA. (1994). 3,4-Methylenedioxymethamphetamine (MDMA, "Ecstasy"): pharmacology and toxicology in animals and humans. *Addiction*. **89**: 539-551.

186. Stephenson CP, Hunt GE, Topple AN, McGregor IS. (1999). The distribution of 3,4-methylenedioxymethamphetamine "Ecstasy"-induced c-fos expression in rat brain. *Neuroscience*. **92**: 1011-1023.

187. Stone DM, Stahl DC, Hanson GR, Gibb JW. (1986). The effects of 3,4-methylenedioxymethamphetamine (MDMA) and 3,4-methylenedioxyamphetamine (MDA) on monoaminergic systems in the rat brain. *Eur J Pharmacol*. **128**: 41-48.

188. Suto N, Austin JD, Tanabe LM, Kramer MK, Wright DA, Vezina P. (2002). Previous exposure to VTA amphetamine enhances cocaine self-administration under a progressive ratio schedule in a D1 dopamine receptor dependent manner. *Neuropsychopharmacology*. **27**: 970-979.

189. Suto N, Tanabe LM, Austin JD, Creekmore E, Vezina P. (2003). Previous exposure to VTA amphetamine enhances cocaine self-administration under a

progressive ratio schedule in an NMDA, AMPA/kainate, and metabotropic glutamate receptor-dependent manner. *Neuropsychopharmacology*. **28**: 629-639.

190. Sutton RE, Koob GF, Le Moal M, Rivier J, Vale W. (1982). Corticotropin releasing factor produces behavioural activation in rats. *Nature*. **297**: 331-333.

191. Swanson LW. (1982). The projections of the ventral tegmental area and adjacent regions: a combined fluorescent retrograde tracer and immunofluorescence study in the rat. *Brain Res Bull*. **9**: 321-353.

192. Swanson LW, Sawchenko PE, Cowan WM. (1981). Evidence for collateral projections by neurons in Ammon's horn, the dentate gyrus, and the subiculum: a multiple retrograde labeling study in the rat. *J Neurosci*. **1**: 548-559.

193. Swerdlow NR, Vaccarino FJ, Amalric M, Koob GF. (1986). The neural substrates for the motor-activating properties of psychostimulants: a review of recent findings. *Pharmacol Biochem Behav*. **25**: 233-248.

194. Taghzouti K, Simon H, Louilot A, Herman JP, Le Moal M. (1985). Behavioral study after local injection of 6-hydroxydopamine into the nucleus accumbens in the rat. *Brain Res*. **344**: 9-20.

195. Tanji J, Hoshi E. (2001). Behavioral planning in the prefrontal cortex. *Curr Opin Neurobiol.* **11**: 164-170.

196. Thiel CM, Muller CP, Huston JP, Schwarting RK. (1999). High versus low reactivity to a novel environment: behavioural, pharmacological and neurochemical assessments. *Neuroscience*. **93**: 243-251.

197. Touyarot K, Venero C, Sandi C. (2004). Spatial learning impairment induced by chronic stress is related to individual differences in novelty reactivity: search for neurobiological correlates. *Psychoneuroendocrinology*. **29**: 290-305.

198. Tzschentke TM. (2001). Pharmacology and behavioral pharmacology of the mesocortical dopamine system. *Prog Neurobiol*. **63**: 241-320.

199. Vale W, Spiess J, Rivier C, Rivier J. (1981). Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and beta-endorphin. *Science*. **213**: 1394-1397.

200. Valentino RJ, Liouterman L, Van Bockstaele EJ. (2001). Evidence for regional heterogeneity in corticotropin-releasing factor interactions in the dorsal raphe nucleus. *J Comp Neurol*. **435**: 450-463.

201. Van Oekelen D, Luyten WH, Leysen JE. (2003). 5-HT2A and 5-HT2C receptors and their atypical regulation properties. *Life Sci*. **72**: 2429-2449.

202. van Riezen H, Schnieden H, Wren AF. (1977). Olfactory bulb ablation in the rat: behavioural changes and their reversal by antidepressant drugs. *Br J Pharmacol*. **60**: 521-528.

203. Varga V, Kocsis B, Sharp T. (2003). Electrophysiological evidence for convergence of inputs from the medial prefrontal cortex and lateral habenula on single neurons in the dorsal raphe nucleus. *Eur J Neurosci*. **17**: 280-286.

204. Vertes RP. (1991). A PHA-L analysis of ascending projections of the dorsal raphe nucleus in the rat. *J Comp Neurol*. **313**: 643-668.

205. Vollenweider FX, Gamma A, Liechti M, Huber T. (1998). Psychological and cardiovascular effects and short-term sequelae of MDMA ("ecstasy") in MDMA-naive healthy volunteers. *Neuropsychopharmacology*. **19**: 241-251.

206. Wagner FA, Anthony JC. (2002). From first drug use to drug dependence; developmental periods of risk for dependence upon marijuana, cocaine, and alcohol. *Neuropsychopharmacology*. **26**: 479-488.

207. Wagner MK. (2001). Behavioral characteristics related to substance abuse and risk-taking, sensation-seeking, anxiety sensitivity, and self-reinforcement. *Addict Behav*. **26**: 115-120.

208. Waselus M, Valentino RJ, Van Bockstaele EJ. (2005). Ultrastructural evidence for a role of gamma-aminobutyric acid in mediating the effects of corticotropin-releasing factor on the rat dorsal raphe serotonin system. *J Comp Neurol*. **482**: 155-165.

209. White FJ, Kalivas PW. (1998). Neuroadaptations involved in amphetamine and cocaine addiction. *Drug Alcohol Depend*. **51**: 141-153.

210. White SR, Harris GC, Imel KM, Wheaton MJ. (1995). Inhibitory effects of dopamine and methylenedioxymethamphetamine (MDMA) on glutamate-evoked firing of nucleus accumbens and caudate/putamen cells are enhanced following cocaine self-administration. *Brain Res*. **681**: 167-176.

211. White SR, Obradovic T, Imel KM, Wheaton MJ. (1996). The effects of methylenedioxymethamphetamine (MDMA, "Ecstasy") on monoaminergic neurotransmission in the central nervous system. *Prog Neurobiol*. **49**: 455-479.

212. Willins DL, Meltzer HY. (1997). Direct injection of 5-HT2A receptor agonists into the medial prefrontal cortex produces a head-twitch response in rats. *J Pharmacol Exp Ther*. **282**: 699-706.

213. Wirtshafter D, Stratford TR, Pitzer MR. (1993). Studies on the behavioral activation produced by stimulation of GABAB receptors in the median raphe nucleus. *Behav Brain Res*. **59**: 83-93.

214. Wise RA, Bozarth MA. (1987). A psychomotor stimulant theory of addiction. *Psychol Rev*. **94**: 469-492.

215. Wong LS, Eshel G, Dreher J, Ong J, Jackson DM. (1991). Role of dopamine and GABA in the control of motor activity elicited from the rat nucleus accumbens. *Pharmacol Biochem Behav*. **38**: 829-835.

216. Yan Q, Reith ME, Yan S. (2000). Enhanced accumbal dopamine release following 5-HT(2A) receptor stimulation in rats pretreated with intermittent cocaine. *Brain Res*. **863**: 254-258.

217. Yan QS. (2000). Activation of 5-HT2A/2C receptors within the nucleus accumbens increases local dopaminergic transmission. *Brain Res Bull*. **51**: 75-81.

218. Yan QS, Yan SE. (2001). Serotonin-1B receptor-mediated inhibition of [(3)H]GABA release from rat ventral tegmental area slices. *J Neurochem*. **79**: 914-922.

219. Yap CY, Taylor DA. (1983). Involvement of 5-HT2 receptors in the wet-dog shake behaviour induced by 5-hydroxytryptophan in the rat. *Neuropharmacology*. **22**: 801-804.

220. Yokel RA, Pickens R. (1973). Self-administration of optical isomers of amphetamine and methylamphetamine by rats. *J Pharmacol Exp Ther*. **187**: 27-33.

221. Zuckerman M. (1979). *Sensation Seeking: Beyond the Optimal Level of Arousal*. Hillsdale, NJ: Laurence Erlbaum Associates, Inc.

VITA

Julie Danielle Ross was born on September 6, 1975 to John Michael Ross and Denise Ann Spitler in Portales, New Mexico. After attending the University of Texas at Austin for her Bachelor of Science degree in molecular biology and minor in German, Ms. Ross matriculated into the M.D.-Ph.D. Combined-Degree Program at the University of Texas Medical Branch in Galveston, Texas. While in graduate school, Ms. Ross received several honors including the Robert Harrison, M.D. Memorial M.D./Ph.D. Scholarship, the James E. Beall II Memorial Scholarship, a National Institute for Drug Abuse M.D./Ph.D. Individual National Research Service Award and induction into the Honor Society of Phi Kappa Phi.

Ms. Ross gained significant teaching experience while at the University of Texas Medical Branch. She served twice as laboratory teaching assistant for the Neuroscience and Human Behavior Course in the Integrated Medical Curriculum, as guest lecturer for effective strategies for USMLE preparations for the Alliance of Internal Medicine, as a USMLE student mentor and participated in the Summer Undergraduate Research Program.

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