FACTORS THAT INFLUENCE THE DOPAMINE NEURON AS REVEALED BY DOPAMINE TRANSPORTER EXPRESSION

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ABSTRACT

The primary focus of the present thesis is the exploration of factors that influence the dopamine (DA) neuron by examining the expression of the dopamine transporter (DAT), a marker of the DA neuron. The secondary focus of this thesis is on the serotonin neuron and in particular the serotonin transporter (SERT), a marker of the serotonin neuron. To this end three distinct and separate models have been employed. The goals of this thesis were: (1) to test the hypothesis that monoamine oxidase inhibition during development alters serotonergic innervation in the cortex and raphe, while not affecting relative DA innervation of nigrostriatal pathway, (2) to test the hypothesis that elevated brain levels of hypoxanthine (Hx) deleteriously affect the DA neuron, and (3) to test the hypothesis that densities of DAT and SERT in brainstem cell body regions distinguish alcohol-preferring vervet monkeys with different behavioral patterns of ethanol consumption.

Alterations in the activity of monoamine oxidase (MAO), a degradative enzyme that plays an important role in regulating levels of monoamine transmitters, may have a profound effect on brain development. The present study investigates relative DA and serotonin innervation of cortical and subcortical areas, measured by DAT and SERT densities, following MAO inhibition (A or B or A+B) in mice throughout gestation and early post-natal development. DAT binding was unaltered within the nigrostriatal pathway. The most significant finding reported here is that the combined MAO-A+B inhibition significantly reduced SERT binding by 25% in both the cortex and raphe nucleus. Lower levels of SERT binding were apparent during the early post-natal period (PND 14), a period during which pups were still exposed to MAO inhibitors in the dam's milk, but also persisted into later life (PND's 35 and 90) after inhibitors were no longer being administered. Persistent effects were restricted to cortex and raphe, suggesting a relative vulnerability of these regions to alterations in monoamine transmitter levels during development.

The second study presents data demonstrating that Hx delivered intracerebroventricularly significantly reduces the number of tyrosine hydroxylase immunoreactive cells (TH-ir) in the substantia nigra by 22% and 30%, at 7 and 21 days,

respectively. After 3 days of Hx administration, striatal DA and serotonin were elevated over control levels by 22% and 25%, respectively, but returned to control levels by 7 days. The serotonin metabolite 5-HIAA was elevated after 3 days of Hx, but levels of DA metabolites were not different from control. Locomotion, a behavior thought to be related to DA transmission, was elevated following Hx treatment, as were presynaptic markers of the DA system such as DAT and TH protein levels. The persistent reduction in TH positive cell numbers suggests that Hx damages or kills DA neurons. The increase in intracellular DA at early time points suggests that Hx might interfere with DA release, possibly by temporarily inactivating DA neurons. These findings are consistent with the hypothesis that Hx, a purine significantly elevated in blood and CSF of Lesch-Nyhan patients, maybe involved in DA dysfunction.

Studies on alcohol abuse have focused on the mesolimbic DA pathway and the serotonergic influence within this pathway. Here we report that abstinent binge-drinking monkeys have significant reductions of SERT binding, and to a lesser extent, DAT binding in the midbrain region, while abstinent heavy-drinking subjects have elevated levels of DAT binding, as compared to controls. Both mesolimbic and nigrostriatal pathways are affected. CSF levels of both HVA and 5-HIAA substantiate the neuroanatomical differences between binge- and heavy-drinking vervets. Taken together, these findings provide a neurochemical profile with which to further distinguish subtypes of alcohol-preferring vervet monkeys.

RÉSUMÉ

La présente thèse a pour but premier l'exploration des facteurs influençant les neurones dopaminergiques (DA) par l'observation de l'expression des transporteurs de dopamine (DAT), constituant le marqueur de ces neurones. Le deuxième point important concerne les neurones sérotoninergiques (SER) et plus particulièrement les transporteurs de sérotonine (SERT), marqueurs pour ces neurones. À cette fin, trois modèles expérimentaux ont été employés. Les principaux buts de cette thèse sont : (1) de tester l'hypothèse indiquant que l'inhibition de la monoamine oxydase durant le développement altère l'innervation sérotoninergique dans le cortex et le raphé. Ce qui n'affecte cependant pas l'innervation DA de la voie nigro-striée. (2) de tester l'hypothèse révélant que l'élévation des niveaux cérébraux d'hypoxanthine (Hx) affecte les neurones DA et (3) de vérifier l'hypothèse qui spécifie que la densité de DAT et de SERT dans la région des corps cellulaires du tronc cérébral permet de distinguer parmi les singes vervets ayant une préférence alcoolique, différents schémas comportementaux associés à la consommation d'éthanol.

Des altérations dans l'activité de la monoamine oxydase (MAO), une enzyme de dégradation qui joue un rôle important dans la régulation des niveaux de neurotransmetteurs, montrent possiblement des effets profonds sur le développement du cerveau. La présente étude tente d'investiguer l'innervation relative dopaminergique et sérotoninergique des aires corticales et sub-corticales, mesurée par les DAT et la densité de SERT, suivi de l'inhibition du MAO (A ou B ou A + B) en utilisant des souris pendant la gestation ou à un stade post-natal du développement. Le couplage des DAT a été inaltéré à l'intérieur de la voie nigro-striée. La découverte la plus significative de cette expérimentation indique que la combinaison MAO- A+B a réduit significativement le couplage SERT de 25% dans le cortex et le noyau raphé. Des niveaux encore plus bas de couplage de SERT ont été notés très tôt durant la période post-natale (PND14), une période durant laquelle les poupons étaient toujours exposés aux inhibiteurs de la MAO dans la composition du lait pour nourrissons. Ces niveaux ont également persistés plus tard lors du développement (PND's 35 et 90) malgré le fait que les inhibiteurs ne leur étaient plus administrés. Les effets persistants étaient restreints au cortex et au raphé, ce

qui suggère une vulnérabilité relative de ces régions à l'altération des niveaux de transmission de monoamine durant le développement.

La seconde étude seconde étude présente des données démontrant que le Hx administré intracérébroventriculairement diminuait significativement le nombre de cellules immunoréactives de tyrosine hydroxylase (TH-ir) dans la substance noire de 22% et 30%, à 7 et 21 jours, respectivement. Après trois jours d'administration de Hx, les niveaux de DA striatale et de sérotonine se sont élevés au-delà des niveaux de contrôle de 22% à 25% respectivement, mais sont retournés au niveau de contrôle après 7 jours. Le métabolite de la sérotonine 5-HIAA était élevé après trois jours d'administration de Hx, mais les niveaux du métabolite DA n'ont pas dérogés du standard de contrôle. La locomotion, un comportement que l'on croyait relié à la transmission de DA, était élevée après le traitement de Hx, tout comme les marqueurs pré-synaptiques du système DA, tel que DAT et les niveaux de protéines TH. La réduction persistante du nombre de cellules TH positives suggère que Hx possède un effet dommageable et même mortel sur les neurones. L'augmentation du taux de DA cellulaire très tôt dans l'expérimentation laisse présumer que Hx pourrait interférer avec la libération de DA, possiblement par une désactivation temporaire des neurones DA. Les résultats sont en accord avec l'hypothèse suggérant que Hx, une purine significativement élevée dans le tissu sanguin et le CSF des patients de Lesch-Nylan, pourrait être impliqué dans la dysfonction de la DA.

Plusieurs études concernant l'abus d'alcool se sont surtout concentrées sur la voie de la DA mésolimbique et sur l'influence sérotoninergique sur cette dernière. Dans cette présente recherche, nous avons démontré que les singes souffrant d'alcoolisme périodique (binge-drinking) dont nous avons stoppé la consommation, ont présenté une réduction significative du couplage du SERT et, à un niveau moindre, du couplage du DAT dans le «mid-brain region», tandis que les singes souffrants d'alcoolisme lourd (heavy drinking) dont nous avons interrompu la consommation, ont eu des niveaux élevés de couple du DAT, en comparaison avec le groupe-témoin. Les voies mésolimbiques et nigro-striées ont toutes les deux été affectées. Les niveaux de CSF des HVA et 5-HIAA viennent confirmer les différences neuroanatomiques entre les deux catégories d'alcoolisme des singes vervets. Lorsque globalement pris en compte, ces résultats

fournissent un outil neurochimique avec lequel l'on pourrait davantage distinguer les deux sous-types d'alcoolisme.

CHAPTER I

FACTORS THAT INFLUENCE THE DOPAMINE NEURON AS REVEALED BY DOPAMINE TRANSPORTER EXPRESSION

Introduction

The dopamine (DA) system orchestrates and modulates behaviors involved with motor and oculomotor function, affect, and cognition, having effects in several discrete neurocircuits and working in concert with several neurotransmitters, including glutamate, GABA, and serotonin. Imbalance of transmitter levels, either in any discrete circuit component or through multiple circuits, may result in aberrant behavior. Despite intense research, many of the factors that influence the DA system remain to be explored. The primary focus of the present thesis is the documentation of some factors that influence the expression of the dopamine transporter (DAT), a cell surface macromolecule that has been identified as a marker of the DA neuron (Kuhar, 1998). Three distinct experiments examine: 1) effects of monoamine oxidase (MAO) inhibition during development, 2) effects of the purine hypoxanthine (Hx) on the nigrostriatal pathway, and 3) brain stem levels of DAT in a model of vulnerability to alcohol abuse. Interest in the potential of these factors to influence the DA system arose from clinical findings in Brunner's syndrome (Brunner et al., 1993a,b), Lesch-Nyhan disease (LND) (Lesch & Nyhan, 1964; Lloyd et al., 1981), and alcoholism (Tiihonen et al., 1995). Evidence from both clinical studies and animal models also suggests a prominent role of serotonin in these disorders (Whitaker-Azmitia et al., 1994; Luthman et al., 1987; Heinz et al., 1998; McBride et al., 1997). Therefore the secondary focus of the experiments is on the serotonin neuron and in particular the serotonin transporter (SERT) which (analogous to DAT) has been identified as a marker of the serotonin neuron (Horschitz et al., 2001; Hall et al., 2004).

During early development, the brain relies on a synchronized series of events for the formation of intact neuronal circuits, and as a consequence, alterations of neurotransmitter levels during critical periods renders the formation of neural circuits vulnerable (Berger-Sweeney & Hohmann, 1997). To examine the potential developmental influence of DA and serotonin, in the first experiment MAO was partially inhibited during embryonic and early post-natal development. Previous behavioral work in our laboratory suggests that MAO inhibition during early development modifies behaviors which rely upon the proper formation of neural circuits involving both DA and serotonin (Mejia et al., 2002), but the neurochemical basis of these behaviors were not

examined in the previous work. In the present study makers of DA and serotonin innervation in cortical and subcortical areas during different developmental periods were examined.

In the second experiments, the effects of pharmacological levels of exogenous Hx on the nigrostriatal DA system were examined. Interest in the effects of Hx on the DA system arise from neurochemical studies of patients with Lesch-Nyhan disease, an inherited disorder in which Hx levels are more than doubled and DA is depleted (Lesch & Nyhan, 1964; Lloyd et al., 1981). The final factor investigated here is the hypothesis that the density of DA and/or serotonin neurons may, in part, subserve vulnerability to alcohol abuse. Drug abuse is an extremely complex process, with at a minimum, periods of initial response, acquisition, relapse, withdrawal, and craving. None of these phases are completely understood, but it is at least certain that DA and serotonin are elevated during initial exposure to many drugs of abuse. Many investigators would argue that identification of vulnerability to excessive drug taking is a key to success in designing effective programs for harm reduction. Neurochemical vulnerabilities to subsequent alcohol abuse in the non-human primate are the focus of the present investigation. Monkeys in this project were screened for alcohol preference and consumption pattern, however since access to alcohol was restricted and consumption quantities carefully monitored, these subjects were not allowed to abuse alcohol thereby minimizing potential neuroadaptations.

Because the questions posed in different experiments cover a very broad range, it is necessary in this introduction to review the neural circuitry, the physiology and pharmacology, and ontology of the DA system as it relates to normal operation of the central nervous system. Thereafter, evidence of DA system dysregulation in Brunner's syndrome, LND and vulnerability to ethanol abuse is reviewed. The introduction to this thesis concludes with an enunciation of the questions and hypotheses that are addressed in the experiments that form the body of work presented here.

Neural Circuitry of the Dopamine System

The DA system can be divided into three major pathways on the basis of cell body origin and projections: mesolimbic, mesocortical, and nigrostriatal. Each of the disorders investigated in the present study involve distinct but overlapping areas of these anatomically-based DA pathways. The aggressive and impulsive behavior displayed following MAO depletion during development is indicative of an abnormal mesolimbic system (Miczek et al., 2002; Cardinal et al., 2000, 2004; Winstanley et al., 2005). The severe motor impairment in LND suggests a prominent dysfunction within the nigrostriatal motor pathway (Lesch & Nyhan, 1964; Visser et al., 2000), whereas the aggressive and self-mutilatory behavior (Lesch & Nyhan, 1967; Hall et al., 2001; Robey et al., 2003) is indicative of a dysfunctional mesolimbic pathway. Finally, accumulating evidence suggests that individuals who are vulnerable to alcohol abuse have an array of cognitive deficits, including higher-order executive functions, suggesting a mesocortical abnormality (Drejer et al., 1985; Schaeffer et al., 1984, 1988; Wilson et al., 1988; Tarter et al., 1989a,b) as well as a hypothetical pre-existing dysfunction in the mesolimbic pathway which may result in an aberrant response to reward (Sher et al., 1991; Gabel et al., 1995; Finn et al., 1997, 2000; Caspi, 2000; Soderstrom et al., 2001).

Each of the three major DA pathways (mesocortical, mesolimbic, and nigrostriatal) originates in the midbrain DA area. Based on projections and cytoarchitecture, the mesocephalon or midbrain DA area of the primate is divided into three areas the retrorubral area, the dorsal tier (comprised of the ventral tegmental area-VTA and dorsal substantia nigra pars compacta-SNc), and the ventral tier comprised of the densocellular region of the SNc and ventral cell columns (Olszewski & Baxter, 1954; Haber & Fudge, 1997; Francios et al., 1999). These cell groups differentially innervate each of the major DA pathways.

The retrorubral area (RRA) has widespread projections to the frontal cortex (medial frontal, precentral, and prefrontal areas), hippocampus, amygdala, and striatum (Jiminez-Castallenos & Graybiel, 1987; Langer & Graybiel, 1989; Gasbarri et al., 1997; Williams & Goldman-Rakic, 1998; Zahm et al., 1999). The dorsal tier (VTA and dorsal SNc) has some of the most extensive mesocortical projections as well as contributing to the nigrostriatal pathway. The VTA and its largest nucleus, the parabrachial pigmented nucleus (PBPG) project to the dorsolateral prefrontal cortex (area 46) with extensive

innervation of limbic-related cortices such as the prelimbic, infralimbic and anterior cingulate. The dorsolateral prefrontal cortex receives substantial projections from the dorsal SNc while the motor cortex innervated by the RRA, dorsolateral PBPG, and dorsal SNc. The SNc has a relatively minimal contribution to the mesocortical pathway with sparse projections to the dorsolateral prefrontal and motor cortices. It is evident that in the primate, discrete midbrain cell populations have preferential cortical innervation, however cortical areas such as the medial prefrontal and dorsolateral prefrontal cortices receive partially overlapping projections from different midbrain regions (Williams & Goldman-Rakic, 1998). The heterogeneity of the mesocortical projections underscores the functional heterogeneity that DA has in the central nervous system.

The principle target of midbrain DA neurons is the striatum, which can be divided into three regions based on cortical and subcortical afferents. The midbrain DA projections to the striatum are a reflection of the functionally orientated innervation of the cortex and amygdala. The ventral medial striatum, or limbic-related striatum, (including the nucleus accumbens) receives DA projections from the dorsal tier of the midbrain (Haber & Fudge, 1997) as well as limbic-related cortical areas (such as the medial prefrontal orbital cortex) and amygdala (Russchen et al., 1985; Selemon & Goldman-Rakic, 1985; Haber et al., 1990, 1995a; Kunishio & Haber 1994; Chikima et al., 1997; Haber & McFarland, 1999; Fudge et al., 2002; Fudge & Haber, 2002). The dorsolateral striatum, or motor-related striatum, receives DA projections from the ventral cell columns of the ventral tier, as well as motor related cortical areas such as the primary motor cortex (Kunzle, 1975; Lynd-Balta & Haber, 1994a,b; Inase et al., 1996, 1999; Takada et al., 1998; Haber et al., 2000).

The striatum (including the nucleus accumbens) is ideally situated to integrate midbrain, cortical and subcortical information. This is achieved through a series of parallel and convergent pathways (Alexander et al., 1986; Haber, 2003). Projections to the striatum respect functional boundaries of motor, affect, oculomotor, and cognition circuits. This parallel processing is carried through the output of the striatum to the globus pallidus and eventually the thalamus (Alexander et al., 1986). Alternatively, projections from the striatum to the substantia nigra form a series of overlapping

feedback loops that allow for a neuroanatomical basis for communication between the circuits (Haber, 2003).

The regulation of the DA neural circuits depends on a proper balance between cortical and subcortical neurochemical systems. The behaviors displayed in Brunner's syndrome, LND, and alcohol abuse suggests that communication within and between the circuits does not function properly. Although the mesotelencephalic DA system is implicated in these disorders the manner in which it is involved at each level remains elusive. This is due in part to a dynamic interaction with other neurotransmitters throughout the circuitry as well as intrinsic physiology and pharmacology of the DA neuron itself.

Physiology and Pharmacology of the Dopamine System

Basic Physiology and Pharmacology of the Dopamine Neuron

Dopamine is synthesized from tyrosine by tyrosine hydroxylase (the rate-limiting step) to DOPA then to DA by aromatic l-amino acid decarboxylase. Dopamine can then be further metabolized to norepinephrine by dopamine-beta-hydroxylase (for further review, Cooper et al., 1996). Once released, DA is catabolized by catechol-O-methyltransferase (COMT) and monoamine oxidase (MAO) into dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA). DOPAC is the main metabolite in rodents, whereas HVA predominates in primates and humans. Mean levels of these metabolites are used as an index of steady-state DA neuronal activity (Bloom, 1996; Cooper et al., 1996).

Dopamine is stored in vesicles until it is released via an influx of calcium ions induced by an action potential (Kelly, 1993; Floor et al., 1995; Cooper et al., 1996). Once released, DA acts on pre- and post-synaptic DA receptors belonging to two families. The D_1 receptor family includes the D_1 and D_5 subtypes whereas the D_2 receptor family includes the D_2, D_3, and D_4 subtypes. These receptors display regional variation. Both D_1 and D_2 receptors are highly concentrated within the striatum and substantia nigra, but are differentially distributed throughout the rest of the brain (Cortes et al., 1989; Camps et al., 1989). The cerebral cortex and amygdala contains a higher

concentration of D_1 as compared to D_2 receptors (De Keyser et al., 1988; Cortes et al., 1989; Camps et al., 1989). However, D_2 receptor concentrations are abundant in the CA1 and CA3 region of the hippocampus whereas the D_1 receptor displays low densities in the hippocampus (Cortes et al., 1989; Camps et al., 1989). The D_3 and D_4 receptor subtypes are much less abundant than the D_2 receptor and are differentially distributed. The D_3 receptor is primarily located primarily within the nucleus accumbens and olfactory tubercle, with low concentrations present in the substantia nigra, striatum, hippocampus, and prefrontal cortex (Levesque et al., 1992). Although it would be tempting to speculate that D_3 receptors are primarily associated with the limbic system, the low concentrations of this receptor in the prefrontal cortex (Levesque et al., 1992) and high levels in the human dorsolateral striatum (Gurevich & Joyce, 1999) would caution against such an assumption. The D_4 receptor, however, is intimately associated with the cortical and limbic DA systems. The D_4 receptor is present in the hippocampus (CA1, CA2, CA3, and dentate gyrus), entorhinal cortex, medial and lateral prefrontal cortices (Primus et al., 1997; Tarazi et al., 1997). Within these regions, D_4 receptors constitute 40-50% of all D_2-like radioligand binding.

Most of the available information concerns the D_1 and D_2 receptors. All of these receptors are coupled to GTP-regulatory proteins, which act to transduce the neurotransmitter signal through modulation of gene expression or activation of other intracellular signaling pathways. The D_1 family is coupled to the stimulatory G_s subunits of the nucleotide regulatory protein, thus stimulating intracellular cAMP production, while the D_2 family is coupled to G_i and other inhibitory subunits to reduce cAMP production. In addition, the D_1 family is considered to be exclusively post-synaptic, whereas most of the D_2 family receptors are located pre-synaptically regulating DA release (Bloom, 1996; Cooper et al., 1996; Emilien et al., 1999) as well as participating in the regulation of DA synthesis (Cass & Gerhardt, 1994; Cooper et al., 1996; Schwarting & Huston 1996; Saiardi et al., 1998).

After release, DA is taken up by high affinity sites, termed DAT (Cooper et al., 1996; Schwarting & Huston, 1996). DAT is synthesized in the cell body then transported by tubulovesicles and inserted into the plasma membrane (Bradbury & Bridges, 1994; Nirenberg et al., 1996). Interestingly, the location of DAT is outside the active zone of

the synapse in the perisynaptic area and requires the diffusion of DA prior to reuptake (Garris et al., 1994; Nirenberg, et al., 1996, 1997; Kuhar, 1998). DAT concentrations are differentially expressed in DA innervated areas with the highest concentrations being in the striatum, followed by substantia nigra (excluding the medial region), with even less in the ventral tegmental area (Hurd et al., 1994; Kuhar, 1998).

The DAT is thought to be the essential regulator of extrasynaptic DA concentrations (Garris et al., 1994; Cass & Gerhardt, 1994; Giros et al., 1996; Chen & Reith, 2000; Cragg & Rice, 2004). In the absence of the DAT, the normal equilibrium between DA release and clearance cannot be maintained (Giros et al., 1996). Also in the absence of DAT the clearance of DA is thought to occur through SERT and the norepinephrine transporters (Hall et al., 2004). DAT is highly regulated by the D_2 receptor and second messengers systems including arachidonic acid (AA), protein kinase C (PKC), cAMP, and alpha-synuclein which act upon the DAT either to inhibit or enhance DA clearance (Kadowaki et al., 1990; Piomelli et al., 1991; Meiegerd et al., 1993; Vial & Piomelli, 1995; Zhang & Reith, 1996; Zhang et al., 1997; Myers et al., 2001; Lee et al., 2001; Wu et al., 2002; Wersinger & Sidhu, 2003).

DAT is found only on DA neurons, within axons, nerve terminals, cell soma, and dendrites (Nirenberg et al., 1996, 1997; Kuhar, 1998), with the latter location providing support for dendritic release of DA (Cheramy et al., 1981; Abercrombie et al., 1998; Cobb & Abercrombie, 2003). Consequently DAT has been proposed to be a specific marker of DA neurons (Kuhar, 1998). Since the first binding assay with cocaine (Reith et al., 1980) demonstrating its affinity for DAT (Kennedy & Hanbauer, 1983; Reith et al., 1985; Ritz et al., 1987; Fugita et al., 1994; Reith et al., 1997), analogs of cocaine have routinely been used for *in vivo* and *in vitro* measurements of the DA neuron (Mash et al., 1996; Wong et al., 1996; Tiihonen et al., 1997, 1998; Tupala et al., 2001). The use of cocaine analogs does give rise to methodological concerns because in many instances these ligands have considerable affinity for the serotonin (Laruelle et al., 1994) and norepinephrine transporters (Okada et al., 1998), as well as DAT. Specific measurement of DAT must occlude the binding of ligand to the other transporters, which is readily accomplished *in vitro* but not necessarily performed *in vivo* (Tiihonen et al., 1997; Heinz et al., 1998).

In addition to being a specific marker of DA neurons and the principal site of DA removal, DAT plays a key role in neurotoxicity and is important in the action of drugs of addiction. 6-hydroxydopamine (6-OHDA) has been known for many years to be a potent neurotoxin toward the DA neuron (Ungerstedt, 1968). This neurotoxin is taken into the neuron through DAT, and then undergoes intracellular auto-oxidation, generating free radicals and hydrogen peroxide (Decker et al., 1993). N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), another toxin which is selective for the DA neuron, is also internalized through DAT. In fact mice lacking DAT are relatively insensitive to this neurotoxin (Gainetdinov et al., 1997). There is some speculation that the relative vulnerability of subpopulations of midbrain DA neurons in Parkinson's disease is related to the differential concentration of DAT present in the various cell body regions (German et al., 1989; Gibb & Lees, 1991; Damier et al., 1999; Hornykiewicz, 2001).

DAT is also important in the molecular action of drugs of addiction and has been dubbed the receptor for cocaine (Kuhar et al., 1990). Cocaine which blocks DA reuptake by DAT, allows DA to remain in the synapse for an extended period of time (Ritz et al., 1987; Pifl et al., 1995). It is evident that DAT, although an excellent marker of the DA neuron, is much more than a marker. This protein is a dynamic, highly regulated structure with an integral role in DA release and uptake, neurotoxicity, and a principle site of action of drugs of abuse (Gainetdinov et al., 1997; Kuhar et al., 1990).

Electrophysiological Properties of Dopamine Neurons

Electrophysiological investigations reveal that DA neurons in the midbrain (VTA and substantia nigra) display a number of firing patterns ranging from inactive to high intensity single spike bursts (Grace & Bunney 1984a,b; Gonon, 1988; Schultz, 1986). The normal physiological firing repertoire of DA neurons is a slow pacemaker pattern with a frequency of 3-8Hz with 1-9 spikes per second (Grace & Bunney 1984a,b; Redgrave et al., 1999). Superimposed on this slow pacemaker or tonic firing pattern are bursts or phasic firing with short duration (<200msec) consisting of 3-4 spikes separated by 60-70msec (Grace & Bunney, 1984a). Intraburst frequency of DA neurons can be in excess of 30Hz (Grace, 1987; Wightman & Robinson, 2002). Although both the substantia nigra and VTA contain bursting neurons, the latter expresses a larger

proportion of burst-firing DA cells (Grenhoff et al., 1986). The balance between tonic and phasic DA transmission is thought to influence extracellular DA concentrations which are regulated by DAT (Wightman & Robinson, 2002). As a consequence, this balance is implicated in the proper functioning of neural circuits involved in executive cognitive functioning (Sawaguchi & Goldman-Rakic, 1991), affect, reward (Grace, 1995, 2000; Schultz, 1998), and movement (Berke & Hymen et al., 2000). Irregularities in DA neuronal firing have been associated with schizophrenia (Grace et al., 1991), stimulant abuse (Grace et al., 1995), and alcoholism (Grace, 2000). Additionally, disruptions in cortical development have also been shown to alter DA neuronal firing within the prefrontal cortex (Lavin et al., 2005).

Tonic release of DA results in a relatively steady-state concentration of extracellular DA (Robinson & Wightman, 2002). Tonic DA release is thought to be partially under the control of excitatory inputs from cortical and limbic areas as well as autoregulation by the DA neuron (Grace, 1991, 1995; Dugast et al., 1997). The extracellular concentrations resulting from tonic release of DA are sufficient to activate D_1 (Richfield et al., 1989) and D_2 receptors as well as DAT (Suaud-Chagny, 1995; Chergui et al., 1994). It is the balance between re-uptake by DAT and activation of the D_2 receptor that is thought to assist in the regulation of extracellular DA (Schmitz et al., 2003; Schultz, 1998). It has been proposed that tonic release of DA suppresses phasic DA release via D_2 receptor activation (Grace, 1991, 1995). Because tonic DA firing is in part under the control of autoreceptor inhibition, elevation of DA via sustained tonic release would also diminish tonic firing and it is at this point where DA neurons are proposed to be most likely to switch to burst firing (Grace, 1991, 1995). In the brief periods where tonic levels are depressed due to autoinhibition, phasic release of DA is facilitated (Grace, 1991, 1995; Phillips et al., 2003; Carelli & Wightman, 2004). Similar to tonic release of DA, extracellular concentrations of DA induced by phasic release are sufficient to stimulate the D_1 (Richfield et al., 1989; Gonon, 1997) and D_2 (Richfield et al., 1989; Dugast et al., 1997) receptors as well as DAT (Chergui et al., 1994). Activation of the D_2 receptor by phasic release of DA also acts as an autoregulator of DA transmission (Dugast et al., 1997).

Tonic and phasic firing patterns result in a differential release of DA into the extracellular space. The relatively small synaptic space (approximately 200nm in length and 10nm in width) allows for extrasynaptic diffusion of DA (Garris et al., 1994). Since microdialysis (200μm) and voltammetry (5-30μm) probes are too large to measure synaptic concentrations of DA, these methods typically represent extrasynaptic DA concentrations. The slow tonic firing pattern of DA results in an extracellular concentration of DA estimated to be anywhere from 10-100nM that can be measured by microdialysis and voltammetry. However, the ability of these methods to dissociate tonic versus phasic DA release remains problematic (Jones, 1993; Suaud-Chagny et al., 1991, 1992; Kawagoe et al, 1992; Wightman & Robinson, 2002). *In vivo* voltammetry suggests that phasic DA release may increase extracellular DA to concentrations ranging from 200-1000nM (Dugast et al., 1994; Suaud-Chagny et al., 1995; Robinson et al., 2001), however the exact amount of DA that escapes the synaptic cleft after phasic stimulation is not clear (Floresco et al., 2003; Phillips & Wightman, 2004). Since DAT activation limits the amount of DA "leakage" into the perisynaptic space (Suaud-Chagny et al., 1991, 1995; Garris et al., 1994) *in vivo* kinetics of DAT versus diffusion rates have been used to estimate that phasically released DA can diffuse approximately 12μm from the release site (Garris et al., 1994; Schultz, 1998; Gonon, 1997). The diffusion of DA from the synaptic cleft occurs rapidly (under 40μsec) and the maximal extrasynaptic concentration is achieved within 75msec following a single impulse (Garris et al., 1994; Schultz, 1998). Following multiple impulses within a 200msec timeframe, elevated extrasynaptic DA concentrations have a potential duration of 600msec before steady-state tonic levels are achieved (Schultz, 1998).

In addition, the DA terminal area also dictates elimination time for extracellular DA (defined as 50% decay of DA oxidant current measured by *in vivo* amperometry) following phasic release. For example, elimination of DA in the striatum occurs nearly twice as rapidly as that in the nucleus accumbens (Suaud-Chagny et al., 1995). Other regional disparities of DA synaptic overflow and elimination are apparent within the mesocorticolimbic pathway. Electrical stimulation of DA fibers, mimicking phasic activity, results in similar release of DA in the medial prefrontal cortex (mPFC), basolateral amygdala (BLA), striatum, and nucleus accumbens, however extrasynaptic

overflow is substantially different between these areas. Rate constants for uptake and release of DA within the mPFC and the BLA are significantly lower than those found in the striatum and nucleus accumbens. Additionally, when normalizing the rate of release and uptake relative to DA innervation, the mPFC has a 10-fold greater rate of release and the BLA has a 10-fold lower rate of uptake, as compared to the striatum and nucleus accumbens. Functionally this means that when DA neurons projecting to the BLA or mPFC are phasically activated, the actions of DA may have a larger temporal and spatial activation of postsynaptic receptors than would be the case for the striatum (Garris & Wightman, 1994).

Neurochemical Interactions within the Dopamine System

The DA neurons are differentially regulated throughout the mesotelencephalic DA system. GABA, glutamate and serotonin all interact with DA neurons to increase or decrease DA release. Because of the complexity of these interactions there are both regulatory and counter-regulatory changes at different places and under various circumstances throughout the mesotelencephalic DA system. Accordingly, changes in one of these neurotransmitters often leads to counter-regulatory alterations in one or more of the other neurotransmitter systems.

One important regulator of DA release, throughout the neuronal circuitry, is glutamate. Glutamate, which is an excitatory neurotransmitter, generally facilitates DA release, however there are instances where glutamate release attenuates DA release, possibly as a consequence of inhibitory interneuron activation. Glutamate receptor agonists (NMDA and kainate) applied locally onto VTA and SNc neurons results in elevated DA release in the mPFC, nucleus accumbens, and striatum (Westerink et al., 1996, 1998). Similarly, glutamate receptor activation in the mPFC, via the corticofugal pathway (Sesack et al., 1989; Sesack & Pickel, 1992), activates firing of VTA DA neurons projecting to the mPFC while inhibiting those projecting to the nucleus accumbens (Takahata & Moghaddam, 2000). Anatomical evidence suggests that the corticofugal projections from the mPFC synapse directly on DA neurons projecting back to the mPFC (Carr & Sesack, 2000) which maybe responsible for the excitatory response of VTA neurons projecting back to the mPFC. However corticofugal projections also

make contact with non-DA cells in the VTA, presumably GABAergic neurons, which project to the nucleus accumbens (Carr & Sesack, 2000), which may be responsible for the inhibitory effects of mPFC activation on DA neurons of the nucleus accumbens. Within the mPFC, locally applied NMDA receptor antagonists elevate, while AMPA receptor antagonists decrease local DA release (Takahada & Moghaddam, 1998).

The effect of glutamate on DA release in the nucleus accumbens is a point of contention. Moghaddam et al. (1990) found that high levels of glutamate (10mM) applied into the nucleus accumbens increases extracellular DA levels. Elevating glutamate by locally inhibiting the glutamate transporter has been shown to both decrease (Taber et al., 1996) and increase (Segovia & Mora, 2001) extracellular DA concentrations in the nucleus accumbens. The ability of glutamate to regulate DA in the nucleus accumbens may be dependent on relative receptor activation since application of low dose (0.1 mM) of NMDA agonists into the nucleus accumbens inhibited DA release, while high dose (1.0mM) application facilitated DA release (Taber et al., 1996). Within the striatum, activation of either NMDA or AMPA receptor agonists results in elevated DA release (Youngren et al., 1993; Westerink et al., 1996; Segovia et al., 1997; Hernandez et al., 2003). Conversely, DA has been shown to elevate glutamate levels in the striatum and nucleus accumbens (Exposito et al., 1999), but attenuates glutamate transmission in the mPFC (Godbout et al., 1991; Pirot et al., 1992; Law-Tho et al., 1994; Harte & O'Connor, 2004).

As is the case with glutamate, GABA has been shown to both stimulate and attenuate DA release throughout the DA circuitry. As GABA is everywhere inhibitory, the phenomenon of GABA-mediated stimulation is also a second-order phenomenon, which is typically termed disinhibition. Activation of GABA receptors in the VTA reduces the firing of DA neurons in the mPFC and nucleus accumbens (Westerink et al., 1996, 1998; Takahata & Moghaddam, 1998). Similarly, activation of GABA-B receptors within the substantia nigra decreases firing of striatal DA neurons (Westerink et al., 1996). Alternatively, activation of GABA-A receptors in the substantia nigra increases striatal DA release (Santiago et al., 1993a,b; Westerink et al., 1996), which in turn elevates striatal GABA release (Exposito et al., 1999). Within the mPFC, activation of GABA-A and B receptors reduces local DA release (Santiago et al., 1993a,b). Dopamine

release in the mPFC does not affect GABA transmission directly, but may potentiate AMPA stimulated GABA release via D_2 receptor activation (Del Arco & Mora, 2002).

Detailed analysis of the functional aspects of DA-GABA-glutamate interaction throughout the mesotelencephalic pathway is beyond the scope of this review, however an example of how this system may be integrated is warranted. The amygdala, which processes signals from the cortex, also exerts influence in both the mPFC and nucleus accumbens. Microstimulation of the BLA results in increased glutamate release in both the mPFC and nucleus accumbens, which in turn elevates DA in the mPFC not the nucleus accumbens. However, when glutamate transmission in the mPFC is attenuated, BLA microstimulation results in increased extracellular DA in the nucleus accumbens suggesting an inhibitory role of mPFC glutamate release on DA release in the nucleus accumbens (Jackson & Moghaddam, 2001). Additionally, stress-induced DA release in the nucleus accumbens is potentiated whereas right mPFC DA is attenuated by the ablation of DA innervation, via 6-OHDA lesions, in the BLA (Stevenson et al., 2003). Stevenson et al. (2003) suggests that the lack of DA tone in the BLA, which is suggested to mediate response to stressful stimuli (LeDoux, 1996), would ultimately result in increased accumbal DA response since the BLA lesion would increase glutamate transmission to the mPFC, thereby decreasing mPFC DA response to physical stress via NMDA or AMPA receptor activation (Del Arco & Mora, 2001). Decreases in DA as a result of 6-OHDA lesions within the mPFC have been shown to enhance stress induced DA transmission within the nucleus accumbens (Deutch et al., 1990) supporting the assertion made by Stevenson et al. (2003). Alternatively, the decrease in DA tone in the BLA following the ablation of DA may disinhibit the direct pathway from the BLA to the nucleus accumbens which would increase DA transmission and affect output from the nucleus accumbens. Dopamine via the BLA and mPFC exert a regulatory tone on nucleus accumbens DA transmission either directly or indirectly. Regulation of DA through the mesocorticolimbic pathway is of utmost importance for normal motivational function. Altered mesocorticolimbic DA response to stimuli such as stress may very well underlie such pathologies such as self-mutilation in Lesch-Nyhan disease where children over-respond to minor stressors (Kelley & Wyandergarden, 1989) and the acquisition and maintenance of drug addiction (Haney et al., 1995; Hurd et al., 1997).

Serotonin and Dopamine Interactions within the Mesotelencephalic Pathway

Like glutamate and GABA, serotonin differentially modulates extracellular DA concentrations throughout the DA pathway. Modulation of the DA system by serotonin is complex since serotonin has been shown to both increase (Tanda et al., 1995; Nomikos et al., 1996; Gervais & Rouillard, 2000) and decrease (Di Mascio et al., 1998; Gervais & Rouillard, 2000) extracellular DA levels within the mesotelencephalic system. The ambiguity is a result of a non-uniform distribution and action of serotonin receptor subtypes (Prisco et al., 1992, 1994; Cameron & Williams, 1995), of which there are 14 identified subsets (Azmitia, 2001).

Co-locolization of serotonin and DA neurons occurs in various brain areas including the striatum (Fuxe, 1965; Pasik & Pasik, 1982; Mori et al., 1985), SNc, SNpr, VTA (Lavoie & Parent, 1990), and amygdala (Sadikot & Parent, 1990; Lavoie & Parent, 1990). Localization of specific serotonin receptors within these areas is a key component for their relative regulation of the DA system. In the dorsal raphe, the 5-HT_{1A} receptor serves as an autoreceptor, but in the VTA, activation of the 5-HT_{1A} receptor enhances both the rate of firing and the frequency of bursting activity of DA neurons (Arborelius et al., 1993; Prisco et al., 1994; Chen & Reith, 1995; Gobert et al., 1995; Lejeune & Millan, 1998). Systemic administration of 5-HT_{1A} receptor agonists increase outflow of DA in the mPFC (Rasmusson et al., 1994; Gobert et al., 1999), while 5-HT_{1A} antagonists decrease DA outflow in the nucleus accumbens and striatum (Nomikos et al., 1996). The 5-HT_{1A} receptor subtype is not uniformly distributed in VTA, with the parabrachial division displaying a higher concentration of this receptor than the midline paranigral division (Doherty & Pickel, 2001). Consistent with this, 5-HT_{1A} agonists exert a more pronounced increase in firing rate and burst activity in the parabrachial nucleus than the paranigral division (Arborelius et al., 1993).

By contrast, systemic application of agonists of the 5-HT_{1B} receptor, which is particularly dense within the mesolimbic pathway (Bruinvels et al., 1994), decreases the basal firing rate of the VTA neurons (Prisco et al., 1994). However direct application of the 5-HT_{1B} agonist CP 93129, into the nucleus accumbens or VTA results in dose-dependent increases of extracellular DA within the nucleus accumbens (Yan & Yan,

2001) and VTA along with decreased GABA concentrations within the VTA (Yan et al., 2004). These data suggest that, within the VTA, activation of the 5-HT_{1B} receptor increases DA release indirectly through disinhibiting tonic GABAergic inhibition of the DA neuron. Likewise activation of the 5-HT_{1D} receptor is thought to facilitate DA transmission in the nucleus accumbens via disinhibition of GABA inhibition in the VTA (Cameron & Williams, 1994, 1995).

The 5-HT_2 receptor family, which densely populates the mesolimbic pathway, especially the terminal region (Abramowski et al., 1995; Lopez-Gimenez et al., 2001), differentially activates mesocorticolimbic versus nigrostriatal DA neurons. Specifically, the 5-HT_{2C} receptor is thought to preferentially decrease mesolimbic and mesocorticolimbic DA transmission (Di Matteo et al., 2000; Di Giovanni et al., 2000; De Deurwaerdere et al., 2004). *In vivo* electrophysiological and microdialysis techniques reveal that 5-HT_{2C} receptor agonists decrease firing rate and bursts of DA neurons in the VTA (Di Matteo et al., 2000; Di Giovanni et al., 2000) and decrease DA release in the mPFC (Millan et al., 1998; Gobert et al., 2000) and nucleus accumbens without affecting striatal release (Gobert et al., 2000; Di Matteo et al., 2000; Di Giovanni et al., 2000). 5-HT_{2C} agonists also induce a slight decrease in basal firing of the substantia nigra and have been reported to have either no effect (Di Giovanni et al., 2000) or to decrease DA outflow in the striatum (Gobert et al., 2000). Additionally, 5-HT_{2c} antagonists elevate basal firing of DA neurons, as well as burst firing, in the VTA with substantially lower effect on neuronal firing in the substantia nigra pars compacta. The increase in VTA firing corresponds to an observed increase in extracellular DA in the nucleus accumbens with little increase in the striatum (Di Matteo et al. 1999, 2000). These results suggest that serotonin, acting via the 5-HT_{2C} receptor, has a selective effect on VTA (as compared to SNc) DA neurons with respect to tonic and phasic inhibitory regulation (Di Matteo et al., 2002).

Conversely, antagonism of the 5-HT_{2A} subtype, located in the VTA on both DA and non-DA neurons (Cornea-Hebert et al., 1999; Doherty & Pickel, 2000), apparently has little effect on basal firing of DA neurons (Gobert & Millan, 1999; Gobert et al., 2000; Pehek et al., 2001; Minabe et al., 2001). However, unlike the 5-HT_{2C} receptor, recent evidence suggests that the 5-HT_{2A} receptor subtype is involved in potentiating

stimulated DA release. Stimulated release of DA by either amphetamine (Porras et al., 2002), potassium (Pehek et al., 2001), or systemic application of 5-HT_2 agonists (Gobert & Millan, 1999) is reduced by antagonists of the 5-HT_{2A} receptor. The 5-HT_{2A} antagonist, M100,907 has also been shown to decrease the degree of burst firing of VTA DA neurons (Minabe et al., 2001) and to block stimulated release of DA in the mPFC when applied either systemically or locally (Pehek et al., 2001). Additionally, the 5-HT_{2A} antagonist SR46349B given systemically attenuates amphetamine-induced release of DA in the nucleus accumbens and striatum (Porras et al., 2002). These data taken together suggest that activation of the 5-HT_{2A} receptor facilitates phasic release of DA whereas the 5-HT_{2C} exerts a tonic and phasic inhibitory regulation of DA neurons in the VTA.

The 5-HT_3 receptor has also been shown to regulate DA transmission despite its fairly sparse distribution within the mesotelencephalic DA system (Palfreyman et al., 1993; Mylecharane, 1996; Morales et al., 1998; Puig et al., 2004). The distribution of this receptor is more prominent in the mesocorticolimbic pathway than the nigrostriatal pathway (Morales et al., 1998; Puig et al., 2004). The activation of this receptor is thought to facilitate DA release within the mesolimbic pathway (De Deurwaerdere et al., 1998). Evidence also demonstrates that blocking the 5-HT_3 receptor eliminated self-infusion of ethanol into the VTA in rodent models of alcohol self-administration, suggesting that this receptor facilitates the reinforcing effects of ethanol within the VTA possibly by blocking ethanol-stimulated release of DA (Campbell & McBride, 1995; Campbell et al., 1996; Rodd-Henricks et al., 2003).

The precise mechanisms through which serotonin receptors regulate DA transmission within the different areas of the mesotelencephalic pathway remain unclear. Systemic, as opposed to local, application of serotonin receptor agonists and antagonists are often employed which non-specifically engages target receptors throughout the brain, and as such, does not allow for the determination of specific roles of neuroanatomically-localized receptors. Additionally, the relative non-selectivity of compounds directed toward serotonin receptors makes interpretation of the literature difficult, although in recent years measures have been taken to remedy this limitation. Given these limitations, it is agreed that regulatory interactions between DA and serotonin throughout the mesotelencephalic pathway are important for the normal behaving mammal and

alterations within this regulatory system are implicated in psychopathologies such as addiction, aggression, and impulsivity (Campbell & McBride, 1995; Ferrari, 2003).

Dopaminergic Systems in the Central Nervous System: Dopamine and Behavior

Researchers have learned much about the roles of DA in normal behavior by studying impairments of behavior in lesioned animals, and in disorders in which DA is significantly altered. Undoubtedly, a profound appreciation for the role of DA and the basal ganglia arises from disease states. Functional implications of the DA system in disease range from psychotic ideation, stress, attention, drug abuse (cocaine, nicotine, and alcohol), motor dysfunction (Parkinson's disease, and Huntington's disease), and a combination of motor and affect dysfunction (LND).

Perhaps the most recognized role DA plays in behavior is its involvement in motor capabilities. The putative involvement of DA in this facet is readily observed in Parkinson's disease. Although the etiology of Parkinson's disease is unknown, there is an established neuropathology associated with the DA system. The behavioral manifestation of bradykinesia, rest tremor, rigidity, and postural instability are linked to the 80-97% loss of DA content with similar reductions in HVA, TH, and DAT (Beal, 2001). Dopamine transmission has also been linked with stereotypies and in particular intense grooming in rodents (Waddington 1986; Murray et al., 1990; Berridge & Aldridge, 2000). It has been shown that D_1 agonists elicit excessive stereotypical grooming in rodents (Berridge & Aldridge, 2000). In fact the hyperdopaminergic DAT knockout mouse displays increased stereotyped and rigidly predictable grooming sequences. This type of overly-rigid and predictable repetitive behavior has been likened to behavior seen in Tourette's syndrome and obsessive compulsive disorder (Berridge et al., 2005).

In addition to its regulatory role in motor activities, DA is also involved in affective (Miczek et al., 2002) as well as cognitive functions (Williams & Goldman-Rakic, 1995; Murphy et al., 1996; Gao et al., 2001; van den Heuvel et al., 2003; Guigoni et al., 2005). The combined functional modalities of DA (i.e. motor, cognition, and affect) are apparent in habit formation (Faure et al., 2005) and are suggested to participate in addictive behaviors (Tiffany, 1990). Cognitive modalities such as mnemonic and

planning functions have been shown to involve dorsal striatum activation (White & Salinas, 2003; van den Heuvel et al., 2003).

Moreover, the process of conditioned learning has a DA component, inasmuch as DA depletion results in defective conditioned learning (Nishii et al., 1998; Faure et al., 2005). Dopamine transmission, particularly in the striatum, has recently been shown to be involved in habit formation. In conditioned stimulus response paradigms, over-training rats to respond (e.g. lever press for food) for a reward renders the response habitual and relatively insensitive to post-training changes such as devaluation of reward (Ikemoto et al., 1999; Yin et al., 2004). Animals with depleted mesostriatal DA (via 6-OHDA lesion) are more sensitive than non-lesioned animals to the devaluation of food reward in a conditioned lever-pressing paradigm, suggesting that an intact DA system is required for habit formation (Faure et al., 2005). Moreover, ablation of the dorsolateral and not the dorsomedial striatum inhibits the formation of habitual behavior (Yin et al., 2004).

There is also evidence that alcohol ingestion by rats is relatively resistant to devaluation in the conditioned-response paradigm, suggesting that habitual behavior maybe involved in addictive disorders (Dickinson et al., 2002). Further supporting the idea of multiple functional modalities in addiction is a recent study involving cocaine self-administration in monkeys. Acute exposure (5 days) to cocaine decreased activity (measured by 2-[^{14}C]deoxyglucose utilization) in the ventromedial (limbic-related) striatum, whereas chronic exposure (over 100 days) led to depressed activity of the associative and dorsolateral (sensorimotor-related) as well as the ventromedial striatum (Porrino et al., 2004). Likewise, the sensorimotor, limbic, and associative areas of the striatum and globus pallidus have been shown to be involved in L-DOPA induced dyskinesia (Guigoni et al., 2005).

Although usually associated with the serotonergic system, aggressive and perhaps even impulsive behavior has also been associated with DA dysfunction (Miczek et al., 2002). A number of lines of evidence suggest that DA is involved in mediation of aggression, however, the mesocorticolimbic system is not specific for this behavior. Increases in DA have been noted in the mPFC and nucleus accumbens in anticipation of aggression in male rodents (Tidey & Miczek, 1996; Ferrari et al., 2003). Subsequent to

aggression, DA is elevated in both the nucleus accumbens and mPFC, whereas serotonin is decreased in the mPFC and not the nucleus accumbens (van Erp & Miczek, 2000). Additionally, in isolated-reared mice, where aggression is heightened, DA in the prefrontal cortex is elevated. When these mice are placed in a resident intruder paradigm, aggressive behavior is attenuated by 5-HT_{1A} agonism or by apomorphine (Matsuda et al., 2001). Anticipation and subsequent response to aggression appears to be dependent on an interactive serotonin/DA mesocorticolimbic system. Likewise, evidence is accumulating which suggests that serotonin/DA interaction within the mesocorticolimbic system is necessary for the control of impulsive behaviors. Although it is hypothesized that there are multiple forms of impulsivity (Winstanley et al., 2004a), an interactive role for the BLA, orbital frontal cortex, and nucleus accumbens has been proposed as an underlying circuit mediating this behavior (Cardinal et al., 2000, 2004). The delayed-discounting paradigm has been employed to measure impulsivity in rodents, in which the animal may obtain immediate small food rewards or delayed large food rewards, where higher responding in the former is a measure of impulsivity. Lesions of the BLA and nucleus accumbens increased impulsive choice, whereas lesions of the orbital frontal cortex increased ability to delay and thus obtain a larger food reward (Winstanley et al., 2004b; Cardinal et al., 2004). Intra-accumbal ablation of DA, via 6-OHDA lesion, does not induce impulsive behavior, but it does prevent impulsivity induced by 8-OH-DPAT, a 5-HT_{1A} agonist (Winstanley et al., 2005). Experiments investigating aggression and impulsivity demonstrate the intricate interaction of DA and serotonin in the mesocorticolimbic pathway in controlling both normal and maladaptive behaviors.

Ontogenesis of the Dopamine and Serotonin Neural Circuits

During early development, the brain relies on a synchronized series of events in which the foundation of neural networks that shape the mature central nervous system are formed (Berger-Sweeney & Hohmann, 1997). Prenatal development represents a time where neuronal morphogenesis, cell migration, and axon guidance occurs. Postnatal development represents a period of synaptogenesis and synapse elimination. Dopamine and serotonin transmission during these periods exerts substantial influence on target neurons during development and in particular serotonin release regulates its own

neuronal development (Lauder et al., 1982; Wallace & Lauder, 1983; Benes et al., 2000; Whitaker-Azmitia 2001).

Since the vast majority of the developmental investigations occur in rodents, it is important to be aware of developmental time-frame differences between mice, rats, and humans in order to accurately compare developmental events between species. Intuitively, human CNS development is more protracted than that seen in the rodent given the gestational time-table (21 days rat; 19 days mouse; 270 days human). At birth, rodents are less neuronally developed than humans are at mid-gestation. Additionally, after two weeks of post-natal development, rodents have the equivalent neuronal development of a 200 gestational day human (Clancy et al., 2001). Even between mice and rats, there exists a 2-3 day discrepancy in developmental time, which is substantial given the relatively short developmental period for these species (Clancy et al., 2001).

Despite species differences in timing, the mammalian brain shows a remarkable similarity in the pattern and sequence of neuronal ontogenesis across species. In both humans and rodents, DA neurons appear relatively early in prenatal development. In the rat, DA cell groups are apparent in the ventral mesencephalon on E13 (Voorn et al., 1988) and E11-12 for the mouse (Marti et al., 2002), while human midbrain DA neurons appear as early as the sixth week of gestation (Pickel et al., 1980; Freeman et al., 1991). In the rat, striatal striosomes (patches) receive DA projections relatively early (E19), and the diffuse DA innervation of both the striatal striosomes and matrix develops by the second post-natal week (Voorn et al., 1988). This pattern of protracted striatal matrix development in the rodent is quite similar to that of the human, in that DA arrival in the striosomes precedes that in the matrix by eight gestational weeks (Brana et al., 1996). It should be noted that although there is selective targeting of the striosomes, the ventral tegmental area and substantia nigra non-selectively target the striatum as a whole during early embryogenesis, but then by birth, the VTA innervates the nucleus accumbens and the substantia nigra targets the dosolateral striatum (Hu et al., 2004). The arrival of DA fibers at the future prefrontal cortex of the rat begins between E15-17, at which time DA is detected in the lateral neocortical primordium and the subplate of the prefrontal cortex anlage. Within two days after birth, DA fibers are apparent in the marginal zone, the future layer 1 of the prefrontal cortex, followed by layer VI on PND4 (Kalsbeek et al.,

1988). Innervation of the striatum and cortex of the rat are apparent only after birth when varicosities and structural changes in DA neuronal organization emerge (Voorn et al., 1988; Kalsbeek et al., 1988). The number of striatal DA varicosities increases considerably until adult levels are attained at PND20 (Voorn et al., 1988). By contrast, the number of DA fibers in the prefrontal cortex continues to increase from birth through PND60, at which time adult levels are achieved (Kalsbeek et al. 1988). It may be that the cortex requires a longer developmental period as a consequence of its complex neuronal network.

The role of DA in development is, in part, mediated by its receptors (primarily DAT, D_1 and, D_2), which appear early in embryonic development in both the striatum and cortex between embryonic days 14-16 (Foster et al., 1988; Guennoun & Bloch, 1991; Le et al., 1992; Reinoso et al., 1996; Jung & Bennett, 1996; Tarazi et al., 1998). The D_3 receptor is hypothesized to be involved in synaptogenesis since it is expressed early in life (PND7-10) in the nucleus accumbens (Stanwood et al., 1997). These receptors dramatically increase after birth, especially from PND7-35 when adult levels are achieved (Le et al., 1992; Jung & Bennett, 1996; Stanwood et al., 1997; Tarazi et al., 1998). Although DA concentrations are relatively low at birth (10% of adult content), it is apparent that DA exerts substantial influence on growth of both midbrain and target neurons (Restani et al., 1990; Le et al., 1992).

Since DA and its receptors are present in target areas such as the striatum and cortex well in advance of the completion of histogenesis, it has been inferred that DA and its receptors play a role in the development of both the cytoarchitecture and neural networks of these terminal areas (Lauder et al., 1982; Schambra et al., 1994; Mack et al., 1991; Reinsoso et al., 1996). Neonatal lesions of the VTA have shown that the loss of DA in the mesocortical pathway has a detrimental effect on the morphogenesis of the prefrontal cortex. When DA is depleted in the mPFC, layer V pyramidal cells had a 30% decrease in dendritic length and branching (Kalsbeek et al., 1989). Moreover, through the use of *in vitro* techniques, the putative role of DA and its receptors during development are being elucidated. Dopamine has been shown to dose dependently increase neurite length of cortical neurons, alternatively physiologically low concentrations of DA decreases soma and dendrite size (Reinoso et al., 1996). Dopamine

has also been shown to inhibit growth cone motility and elongation of neurites (McCobb et al., 1988). These differential effects of DA on neurites may be attributable to DA concentrations used in different experimental procedures or the differential expression of DA receptor subtypes present.

The location and subtype of DA receptors are important because, through their activation, DA exerts its morphogenic actions. For example, activation of the D_1 receptor promotes striatal neuronal morphogenesis (examined in tissue culture with samples taken at E17) by increasing growth cones, neurite length, and neuronal aborization (Schmidt et al., 1996). In the developing rat striatum (E17), D_1 signaling controls functional differentiation of GABA neurons by decreasing GAD67 mRNAs, increasing GAD65 expression and increasing GABA synthesis (Kuppers et al., 2000). In contrast to its role in the striatum, D_1 receptor activation in the developing cortex (examined in tissue culture with samples taken at E16-18) has been shown to inhibit neurite outgrowth and enhance neuronal maturation (Todd, 1992; Reinoso et al., 1996). The D_2 agonist, quinpirole, has been shown to increase the length of neurites and the number of branch points per neurite (Todd, 1992; Reinoso et al., 1996).

Similar to DA, serotonin has been implicated in the developmental process of its target areas (Lauder et al., 1982; Wallace & Lauder, 1983). Serotonergic cells are some of the earliest to be generated (E11) in rodent (Wallace & Lauder, 1983) and (G5-12 weeks) in the human raphe nuclei (Sundstrom et al., 1993; Herlenius & Lagercrantz, 2001). In the rat, axon collaterals from the raphe reach the neocortex by E17 (Wallace & Lauder, 1983; Lidov & Molliver, 1982a,b) then display a protracted innervation of the cortex extending from E19-PND21 (Lindov & Miller, 1982b). Similar to DA, serotonin levels increase sharply after PND6 and double from birth to the juvenile stage in rats (Restani et al., 1990).

Fluctuations in serotonin receptor densities throughout development suggest an ontogenetic role in the development of serotonin neurons and target areas. Candidate receptors for developmental control are the 5-HT_{1B}, 5-HT_2, and 5-HT_{1A} receptors. The 5-HT_{1A} receptor develops rather early appearing around E12, peaking at E15, then declines to adult levels in the rat brain (Hillion et al., 1993). This pattern is also seen in human development where the peak 5-HT_{1A} densities occur early between G16-24 weeks and

subsequently declines (Bar-Peled et al., 1991). It is believed that this receptor subtype influences the target cell differentiation and autoregulation of serotonin neurons (Whitaker-Azmitia et al., 1987; Sikich et al., 1990; Azmitia, 2001). The 5-HT_2 receptor is first detectable by immunohistochemisty at E19, and by PND4-7 there is an elaborate cortical distribution of these receptors with increasing density through PND20 at which point these receptors subsequently recede (Morilak & Ciaranello, 1993). The 5-HT_2 receptor has been proposed to be involved in neuronal proliferation (Brezun & Daszuta, 2000; Azmitia, 2001), however due to its arrival during the early post-natal period it is an unlikely candidate for this action (Morilak & Ciaranello, 1993). This receptor may however, play a role in early plasticity of neuronal development since the 5-HT_2 receptors (both A and C subtypes) are sensitive to a number of external factors, including maternal stress, which induces a long-term increase in the levels of these receptors in the offspring (Peters, 1988). Additionally, the 5-HT_2 receptors appear during rapid synaptogenesis of the cortex, adding temporal support to a possible role in this process. Densities of SERT throughout development are dynamic. Qualitative data demonstrates that SERT first appears in the midbrain of the mouse by E13 (E15 of the rat), then progressing to the cerebral cortex by E16 and striatum, amygdala, hypothalamus, and substantia nigra between E17-18 (Bruning et al, 1997). By PND7, the distribution of SERT resembles that seen in the adult (Zhou et al., 2000). Also during embryogenesis, there is a transient expression of SERT (starting on E13) on non-serotonin neurons thought to be thalamocortical (Bruning & Liangos, 1997; Zhou et al., 2000; Verney et al., 2002). The role of SERT in development should not be underestimated, since it is the primary mechanism for removal of serotonin from the synapse and ultimately controls extra-neuronal serotonin availability.

Similar to DA, serotonin influences target cells and neural networks during development. In organotype cell culture from newborn rats, physiological concentrations of serotonin has been shown to stimulate proliferation of glial cells, promote neuronal differentiation and synaptogenesis of cortical neurons, and increase spontaneous firing of cortical neurons (Chubakov et al., 1986). Serotonin has also been shown to inhibit growth cone motility and elongation of neurites (McCobb et al., 1988). Further, serotonin displays both a positive and negative autoregulation involving its own neuronal

terminal density, particularly in the cortex (Whitaker-Azmitia, 2001). The autoregulatory action of serotonin occurs through a direct and indirect pathway. The indirect pathway involves the astroglial-derived S-ß100 protein, which is stimulated by serotonin through the 5-HT_{1A} receptor, as well as metabotropic glutamate receptors and adenosine-1 receptors (Whitaker-Azmitia et al., 1990a; Ciccarelli et al., 1999; Ahlemeyer et al., 2000). S-ß100 has been shown to increase neurite outgrowth of serotonin neurons (Azmitia et al., 1990; Liu & Lauder, 1992). Interestingly, S-ß100 inhibits the spatial expansion of tyrosine-hydroxylase immunoreactive (TH-ir) neurites in the midbrain during early embryonic (E14) development. However, both DA and serotonin cell body growth is stimulated by insulin-like growth factor II (IGF-II) which also promotes neurite outgrowth of TH cells (Liu & Lauder, 1992). Although physiological concentrations of serotonin promote synaptogenesis, Chubakov et al. (1986) found that under-stimulation (serotonin depletion) and over-stimulation (5-methoxytryptamine, general serotonin receptor agonist) during embryonic development in the rat (E12-17) results in decreased 5-HT_{1A} receptor densities (Whitaker-Azmitia et al., 1987; Lauder et al., 2000). It has also been shown that early post-natal depletion of serotonin by 5-methoxytryptamine or 5-HT_{1A} antagonism decreases dendritic synaptogenesis of the hippocampus resulting in learning deficits (Mazer et al., 1997; Faber & Haring, 1999). The direct pathway of serotonergic development is an inhibitory action of growth initiated by excess serotonin availability (Whitaker-Azmitia & Azmitia, 1986; Shemer et al., 1991), mediated through the 5-HT_{1B} autoreceptor (Whitaker-Azmitia 2001).

Of equal importance is the suggestion that serotonin exerts a negative influence on the developing DA system in both the midbrain and prefrontal cortex (Benes et al., 2000). Evidence for this idea is derived from neonatal serotonin lesions which show that, in the absence of serotonin, TH-ir fibers increase in both the midbrain (VTA and substantia nigra) and in the prefrontal cortex. Interestingly the increase in prefrontal cortex occurs primarily in layers V and VI where copious amounts of DA fibers typically reside (Bolte Taylor et al., 1998). Likewise, when DA is lesioned during early post-natal development, serotonergic fibers are significantly increased in the striatum (Reader et al., 1995). Furthermore, abnormal activation of D_1 receptor during the embryonic period results in reduced SERT binding in the brainstem (Whitaker-Azmitia et al., 1990b).

These data suggest an interdependence of DA and serotonin during development (Whitkaer-Azmitia et al., 1990b).

The development of DA and serotonin does not cease after weaning in the rodent or after early childhood in humans. Evidence suggests that both neurotransmitters continue to change and develop well into adolescence across mammalian species which is necessary of the maturation of the central nervous system (Goldman-Rakic & Brown, 1982; Seeman et al., 1987; Segawa, 2000; Kalsbeek et al., 1988; Le et al., 1992). During development there is an overgrowth of DA and serotonin pathways resulting in more synaptic connections than needed in the mature central nervous system. During the adolescent and peri-adolescent stage in rodents, as well as humans, there is substantial regional pruning of the DA system. As a result, synaptic connections which are not needed are pruned. In fact, it is estimated that synaptic density is decreased up to 40% in the frontal cortex in the transition period from childhood to adulthood (Huttenlocher et al., 1979; Rakic et al., 1986). There is evidence that serotonin terminals are maximal during early development only to be pruned during early adolescent development (Galineau et al. 2003). Dopamine terminals in both the striatum and frontal cortex peak during adolescence in rodents (between PND35-60), with DAT peaking around 35PND (Tarazi et al., 1998), TH activity by PND 49, DA content by PND 56 (Le et al., 1992), DA fiber density in the prefrontal cortex by PND60 (Kalsbeek et al., 1988), and striatal $D_{1/2}$ receptors by PND40 (Teicher et al., 1995). During the adolescence/adult transition period (PND60-120) of the rat, there is a significant pruning of D_1 and D_2 receptors in the striatum, but not in the nucleus accumbens (Teicher et al., 1995; Andersen et al., 2000). In the prefrontal cortex, D_1 and D_2 receptors are pruned from PND40 through PND120 (Andersen et al., 2000). By contrast, D_2 levels are lowest at PND60 for the striatum and PND80 in the prefrontal cortex. Likewise, the pruning process for the D_1 receptor is protracted in the prefrontal cortex with a nadir at PND100 compared to PND80 for the striatum (Andersen et al., 2000; Teicher et al., 1995). This protracted pruning process in the prefrontal cortex is perhaps a consequence of the longer developmental time of the cortex compared to that of the striatum (Andersen et al., 2000).

In comparing developmental processes between DA and serotonin neurons, data suggests that adult concentrations of serotonin receptors reach adult levels earlier than do

markers of DA. The maturation of these two neurotransmitter systems alludes to their relative roles and vulnerabilities during development. The arrival and subsequent maturation of DA and serotonin neurons corresponds to synaptogenesis and differentiation of the cortex, which begins around E16 and is carried through well into adolescence and adulthood. The innervation and maturation of serotonin neurons in the cortex by E17 (Wallace & Lauder, 1983; Lidov & Molliver, 1982a,b) and adult pattern by PND28 (Morilak & Ciaranello, 1993; Galineau et al. 2003), indirectly suggests that serotonergic influence on cortical development has a relatively short critical period coinciding with rapid synaptogenesis of the cortex. Alternatively, DA neurons reach the cortex by E15-17 (Kalsbeek et al., 1988), but adult levels are not established until PND120 (Anderson et al., 2000). This protracted developmental period of innervation (up to PND60) and subsequent pruning (up to PND120) of DA neurons in the cortex suggests that DA plays a role in the differentiation and maturation of cortical neural circuits.

The developmental period represents a critical phase during which disturbances affecting DA and serotonin concentrations may result in profound behavioral and cognitive consequences. Inherited diseases such as LND where DA is depressed during post-natal development (Lloyd et al., 1981), and Brunner's syndrome (Brunner et al., 1993) where serotonin is elevated during pre- and post-natal development, are cases in point where monoaminergic alterations during development results in profound behavioral abnormalities including aggression and impulsivity (Lesch & Nyhan, 1964; Brunner et al., 1993). It has been argued that the developmental abnormalities in LND are a result of a neurochemical lesion leading to an impairment of aborization in the nigrostriatal pathway (Baumeister & Frye, 1985). A lack of DA during post-natal development would likely affect the density of D_1 receptors (Frohna et al., 1995) and in turn would have profound effects on neuronal arborization (Schmidt et al., 1996) as well as GABA synthesis (Kuppers et al., 2000). Furthermore, excess pruning within the cortex is also a possibility due to the lack of DA innervation (Huttenlocher et al., 1979; Rakic et al., 1986), however, neuroanatomical evidence for this assertion is currently unavailable. Alternatively, monoamine oxidase inhibition during development would raise serotonin concentrations. Elevated serotonin during development would lead to

decreases in 5-HT_{1A} receptor densities (Whitaker-Azmitia et al., 1987; Lauder et al., 2000), which would reduce target cell differentiation during early pre-natal development (Whitaker-Azmitia et al., 1987; Sikich et al., 1990; Azmitia, 2001). Elevated negative autoregulation of the serotonin neuronal terminal field, particularly in the cortex, would also be an expected consequence of increase serotonin concentrations during development (Whitaker-Azmitia, 2001).

It is evident that both DA and serotonin are factors in the development of target tissue including the cortex and striatum. Disturbances in the concentrations of these monoamines may have dramatic effects on neural networks resulting in abhorrent behaviors. Although there are data to support this notion, there remain important gaps concerning the development of the DA and serotonin systems. This is particularly evident in situations where these neurotransmitters are elevated, as opposed to depressed, during development (Berger-Sweeney & Hohmann, 1997).

MAO Inhibition During Development

In 1993, Brunner et al. described a kindred in which affected males display violent impulsive and aggressive behaviors as well as mild mental retardation (Brunner et al., 1993a,b). Genetic analysis of affected males demonstrated a point mutation of the monoamine oxidase A (MAO-A) gene (Brunner et al., 1993a,b) in affected family members, resulting in a complete deficiency of enzymatic activity (Brunner et al., 1993b). Historically, low levels of serotonin and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) have been thought to be associated with aggression and impulsivity (Brown et al., 1982; Brown & Linnoila, 1990; Kruesi et al., 1990; Soderstrom et al., 2001). However, in affected individuals in the kindred described by Brunner et al (1996) serotonin levels were significantly elevated, leading to an intriguing exception to the low serotonin-high aggression paradigm.

In the mammalian brain MAO comes in two forms, A and B, which have substrate specificity for the degradation of DA and serotonin. In the primate (human and non-human), MAO-A preferentially degrades serotonin, while MAO-B preferentially degrades DA (Glover et al., 1977; Kaseda et al., 1999; Lakshmana et al., 1998). In the mouse, MAO-A effectively catalyzes the degradation of both DA and serotonin under

basal conditions. Evidence for this assertion is derived from MAO knockout mice, with MAO-A knockout mice displaying elevated levels of serotonin and DA (Cases et al., 1996), while MAO-B knockout mice display normal basal levels of DA (Fornai et al., 1999). However, when L-Dopa is administered systemically, raising DA levels, MAO-B knockout mice display a larger increase of DA than wild-type control mice (Fornai et al., 1999). This suggests that, under stimulated conditions, MAO-B plays an important role in the degradation of DA. Additionally, the MAO-B inhibitor, deprenyl, does not affect basal levels of DA (Ingram et al., 1993; Steyn et al., 2001). Similar to the mouse, MAO-A preferentially degrades serotonin and DA in the rat (Wachtel & Abercrombie, 1994; Lamensdorf et al., 1996) and MAO-B has little effect under basal conditions (Wachtel & Abercrombie, 1994; Brunnan et al., 1995; Lamensdorf et al., 1996).

There are a number of clinically used compounds that inhibit MAO activity and elevate serotonin availability, but in general, elevated aggression is not associated with the use of MAO inhibitors (Kaplan & Sadock, 1998). Indeed, irreversible MAO-A inhibition by clorgyline has been shown to be clinically effective as both an antidepressant and anti-anxiety drug in humans (Lipper et al., 1979; Pare, 1985). Behaviorally, MAO inhibitors such as clorgyline (MAO-A inhibitor) and deprenyl (MAO-B inhibitor) do not induce abhorrent behaviors such as aggression in adult animals. On the contrary, clorgyline administration has been shown to attenuate footshock-induce aggression in adult rats (Datla et al., 1991) and deprenyl attenuates impulsive behavior in spontaneously hyperactive rats (Boix et al., 1998). Locomotor activity has been reported to be increased (Barbelvien et al., 2001) and decreased (Popova et al., 2000) following clorgyline treatment (Engberg et al., 1991). Increased locomotor activity has also been reported following deprenyl treatment (Engberg et al., 1991). There are a number of receptor densities which change after MAO-A inhibition including a decrease in 5-HT_{1C} binding in the hypothalamus and striatum (Hulihan-Giblin et al., 1994), no effect on 5-HT_{1A} densities in the raphe or terminal areas (Hensler et al., 1991), and a decrease in 5-HT_2 binding in the frontal cortex (Twist et al., 1990; Goodnough & Baker, 1994). Neither MAO-A nor MAO-B inhibition, by clorgyline and deprenyl respectively, alters DAT or SERT binding densities in adult animals (Scheffel et

al., 1996; Yeghiayan et al., 1997; Innis et al., 1999; Lamensdorf et al., 1999; Fowler et al., 2001).

The clinical findings by Brunner et al. (1993a,b) cannot be trivially explained by MAO inhibition, since it is clear that inhibition by clorgyline or deprenyl in the adult does not result in aggressive and impulsive behavior, nor does it result in borderline mental retardation. Perhaps having diminished MAO activity during development is a critical factor in the behavioral abnormalities reported by Brunner et al. (1993a,b). In an attempt to understand this process, MAO-A, MAO-B, and MAO-A/B knockout mice have been generated (Cases et al., 1995; Fornai et al., 1999; Chen al., 2004). MAO-A and MAO-A/B knockout mice demonstrate enhanced male aggression in the resident-intruder paradigm, while MAO-B mice do not display this behavior (Cases et al., 1995; Grimsby et al., 1997; Chen et al., 1999; Holschnieder et al., 1999; Chen et al., 2004). These behavioral findings lend support to a developmental process in which the absence of MAO-A activity leads to aggressive behavior (Cases et al., 1995; Kim et al., 1997; Chen et al., 2004). Neurochemically, MAO-A and MAO-A/B knockout mice have significantly elevated serotonin concentrations along with a less pronounced increase in norepinephrine, and DA during development and attenuate to control levels by adulthood (Cases et al., 1995; Chen et al., 2004).

To date, relatively few studies are available which examine the neuroanatomical consequences of elevated serotonin during development resulting from MAO depletion. Densities of 5-HT_2 receptors, which appear during rapid synaptogenesis of the cortex (Morilak & Ciaranello, 1993), are decreased in the frontal cortex of MAO-A knockout mice (Shih et al., 1999). Behaviorally the systemic administration the 5-HT_{2A} antagonist ketanserin, was able to abolish aggressive behavior in these mice (Shih et al., 1999). Additionally, SERT binding is lower in MAO-A knockout mice as compared to wildtype controls in the dorsal raphe nucleus (Owesson et al., 2002). These data suggest that the aggressive behavior in MAO-A knockout mice is, at least in part, due to an altered mesocorticolimbic pathway involving both DA and serotonin.

Additionally the 5-HT_{1A} receptor is reduced in the hippocampus of MAO-A knockout mice (Shih et al., 1999). This receptor density reduction maybe a direct consequence of elevated serotonin concentrations during development which has been

shown to suppress 5-HT_{1A} receptor densities (Whitaker-Azmitia et al., 1987; Lauder et al., 2000). Functionally, a reduction in 5-HT_{1A} receptor density may result in decreased dendritic synaptogenesis in hippocampus leading to impaired learning (Mazer et al., 1997; Faber & Haring, 1999). Also as a direct consequence of elevated serotonin levels during development, MAO-A knockout mice display impaired formation of the barrel field of the somatosensory cortex, which is a somatotopic representation of the whiskers in the mouse (Cases et al., 1995, 1996).

The complete absence of MAO-A/B activity is not required for behavioral and neurochemical alterations. Inhibition of MAO-A/B by clorgyline and deprenyl throughout gestation and early post-natal development has been shown to induce pervasive aberrant behavior in both mice and rats (Whitaker-Azmitia et al., 1994; Mejia et al., 2002). MAO-A/B inhibited rats were behaviorally characterized by impulsivity measured by the passive avoidance paradigm, visual impairments, and aggressive behavior toward cage-mates and handlers (Whitaker-Azmitia et al., 1994). MAO-A/B inhibited mice also exhibit an elevated aggressive behavior measured by the resident intruder paradigm and a tendency for impulsive behavior measured by the differential reinforcement of low rate responding paradigm (Mejia et al., 2002).

Neuroanatomically, MAO-A/B inhibited rats present normal DA innervation measured by DAT autoradiography and altered cortical, hippocampal, and caudate serotonin innervation measured by relative SERT densities (Whitaker-Azmitia et al., 1994). The densities of SERT in the cortex were reduced at PND5, elevated at PND15, and reduced at PND30 as compared to control animals. Hippocampal and caudate SERT densities were elevated at PND5 and were not different from control values at PND15 or PND30 (Whitaker-Azmitia et al., 1994). However, this study does not address long-term consequences of MAO deprivation on either DAT or SERT densities in the cortex, hippocampus, or caudate and provides no data on the midbrain region. It would be expected that the cell body region of the serotonergic system, the raphe nuclei, would undergo alterations given the pronounced changes in the terminal field.

Continuous deprivation of MAO during development provides a reliable and reproducible model in which to investigate pervasive aggressive and impulsive behaviors (Whitaker-Azmitia et al., 1994; Mejia et al., 2002; Chen et al., 2004). The complex

effects on the developing neurotransmitter systems by MAO inhibition are likely to be principally responsible for the resulting aberrant behavior (Brunner, 1996). Certainly, previous work clearly demonstrates neuroanatomical and behavioral abnormalities following MAO inhibition during development. However work in the rat model did not examine the cell body regions of DA and serotonin. Furthermore, the previous neuroanatomical analysis did not extend into adulthood. There is substantial evidence that the DA and serotonin neuronal systems are still developing well after PND30. The present investigation examines the relative DA and serotonin innervation of cortical and subcortical areas, measured by DAT and SERT densities, following MAO inhibition in mice throughout gestation and early post-natal development. The working hypothesis for this study is that MAO inhibition during development reduces serotonin innervation, as revealed by SERT binding densities at different developmental periods, in various brain regions. Since the primary deamination of serotonin occurs through the action of MAO, it is hypothesized that MAO-A/B inhibited mice will display a reduction in SERT binding in the cortex and raphe throughout the development and into adulthood. It is also hypothesized that SERT binding in the hippocampus will be unaffected at the developmental time-points investigated in the present study. Additionally, it is hypothesized that relative innervation of DA will be largely unaffected by MAO inhibition since deamination of catecholamines also occurs through catechol-*O*-methyl transferase (COMT) thus reducing the potential increases DA following MAO inhibition (Eisenhofer et al., 1994, 1996; Lenders et al., 1996). Furthermore it is hypothesized that relative DAT and SERT densities will follow a similar pattern between MAO inhibited animals and control animals.

Hypoxanthine and the Nigrostriatal Dopamine System

Under normal conditions the purine cycle is a highly regulated system of synthesis, recycling, and degradation (Seegmiller, 1975; Kelley et al., 1975; Holmes et al., 1975). In tissue where xanthine oxidase is present (i.e. the liver), the end product of purine metabolism is uric acid; and since the brain is relatively devoid of xanthine oxidase, the majority of uric acid is produced outside of the brain (Rosenbloom et al., 1967). The end product of purine metabolism in the brain is generally considered to be

Hx. In healthy individuals, this compound is recycled by the enzyme hypoxanthine-phosphoribosyltransferase (HPRT). IMP, the product of this reaction, regulates *de novo* synthesis of purines through negative feedback inhibition. Unsalvaged Hx is then further oxidized by xanthine oxidase to form xanthine, or ultimately, uric acid (Seegmiller, 1975; Rosenbloom et al., 1967; Kelley et al., 1975; Holmes et al., 1975).

Lesch-Nyhan disease (LND) is an X-linked inherited disorder of purine metabolism (Nyhan, 1973), which is traced to a total or nearly total deficiency in HPRT activity. As a result of this enzymatic deficiency, *de novo* synthesis goes unregulated (Rosenbloom et al., 1967) leading to an overproduction of Hx, which in turn causes an increase of Hx in the brain and of uric acid in the periphery (Lesch & Nyhan, 1964; Rosenbloom et al., 1967; Nyhan, 1973). As a point of reference, patients with LND have cerebrospinal fluid (CSF) Hx concentrations four times higher than normal and plasma uric acid levels double that found in controls (Sweetman, 1968; Rosenbloom et al., 1967).

There is a well understood sequence of events leading from the deficiency of HPRT to hyperuricemia, urinary tract stone disease, and nephropathy, which eventually causes renal failure or other related complications. In the periphery, elevated levels of uric acid are directly related to the array of kidney related problems faced by LND patients (in fact orange uric acid crystals in the diapers of these patients is often the first noticed indicator that leads the physician to the diagnosis of LND). In rare cases, kidney failure may lead to the death of the patient, but more commonly the patient dies of aspiration pneumonia secondary to the neurological impairments (Kelley & Wyndgaarden, 1989; Nyhan, 1973; Lesch & Nyhan, 1964). The neurobehavioral manifestations of this disorder include choreoathetosis, spastic cerebral palsy, self-mutilatory behavior, and aggressiveness; these manifestations are not readily explained as purine regulatory anomalies (Lesch & Nyhan, 1964; Nyhan, 1973). Despite the gaps of our knowledge concerning the neuropathogenesis of LND, neurochemical abnormalities within the basal ganglia suggest this area is responsible for the behavioral characteristics associated with LND despite the absence of consistent observable lesions (Lloyd et al. 1981). More specifically, post-mortem biochemical analysis in LND patients has documented reduced levels of DA in striatal tissue (Lloyd et al., 1981).

Neurochemistry of Lesch-Nyhan Disease

Clinical evidence supports the notion that a wide range of neurochemical abnormalities marks LND, including GABA, serotonin, norepinephrine, and DA. In one study, levels of glutamic acid decarboxylase, the enzyme which converts glutamic acid to GABA, were normal in three post-mortem brains of LND (Lloyd et al., 1981), but later, Rassin et al. (1982) found GABA to be increased in the putamen. It has also been reported that Hx binds to the benzodiazepine receptor (Asno & Spector, 1979), which in turn modulates the GABA-A receptor. At high concentrations, perhaps Hx interferes with the ability of the benzodiazepine receptor to modulate GABA receptors (Kish et al., 1985).

Changes in serotonin have also been observed in LND, although the data are inconsistent across studies. In post-mortem tissue of three patients both serotonin and 5-HIAA, the main metabolite of serotonin, were increased (Lloyd et al., 1981). CSF 5-HIAA was increased in three out of five patients in one study (Jankovic et al., 1988), but was within the normal range in four patients of different ages in a separate investigation (Silverstein et al., 1985). Norepinephrine has also been studied in LND with conflicting results. In six patients plasma dopamine-β-hydroxylase (DBH) activity was elevated and an absence of pressor response to sympathetic stimulation was reported (Rockson et al., 1974). In another study fourteen patients were shown to have significantly low plasma DBH, and normal levels of norepinephrine, but under postural stress, norepinephrine levels increased less than in normal subjects (Lake & Zeigler, 1977). Low CSF levels of MHPG (3-methoxy-4-hydroxy-phenylglycol), the main metabolite of norepinephrine, were also reported, but there was a marked decrease in only two out of five patients (Jankovic et al., 1988). Lloyd et al., (1981) found no significant abnormality in norepinephrine levels in the nucleus accumbens or substantia nigra.

Of all the neurochemical changes observed in LND, the most consistent and significant change is a decrease of DA and associated neuronal proteins (Lloyd et al., 1981; Silverstein et al., 1985; Jankovic et al., 1988; Wong et al., 1996; Earnst et al., 1996; Endres et al., 1997; Saito et al., 1999). These abnormalities have been observed in all LND subjects that exhibit self-mutilitory behavior. Post-mortem biochemical analysis

found reduced concentrations of DA and its metabolite, homovanillic acid (HVA), in the caudate, putamen, external pallidum, and nucleus accumbens in LND patients compared to control subjects (Lloyd et al., 1981). Reduced HVA levels in CSF of LND patients have also been reported (Silverstein et al., 1985; Jankovic et al., 1988). Neuroanatomically, Wong et al. (1996) found a marked reduction of DAT in the striatum through the use of positron emission tomography (PET). In another PET study using F^{18}-fluorodopa, Earnst et al. (1996) found that presynaptic accumulation of this tracer was decreased in the caudate, putamen, frontal cortex, VTA, and substantia nigra in LND patients compared to control subjects. The uptake of the tracer F^{18}-fluorodopa is used to measure *in vivo* DA neuronal activity where a low ratio of specific to nonspecific radioactive counts indicates a decrease in dopa decarboxylayse activity and DA storage. The findings of abnormally low F^{18}-fluorodopa uptake in LND patients (Earnst et al., 1996) maybe attributable to a decrease of DAT (Endres et al., 1997) but may also reflect a decrease of DA terminals and cell bodies (Earnst et al., 1996). The volume of the caudate nucleus of LND patients has also been found to be reduced by 30% via MRI analysis, which suggests a change specific to the DA terminal field in LND (Wong et al., 1996; Harris et al., 1998).

Model Systems of Lesch-Nyhan Disease

The gap in our understanding between the enzymatic deficiency seen in LND and the resultant neurochemical, behavioral, and cognitive manifestations of LND in combination with limited success in treatments underscores the need for further understanding of the underlying neurochemical and neuroanatomical abnormalities of LND. An effective model which mirrors the neurochemical alterations seen in LND would provide a substrate in which to investigate the neuroanatomical and behavioral consequences of these alterations. Model systems used to investigate the basis of neuropathology in LND include genetic mouse models (HPRT knockouts), neonatal 6-OHDA lesions and tissue culture studies (Breese et al., 1984; Bitler & Howard, 1986; Kuehn et al., 1987; Jinnah et al., 1994; Yeh et al., 1998). These model systems are designed to explain distinct aspects of LND in which they have been particularly beneficial.

Neonatal Lesion Model

6-OHDA lesions have been used to study the neuroanatomy and neurochemistry of the DA system in relationship to DA depletion and motor impairments (Ungerstedt, 1968; Ungerstedt, 1971; Schwarting & Huston, 1996). 6-OHDA is taken up into the DA neuron by the DAT where it undergoes auto-oxidation, generating free radicals and hydrogen peroxide (Decker et al., 1993). These compounds are believed to interfere with the mitochondrial respiratory chain (Glinka et al., 1997) resulting in cytotoxicity at the DA nerve terminals which leads to DA depletion and behavioral abnormalities directly related to the motor system (Schwarting & Huston, 1996). Bilateral lesions of the striatum in adult rats results in receptor supersensitivity to apomorphine which suggests changes in the receptor sensitivities. Moreover apomorphine causes a marked increase in locomotion and stereotypies which is thought to be a result of supersensitive receptors (Kelley et al., 1975). In adult rats, 6-OHDA lesions are used as models of Parkinson's disease due to the resultant loss of DA (Moy et al., 1997).

When 6-OHDA is administered neonatally, the behavioral and neuroanatomical outcome is markedly different and as a consequence, has been proposed as an animal model of LND. Neurochemically, adult and neonatally lesioned animals both show a marked decrease in DA and its metabolites. Adult lesioned animals display supersensitivity at the D_2 receptor whereas neonatally lesioned animals have a D_1 supersensitivity as measured by locomotor activity (Moy et al., 1997). A striking difference between neonatally and adult lesioned animals occurs when a mixed $D_{1/2}$ agonist is administered. The adult lesioned group demonstrates hyperactivity whereas the neonatal lesioned animal displays self-mutilatory behavior (Breese et al., 1984a,b). This self-mutilatory behavior, resulting from the administration of mixed DA agonists following neo-natal DA lesion, is prevented by the application of a D_1 receptor antagonist (Breese et al., 1984b, 1990). These data suggest that the timing of insult to the DA system is in part responsible for the affective dysfunction in LND and that the D_1 supersensitivity plays a role in this dysfunction.

In addition to the alterations in DA functioning, there is a serotonergic hyperinnervation striatum in the neonatally lesioned animal accompanied by elevated serotonin, 5-HIAA, and SERT concentrations (Luthman et al., 1987). Along with the

hyperinnervation of serotonin there are changes in serotonin receptors within the basal ganglia. The striatum, substantia nigra and globus pallidus all demonstrate increases in 5-HT_{1B} and 5-HT_{1nonAB} receptors. Additionally, in the rostral striatum there is an increased 5-HT_{2A} receptor concentration (Radja et al., 1993b; El Mansari et al., 1994). Functionally this serotonin innervation is important since 5-HT_{2A} receptor antagonists reduce hyperactivity in the neonatally lesioned animals (Luthman et al., 1991).

The importance of this model is that despite a similar loss in DA, the developmental period in which an insult is introduced has significant influence in the resultant neurochemical and behavioral response of the DA system. This model also demonstrates the role serotonin has in the regulation of the DA system in response to neurochemical insults in neonatal and adult animals. This model helps to explain the behavioral differences seen between Parkinson's disease and LND. Although DA depletion is similar between these two diseases the behavioral differences are likely due to neonatal versus adult onset of the loss.

Genetic Mouse Model

In an attempt to mimic LND, a transgenic HPRT knockout ($HPRT^-$) mouse has been produced (Kuehn et al., 1987; Dunnett et al., 1989; Jinnah et al., 1991; Jinnah et al., 1993, Jinnah et al., 1994). These mice, like LND patients, are devoid of HPRT activity and demonstrate neurochemical abnormalities. Dopamine depletions ranging from 20-60% have been reported (Dunnett et al., 1989; Jinnah et al., 1994) with the largest depletions being within the striatum (Jinnah et al., 1994). Reductions of DA concentrations are reported in the striatum, olfactory tubercle, olfactory bulb, prefrontal cortex, substantia nigra, and sensorimotor cortex (Dunnett et al., 1989; Jinnah et al., 1994). No differences in HVA were found in the striatum (Dunnett et al., 1989; Jinnah et al., 1994) or nucleus accumbens, but decreases of HVA were found in the cortex, diencephalon, midbrain, brainstem, and hippocampus (Jinnah et al., 1994). Reduced DOPAC was also reported in the striatum, diencephalon, and midbrain of HPRT knockout mice (Jinnah et al., 1994). These decreases were accompanied by a decrease in striatal and cortical tyrosine-hydroxylase activity and a decrease in forebrain concentrations of DAT (Jinnah et al., 1994). These neurochemical and neuroanatomical

data suggest that HPRT knockout mice display DA deficiency throughout the mesotelencephalic pathways. Evidence suggests oxidative stress may play a role in this model since there is a greater reduction of striatal DA concentrations after 6-OHDA challenge in $HPRT^-$ as compared to controls ($HPRT^+$). Such vulnerability may in part explain the neurochemical abnormalities seen in the $HPRT^-$ mice. However, individual markers of oxidative stress were not elevated in the knockout mice (Visser et al., 2002).

Behaviorally, HPRT knockout mice do not display any spontaneous abnormalities, however, they are more sensitive to amphetamine stimulated stereotypical behavior and locomotor activity (Jinnah et al., 1991). Even when challenged with pharmacological agents known to induce self-injurious behavior in rats, HPRT knockout mice did not display this behavior at a greater level than wild-type mice (Kasim & Jinnah, 2002). Although the HPRT knockouts share the same etiology as LND, they do not exhibit any spontaneous neurobehavioral abnormalities and do not exhibit elevated levels of Hx which are characteristic of LND (Jinnah et al., 1993). It has been suggested that in HPRT knockout mice, Hx is converted to inosinic acid via the purine nucleoside phosphorylase and inosine kinase enzymatic pathways (Allsop & Watts, 1990).

In Vitro Models

In an attempt to determine the cellular underpinnings of LND, numerous cell culture experiments have been undertaken. The majority of these studies involve examining the developmental process and DA/HPRT relationships in HPRT-deficient cell lines. In HPRT-deficient non-DA neuroblastoma cells, cellular adhesion was increased resulting in an increased differentiation with a decrease in proliferation. These cells also have an increased morphological complexity due to a decreased neurite growth. Additionally, within these cells, Hx elevates levels of secondary and tertiary branching (Stacey et al., 2000; Connolly, 2001; Connolly et al., 2001; Ma et al., 2001). These data suggest an inherent problem within HPRT deficient cells, however it does not explain why the DA system is preferentially affected by this deficiency. In a different HPRT deficient cell line, one of which contains DA (PC12 $HPRT^-$), cells did not undergo neuronal differentiation in the presence of DA. If the DA content is reduced to less than 8% of normal levels, cells underwent normal differentiation indicating that DA acts as a neurotoxin in the HPRT deficient cells (Yeh et al., 1998). In an attempt to look

specifically at the relationship between Hx and DA, Poulsen et al. (1993) found that Hx increased spontaneous DA outflow in a dose and time dependent manner in striatal synaptosomes. The DA release was prevented by catalase, again suggesting Hx plays a role in oxidative stress (Poulsen et al., 1993). Furthermore Hx has been found to inhibit Na^+, K^+-ATPase activity in striatal synaptic plasma membrane of neonatal rats (Bavaresco et al., 2004) which is associated with oxidative stress and apoptosis (Wang et al., 2003). These studies taken together suggest a possible synergistic relationship between DA and Hx, however few studies have focused on this relationship.

Hypoxanthine Hypothesis of Dopamine Dysfunction in LND

These three model systems have served a valuable role in our knowledge of the neuropathology of LND. The neonatal 6-OHDA model places an emphasis on LND as a neurodevelopmental disorder and reinforces the relationship between DA lesions and self-mutilatory behavior. The HPRT knockout mouse provides a model in which to examine more closely the pathophysiology of LND and the resulting DA loss. Although DA is decreased in this model, behavior and Hx levels remain normal. This may be due to species differences in purine salvage pathways between humans and mice with one controversial suggestion being that adenine phosphoribosyltransferase (APRT) plays a compensatory role (Wu & Melton, 1993; Engle et al., 1996; Edamura & Sasai, 1998; Jinnah et al., 1999). Alternatively, Hx might be converted to inosinic acid via the purine nucleoside phosphorylase and inosine kinase enzymatic pathways (Allsop & Watts, 1990). Cell culture studies have been able to answer specific questions in isolated cells and in this model system have been quite useful in demonstrating that HPRT deficient cells have abnormal growth patterns which could alter neural circuits. In addition these studies draw attention to the role that Hx may play in the pathogenesis of LND, although this hypothesis has been largely ignored (personal communication Jinnah, H. & Breese, G.).

The major challenge to our understanding of the pathogenesis of this disease lies in defining the relationship between an enzymatic deficiency which results in increased purine metabolism and the observed DA dysfunction. So the question remains, is there a relationship between elevated Hx levels and DA dysfunction, and if so, to what extent does Hx affect the DA system? In an attempt to determine the role of Hx in LND,

Palmour et al. (1989) examined rats treated unilaterally with Hx. To do this, cannuli packed with solid Hx were stereotaxically placed in the caudate or lateral ventricle. Animals were pharmacologically challenged with apomorphine and observed for rotational behavior. It was found that Hx-treated animals displayed an increase in rotational behavior over a course of three weeks. This coincided with decreases in whole brain levels of DA and DOPAC. In an attempt to replicate and expand on this research, Burke et al. (1999) used the same implantation method restricted to the caudate. These animals did not display rotational behavior to the extent previously reported (possibly due to the use of a different species of rat), but histopathological analysis suggested a neurotoxic effect of Hx. Fluoro-Jade, a stain for neurodegeneration (Schmeud et al., 1997), revealed extensive damage to the fiber bundles within the striatum with up to 90% of these fiber bundles staining positive. However the delivery method employed by Burke et al. (1999) and Palmour et al. (1989) was inconsistent and unreliable, and thus a new method had to be employed in order to determine the effects of controlled administration of Hx on the DA system. It was decided that an Alzet osmotic mini-pump would be able to overcome the limitations of the packed cannula. The pumps employed in this project have a constant delivery of 2.5μl/hour that delivers 40.8μg of Hx per day.

The investigation of the effects of Hx on the DA system provides a unique perspective, which is complementary to developmental models of LND. The purpose of this investigation is to study the effects of high levels Hx on the mammalian brain with relevance to LND. The present study tests the working hypothesis that excess Hx deleteriously affects the DA system. Specific hypotheses tested within this study are: 1) excess Hx induces DA neuronal death, 2) excess Hx causes a reduction in DA and its metabolites, and 3) excess Hx alters markers of the DA neuron, specifically DAT and tyrosine hydroxylase. It should be noted that this is not a model of LND per se, but is designed to specifically examine the relationship between high concentrations of Hx and DA neuronal dysfunction.

Dopamine and Drugs of Abuse

Concepts of Drug Abuse

Drugs of abuse including nicotine, cocaine, amphetamine, and alcohol have all been shown to increase DA, and to a lesser extent, serotonin release (Imperato & DiChiara, 1986, 1988; DiChiara & Imperato, 1986; Wise, 1996; Boileau et al., 2003). Although these drugs share in their propensity to increase extracellular DA, the extent to which DA plays a mechanistic role in the development of drug abuse and addiction remains contentious (Berke & Hyman, 2000). Dopamine release is thought to subserve the rewarding effects of both natural (food, water, sex) stimuli and drugs of abuse (Schultz et al., 1993; Wise, 2002; Berridge, 2004). Whether this is primary or secondary to the complex interactions of multiple neurotransmitters within the different areas of the mesocorticolimbic pathway is not known (Jentsch & Taylor, 1999; Berke & Hyman, 2000).

The mesocorticolimbic DA reward pathway did not develop for the pleasurable effects of potentially addictive drugs, but rather signals natural rewards such as food. In fact much of what we know about the reward pathway and the role DA plays in reward is derived from using palatable food as well as drugs of abuse as unconditioned reinforcers (Berridge, 2004; Wise, 2002; Robinson & Berridge, 2003; Schultz et al., 1993; Kelley & Berridge, 2002). However, an important difference here is that drugs of abuse such as cocaine have the ability to raise DA levels up to five-fold whereas natural food rewards raise DA levels by 1.5-fold (Hernandez and Hoebel, 1988). The dramatic rise in DA levels via drug ingestion such as heroin or cocaine is thought by many to subserve their addictive properties (Wise, 2002). Although the leading concepts regarding the progression from recreational to abusive use of drugs are varied, each of these hypotheses - hedonic allostasis (Koob & Le Moal, 1997; Koob 2003), incentive-sensitization (Berridge & Robinson, 1998; Kelley & Berridge 2002; Berridge 2004), and over-learned habits involving aberrant learning (Tiffany, 1990; Berke & Hyman 2000; Ito et al., 2002) – indicate alterations within in the mesocorticolimbic DA pathway. Each of these theories implicate a role of DA in reward, however reward is not a unitary phenomena, instead it is composed of multiple components. Berridge and Robinson (1998) identified three such components which begins with a hedonic activation or a "liking" of the stimuli; followed by associative learning between the stimuli and the hedonic effect; and

finally the attribution of incentive salience where the mesocorticolimbic circuit becomes hypersensitive to the drug effects and drug-associated stimuli such that it causes a pathological "wanting" of the drug (Berridge & Robinson, 1998; Robinson & Berridge 2003).

These theoretical concepts of drug addiction rely on data obtained from conditioning normal animals to self-administer drugs (Koob & LeMoal, 1997; Kelley & Berridge, 2002; Wise, 2002) and do not necessarily take into account inherent differences that render individuals vulnerable to drug abuse. However, human data obtained from individuals with a family history of alcoholism and longitudinal developmental studies suggest that for some individuals the mesocorticolimbic system is dysfunctional prior to substance abuse (Cloninger et al., 1988; Tarter et al., 1989a,b; Sher et al., 1991; Finn et al., 1997, 2000; Caspi 2000). Since human data suggests that certain individuals have an inherent vulnerability to alcohol abuse, the current investigation is concerned with what Berridge and Robinson (1998) define as the first component of reward, that is hedonic activation leading into the acquisition phase of drug abuse. More specifically, it is the objective of the present investigation to identify markers of the midbrain DA and serotonin neurons associated with alcohol preference in vervet monkeys.

Neural Mechanisms of Addiction

Modulation of the DA system appears to be a critical factor in the acquisition, continuation, and relapse into drug addiction. Of these, the acquisition phase is the one of concern for the present study. The pharmacological influence of drugs of abuse such as alcohol, cocaine, and amphetamine on DA [and serotonin] within the affective circuit has placed these neurotransmitters, along with the mesocorticolimbic pathway at the forefront of research investigating the vulnerability to addiction. Changes in the physiological properties of the DA system have been proposed to be involved in the evolution from recreational to compulsive drug taking behavior (Grace, 1995, 2000; Bonci et al., 2003).

As discussed in a previous section, DA neurons display a pacemaker-like firing pattern as part of its normal physiological firing repertoire. Superimposed on this tonic firing pattern are high frequency bursts/short duration bursts (Grace & Bunney, 1984a,b). The transition from tonic to phasic firing may be induced by unconditioned rewards,

either natural (i.e. sex, food, and water) or drug (Hollerman & Schultz, 1998; Schultz, 1998; Careli & Wondolowski, 2003; Carelli et al., 2004). However, natural and drug rewards have been shown to elicit responses from different neuronal populations within the nucleus accumbens (Bowman et al., 1996; Carelli & Wondolowski, 2003). This differential activation of the nucleus accumbens to natural versus drug rewards maybe in part responsible for the process that leads to compulsive drug seeking behavior proposed by Wise (2002).

In accordance to the incentive salience (Berridge & Robinson, 1998) and the over-learned habits involving aberrant learning (Tiffany, 1990; Berke & Hyman 2000; Ito et al., 2002) models of addiction, phasic release of DA in the nucleus accumbens shifts from the receipt of the reward to the conditioned stimulus which predicts the reward (Schultz, 1998). In rats trained to self-administer cocaine, extracellular DA concentrations in the nucleus accumbens increase approximately 10 times basal levels within 100ms of the presentation of the conditioned stimuli (Phillips et al., 2003) suggesting that the phasic release of DA is involved in the learned association between cues and drug-seeking behavior. It has been proposed that in the acquisition phase of drug abuse, changes in the firing pattern of DA neurons lead to enduring adaptations and subsequent compulsive drug-seeking behavior after multiple drug administrations (Grace, 1995, 2000; Bonci et al., 2003).

The homeostatic steady-state balance between tonic and phasic DA release and subsequent extracellular DA levels has been proposed to be disrupted by repeated exposure to addictive drugs, resulting in an allostatic steady-state rendering an individual vulnerable to relapse (Grace, 1995, 2000; Bonci et al., 2003). Addictive drugs such as amphetamine, cocaine, and alcohol exert their rewarding effects through the mesolimbic DA pathway, albeit through different mechanisms. Amphetamine and cocaine act directly, blocking DAT, while alcohol is thought to act through multiple neurotransmitter systems including GABA and glutamate to elevate extracellular levels of DA (Deitrich et al., 1989; Ticku, 1990; Wirkner et al., 1999; Maiya et al., 2002). As would be expected, cocaine and amphetamine have the ability to raise extracellular levels of DA much more effectively than alcohol. A model of altered phasic/tonic release of DA has been proposed whereby each of these drugs induces a rapid increase in extracellular DA,

corresponding to phasic activity, leading to an increase in tonic levels of extracellular DA (Grace, 1995, 2000; Onn & Grace, 2000). The proposed prolonged increase in tonic DA release increase would lead to autoreceptor activation causing a decrease in phasic DA release to below basal levels, resulting in a dysphoric mood (Grace, 1995). Phasic release can once again be achieved by repeated drug ingestion (Grace, 1995, 2000).

It is proposed that repeated administration of the drug would cause a new steady-state where phasic release of DA is counterbalanced by an increase in tonic DA levels resulting in autoreceptor desensitization. Upon withdrawal of the drugs, dysphoria would ensue as a result of continued inhibition of phasic DA release by long-lasting elevation in tonic levels of DA. In withdrawal, the increased tonic levels of DA are in part mediated by increased corticoaccumbal glutamate transmission (Grace, 1995, 2000; Onn & Grace, 1995). Postsynaptic receptors are also sensitized due to the lack of phasic stimulation (Grace, 1995). Upon presentation stimuli that activates corticoaccumbal glutamate, such as a priming low dose of the targeted drug or drug related-stimulus or stress, would further increase tonic levels and exacerbate the attenuation of phasic neuronal firing. In order to re-establish phasic release additional drug would need to be taken thereby increasing the potential for relapse (Grace, 2000). The tonic/phasic hypothesis of drug addiction dictates that there need not be an alteration in absolute concentrations of DA for relapse to occur (Grace, 1995). Although Grace (1995, 2000) proposes that cortical glutamate and accumbal GABA are principally responsible for the dysregulation of tonic and phasic DA levels, there exist other factors that influence DA transmission throughout the mesocorticolimbic pathway which maybe responsible for dysregulation in drug addiction (Tanda et al., 1995; Nomikos et al., 1996; Di Mascio et al., 1998; Gervais & Rouillard, 2000; Floresco et al., 2003).

Although the dysregulation between tonic and phasic DA release is proposed as a neuroadaptation to repeated drug use leading to craving and relapse, recent evidence suggests that abnormal DA transmission is associated with vulnerability to drug abuse. Evidence suggests that elevated basal firing rates and bursting activity of DA neurons in the VTA is associated with subsequent acquisition of cocaine use in rats (Marinelli & White, 2000). Elevated basal firing and bursting activity was also present in the substantia nigra but not to the same degree as seen in the VTA (Marinelli & White, 2000.

Historical Perspectives of Alcohol Abuse

The consumption of alcohol has its roots in our evolutionary frugivory history. The presence of ethanol in fruits coincides with ripeness and sugar content and with a potentially higher caloric content. As a result it would have been advantageous to consume ripe fruit that has started to ferment (Dudley, 2000). Voluntary alcohol intake has been noted in many species including birds, baboons, elephants, and monkeys (Marias, 1969; Ervin et al., 1990; Juarez et al., 1993; Dudley, 2000). It has been hypothesized that the excessive consumption of alcohol is due to an advantageous ancestral trait that has become disadvantageous due to the abundant access of nutrition (Cloninger, 1987; Dudley, 2000). Although the neurological consequences of ethanol use (sedative, tolerance, anxiolytic, and dependence) may be important factors in the development and sustenance of alcohol abuse, the data presented here concentrate on the inherent neurochemical differences between those who abuse alcohol and those who do not display this behavior.

The diagnostic and statistical manual of mental disorders, fourth edition (DSM IV), describes two types of alcohol use disorders: abuse and dependence. Individuals with alcohol dependence display signs of alcohol-related compulsive behavior along with tolerance to- and withdrawal from- alcohol. These individuals will continue to drink despite the aversive reaction to withdrawal and tend to devote a considerable amount of time to the consumption of alcohol even after adverse physical complications develop (e.g. liver disease). Alcohol abuse shares similar social signs as alcohol dependence, however it does not include the criteria of tolerance, withdrawal, or compulsive use of alcohol. Individuals who abuse alcohol may start to neglect responsibilities (e.g. job or school performance/attendance suffers, child care may be neglected), alcohol related legal troubles appear (e.g. drunk driving, alcohol related aggression), or have interpersonal problems (e.g. child abuse, arguments with loved-ones).

In an examination of genetic and environmental factors underlying alcohol abuse, Cloninger et al. (1981) described the subdivision of alcoholics based on a large adoption study. Within this population, two extreme types of alcoholism (in addition to an undefined middle range), termed type 1 and type 2, emerged (Cloninger et al., 1981). Type 1 alcoholics exhibit onset of clinical symptoms after 25 years of age, have

psychological dependence pertaining to the loss of control, and feelings of guilt and fear about their dependence (Cloninger et al., 1981). These individuals are low novelty seekers but avoid harm and are highly dependent on external validation (Sigvardsson et al., 1982; Cloninger et al., 1987; Cloninger et al., 1988; Buydens-Branchey et al., 1989a). Type 2 alcoholics are described as those who are high novelty seekers, are not necessarily reward dependent or harm avoiding, do not express guilt or fear about their alcoholism, have an inability to abstain (spontaneous alcohol-seekers), and are frequently arrested (often due to fighting as a result of drinking). Such persons often have a biological father who also severely abused alcohol (Cloninger et al., 1981; Sigvardsson et al., 1982; Cloninger et al., 1987; Cloninger et al., 1988; Buydens-Branchey et al., 1989a). Although this classification system is frequently used in neurobiological studies of alcoholism, it does not fully represent the heterogeneity of alcoholism and there are indications that subgroups exist even within these two categories (Penick et al., 1990; Lamparski et al., 1991; Tiihonen et al., 1997; Kovac et al., 2001). Additionally, not all criteria are used or equally weighed in the classification of individuals as either Type 1 or Type 2 alcoholics (Fils-Aime et al., 1996; Virkkunen et al., 1996; Tiihonen et al., 1997; Heinz et al., 2001).

Neurochemical Features of Human Alcoholism

Studies focusing on neurochemical markers of human alcoholism have employed a number of techniques including *in vivo* imaging (single-photon emission computed tomography-SPECT, positron emission tomography-PET, and functional magnetic resonance imaging-fMRI), post-mortem *in vitro* imaging, and examination of cerebral spinal fluid (Braus et al., 2001; Bergström et al., 2001; Ratsma et al., 2002). On the basis of evidence, some investigators have suggested that there are alterations in the DA and serotonin pathways, which differentiate alcoholics from healthy controls. The majority of these studies utilize Cloninger's classification (Cloninger et al., 1981), to suggest that type 2 alcoholism is primarily characterized by a deficiency in the serotonin system whereas type 1 is related to deficiencies in the DA system (Buydens-Branchey et al., 1989b; Tiihonen et al., 1995; Tiihonen et al., 1997; Tiihonen et al., 1998; Laine et al.,

1999; Repo et al., 1999; Tupala et al., 2000; Kuikka et al., 2000; Laine et al., 2001; Braus et al., 2001; Heinz et al., 2001; Tupala et al., 2001a,b; Tupala et al., 2003a,b).

Within the midbrain, striatum, and nucleus accumbens, SPECT and post-mortem studies have shown lower DAT densities in abstinent type 1 alcoholics, as compared to control subjects (Tiihonen et al., 1995; Tiihonen et al., 1997; Repo et al., 1999; Tupala et al., 2000; Tupala et al., 2001a,b; Tupala et al, 2003a). The D_2 receptor is also low in the nucleus accumbens and amygdala in patients, but there are no differences in the D_1 or D_3 receptor levels, as compared to controls (Hietala et al., 1994; Tupala et al., 2001b; Tupala et al., 2003a). These differences, present in the absence of alcohol, have been suggested to be putative markers of type 1 alcoholism (Repo et al., 1999) and are not seen in type 2 alcoholism (Tiihonen et al., 1997; Tupala et al., 2001b; Tupala et al., 2003a). Furthermore type 1 alcoholics have a more uniform distribution of DAT throughout the striatum, in contrast to the normally patchy pattern of distribution. On the basis of this evidence, a hypoactive DA system has been hypothesized to characterize type 1 alcoholism (Tupala et al., 2001a).

By contrast, serotonin dysfunction is typically associated with Cloninger's type 2 classification (Cloninger et al., 1981). SPECT imaging analysis suggests reduced SERT densities in the midbrains of alcoholics as compared to control subjects (Tiihonen et al., 1997; Heinz et al., 1998a; Heinz et al., 2001). There is also an observed increase in heterogeneous striatal distribution of DAT in type 2 alcoholism as compared to type 1 and controls. This increased "spotty" distribution may represent a dysregulated or even hyperactive DA system in the absence of an overall change in striatal DAT or DA receptor concentrations (Kuikka et al., 1998; Tupala et al., 2001a).

Some studies report heterogeneity of neurochemical patterns of DA and serotonin in Type 1 and Type 2 alcoholics. For example, differences in presynaptic DA are not homogeneous amongst all diagnosed type 1 alcoholics. 6-[^{18}F]-fluorodopa (FDOPA) uptake, used as a marker for presynaptic DA synthesis, is elevated as compared to control subjects (range from 9-36%) in the striatum of some patients with type 1 alcoholism, indicating increased DA function. However, in a subset of type 1 diagnosed alcoholics FDOPA uptake is reduced up to 60% compared to controls, suggesting that this classification encompasses multiple subgroups of alcoholism (Tiihonen et al., 1998).

Heterogeneity among Type 1 alcoholics where *in vivo* SPECT studies demonstrate both low (Heinz et al., 1998a) and unaltered (Tiihonen e t al., 1997) SERT densities in the midbrain. Studies involving type 2 suggest a subset of type 2 subjects display reduced midbrain DAT similar to those seen in type 1 subjects (Tiihonen et al., 1997). Additionally, abstinent type 2 alcoholics display a lower 5-HIAA CSF concentration than type 1 and controls with no differences in HVA (Virkkunen et al., 1995; Fils-Aime et al., 1996; Hibblen et al., 1998). However, in a subset of type 2 alcoholics, both HVA and 5-HIAA are decreased (Fils-Aime et al., 1996; Virkkunen et al., 1996). Type 1 alcoholics have been reported not to show differences in monoamine metabolites (Kaakkola et al., 1993), although a subset that is diagnosed with depression demonstrates a lower level of HVA (Sher et al., 2003).

Studies involving human alcoholism, although informative, have inherent confounds (Goethals et al., 2001). At the time of study, individuals are either recently abstinent (*in vivo* imaging studies), still under the influence of alcohol (post-mortem imaging studies), or taking another psychoactive drug such as nicotine, benzodiazepines, diazepam, and cannabinoids (Laine et al., 1999; Tiihonen et al., 1997; Goethals et al., 2001). Drugs such as nicotine can alter DA levels through its inhibitory actions on MAO (Fowler et al., 1996, 1998). Additionally, although there are measured variations of serotonin and DA markers between Type 1, Type 2, and control subjects it is impossible to determine if these differences are a consequence of or if these alterations precede addiction. Furthermore, methodological concerns also arise when SERT and DAT *in vivo* because of the commonly used ligand, ßCIT. Although ßCIT does bind to SERT, it also binds to DAT (Laruelle et al., 1994) and norepinephrine transporters (Okada et al., 1998). Moreover the substantial quantities of both norepinephrine (Ordway et al., 1997) and DAT (Peyron et al., 1995) in the raphe nucleus make it unlikely that specific SERT signals can be discriminated. Therefore, it would be necessary to either occlude both DAT and norepinephrine transporters prior to scanning or to perform two scans with the second occluding the SERT (Tiihonen et al., 1997). This procedure is not necessarily utilized (Heinz et al., 1998a,b., 2000, 2001, 2002), however these studies do demonstrate altered monoamine transporters.

Further complicating the interpretation of clinical findings is that alcoholism is often comorbid with other psychiatric disorders such as anti-social personality disorder and depression (Tiihonen et al., 1995; Heinz et al., 1998a,b; Hallikainen et al., 1999; Kuikka et al., 2000; Goethals et al., 2001; Tupala et al., 2001a,b; Heinz et al., 2002; Sher et al., 2003; Tupala et al., 2003b). Although Cloninger's typology of alcoholism (Cloninger et al., 1981; Cloninger et al., 1987; Cloninger et al., 1988) is designed to facilitate the study of alcoholism, difficulties and confusion arise with diagnosis since alcoholism is a heterogeneous disorder. The misclassification or inability to clearly define the subtypes of alcoholism along with co-morbidity with other disorders leads to conflicting reports within the literature (Penick et al., 1990; Lamparski et al., 1991; Tiihonen et al., 1997; Laine et al., 1999; Tupala et al., 2000; Goethals et al., 2001). Furthermore, the weighting of certain criterion to determine classification complicates the interpretation of reported findings (Fils-Aime et al., 1996; Virkkunen et al., 1996; Tiihonen et al., 1997; Heinz et al., 2001). Additionally, clinical subgroups are not always as well-defined and homogenous as would be desired, and further that, even with imaging, there are no longitudinal studies (Tiihonen et al., 1997; Laine et al., 1999; Heinz et al., 2001).

Model Systems of Alcohol Abuse

The development of animal models of alcoholism has provided a set of tools, which, to some extent, address the methodological limitations of clinical studies of alcohol abuse and dependence. Theoretical criteria have been proposed in order to define the validity of animal models of alcohol abuse (Lester & Freed, 1973; McMillen, 1997). These criteria include voluntary oral ingestion of alcohol directed to its intoxicating effects in the presence of other palatable fluids and the maintenance of intoxication over an extended period of time (Lester & Freed, 1973).

Rodent Models

Several lines of alcohol-preferring rodents have been developed over successive generations by mating extremes of the normal ethanol-drinking distribution (Erikson et

al., 1968; Mardones et al., 1983; Li et al., 1993; Colombo et al., 1997; Murphy et al., 2002). Because the neurochemical differences between alcohol-preferring and non-preferring mammals are the focus of the current work, the Indiana preferring/non-preferring (P/NP) inbred rat lines will be examined since they have been extensively studied (Murphy et al., 2002). P rats will typically drink 5.0-9.5 grams of ethanol/kg/day across the different preferring lines, whereas the NP lines will drink on average 0.5 grams of ethanol/kg/day (Murphy et al., 2002). Screening for alcohol preference is generally performed by providing ethanol as the sole liquid for a period of days (priming), followed by a free-choice paradigm where alcohol and water are available (Dyr et al., 1993).

The Indiana alcohol-preferring rat line has also been useful in elucidating the roles of DA and serotonin in alcohol and drug abuse. The alcohol-preferring line displays lower levels of serotonin and 5-HIAA, the principal metabolite of serotonin, in the cerebral cortex, hippocampus, striatum, hypothalamus, and thalamus as compared to the non-preferring line (Murphy et al., 1982). Preferring rats also have fewer serotonergic fibers projecting to the anterior frontal cortex, nucleus accumbens, and ventral hippocampus, possibly as a consequence of the decrease in serotonergic neurons within the dorsal and median raphe nuclei (Zhou et al., 1991a, 1991b, 1991c). Serotonergic receptors are also altered in the preferring line. 5-HT_{1B} receptor densities are lower in the cingulate cortex, septum, and lateral amygdala (McBride et al., 1997) and densities of 5-HT_{2C} receptors are higher in the hippocampus and amygdala, leading to an increase of phosphoinositide hydrolysis and putative receptor supersensitivity (Pandey et al., 1996). The postsynaptic 5-HT_{1A} receptor is generally higher within the cortex, namely the medial prefrontal, frontal, parietal, cingulate, temporal, and entorhinal cortices and posterior hippocampus. However, the concentration of 5-HT_{aA} receptors is lower in the raphe nucleus where it is considered to be a presynaptic autoreceptor (McBride et al., 1994). Within the amygdala, lateral and posteromedial cortical nuclei, the 5-HT_3 receptor is lower; however this receptor is not different between lines in the frontal, cingulate, parietal, and entorhinal cortices (Ciccocioppo et al., 1998). It is becoming increasingly clear that the 5-HT_{1A} receptor is important in alcoholism as it mediates, to some extent, the ethanol induced release of serotonin and DA in the nucleus accumbens (Yoshimoto et al., 1992a,b). 5-HT_3 receptor antagonists also have been

shown to acutely decrease ethanol intake in a variable access paradigm which supports a pattern specific affect of the 5-HT_3 receptor-mediated ingestion of alcohol (McKinzie et al., 1998). In an intracranial ethanol infusion paradigm, 5-HT_3 receptor antagonists eliminated self-infusion of ethanol into the VTA, suggesting that this receptor maybe involved in the reinforcing effects of ethanol within the VTA (Rodd-Henricks et al., 2003).

Along with alterations within the serotonin system, the Indiana alcohol-preferring line displays lower DA content within the cerebral cortex, nucleus accumbens and anterior striatum (Murphy et al., 1982; McBride et al., 1995; Zhou et al., 1995). It has also been shown that the VTA sends fewer collaterals to the nucleus accumbens in preferring versus non-preferring rats resulting in a lower concentration of D_2 receptors in the ventromedial striatum (McBride et al., 1993, 1995; Zhou et al., 1995). Additionally D_2 receptors are lower in striatum proper and VTA, but no differences in the D_1 receptor have been reported for alcohol-preferring line (McBride et al., 1993, 1997). The strain differences in serotonin and DA throughout cortical and subcortical areas involved in the limbic system suggests a direct relationship between the affective circuit of the basal ganglia and alcohol abuse.

While the rodent model of alcohol abuse provides an inexpensive model in which to study neurochemical markers of alcohol abuse and pharmacological effects of ethanol, it is not without drawbacks. Since the human form of alcohol abuse incorporates a multitude of complex behaviors including a cognitive as well as limbic components, which contributes to the multiple forms of alcohol abuse, rodent models are limited in their ability to provide insights to the human condition (McMillen et al., 1998; Spanagel, 2000; Tabakoff & Hoffman, 2000; Grahame, 2000; Murphy et al., 2002). Additionally the basal ganglia neuroanatomy of the rat is substantially different from that of the primate including the mesocortical pathway, which is implicated in alcohol abuse. In order to determine the neurochemical underpinnings of alcohol abuse in humans, it is necessary to examine the neurochemistry of the different subtypes of alcohol abuse in non-human primates; often these investigations are guided by findings from the rodent models.

Non-Human Primate Models

Although the etiology of alcohol abuse is unknown in humans, the likelihood of alcohol preference in non-human primates sharing some aspects of the same etiology is substantial. These similarities make the non-human primate model of alcohol preference an invaluable tool in which to investigate the neurochemical underpinnings of alcohol abuse (Gerald & Higley, 2002; Grant & Bennett, 2003). There are two prominent non-human primate models of voluntary ethanol consumption, involving rhesus macaques and African green vervet monkeys (Higley et al., 1996a,b; Ervin et al., 1990). Although both models examine alcohol preference, the natural history of the two models differs substantially and therefore will be reviewed separately.

The rhesus model is derived from early studies of rhesus macaques separated at birth from their mothers (Suomi et al., 1970, 1971), and thus intrinsically focuses on early life experiences as predictors of later alcohol consumption (Higley et al., 1991). More recently, there has been attention to identifying the gene-environment interactions leading to alcohol abuse in this population. The basic paradigm is comparison between monkeys separated from their mothers at birth and raised in peer groups, and a matched set of individuals raised by their mothers until the age of 7 months, at which time they are housed with the peer-raised group until the time of study, which is typically 4 years of age (Higley et al., 1991; Higley et al., 1996a,b). The peer-reared group displayed a basal dampening of exploration and higher level of fear behaviors as compared to their mother-raised conspecifics (Higley et al., 1991). At 50 months of age the subjects were given access to a solution containing 7% ethanol, sweetened with 30% aspartame for 1 hour for 4 days per week for 8 weeks. The peer-raised group consistently drank more than the mother-raised group (with the exception of chronic stress defined as prolonged social separation) with rates averaging from 0.4-1.0 g ethanol/kg consumed (Higley et al., 1991). Baseline 5-HIAA CSF values were consistently lower in the peer-raised group as compared to the mother-reared group (Higley et al., 1996a). Additionally low levels of 5-HIAA are associated with lower levels of social interaction and lower social rank regardless of rearing style; however the peer-raised group had a higher degree of aggression as compared to the mother-reared group (Higley et al., 1996b). As a result of early environmental influences, low CSF 5-HIAA, elevated aggression, decreased social

interaction, and elevated ethanol consumption, peer-reared rhesus monkeys have been proposed as a model of type 2 alcoholism (Higley et al., 1996a,b).

As with human studies, the SERT in the raphe of rhesus monkeys has been measured using SPECT (Heinz et al., 1998a; Heinz et al., 1998b, Heinz et al., 2002, Heinz et a., 2003). To this end a ligand that binds to SERT, [^{123}I]ß-CIT, is used as a marker for serotonergic activity. In rhesus monkeys, this group found a negative correlation between SERT densities in the raphe and CSF 5-HIAA (Heinz et al., 1998). SERT in the raphe is positively correlated with alcohol intake, but alcohol intake is not correlated with aggression (Higley et al., 2003). Co-relational studies comparing midbrain SERT availability and aggression resulted in mixed findings (Heinz et al., 1998, 2003). This model has a restricted focus on the environmental and neurobiological factors leading to type 2 alcoholism with primary attention on the serotonergic system.

The vervet alcohol model is a population-based naturalistic model, using a large sample size (n>1200 individuals) and takes into account the probability that there are multiple subtypes of alcohol abuse in monkey as in man (Ervin et al., 1990; Palmour et al., 1997). Alcohol preference testing in this model is voluntary and does not involve priming or behavioral coercion of any type. As is the case for most other experimental studies of alcohol consumption, alcohol consumption is measured through a two-bottle choice test. In the standard screening paradigm, there is free access to either tap water or 0.5% sucrose with and without 10% ethanol. In the second week of testing, alcohol is available on a limited access schedule, typically 4 h per day (Ervin et al., 1990; Palmour et al., 1998). Based on this preference test, four distinct groups emerge based on quantity and patterns of consumption, of which two would be considered to be heavy drinkers. Both heavy drinkers (about 15% of the population) and binge drinkers (about 5% of the sample) drink in excess of 6 g ethanol/kg/day. The other two groups would not be considered to abuse alcohol and represent the majority of the population, as is the case in humans. "Social-drinkers" (about 56% of the population) typically consume <3.5 g ethanol/kg/day, and the alcohol-avoiding individuals (about 23% of the population) drink less than <2.0 g ethanol/kg/day (Palmour et al., 1997). The heavy- and binge-drinkers are not differentiated by the quantity of ethanol consumed during a 24-hour period, but rather by patterns of consumption, most evident in the restricted access paradigm. Binge-

drinkers will repeatedly drink to intoxication within two hours upon alcohol availability often to the point of alcohol-induced coma. During the restricted access paradigm 90% of fluid intake in binge-drinkers is ethanol whereas in heavy-drinkers this percentage drops to 50%. Heavy drinkers rarely drink to the point of coma under either paradigms (Palmour et al., 1997).

Monkeys that drink large quantities will continue to drink increasing concentrations of ethanol and will increase consumption over time, and show withdrawal signs. Neurochemical data obtained from heavy-drinking subjects indicates that relatively low levels of HVA at baseline increase under acute alcohol conditions, and decrease to control levels under chronic alcohol conditions (Ervin et al., 1990; Mash et al., 1996) with no other differences in metabolites. More recent data shows that naïve animals which go on to drink larger quantities of beverage ethanol show a reduced ethanol-stimulated release of CSF HVA, as compared to individuals who drink more moderately (Palmour et al., submitted).

Post-mortem neurohistochemical studies in selected individuals show that heavy-drinking monkeys exhibit high baseline levels of striatal and mesolimbic DAT as compared to alcohol-avoiding conspecifics (Mash et al., 1996). It was further shown that DAT expression is modulated by ethanol exposure leading to a suggestion that the DAT densities might be a phenotypic marker of alcohol preference (Mash et al., 1996). The previous study did not effectively address whether differences in DAT densities were restricted to the DA terminal field or were pervasive throughout the DA system. One way to determine the pervasiveness of DAT alterations is to specifically examine the midbrain DA region. The current study tests the working hypothesis that densities of DAT varies in the midbrain DA cell body regions in relationship to four patterns of ethanol consumption in abstinent vervet monkeys. In addition, the current analysis has been extended to include all 3 classes of drinkers [heavy-drinkers (>7.5g ethanol/kg/day under voluntary access), binge-drinkers (those who drink to intoxication within 2h of ethanol availability) and social drinkers (>2g and <5g ethanol/kg/day) in order to examine the possibility that there are simple linear correlations between neurohistochemical markers and levels of CSF neurotransmitter metabolites. Additionally since clinical evidence suggests that there is a serotonergic component to

alcohol abuse, this study also tests the hypothesis that SERT densities vary in relationship to four patterns of ethanol consumption in abstinent vervet monkeys.

Conclusion

The primary focus of the present thesis is the exploration of factors that influence the expression of DAT, a protein that has been identified as a marker of the DA neuron (Kuhar, 1998). To this end three distinct and separate models have been employed. First, previous behavioral work from our laboratory suggests that MAO inhibition during development alters behaviors that are thought to be regulated by neuroamines (Mejia et al., 2002). Because the largest change in behavior occurred when both MAO-A and MAO-B were inhibited during development, an important question was to evaluate the relative changes in both DA and serotonergic neurons in several target areas. As a first step, we used SERT and DAT surrogate markers of the serotonin and DA neurons, respectively. In the current investigation the working hypothesis was that MAO inhibition during development would preferentially alter SERT binding densities, but have minimal effects on DAT binding. In a second set of studies, we examined the effects of pharmacological levels of Hx [a metabolite elevated to pharmacological levels in the inherited disorder known as Lesch-Nyhan disease (Lesch & Nyhan, 1964, Sweetman, 1968; Rosenbloom et al., 1967)] on DAT and other markers of the DA neuron. The working hypothesis was that, as is the case in LND (Lloyd et al., 1981), Hx would damage nigrostriatal DA neurons. Finally, human studies suggest that there exist predisposing factors that render certain individuals vulnerable to alcohol abuse (Cloninger et al., 1988; Caspi 2000). Previous histochemical work from our group has shown that heavy-drinking monkeys exhibit high baseline levels of striatal and mesolimbic DAT as compared to alcohol-avoiding conspecifics (Mash et al., 1996). The final set of experiments tests the working hypothesis that densities of both DAT and SERT also vary in brainstem cell body regions in relationship to four patterns of ethanol consumption in abstinent vervet monkeys. To accomplish these objectives, the DAT binding assay designed by Coulter et al. (1995) was employed, however this assay was not optimized for labeling of SERT. In collaboration with Paul Clarke (McGill University), we optimized the DAT binding protocol for SERT (*please see Technical*

Note). These experiments examine midbrain DA neurons that are part of the mesolimbic, mesocortical, and nigrostriatal pathways.

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CHAPTER II

TECHNICAL NOTE

Preface

The objective of this thesis was to identify factors that influence the DA neuron, in part by examining DAT expression. Since the factors being investigated in the present thesis are known to also have a serotonergic component it was necessary to carry out experiments that examine the serotonin neuron. Therefore the secondary focus of the experiments is on the serotonin neuron and in particular the serotonin transporter (SERT) which (analogous to DAT) has been identified as a marker of the serotonin neuron (Horschitz et al., 2001; Hall et al., 2004). To accomplish these objectives, the DAT binding assay designed by Coulter et al. (1995) was employed, however this assay was not optimized for labeling of SERT. This chapter details the process the optimization of the DAT binding assay (Coulter et al., 1995) to reliably measure SERT binding.

Introduction

Transporters are key in regulating synaptic concentrations of their respective neurotransmitters (Giros et al., 1996; Chen & Reith, 2000). Re-uptake transporters such as DAT and SERT serve as target sites for drugs of abuse (Little et al., 1993; Mash et al., 2000; Hall et al., 2004), introduction of neurotoxins into the neuron (Decker et al., 1993; Gainetdinov et al., 1997), and ligand binding for psychopharmacological intervention in a range of psychiatric disorders (Claghorn et al., 1992). In the adult, DAT is expressed only on DA neurons (Nirenberg et al., 1996, 1997; Kuhar, 1998) and SERT, on serotonin neurons (Zhou et al., 2000) and as such have been proposed as specific markers of neurons that synthesize DA and serotonin, respectively (Kuhar, 1998; Horschitz et al., 2001).

Specificity of ligands that bind to DAT and SERT overlap, so it is essential to have an assay that clearly delineates each molecule. Quantification of both DAT and SERT is particularly germane to the experimental models tested in this thesis. In the first set of experiments, both DA and serotonin development are potentially affected by monoamine oxidase inhibition during development (Brunner et al., 1993; Whitaker-Azmitia, 1994). Therefore, quantifying DAT and SERT in this model would represent relative innervation of the regions of interest and indicate the relative sensitivity of these neurons to developmental stressors. Second, in order to test the relative specificity of Hx on the DA system it was necessary to rule out long-term changes in serotonin innervation of the striatum, since typical lesion experiments involving the nigrostriatal pathway indicates a robust serotonergic response (Zhou et al., 1991; Maeda et al., 2003). The final model investigated in this thesis tests the hypothesis that DAT and SERT are differentially expressed in the midbrain in relationship to different patterns of ethanol consumption in vervet monkeys. Evidence suggests that alterations in DA and serotonin, along with their respective markers, may play a role in the vulnerability to subsequent alcohol abuse (Mash et al., 1996, Tiihonen et al., 1995; Virkkunen et al., 1995, 1996; Heinz et al., 1998).

Since the first binding assay with cocaine (Reith et al., 1980) which demonstrated its affinity to DAT (Kennedy & Hanbauer, 1983; Reith et al., 1985; Ritz et al., 1987;

Fugita et al., 1994; Reith et al., 1997), analogs of cocaine have routinely been used for *in vivo* and *in vitro* measurements of the DA neuron (Mash et al., 1996; Wong et al., 1996; Tiihonen et al., 1997, 1998; Tupala et al., 2001). [^{125}I]RTI-55 (3ß-(4-iodophenyl)-tropane-2-carboxylic acid methyl ester), a potent analogue of cocaine, has successfully been used to quantify DAT and SERT densities (Boja et al., 1991; Boja et al., 1992, Rothman et al., 1998). In the literature there are many variants on the basic [^{125}I]RTI-55 binding assays for DAT and SERT involving different buffer (e.g. anion, cation or sucrose concentrations, pH) ligand concentrations, incubation temperature and time, and tissue thickness (Kuhar & Unnerstall, 1990; Boja et al., 1991, 1992; Reith & Coffey, 1993; Staley et al., 1994; Coulter et al., 1995; Rothman et al., 1998; Mash et al., 2000). The method of Coulter et al. (1995) was employed in the present experiments to quantify the DAT because this particular method is well characterized for incubation time, buffer, and temperature and a detailed comparison between binding to this ligand and to the tritiated WIN35428, another commonly used DAT ligand, has been published (Coulter et al., 1995). It is also known that citalopram effectively occludes SERT to the point that less than 2% of binding is attributable to SERT, with a concomitant loss of DAT binding sites less than 1% (Coulter et al., 1995). Certain modifications to this protocol were employed in the present study. Most notably, GBR 12909 was substituted for cocaine to define non-specific binding sites, since GBR12909 has a higher affinity for DAT than does cocaine (Rothman et al., 1998). This method for measuring DAT has been used repeatedly (Grant & Clarke, 2002; Pradhan et al., 2002; Sellings & Clarke, 2003), but the conditions, which would allow estimation of SERT binding, were not established. Because it is known that RTI-55 has a high affinity for both DAT and SERT (Coulter et al., 1995; Rothman et al., 1998), in collaboration with the laboratory of Paul Clarke (McGill University), we developed the appropriate conditions for SERT binding under the Coulter et al. (1995) protocol.

Methods

Subjects:

Adult Long-Evans rats (250-275 grams) from Charles River Laboratories were group housed and provided with food and water *ad libitum.* Animals were kept under a 12-hour light/dark cycle. All animal protocols were approved by the McGill University Animal Care and Use Committee in accordance to the Canadian Council on Animal Care.

Brain Preparation:

Autoradiography: Animals to be used for autoradiological analysis were sacrificed by decapitation, the brains quickly removed and then frozen at –80°C in 2-methylbutane. Serial sections from the same animals were used to autoradiographically identify levels of DAT and SERT.

Dopamine Transporter Autoradiography:

Coronal sections (20μm) were thaw mounted on gelatin-coated slides and stored at –80°C. Quantification of DAT binding sites was performed by incubating sections in a sub-saturating concentration of ^{125}I-RTI-55, a high affinity analogue of cocaine, according to standard protocols (Boja et al., 1992; Coulter et al., 1995; Sellings & Clarke, 2003). Briefly, sections were thawed and incubated for two hours at room temperature with 10pM ^{125}I-RTI-55 (NEN/Perkin-Elmer) diluted in a buffer containing 10 mM sodium phosphate, 120 mM sodium chloride, 0.1M sucrose and 50 nM citalopram (pH7.4). Citalopram was used to mask SERT during both incubation and washing. The sections were washed 3 times in buffer at 4°C, dipped in double deionized water to remove buffer salts, dried and exposed to BioMax MS film for 3 days along with a ^{125}I radioactive standard. Non-specific binding was defined as residual ^{125}I-RTI-55 bound in the presence of 10 μM GBR12909.

Serotonin Transporter Autoradiography:

Experiment 1: Quantification of SERT binding sites was performed under the same buffer conditions used for DAT (10 mM sodium phosphate, 120 mM sodium

chloride, and 0.1M sucrose, pH7.4). Sections were incubated with 10pM ^{125}I-RTI-55, with or without 100nM citalopram, or 10μM GBR 12909, or both counterligands.

Experiment 2: Buffer conditions were identical to experiment 1 with a few notable exceptions. Based on Rothman et al., (1998), the selectivity of GBR12935 DAT is superior to that of GBR12909 and was subsequently used to mask DAT. A range of concentrations of GBR12935 (100-3000nM) was used to occlude DAT while maintaining optimal SERT binding. Non-specific binding was defined as residual ^{125}I-RTI-55 bound in the presence of 100nM citalopram (Rothman et al., 1998).

Results

Experiment 1: ^{125}I-RTI-55 binding alone provided dense non-specific labelling of the entire section. In the presence of 10μM GBR12909, binding dropped almost to the background levels seen when ^{125}I-RTI-55 was co-incubated with 10μM GBR12909 and 50nM citalopram. It was concluded that GBR12909 did not bind selectively enough to DAT to provide a reliable measurement of SERT.

Experiment 2: The concentration of 100nM GBR12935 has been reported to effectively block the high affinity DAT site while not affecting SERT binding (Rothman et al., 1998). However, this concentration does not fully displace ^{125}I-RTI-55 from the low affinity DAT site that is abundant in the striatum (Rothman et al., 1998). Consequently, increasing concentrations of GBR12935 were used to fully displace ^{125}I-RTI-55 from DAT while maintaining SERT binding. These results confirm those of Rothman et al. (1998) in that 100nM GBR12935 failed to totally displace ^{125}I-RTI-55 from the caudate DAT (46.5% non-specific binding), however displacement from cortical DAT was within acceptable range (7% non-specific binding; *table 1*). The next concentration of 300nM GBR12935 also yielded DAT binding background in the striatum (14% non-specific binding; *table 1*). The concentrations of 1000nM and 3000nM GBR12935 provided excellent occlusion of DAT in the caudate (less than 0.5% non-specific binding; *table 1*), however at the higher concentration of GBR12935 striatal SERT binding was also substantially decreased (50% reduction compared to the 100nM GBR12935 concentration; *table 1*). Consequently, the concentration of 1μM GBR12935 was chosen for subsequent studies to fully occlude DAT while maintaining a satisfactory label of SERT. These studies were subsequently replicated, expanded upon, and reported (Pradhan et al., 2002).

Discussion

The present study was primarily concerned with labelling of SERT while minimizing non-specific binding to DAT. In the subsequent study (Pradhan et al., 2002) the focus is on the relative binding to DAT and SERT under these conditions. Pradhan et al. (2002) found that in order to completely isolate SERT binding from DAT binding there was a specific reduction of 20% in striatal and up to 70% in cortical binding to SERT. Despite reductions of specific binding to SERT in cortical and striatal areas, this method allows for reliable multiple comparisons between treatment groups for both DAT and SERT. Furthermore, the binding distribution of SERT with ^{125}I-RTI-55 in the current study closely resembles what is found with [^{3}H] citalopram (Descarries et al., 1995) and [^{3}H] peroxitine (Dewar et al., 1991). There are certain advantages of using an iodinated ligand such as RTI-55 as compared to tritiated ligands such as citalopram. For example the time of film exposure is greatly reduced (3 days for ^{125}I-RTI-55 compared to months with tritiated ligands) and the risk of tissue quenching is greatly reduced (Boja et al., 1991).

Technical considerations when using ^{125}I-RTI-55 for labelling DAT and SERT should be taken into account, particularly buffer composition, incubation time and temperature, and the use of ligands to define non-specific binding (Kuhar & Unnerstall, 1990). For example the addition of sucrose to the buffer enhances ligand binding to DAT, which is independent of the isotonicity of the buffer, itself (Coffey & Reith, 1994). There are also cation and anion requirements for the assay buffer. A range of 20 to 50nM Na^+ is needed for maximal binding of the ligand to the transporter. Higher concentrations of Na^+ do not affect the B_{max}, however the K_D is decreased. This K_D shift can be normalized with the addition of I^- anion. Other anions such as F^-, Cl^-, or Br^- have little or no effect on binding within this assay. However K^+, even at low concentrations, detrimentally affects DAT binding (Reith & Coffey, 1993). The method employed in the present experiment (Coulter et al., 1995) uses a buffer condition with high sodium and moderate sugar content. This method provides comparable binding distribution to that found with ^{3}H-WIN-35428. However binding levels with ^{125}I-RTI-55 were higher than that found with ^{3}H-WIN-35428 in most brain areas with the exception of the lateral

striatum, olfactory tubercle, and caudal substantia nigra pars reticulata (Coulter et al., 1995).

The binding methods described in this technical note are with its limitations since GBR12935 is only moderately selective for DAT. As reported by Pradhan et al. (2002) at the concentration of 1μM GBR12935, we had to accept some loss in the selectivity of ^{125}I-RTI-55 for SERT in order to satisfactorily occlude DAT binding in the striatum. Also, the sodium chloride concentration used for both DAT and SERT binding conditions may in fact reduce the binding to the high affinity sites of both transporters (Staley et al., 1994). At the concentrations used here, we would expect that the K_D be reduced which would affect the high affinity binding site, however the B_{max} would remain unchanged suggesting an accurate measurement of the low affinity site (Reith & Coffey, 1993). Additionally, RTI-55 binding suggests the low affinity binding site for DAT is more prevalent than the high affinity site within the nigrostriatal pathway whereas the high affinity site is more prevalent in the cortex (Boja et al., 1992). Given these limitations, the SERT binding assay developed here using DAT binding conditions (Coulter et al., 1995) provides a useful technique for reliably examining the low affinity site for both SERT and DAT.

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Table 1

Inhibition of [^{125}I RTI-55 Binding by GBR 12935

GBR12935 (nM)	Striatum	% Non-specific	Cortex	% Non-Specific
100	0.958±0.07	46.5%	0.784±0.07	7%
300	0.693±0.07	14.4%	0.618±0.03	2.2%
1000	0.574±0.07	<1%	0.583±0.09	<1%
3000	0.443±0.07	<1%	0.433±0.05	<1%

Mean±Std Dev of displaceable binding to SERT by ^{125}I RTI-55 (fmol/mg tissue, n=3 sections).

CHAPTER III

PERVASIVE NEUROCHEMICAL EFFECTS OF GESTATIONAL EXPOSURE TO MONOAMINE OXIDASE INHIBITORS IN MICE

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Preface

The DAT and SERT binding assays detailed in the previous chapter were employed as surrogate markers of the DA and serotonin neurons, respectively, to determine the effects on the DA and serotonin neurons following MAO inhibition during development. Previous research from this laboratory shows that combined inhibition of MAO-A and B activity during murine development results in aggressive and impulsive-like behavior. Selective inhibition of either MAO-A or MAO-B produced lower intensity behavioral alterations (Mejia et al., 2002). The present study examines the neurochemical substrate for these behavioral alterations. Specifically, the present investigation tests the hypothesis that there are regional reductions in serotonin innervation, as measured by SERT binding in developmentally inhibited mice. Furthermore it is hypothesized that relative innervation of DA will be minimally affected by MAO inhibition, in part, because deamination of catecholamines also occurs through catechol-*O*-methyl transferase (COMT).

Contribution by authors:

Burke, MW: Co-design of study, execution of experiments (behavioral and histological), supervision of M. Fillion, analysis of data, and preparation of manuscript.

Fillion, M: Undergraduate student, co-design of study, execution of experiments and surgical preparation.

Mejia, J: Co-design of study, and surgical preparation.

Ervin, FR: Supervision of experiments and preparation of manuscript.

Palmour, RM: Co-design of study, supervision of experiments, analysis of data and preparation of manuscript.

PERVASIVE NEUROCHEMICAL EFFECTS OF GESTATIONAL EXPOSURE TO MONOAMINE OXIDASE INHIBITORS IN MICE

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ABSTRACT

Numerous factors are involved in the ontogenesis of neurotransmitter systems, least of which are neurotransmitters, such as dopamine and serotonin, themselves (Pendleton et al., 1998). Alterations in the activity of monoamine oxidase (MAO), which regulates levels of these transmitters, may have a profound effect on brain development. Previous research from this laboratory shows that combined inhibition of MAO-A and B activity during murine development results in aggressive and impulsive-like behavior (Mejia et al., 2002). The present study investigates relative dopamine and serotonin innervation of cortical and subcortical areas, measured by dopamine (DAT) and serotonin transporter densities (SERT), following MAO inhibition (A or B or A+B) in mice throughout gestation and early post-natal development. DAT binding was unaltered within the nigrostriatal pathway. The major finding reported here is that the combined MAO-A+B inhibition significantly and specifically reduced SERT binding by 25% in both the cortex and raphe nucleus. Lower levels of SERT binding were apparent during the early post-natal period (PND 14), during which pups were still exposed to MAO inhibitors in the dam's milk, but also persisted into later life (PND's 35 and 90) after inhibitors were no longer being administered. Persistent effects were restricted to cortex and raphe, suggesting a relative vulnerability of these regions to early insult.

Introduction

Low levels of serotonin and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) have long been thought to be associated with aggression and impulsivity (Brown et al., 1982; Brown & Linnoila, 1990; Kruesi et al., 1990; Soderstrom et al., 2001). In 1993, Brunner et al (1993a, b) described a Dutch kindred in which certain males displayed mild mental retardation, aggressive, violent, and impulsive behavior. Genetic analysis showed that all affected males had a point mutation of the monoamine oxidase A (MAO-A) gene (Brunner et al., 1993a,b) resulting in a complete deficiency of enzymatic activity (Brunner et al., 1993b). In affected individuals, levels of serotonin and serotonin metabolites were abnormally high, thus providing an interesting exception to the low serotonin and elevated aggression notion. The nature of the relationship between impulsive aggressive behavior and MAO deficiency was further established in murine models genetically engineered to delete the MAO-A and/or MAO-B genes (Cases et al., 1995; Fornai et al., 1999; Chen et al., 1999, 2004).

Male MAO-A knockout mice exhibit enhanced aggression together with increases in monoamines (serotonin, norepinephrine, and dopamine) during development. These monoamine concentrations return to control levels by adulthood (Cases et al., 1995). More recently, it has been shown that dorsal raphe levels of the SERT are lower in MAO-A knockout mice as compared to wildtype controls (Owesson et al., 2002). By contrast, MAO-B knockout mice display neither aberrant behavior nor detectable changes in monoamines, with the exception of increased levels of phenylethylamine (Grimsby et al., 1997; Chen et al., 1999; Holschnieder et al., 1999). This is not surprising since MAO-A is principally responsible for the catabolism of monoamines in the mouse under normal physiological conditions (Fornia et al., 1999). MAO-A/B double knockout mice have even higher levels of serotonin than MAO-A knockouts, and also have increases levels of dopamine and norepinephrine. Similar to the MAO-A knockout mice, MAO-A/B knockout mice display increased aggression, but they also demonstrate anxiety-like behaviors (Chen et al., 2004).

Partial inhibition of MAO-A/B by clorgyline and deprenyl throughout either gestation or gestation and early post-natal development also induce pervasive aberrant

behavior in both mice and rats (Whitaker-Azmitia et al., 1994; Mejia et al., 2002). Gestational (E2-birth) or gestational plus post-natal (E2-PND 30) inhibition of MAO-A+B in rats increased impulsivity-related behaviors in the passive avoidance paradigm, aggressive behavior toward cage-mates and handlers and impaired visual function. By PND 30, both groups of animals presented normal dopamine innervation, as measured by DAT autoradiography, and a reduction of cortical serotonin innervation, as measured by a relative decrease in SERT densities (Whitaker-Azmitia et al., 1994). Inhibition of MAO-A/B in mice during gestation and nursing (E3-PND 21) results in elevated aggressive behavior (measured by the resident intruder paradigm) and a tendency toward impulsive responding (measured by the differential reinforcement of low rate responding paradigm) that extends beyond the point of MAO inhibition (Mejia et al., 2002).

Deprivation of MAO during development provides a reliable and reproducible model in which to investigate pervasive aggressive and impulsive behaviors (Whitaker-Azmitia et al., 1994; Mejia et al., 2002; Chen et al., 2004). However it would be erroneous to draw a direct casual relationship between MAO inhibition and aggressive behavior. The complex effects of MAO inhibition on the developing neurotransmitter systems are likely to be principally responsible for the resulting aberrant behavior (Brunner, 1996). The present model investigates relative dopamine and serotonin innervation of cortical and subcortical areas, measured by DAT and SERT densities, following MAO inhibition (A or B or A+B) in mice throughout gestation and early post-natal development. It is hypothesized that relative innervation of dopamine will be minimally affected by MAO inhibition because deamination of catecholamines also occurs through catechol-*O*-methyl transferase (COMT). As a consequence, MAO inhibitor-induced increases in dopamine are not likely to be as high as serotonin (Eisenhofer et al., 1994, 1996; Lenders et al., 1996). Since the primary deamination of serotonin occurs through the action of MAO, it is hypothesized that MAO-A/B inhibited mice will display a reduction of SERT binding similar to what is seen after inhibition of MAO-A/B in rats (Whitaker-Azmitia et al., 1994).

Materials and Methods

Subjects

CD1 timed-pregnant mice (n=4/group) were purchased from Charles River Laboratory and were received one day after impregnation. After one day of acclimation, the animals were weighed and minipumps were prepared for each animal. The following day, under ketamine/xylazine anaesthesia the pregnant dams were implanted with ALZET osmotic minipumps (model 1002; two week pump) filled with clorgyline (MAO-A inhibited group), L-deprenyl (MAO-B inhibited group), a combination of clorgyline and L-deprenyl (MAO-AB inhibited group), or 0.9% sterile saline (control group). The delivery rate for each pump was 0.25 μL/hour so that each dam would receive 0.25 mg/kg/day deprenyl, 1mg/kg/day clorgyline, or the two drugs in combination. The animals were subjected to MAO inhibition *in utero* and then to a lower level of MAO inhibition due to the presence of the inhibitors in the dams' milk. In order to maintain MAO inhibition for a total of 6 weeks (3 weeks of foetal development and 3 weeks of post-natal development) the minipumps had to be replaced. To prevent interference with the lactation period, the initial pumps were surgically replaced on E17, two days before parturition (ALZA model 2004; four week pump). Male pups from each treatment group were randomly assigned to the post-natal (PND) 14, 35, and 90 day groups. While all groups received MAOI's post-natally, the PND 14 group still had active inhibition at the time of sacrifice whereas MAOI's were withdrawn at PND 21 for the other groups. All animal protocols were approved by the McGill University Animal Care and Use Committee in accordance to the Canadian Council on Animal Care.

Behavioral Assessment

Neurological Assessment: Neurological evaluations were performed as previously described (Mejia et al., 2002). Briefly, a random sample of the pups (n = 51) from both the PND 35 or PND 90 time points, were evaluated between 30 and 40 post-natal days according to standard procedures (McLearn, 1970) in order to identify early signs of neurological impairment.

Behavioural Tests: Mouse pups assigned to the PND 90 time point (n = 6/treatment group) were behaviourally tested between 30 and 40 days of post-natal life in an Omnitech animal activity monitor between 8:30AM and 11:30AM for three minutes in order to determine activity levels and detect aberrant locomotor behaviour such as excessive stereotypies.

Brain Preparation

Mouse pups were sacrificed at 14, 35, or 90 days of age (n = 6/treatment group/time point) by cervical dislocation followed by decapitation. The brains were then quickly removed and frozen at –80°C in 2-methylbutane. Serial saggital sections (20 μm) were used to identify levels of DAT and SERT autoradiographically.

Dopamine Transporter Autoradiography

Saggital sections (20 μm) were thaw mounted on gelatin-coated slides and stored at –80°C. Quantification of DAT binding sites was performed by incubating sections in a sub-saturating concentration of ^{125}I-RTI-55, a high affinity analogue of cocaine, according to standard protocols (Coulter et al., 1995; Pradhan et al., 2002). Briefly, sections were thawed and incubated for two hours at room temperature with 10pM ^{125}I-RTI-55 (NEN/Perkin-Elmer) diluted in a buffer containing 10 mM sodium phosphate, 120 mM sodium chloride, 0.1 M sucrose, and 50 nM citalopram (pH 7.4). Citalopram was used to mask SERT during both incubation and washing. The sections were washed 3 times in buffer at 4°C, dipped in double deionized water to remove buffer salts, dried and exposed to BioMax MS film for 3 days along with a ^{125}I radioactive standard. Non-specific binding was defined as residual ^{125}I-RTI-55 bound in the presence of 10 μM GBR12909.

Serotonin Transporter Autoradiography

Quantification of SERT binding sites was performed under the same conditions used for DAT, with a few notable exceptions. DAT was occluded with 1 μM GBR12935 in the incubation and wash buffer rather than citalopram. The concentration of labelled ligand (10 pM ^{125}I-RTI-55) remained the same. Non-specific binding was defined as

residual ^{125}I-RTI-55 bound in the presence of 100nM citalopram (Rothman et al., 1998; Pradhan et al., 2002).

Image Analysis

Quantification of the autoradiographic studies was performed using the public domain NIH Image program (developed at the U.S. National Institute of Health and available on the internet at http://rsb.info.nih.gov/nih-image/). Optical density readings from autoradiographs were transformed into fmol/mg bound using ^{125}I microscale standards (Amersham), which were exposed on each film.

Data Analysis

Data analysis was performed on StatView® statistical program. Unless otherwise specified a one- or two-way analysis of variance test (for group contrasts) was performed as appropriate, followed by a Fisher's post-hoc test.

Results

Neurological test: Scores obtained on the neurological tests confirm previous results (Mejia et al., 2002) in that neurological development did not differ ($F_{3,47} = 1.196$, $p = 0.322$) between the four groups.

Open field test: The open field test showed no significant effect of treatment on locomotor activity ($F_{3,20} = 1.12$, $p = 0.366$; *table 1*). There was also an absence of treatment effect on stereotypies ($F_{3,20} = 1.55$, $p = 0.234$). Exploratory patterns, defined as time spent in the center of the monitor ($F_{3,20} = 0.681$, $p = 0.574$) and margin time ($F_{3,20} = 0.681$, $p = 0.574$), were not altered by MAO inhibition.

Histological Analysis: The expression of SERT and DAT differed across the treatment groups at each time point investigated. A two-way ANOVA failed to detect a time and treatment interaction in any region, however there were main effects of both time and treatment. Regardless of treatment, DAT and SERT densities tended to increase over time in all measured areas in agreement with other developmental studies (Coulter et al., 1997; Tarazi et al., 1998; Zhou et al., 2000; Galineau et al., 2004). The present study demonstrates that SERT *(figure 1)* and DAT typically reached adult levels by 35 post-natal days with the exception of some brain stem regions (substantia nigra: DAT; raphe area: SERT) in which adult levels were reached by 14 PND ($F_{3,59}=0.942$, $p>0.05$ and $F_{2,51}=2.962$, $p>0.05$ respectively; *figures 1 and 2*). As a point of reference, SERT concentrations in terminal areas peak from 14-21 PND then steadily decrease from 21-35 PND (Galineau et al., 2004). These time points were not part of the current study, but the distribution and relative densities of SERT on PNDs 14, 35, and 90 of the current study resemble those found in other studies (Tarazi et al., 1998).

Inhibition of MAO with deprenyl (MAO-B) or deprenyl + clorgyline (MAO-A/B) significantly reduced SERT binding in the raphe ($F_{3,51} = 6.065$, $p<0.001$; *figure 1e*), with pairwise differences between groups AB versus C ($p<0.0001$), C versus B ($p<0.01$), and AB versus A ($p<0.01$). Cortical reductions of SERT were also apparent after MAO inhibition ($F_{3,52} = 3.588$, $p<0.02$; *figure 1d*) with significant pairwise differences between

groups AB versus C ($p<0.0005$), AB versus B ($p<0.05$), and AB versus A ($p<0.05$). MAO inhibition did not alter SERT densities in the hippocampus ($F_{3,54} = 0.37$, $p = 0.78$; *figure 1a*), striatum ($F_{3,54} = 1.96$, $p = 0.13$; *figure 1b*), or substantia nigra ($F_{3,57} = 1.64$, $p = 0.19$; *figure 1c*) for any of the groups or time points.

The effects of MAO inhibition during early development on DAT were much less salient than those reported for SERT. Two-way ANOVA failed to detect significant differences in DAT densities between groups (*figure 2*) in the nucleus accumbens ($F_{3,52} = 0.345$, $p = 0.79$; *figure 2a*), striatum ($F_{3,61} = 0.756$, $p = 0.52$; *figure 2b*), or substantia nigra ($F_{3,59} = 0.942$, $p = 0.43$; *figure 2c*). It should be noted that MAO-A inhibition, as well as combined MAO-A/B inhibition, did tend to attenuate DAT densities in the substantia nigra during drug administration (PND 14).

Discussion

MAO inhibitors are commonly used in psychopharmacology and do not seem to cause any increase in aggressive behavior in adults (Kaplan & Sadock, 1998). The developmental period represents a time in which alterations in neurotransmitters may have long-term deleterious consequences (Berger-Sweeney & Hohmann, 1997). Numerous factors are involved in the ontogenesis of neurotransmitter systems, least of which are neurotransmitters, such as dopamine and serotonin, themselves (Pendleton et al., 1998). One of these factors is the level of activity of the degradative enzyme MAO (Whitaker-Azmitia, 2001).

Previous research from this laboratory shows that combined inhibition of MAO-A and B activity during murine development results in aggressive and impulsive-like behavior (Mejia et al., 2002). Selective inhibition of either MAO-A or MAO-B produced lower intensity behavioral alterations. The present study examines the neurochemical substrate for these behavioral alterations. Specifically, the present investigation tests the hypothesis that there are regional reductions in serotonin innervation, as measured by SERT binding, in the brains of mice treated during development with inhibitors of MAO. The most important finding reported here is that the combined MAO-A/B inhibition significantly and specifically reduced SERT binding in the cortex and raphe nucleus throughout developmental and into adulthood. Lower levels of SERT binding were apparent during the early post-natal period (PND 14), during which time pups were still exposed to MAO inhibitors in the dam's milk, but this effect also persisted into later life (PND's 35 and 90) after inhibitors were no longer being administered. Persistent effects were restricted to cortex and raphe, suggesting a relative vulnerability of these regions to early insult. This pattern of reduced SERT binding is consistent with the behavioral changes previously described in this model (Mejia et al., 2002). MAO-A/B and A inhibition tended to reduce DAT binding only in the substantia nigra and only in the perinatal period, possibly as a direct consequence of the concurrent presence of MAO inhibitors.

The present studies used the same protocol for developmental inhibition of MAO activity as that reported previously (Mejia et al., 2002). In that study, the extent of MAO

inhibition was evaluated directly in radiochemical assays of mouse brains collected from PND 21 pups. Treatment with clorgyline (MAO-A group) resulted in 25% and 29% inhibition of MAO-A and MAO-B respectively, while deprenyl treatment (MAO-B) group resulted in a lower overall inhibition of MAO-A and MAO-B (15% and 28% respectively) than did clorgyline treatment. The combined clorgyline plus deprenyl treated group (MAO-A/B group) yielded the highest level of inhibition of both MAO-A (41% inhibition) and MAO-B (42% inhibition) enzymes (Mejia et al., 2002). The present findings suggest that for alterations in serotonin innervation to occur during development, the critical level of MAO inhibition is a reduction of 25-40% below normal activity.

There are conflicting reports regarding the ontogeny of SERT binding. Tarazi et al. (1998) examined SERT densities in the striatum of rats at 7 developmental time-points ranging from PND 7-60 and found that SERT increases steadily throughout development. However, Galineau et al. (2003) describe a tri-phasic pattern of development in rats where SERT binding in the raphe is maximal between PND 0-14, receding between PND 14-28, followed by a plateau in binding through adulthood. Control samples drawn from the present study suggest a SERT ontogeny, in mice, which best resembles the developmental profile described by Tarazi et al. (1998). SERT densities in the raphe are near adult levels by PND 14, and all other areas examined SERT densities increase through to adulthood (Tarazi et al., 1998).

Neurological assessment of pups used in the present study, like those reported by Mejia et al. (2002), showed normal development. Likewise, motor activity was not different between experimental groups in either the current or previous study (Mejia et al., 2002). Additionally, there were no differences between groups with respect to other behavioral measurements such as stereotypies and exploratory behavior.

Comparison with MAO-A/B Rat Model

There are a number of differences between MAO-inhibited rats (Whitaker-Azmitia et al., 1994) and MAO-inhibited mice (Mejia et al., 2002, and present study) which may be due to the different degree and time course of inhibition of MAO, or to species differences, or to other unidentified variables. Two time-course conditions were employed by Whitaker-Azmitia et al. (1994), with MAO activity inhibited either from

E2-parturtition or E2-sacrifice, with sacrifice at PND's 5, 15, and 30. In contrast, MAO activity in the mouse was inhibited from E3-PND 21, followed by sacrifice at PND's 14, 21, 35 or 90. In the Whitaker-Azmitia study (Whitaker-Azmitia et al., 1994), inhibition of rat brain MAO activity approached 60%, while a maximum of 40% inhibition was reported in the mouse pups (Mejia et al., 2002). MAO inhibited rats were reported to be hyperactive (Whitaker-Azmitia et al., 1994), but this was not the case in mice. Blunted growth (measured by weight) was noted in MAO-A/B inhibited rats (Whitaker-Azmitia et al., 1994), but this is not characteristic of MAO-A/B inhibited mice (Mejia et al., 2002). In both regimens, the animals exhibited aspects of impulsive behavioral responding (Whitaker-Azmitia et al., 1994; Mejia et al., 2002).

Neuroanatomical evaluations of SERT and DAT in MAO-inhibited rats focused on early development at PND's 5, 15, and 30. SERT binding was elevated at PND 5 in the hippocampus and caudate, but reduced in the cortex. Hippocampal and caudate densities of SERT were not different from control levels at either PND 15 or 30 (Whitaker-Azmitia et al., 1994). In MAO-inhibited mice, hippocampal SERT densities, although somewhat lower than control levels, were not statistically distinct at any time point. In rat cortex, SERT densities followed a triphasic pattern with reduction at PND 5, elevation at PND 15 and reduction at PND 30, as compared to control values (Whitaker-Azmitia et al., 1994). In the MAO A/B-inhibited mouse, SERT densities in the cortex and raphe were lower than those of controls at all time points studied. In the rat, as in the mouse, there were no alterations in DAT at any time point, suggesting that MAO inhibition during development has little effect on dopamine innervation (Whitaker-Azmitia et al., 1994 and present study).

Factors involved in the Ontogeny of Dopamine and Serotonin

The developmental period represents a critical phase during which disturbances affecting dopamine and serotonin concentrations may result in profound behavioral and cognitive consequences. In the developing brain, monoamine transmitters appear very early along with their cell bodies and have been proposed to possess trophic functions on target areas (Lauder et al., 1982). Dopamine and serotonin are factors in the development of target tissue including the cortex and striatum. Disturbances in the concentrations of

these monoamines may have dramatic effects on neural networks resulting in abhorrent behaviors. The present study assumes that MAO inhibition primarily increases serotonin levels, but does not rule out elevations in dopamine, norepinephrine or other amine neurotransmitters (Cases et al., 1995).

In comparing developmental processes between dopamine and serotonin neurons, there is a consensus that adult concentrations of serotonin receptors reach adult levels earlier than do markers of dopamine. The maturation of these two neurotransmitter systems alludes to their relative roles and vulnerabilities during development. The arrival and subsequent maturation of dopamine and serotonin neurons corresponds to synaptogenesis and differentiation of the cortex, which begins around E16 and is carried through well into adolescence and adulthood. The innervation and maturation of serotonin neurons in the cortex by E17 (Wallace & Lauder, 1983; Lidov & Molliver, 1982a,b) and adult pattern by PND 28 (Morilak & Ciaranello, 1993; Galineau et al. 2003), indirectly suggests that serotonergic influence on cortical development has a relatively short critical period coinciding with rapid synaptogenesis of the cortex. By contrast, dopamine neurons reach the cortex by E15-17 (Kalsbeek et al., 1988), but adult levels are not established until PND 120 (Andersen et al., 2000). This protracted developmental period of innervation (up to PND 60) and subsequent pruning (up to PND 120) of dopamine neurons in the cortex suggests that dopamine plays a role in the differentiation and maturation of cortical neural circuits.

During development, serotonin displays both positive and negative autoregulation involving its own neuronal terminal density, particularly in the cortex (Whitaker-Azmitia, 2001). The autoregulatory action of serotonin occurs through both direct and indirect pathways. The indirect pathway involves the astroglial-derived S-ß100 protein, which is stimulated by serotonin through the 5-HT_{1A} receptor, as well as metabotropic glutamate receptors and adenosine-1 receptors (Whitaker-Azmitia et al., 1990; Ciccarelli et al., 1999; Ahlemeyer et al., 2000). S-ß100 has been shown to increase neurite outgrowth of serotonin neurons (Azmitia et al., 1990; Liu & Lauder, 1992). Interestingly, S-ß100 inhibits the spatial expansion of tyrosine-hydroxylase immunoreactive (TH-ir) neurites in the midbrain during early embryonic (E14) development (Liu & Lauder, 1992). Although physiological concentrations of serotonin promote synaptogenesis, Chubakov

et al. (1986) found that under-stimulation (serotonin depletion) and over-stimulation (5-methoxytryptamine, general serotonin receptor agonist) during embryonic development in the rat (E12-17) results in decreased 5-HT_{1A} receptor densities (Chubakov et al., 1986; Whitaker-Azmitia et al., 1987; Lauder et al., 2000). In the direct pathway, growth is inhibited by excess serotonin availability (Whitaker-Azmitia & Azmitia, 1986; Shemer et al., 1991); this is mediated through the 5-HT_{1B} autoreceptor (Whitaker-Azmitia 2001).

Elevated serotonin during development would be expected to lead to decreases in 5-HT_{1A} receptor densities (Whitaker-Azmitia et al., 1987; Lauder et al., 2000), which would reduce target cell differentiation during early pre-natal development (Whitaker-Azmitia et al., 1987; Sikich et al., 1990; Azmitia, 2001). Elevated negative autoregulation of the serotonin neuronal terminal field, particularly in the cortex, would also be an expected consequence of increased serotonin concentrations during development (Whitaker-Azmitia, 2001). Data obtained in the present study underscore the scenario where increased serotonin during development leads to decreased serotonin innervation of the cortex. This decreased innervation of the cortex may underlie the behavioral abnormalities, such as aggression and impulsivity reported by our laboratory (Mejia et al., 2002). The regional reductions in SERT binding warrants further investigation into potential changes in serotonin receptors and developmental co-factors such as S-ß100.

Although data from DAT binding suggest MAO inhibition did not permanently alter dopamine innervation, it is possible that there is elevated dopamine innervation in the cortex as a result of depressed serotonin innervation (Taylor et al., 1998; Benes et al., 2000). Additionally recent data from *in vivo* electrophysiological recordings suggests that insults to the cortex during development alter the responsiveness of cortical neurons to ventral tegmental stimulation (Lavin et al., 2005). Even though the current study did not find significant differences of DAT binding as a result of MAO inhibition during development, a dopaminergic component to the aggressive and impulsive behavior cannot be ruled out (Tidey & Miczek, 1996; (van Erp & Miczek, 2000; Ferrari et al., 2003; Cardinal et al., 2000, 2004).

Conclusions

The major finding reported here is that the combined MAO-A/B inhibition significantly and specifically reduced SERT binding by 25% in the cortex and raphe nucleus throughout developmental and persists into adulthood. This pattern of reduced SERT binding is consistent with the behavioral changes previously described in this model (Mejia et al., 2002). The developmental patterns of SERT densities are similar for all treatment groups, however the MAO-A/B group fails to obtain the levels seen in the control animals. Furthermore the present findings suggest that a partial inhibition of 25-40% of MAO activity is required for alterations in serotonin innervation to occur during.

The roles of serotonin and dopamine in development raise important issues regarding the ingestion of psychoactive drugs during pregnancy. The current study not only warns about the long-term effects of the use of anti-depressants during pregnancy, but also of other drugs such as cocaine, nicotine, and ethanol which increases dopamine and serotonin transmitter levels (DiChiara & Imperato, 1986; Fowler et al., 1996; Song et al., 2002). Prenatal cocaine exposure in rats results in altered dopamine (Akbari & Azmitia, 1992) and serotonin innervation in the cortex and hippocampus (Azmitia et al., 1990), changes in receptors (Koff & Miller 1994; Choi et al., 1998; Jones et al., 2000; Johns et al., 2002), and reduced growth factors such as S-ß100 (Akbari et al., 1994). These data indicate that although levels of dopamine and serotonin are important factors in development, there exists a critical concentration of these transmitters at which development is hindered, which corresponds to critical periods during development. These data should provide a note of caution to the administration of any psychoactive drug that significantly changes levels of dopamine or serotonin during development.

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Table 1 Motor Activity at 35 PND per Experimental Group

Group	Locomotor Activity	Stereotypy Count	Margin Time	Center Time
A	1626 ± 135 cm	20.0 ± 2.1	157.3 ± 4.1 sec	21.7 ± 4.1 sec
B	1732 ± 173 cm	22.2 ± 2.4	150.2 ± 3.7 sec	28.8 ± 3.7 sec
AB	1571 ± 156 cm	18.3 ± 2.3	153.2 ± 4.1 sec	25.8 ± 4.1 sec
C	1970 ± 198 cm	15.5 ± 2.2	151.3 ± 3.2 sec	27.7 ± 3.2 sec

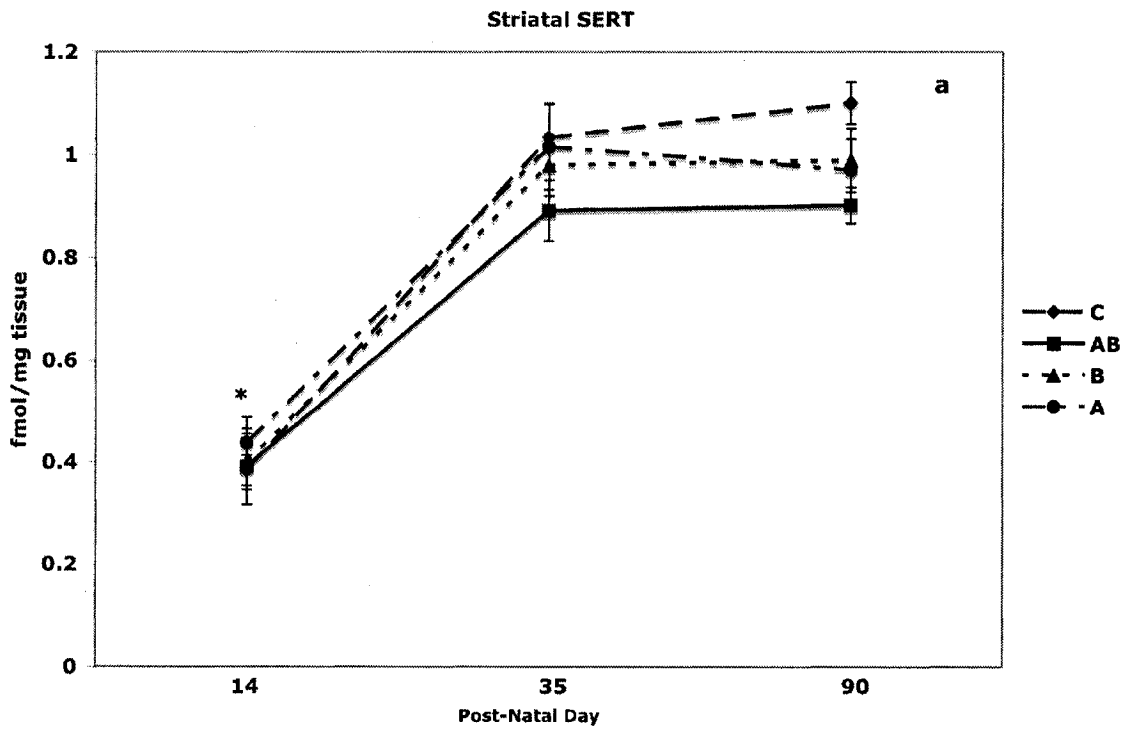
Average locomotor activity ($F_{3,20} = 1.12$, $p = 0.366$) and stereotypies ($F_{3,20} = 1.55$, $p = 0.234$) were not different between experimental groups. Time spent along the sides (margin time; $F_{3,20} = 0.681$, $p = 0.574$) or in the center of the open field did not vary across groups ($F_{3,20} = 0.681$, $p = 0.574$). Data are presented as mean ± standard error. A, monoamine oxidase (MAO) A enzyme-inhibited mice (n = 6); B, MAO-B enzyme-inhibited mice (n = 6); AB, MAO-A and MAO-B enzyme-inhibited mice (n = 6); C, control animals (n = 6).

Figure Captions:

Figure 1 A two-way ANOVA revealed a main effect of time in SERT binding in each region measured. Regional SERT binding follows a developmental curve whereby densities are relatively low at PND 14 and statistically reach adult levels by PND 35 (PND 14 vs 35, $p<0.0001$; PND 35 vs 90, $p>0.05$). The exception is in the raphe where adult levels are apparent as early as PND 14 (PND14 vs 35 vs 90, $p>0.05$). There were no differences in relative SERT densities in the (a) striatum, (b) hippocampus or (c) substantia nigra between treatment groups. MAO inhibition significantly reduced SERT binding in the (d) cortex ($F_{3,52} = 3.588$, $p<0.02$) with pairwise differences between AB and control ($p<0.0005$), AB and B ($p<0.05$), and AB and A ($p<0.05$). SERT binding was also reduced in the (e) raphe following MAO inhibition with pairwise differences between AB and control ($p<0.0001$), B and control ($p<0.05$), and AB and A ($p<0.05$). Group A (*n =6/time point*) was treated with clorgyline; group B (*n =6/time point*), with deprenyl; group AB (*n =6/time piont*), with clorgyline and deprenyl; and group C (*n =6/time point*), with saline. Data are presented as mean $\pm$ standard error. (**p<0.05 between time points; **p<0.05 AB vs C, B vs C, AB vs A).*

Figure 2 Regional DAT binding follows a developmental curve whereby densities are relatively low at PND 14 and statistically reach adult levels by PND 35 (PND 14 vs 35, $p<0.0001$; PND 35 vs 90, $p>0.05$). The exception is in the substantia nigra where adult levels are apparent as early as PND 14 (PND 14 vs 35 vs 90, $p = 0.35$). There were no differences in relative DAT densities in the (a) nucleus accumbens, (b) striatum or (c) substantia nigra between treatment groups. MAO-A/B and A inhibition tended to decrease DAT binding, apparent at PND 14, but did not reach statistical significance. Group A (*n = 6/time point*) was treated with clorgyline; group B (*n = 6/time point*), with deprenyl; group AB (*n =6/time point*), with clorgyline and deprenyl; and group C (*n = 6/time point*), with saline. Data are presented as mean $\pm$ standard error. (**p<0.05 between time points).*

Figure 1


Striatal SERT
1.2
1
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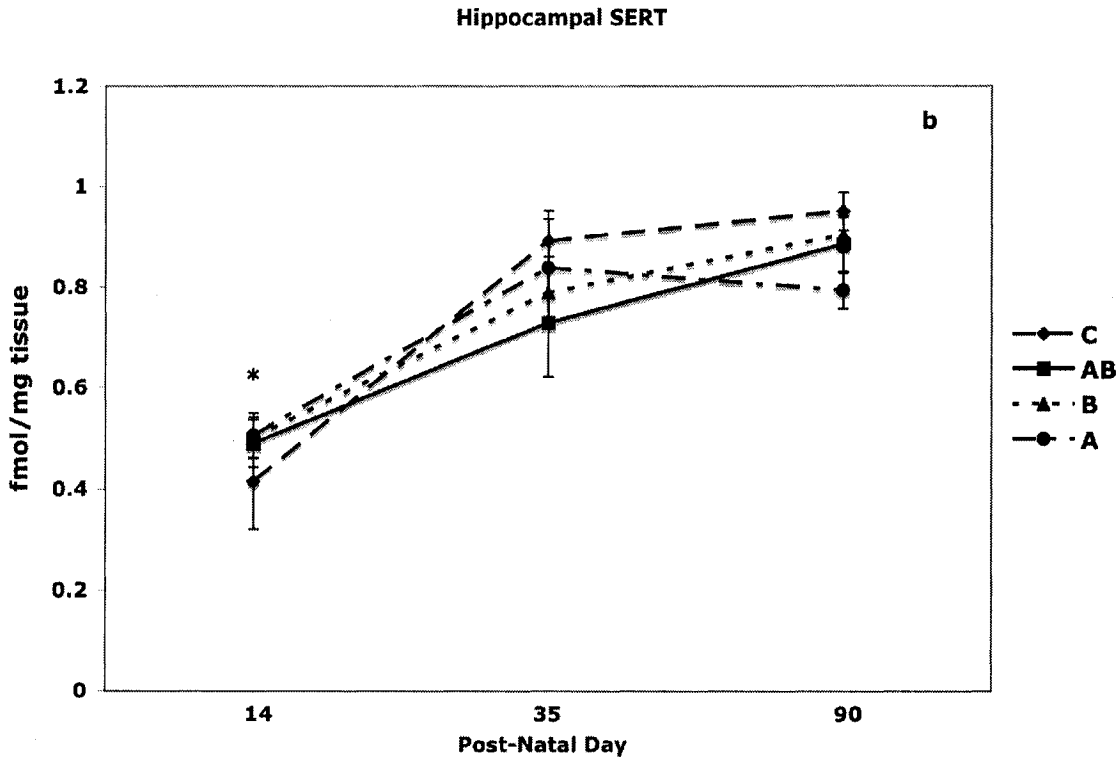
fmol/mg tissue
14
35
90
Post-Natal Day
a
*
C
AB
B
A


Hippocampal SERT
b
1.2
1
0.8
0.6
0.4
0.2
0
fmol/mg tissue
*
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35
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Post-Natal Day
C
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B
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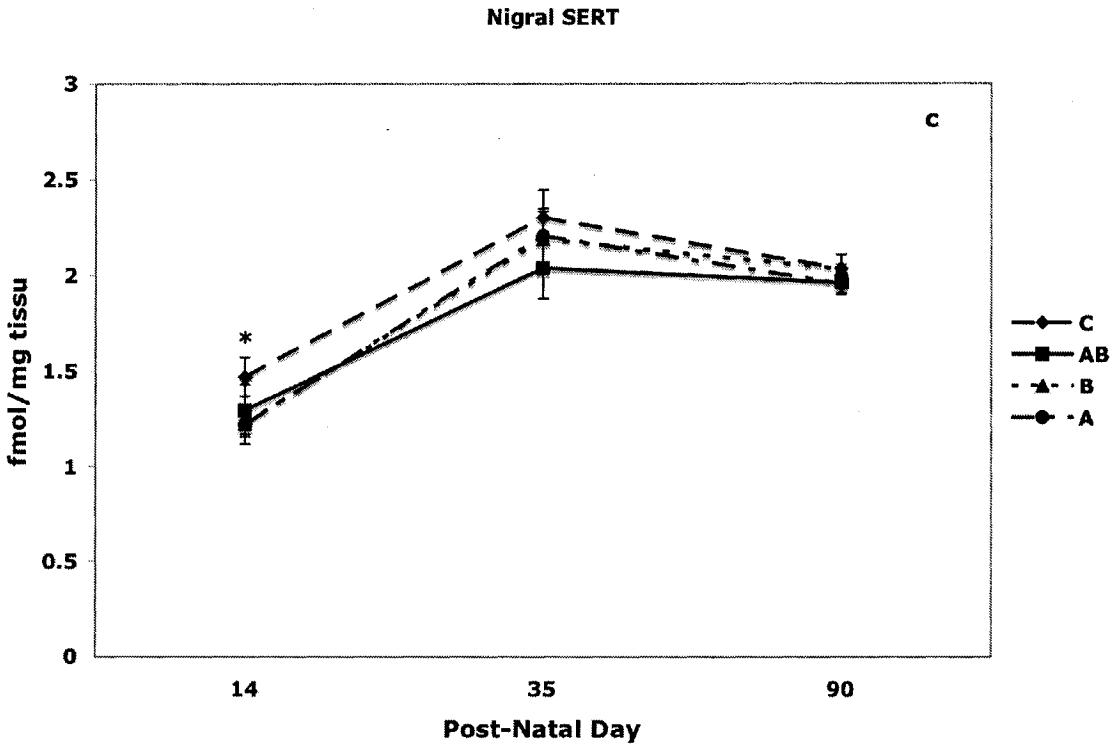
Nigral SERT
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Post-Natal Day
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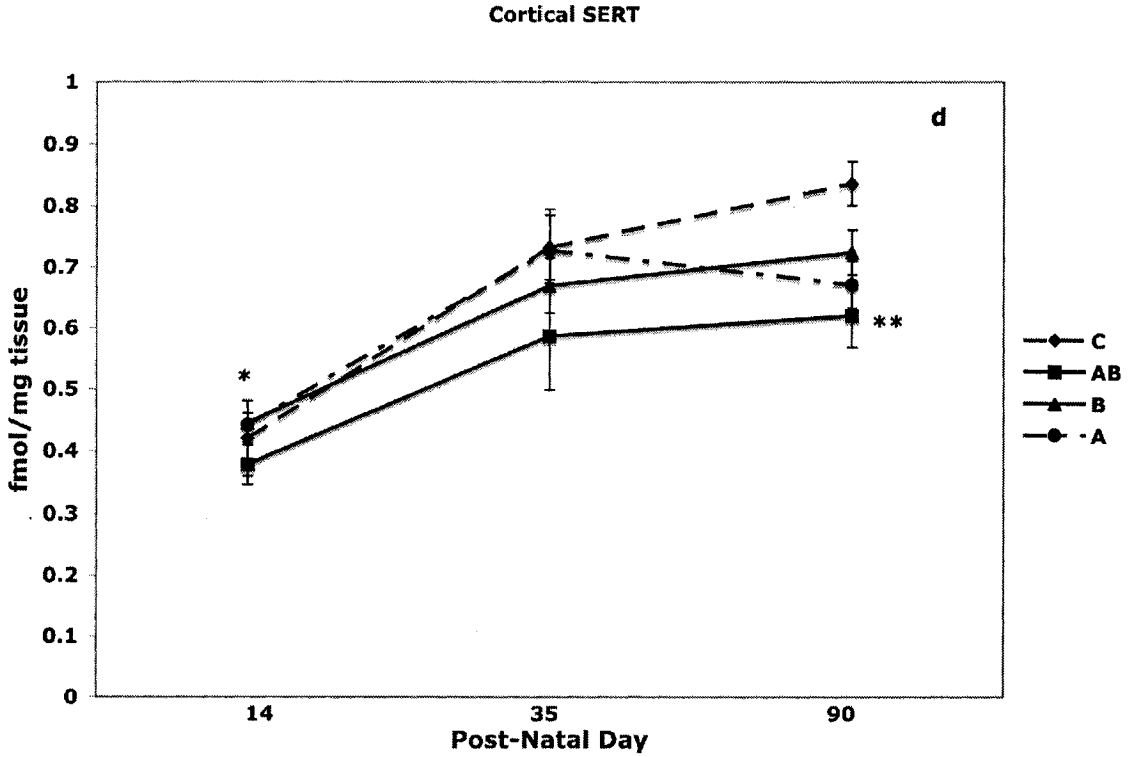
Cortical SERT
d
fmol/mg tissue
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90
Post-Natal Day
C
AB
B
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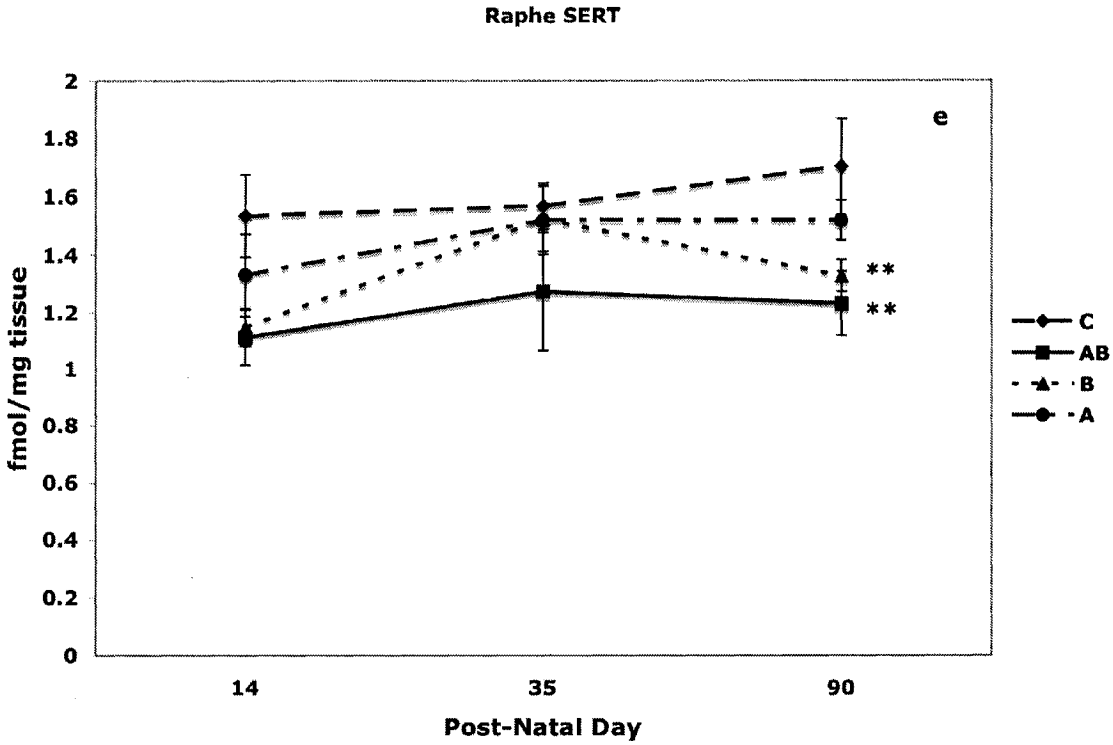
Raphe SERT
e
2
1.8
1.6
1.4
1.2
1
0.8
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fmol/mg tissue
**
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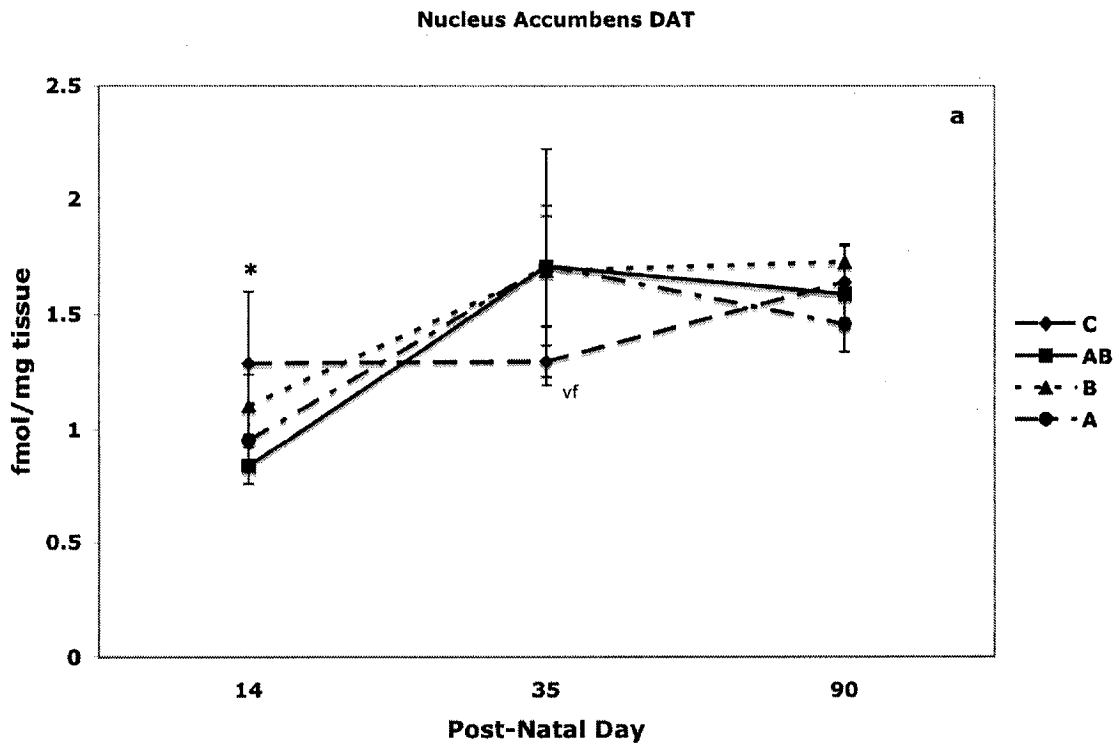
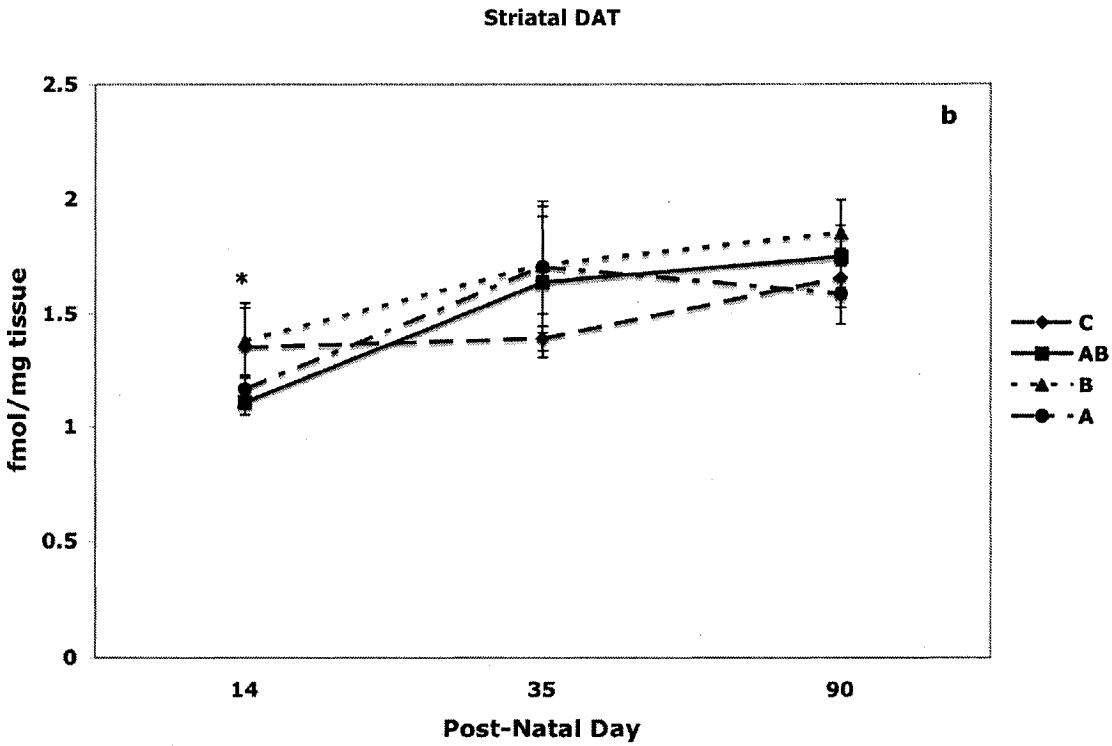
14
35
90
Post-Natal Day
C
AB
B
A

Figure 2


Nucleus Accumbens DAT
a
2.5
2
1.5
1
0.5
0
fmol/mg tissue
*
vf
14
35
90
Post-Natal Day
C
AB
B
A


Striatal DAT
b
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0.5
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fmol/mg tissue
*
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90
Post-Natal Day
C
AB
B
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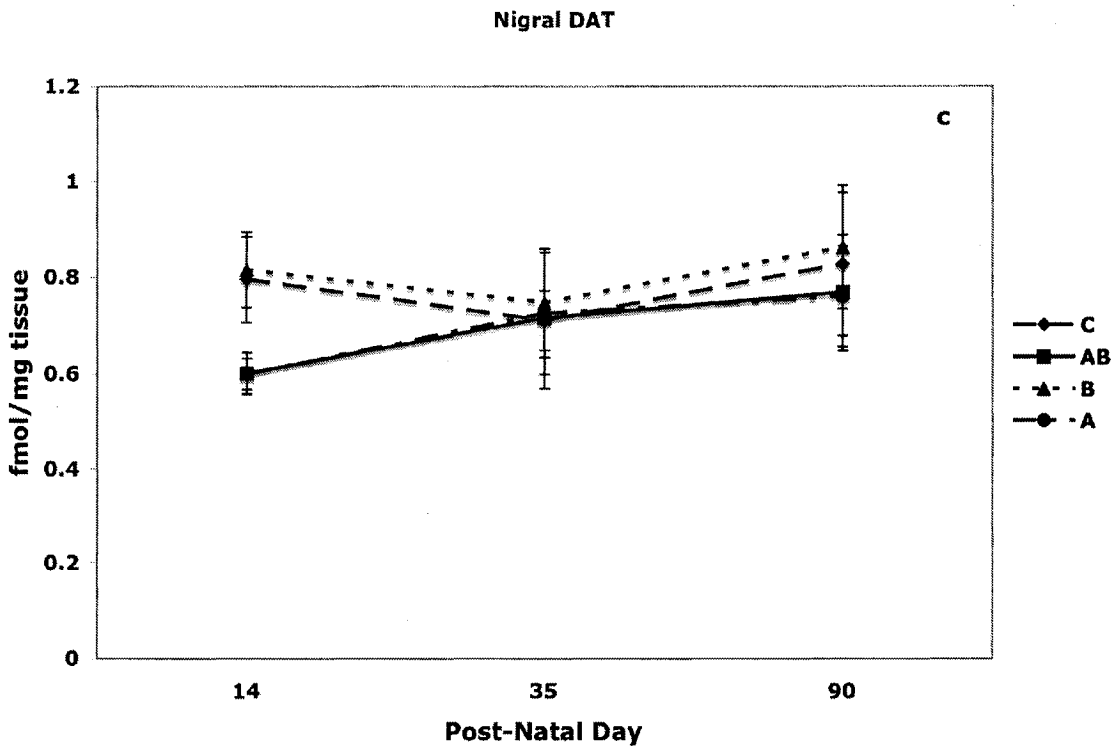
Nigral DAT
C
1.2
1
0.8
0.6
0.4
0.2
0
fmol/mg tissue
14
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CHAPTER IV

IS HYPOXANTHINE TOXIC TO DOPAMINE NEURONS?

Toward an Understanding of Lesch-Nyhan Disease

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Preface

In the previous chapter, MAO inhibition during gestational and early postnatal development was identified to deleteriously affect SERT binding in the cortex and raphe while having little effect on the nigrostriatal DA system. This chapter presents experimental work that examines the effects of high levels of Hx on the nigrostriatal DA pathway. Interest in the effects of Hx on the DA system arises from LND, where Hx levels are more than doubled and DA is depleted (Lesch & Nyhan, 1964; Lloyd et al., 1981). The focus on the nigrostriatal DA pathway is grounded in extensive evidence showing that patients with LND uniformly have a depletion of DA in the striatal terminal field (Lloyd et al., 1981; Wong et al., 1996; Earnst et al., 1996; Endres et al., 1997; Saito et al., 1999). However, a connection between high levels of Hx and alterations in the DA neuron has yet to be established. The present study tests the hypothesis that Hx deleteriously affects the DA neuron *in vivo*.

Contribution by authors:

Burke, MW: Co-designed study, execution of experiments, analysis of data, preparation of manuscript.

Ervin, FR: Supervision of experiments and preparation of manuscript.

Baker, GB: Biochemical analysis of brain amines.

Palmour, RM: Co-design of study, supervision of experiments, analysis of data and preparation of manuscript.

IS HYPOXANTHINE TOXIC TO DOPAMINE NEURONS?

Toward an Understanding of Lesch-Nyhan Disease

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ABSTRACT

Patients with Lesch-Nyhan disease (LND), which results from deficiency of the purine salvage enzyme hypoxanthine-guanine phosphoribosyltransferase (HPRT), exhibit severe movement disorders and compulsive self-mutilatory behavior, thought to be related to loss of dopamine (DA) terminals throughout the striatum. The current study explores the hypothesis that hypoxanthine (Hx), a purine significantly elevated in blood and CSF of LND patients, might alter neurochemical or behavioral markers of DA metabolism if administered in high doses to experimental animals. Rats were treated with either a 5mM solution of Hx or artificial CSF (control group) over the course of 21 days. Hypoxanthine treatment significantly reduced the number of tyrosine hydroxylase immunoreactive cells (TH-ir) in the substantia nigra by 22% and 30%, at 7 and 21 days, respectively. After 3 days of Hx administration, striatal DA and serotonin were elevated over control levels by 22% and 25%, respectively, but returned to control levels by 7 days. The serotonin metabolite 5-HIAA was elevated after 3 days of Hx, but levels of DA metabolites were not different from control. Locomotion, a behavior thought to be related to dopaminergic transmission, was elevated following Hx treatment, as were presynaptic markers of the DA system such as the dopamine transporter (DAT) and TH protein levels. The persistent reduction in TH positive cell numbers suggests that Hx damages or kills DA neurons. The increase in intracellular DA at early time points suggests that Hx might interfere with DA release, possibly by temporarily inactivating DA neurons.

Introduction

Lesch-Nyhan disease (LND) is an X-linked inherited disorder of purine metabolism (Nyhan, 1973), characterized by a total deficiency in HPRT activity. As a result of this enzymatic deficiency, *de novo* synthesis goes unregulated (Rosenbloom et al., 1967) and Hx is overproduced. Accordingly, Hx accumulates in the brain and uric acid builds up in the periphery (Lesch & Nyhan, 1964; Rosenbloom et al., 1967; Nyhan, 1973). Patients with LND have cerebrospinal fluid (CSF) Hx concentrations four times higher than normal and plasma uric acid levels double those found in controls (Sweetman, 1968; Rosenbloom et al., 1967).

The sequence of events leading from the deficiency of HPRT to hyperuricemia, urinary tract disease and nephropathy is well established (Kelley & Wyndarrden, 1989; Nyhan, 1973; Lesch & Nyhan, 1964). This disorder is also manifested behaviorally through choreoathetosis, spastic cerebral palsy, self-mutilatory behavior and aggressiveness; these manifestations are not readily explained (Lesch & Nyhan, 1964; Nyhan, 1973; Visser et al., 2000). There is no consistent evidence for anatomical lesions within the brain of LN patients leading most authorities to conclude that neurochemical abnormalities are the primary cause for the behavioral characteristics associated with this disorder (Lloyd et al., 1981; Wong et al., 1996).

Clinical and biochemical evidence from patients with LND indicates abnormalities of GABA, serotonin, norepinephrine, and DA levels and function. However, of all the neurochemical changes observed in LND, the most consistent and significant change is a decrease of DA, its metabolites and DA-associated proteins (Lloyd et al., 1981; Silverstein et al., 1985; Jankovic et al., 1988; Wong et al., 1996; Earnst et al., 1996; Endres et al., 1997). The volume of the caudate nucleus of LND patients has also been found to be reduced by 30%, which suggests a change specific to the DA terminal field in LND (Wong et al., 1996).

Model systems used to investigate the basis of neuropathology in LND include genetic mouse models (HPRT knockouts), neonatal 6-hydroxydopamine lesions and tissue culture studies (Breese et al., 1984; Bitler & Howard, 1986; Kuehn et al., 1987;

Jinnah et al., 1994; Yeh et al., 1998). HPRT knockouts share the same etiology as LND, but do not exhibit any neurobehavioral abnormalities nor do they exhibit high levels of Hx as seen in LND. These mice do, however, have a reduction both of DA levels, and of DA uptake sites in the forebrain (Jinnah et al., 1993; Jinnah et al., 1994; Jinnah et al., 1990). Rodents neonatally lesioned with 6-hydroxydopamine show that the age of insult results in differential effects on behavior, neurochemistry, and neuropathology (Breese et al., 1984). Neither of these animal models addresses the potential role of elevated Hx levels in LND. Despite successes with these models, they do not provide an explanation of the metabolic pathway leading to the observed neurochemical and behavioral abnormalities found in patients.

Although Hx has received little attention in LND, it is considered to be a significant factor in other disorders such as stroke, multiple sclerosis, myelopathy, epilepsy, and viral meningitis (Stover et al., 1997; Marklund et al., 2000; Akdemir et al., 2001). Data collected from these disorders demonstrates that Hx can play a role in oxidative tissue injury. Under normal conditions there is an ATP-dependent regulation of extracellular glutamate, but in stroke and other irritative brain disorders, increases in Hx produce a rapid degradation in energy regulation leading to glutamate-mediated excitotoxicity (Stover et al., 1997).

The present study tests the working hypothesis that excess Hx deleteriously affects the DA system. Preliminary research in our laboratory suggests that Hx, when administered in high doses to adult animals, produces behavioral changes consistent with alterations in DA and possible neurodegeneration (Palmour, 1989; Burke et al., 1999). An important limitation of the previous research on this topic was an inconsistent, slow and unreliable delivery system. Using an Alzet mini-pump to administer a consistent and reliable delivery of Hx, it possible to characterize the effects of Hx on specific neuronal systems through behavioral, anatomical and neurochemical studies. Specific hypotheses tested with this delivery system are 1) excess Hx induces DA neuronal death as measured by TH-ir, 2) excess Hx causes a reduction in DA and its metabolites, and 3) excess Hx alters markers of the DA neuron, specifically DAT and TH.

Methods

Subjects:

Young adult Long-Evans rats (250-275 grams; 2 months of age) from Charles River Laboratories were group housed and provided with food and water ad libitum. Animals were kept under a 12 hour light/dark cycle. All animal protocols were approved by the McGill University Animal Care and Use Committee in accordance to the Canadian Council on Animal Care.

Surgery:

Under ketamine/xylazine anesthesia (60mg/kg, 6mg/kg IM respectively) ALZET minipumps were placed subcutaneously in the mid-scapular region with a catheter attached to a brain infusion cannula. ALZET osmotic pumps were filled with a solution of Hx in artificial CSF for experimental animals or artificial CSF for control animals. The brain infusion cannula was then placed stereotaxically into the left ventricle (A-P +0.5, M-L –1.3) with the tip entering the ventricle at the point where the left ventricle meets the third ventricle (-0.40 through –0.80 bregma, verified post-mortem for each animal). The brain infusion cannula was secured using skull screws and dental cement. The solubility of Hx in artificial CSF limited the final concentration to 5mM Hx in the minipump. Hypoxanthine was delivered at a rate of 2.5 μl/hour corresponding to 1.7 μg Hx/hour or 40.8 μg Hx/day.

Behavioral Assessment:

Animals (n=94) were tested on 3, 7, and 21 days post-surgery. On the day of testing animals were removed from their home cages, placed in a clean cage, and individually transferred to the behavioral assessment room. Behavioral assessments were performed between 6:00 AM and 11:30 AM with the lights turned off. Motor activity was measured in an Omnitech animal activity monitor; the dependent measures were total distance traveled, number of movements, stereotypies and circling behavior in a 60 minute period. After each test, animals were returned to their home room and the

monitor was cleaned and disinfected. Following behavioral testing, animals were sacrificed and their brains prepared for post-mortem analysis, as described below.

Brain Preparation:

Biochemistry: Animals destined for biochemical analysis (n=18) were decapitated, brains quickly removed at which time the striatum was dissected at 4°C and immediately frozen at -80°C.

Immunohistochemistry: Under ketamine/xylazine anaesthesia (100 mg/kg, 10 mg/kg IM respectively), animals used for immunohistochemistry (n=23) were intracardially perfused with 0.1M PBS for 5 minutes followed by 4% paraformaldehyde (pH 7.4) for an additional 5 minutes. Brains were post-fixed overnight and cryoprotected in a 30% sucrose solution for 3-5 days then frozen at –80°C in 2-methylbutane.

Autoradiography: Animals to be used for autoradiological analysis (n=35) were sacrificed by decapitation, the brains quickly removed and then frozen at –80°C in 2-methylbutane. Serial sections from the same animals were used to autoradiographically identify levels of DAT, the serotonin transporter (SERT), TH, and glutamic acid decarboxylase (GAD).

Western Blot Analysis: Animals to be used for Western blot analysis (n=55) were sacrificed by decapitation, the brains quickly removed and then frozen at –80°C in 2-methylbutane. The striatum and substantia nigra were dissected at –20°C and stored at -80°C.

Biochemistry:

Striatal levels of DA, dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), serotonin, 5-hydroxyindoleacetic acid (5-HIAA), and NE were measure by high-pressure liquid chromatography (HPLC). Samples were homogenized in ice-cold 0.1 N perchoric acid containing ascorbic (0.05 mM) and EDTA (20 mg) and were subsequently centrifuged to remove the precipitated protein. The resultant supernatants were used for

analysis of biogenic amines and their metabolites by HPLC with electrochemical detection, using the procedure of Baker et al. (1987).

Immunohistochemistry:

Frozen brains were sectioned at 40 μm in a Lipshaw cryostat and stored for no more than 2 days in PBS (4°C, 24-well plates) until immunohistochemistry could be performed. Immunohistochemistry of TH was conducted according to standard immunostaining protocols (Eisch & Marshall, 1998; Liu & Graybiel, 1992; Nobrega et al., 1999). Briefly, free-floating sections were pretreated with 3% H_2O_2 and 10% methanol in 0.1M TBS, then partially dehydrated, blocked in 5% normal goat serum, washed in TBS then incubated in mouse anti-TH (Sigma; 1:10000 dilution) for 2 days. After washing with TBS, sections were incubated with biotinylated horse anti-mouse IgG (Vector; 1:200) for 1 hour, followed by two additional washes, then a 30-minute incubation with Vectastain Elite ABC kit (Vector) for 30 minutes. The sections were then developed by incubation with 3,3'-diaminobenzidine, washed, slide mounted, rinsed with 95% EtOH, and cleared with xylene. The cells from a minimum of six pair-wise matched sections, taken through the middle of the substantia nigra pars compacta (range from –5.6 through –5.8 bregma) from control and experimental animals were counted under a 16x objective (Watson & Paxinos, 1986).

Dopamine Transporter Autoradiography:

Coronal sections (20μm) were thaw mounted on gelatin-coated slides and stored at –80°C. Quantification of DAT binding sites was performed by incubating sections in a sub-saturating concentration of ^{125}I-RTI-55, a high affinity analogue of cocaine, according to standard protocols (Coulter et al., 1995; Pradhan et al., 2002). Briefly, sections were thawed and incubated for two hours at room temperature with 10p M ^{125}I-RTI-55 (NEN/Perkin-Elmer) diluted in a buffer containing 10 mM sodium phosphate, 120 mM sodium chloride, and 0.1M sucrose and 50 nM citalopram (pH7.4). Citalopram was used to mask SERT during both incubation and washing. The sections were washed 3 times in buffer at 4°C, dipped in double deionized water to remove buffer salts, dried and exposed to BioMax MS film for 3 days along with a ^{125}I radioactive standard. Non-

specific binding was defined as residual ^{125}I-RTI-55 bound in the presence of 10 μM GBR12909.

Serotonin Transporter Autoradiography:

Quantification of SERT binding sites was performed under the same conditions used for DAT, with a few notable exceptions. DAT was occluded with 1 μM GBR12935, rather than citalopram in the incubation and wash buffer. The concentration of labelled ligand (10 pM ^{125}I-RTI-55) remained the same. Non-specific binding was defined as residual ^{125}I-RTI-55 bound in the presence of 100nM citalopram (Rothman et al., 1998; Pradhan et al., 2002).

Immunoautoradiography:

Serial coronal sections adjacent to those used for DAT and SERT autoradiography were used to determine levels of TH and GAD by immunoautoradiography (Izenwasser et al., 1999). Briefly, sections were fixed in 6% paraformaldehyde, 20% EtOH, 20% ethylene glycol, 10% glycerol, and 0.32M sucrose in PBS for 1 hour at 20°C. The sections were then washed in PBS and 0.3% tween, blocked and exposed to either mouse anti-TH (Sigma; 1:10000 dilution) or rabbit anti-GAD 65-67 antibodies (Sigma; 1:10000 dilution) overnight at 4°C. They were then washed and incubated with 0.3 μCi/10ml of either ^{125}I rabbit anti-mouse (for TH; NEN/Perkin-Elmer) or ^{125}I goat anti-rabbit secondary antibody (for GAD; NEN/Perkin-Elmer) plus 1% BSA, 5% rabbit or goat serum, and 0.05% NaN_3 for one hour. The sections were then washed 3 times, dried and exposed to BioMax MS film for 6 days along with an ^{125}I radioactive standard.

Western Blot Analysis:

Tissue was lysed (50 mM Tris pH 7.4, 1% NP-40, 0.25% sodium dexoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM PMSF, and protease inhibitor cocktail tablet) and incubated for one hour at 4°C. The supernatant and pellet were separated by centrifuged at 14,000g for 10 minutes and frozen at -80°C. Supernatant protein was measured (BioRad Bradford Protein assay) and 20μg aliquots were then diluted in an SDS buffer

(0.125 M Tris pH 6.8, 6% SDS, 15% glycerol, 6% β-mercaptoethanol, and 60 mg bromophenol blue), boiled for 4 minutes, separated electrophoretically through an 8% SDS-polyacrylamide gel, and electrotransferred to nitrocellulose membrane (Hybond-P, Amersham). The blots were then blocked for 1hr in PBST (160 g NaCl, 4.0 KCl, 28.8 g Na_2HPO_4, 4.8 g KH_2PO_4, 20 ml Tween-20 and 980 ml H_2O, pH 7.4) plus 10% milk powder, and exposed to one of the following antibodies: rabbit anti-GAD 65-67 (Sigma; 1:10000), mouse anti-HSP-70 (SIGMA; 1:10000), rabbit anti-caspase-3 (Chemicon; 1:1000), or rabbit anti-heme oxygenase-1 (Sigma; 1:1000) for 1hr at room temperature. After primary antibody incubation, the blots were washed in PBST and incubated in anti-rabbit IgG or anti-mouse IgG secondary antibody (1:4000; SIGMA). The blot was then visualised using chemiluminescence (ECL; Amersham). As an internal standard, β-actin was measured with a mouse anti-β-actin (Sigma; 1:10000) on all Western blots.

Image Analysis:

Quantification of the autoradiography studies was performed using the public domain NIH Image program (developed at the U.S. National Institute of Health and available on the internet at http://rsb.info.nih.gov/nih-image/). Optical density readings from autoradiographs were transformed into fmol/mg bound using ^{125}I microscale standards (Amersham), which were exposed on each film. Western blot optical density readings were performed using a Kodak density step tablet.

Data Analysis:

Data analysis was performed on StatView® statistical program. Unless otherwise specified a one or two-way analysis of variance test (for group contrasts) or linear regression was performed as appropriate followed by a Fisher's post-hoc test.

Results

Effects of Hypoxanthine on the Dopamine Neuron:

Effects of Hx on Presynaptic Dopamine Markers:

The principle finding in the present study is that Hx exposure significantly altered the number of TH-ir cells in the substantia nigra (*figure 1*); there were principal effects of both treatment ($F_{2,19}$ = 35.85, p<0.0001) and time ($F_{2,19}$ = 6.6, p<0.02). Reduced TH-ir cells were apparent after 7 days of Hx administration, with a continued decrease in cell numbers by 21 days of Hx treatment. This decrease was accompanied by changes in both TH protein and DAT concentrations as revealed by autoradiography. Specifically, Hx treatment tended to increase TH protein in both the striatum ($F_{2,27}$=3.988, p=0.056 *table 1*) and substantia nigra ($F_{2,24}$=3.769, p=0.063; *table 1*). Hx treatment did not have a significant effect on striatal DAT ($F_{2,29}$=0.376, p=0.55 *table 1*), but did increase nigral levels of DAT ($F_{2,25}$=5.567, p=0.026 *table 1*).

Effects of Hx on Biogenic Amines and Metabolites

After three days of intraventricular administration of Hx, a one-way ANOVA indicates that striatal levels of DA ($F_{2,15}$ = 8.52, p<0.005; 3d Hx vs control, p<0.02; 3d Hx vs 7d Hx, p=0.001 *figure 2*) and serotonin ($F_{2,15}$ = 4.97, p<0.03; 3d Hx vs control vs 7d Hx, p<0.02 *figure 2*) were significantly elevated. Alterations in serotonin (7d Hx vs control, p=0.8; 3d Hx vs control vs 7d Hx, p<0.02) and DA (7d Hx vs control, p=0.18) levels were not evident after 7 days of Hx administration. Additionally, Hx did not produce any changes in norepinephrine ($F_{2,15}$ = 0.22, p=0.81).

Levels of 5-HIAA, the principal metabolite of serotonin, were also elevated after 3 days of Hx treatment ($F_{2,15}$ = 13.5, p=0.0004; 3d Hx vs control vs 7d Hx, p<0.0005 *figure 2*). Hx also had an effect on DOPAC, the principle metabolite of in the rat ($F_{2,15}$ = 9.34, p<0.003), however it did not follow the pattern of DA levels. Instead there was a trend for an increase of DOPAC following 3 days of Hx treatment (3d Hx vs control;

p<0.08), however after 7 days of Hx treatment the levels of DOPAC were significantly decreased (7d Hx vs control, p=0.03; 7d Hx vs 3d Hx, p=0.0006). Additionally, Hx was not observed to have an effect on HVA, a metabolite of DA ($F_{2,15}$ = 1.85, p=0.19; *figure 2*). These data indicate that the elevation in serotonin is accompanied with an increase in its metabolites, however this is not the case with DA and its metabolites after Hx treatment at either time point. These data suggest an Hx-induced intraneuronal accumulation of DA at 3 days followed by a normalization of internal DA stores. Although internal concentrations are normalized at 7 days, the DA neuronal release appears to be depressed as suggested by the low DOPAC concentrations at 7 days.

Other Neurotransmitter Systems:

In order to explore the specificity of Hx effects on multiple neurotransmitters, we examined levels of SERT and glutamic acid decarboxylase (GAD) at 7 and 21 days post-surgery. SERT was not significantly altered by Hx treatment in either the striatum ($F_{2,20}$=0.317, p=0.58) or substantia nigra ($F_{2,20}$=0.505, p=0.49) despite a significant increase in transmitter levels at 3 days post-surgery. Hx administration tended to decrease striatal levels of GAD protein, as measured by immunoautoradiography, but this effect but did not reach statistical significance ($F_{2,20}$=3.616, p=0.08) over time ($F_{2,20}$=3.679, p=0.07). There was no evidence of Hx-related changes of GAD within the substantia nigra ($F_{2,14}$=0.048, p=0.83). Western blot analysis confirmed the absence of Hx-related change of GAD at any time point.

Oxidative stress analysis:

To test the possibility that the effects of Hx were mediated by oxidative stress, we examined a sample of oxidation-related markers. We failed to identify any detectable Hx-related alteration in these proteins through the use of Western blots. Specifically, caspase 3p20 (striatum-$F_{2,43}$=0.572, p=0.45; nigra-$F_{2,47}$=0.829; p=0.37), HSP-70 (striatum-$F_{2,49}$=0.244, p=0.62; nigra-$F_{2,46}$=0.007; p=0.93) and HO-1 (striatum-$F_{2,46}$=0.018, p=0.89; nigra-$F_{2,28}$=0.595; p=0.45) were not significantly different between Hx treated and controls at any of the tested time points. This was true for both the striatum and substantia nigra.

***Behavioural analysis*:**

Locomotor activity, defined as centimeters travelled by the animal, was measured over a 60-minute period. There was a main effect of treatment on activity ($F_{2,89} = 6.4$; $p = 0.013$; *figure 3a*), with Hx significantly increasing locomotor activity. A time effect did not reach significance ($F_{2,89} = 2.842$; $p=0.064$) nor was a time and treatment interaction evident ($F_{2,89} = 2.104$; $p = 0.13$).

Stereotypies, defined as repeated behaviour such as self-grooming or head bobbing, were positively correlated with locomotor activity in both control and treated animals ($F_{2,93} = 280.9$; $p<0.0001$). There was a main effect of treatment with Hx treatment significantly increasing the number of stereotypies ($F_{2,89} = 6.64$; $p = 0.013$; *figure 3b*). A time effect was not evident ($F_{2,86} = 2.788$; $p = 0.067$). The number of movements, defined as discrete movements separated by a rest period of one second, was also influenced by Hx treatment ($F_{2,86} = 4.09$; $p<0.05$; *figure 3c*) and was positively correlated with locomotion ($F_{2,93} = 36.78$; $p<0.0001$). The fact that there was no significant circling behaviour ($F_{2,88} = 2.606$, $p=0.11$ ipsilateral; $F_{2,86} = 0.08$, $p=0.78$ contralateral), despite a cannula placement in the left lateral ventricle, suggested that Hx induced bilateral effects as opposed to unilateral. Of particular note, a subset of animals treated for 21 days displayed a minor form of self-mutilation (5/6 animals vs 1/4 for control animals) mainly occurring on the inner thigh.

Discussion

The DA system has long been considered to be a major player in the pathogenesis of LND. Clinical evidence indicates low brain and cerebrospinal fluid levels of DA and metabolites, low levels of TH in post-mortem striatal tissue, decreased striatal volume, and low DAT levels (Lloyd et al., 1981; Earnst et al., 1996, Endres et al., 1997; Wong et al., 1996). Hx, which is elevated by as much as 400% in patients with LND, has received relatively little attention as a factor that might subserve the neurochemical and behavioural manifestations of the disorder. The current investigation, which uses osmotic mini-pumps to deliver Hx into the ventricle, provides evidence that Hx induces biochemical and immunohistochemical changes within the system.

The principle finding of the present study is that Hx reduces TH-ir positive cells in the substantia nigra, suggesting that Hx may induce DA neuronal cell death. Furthermore, the longer the exposure to Hx the greater the loss of DA neurons, as illustrated by the continued decrease of TH-ir positive cells from 7 to 21 days exposure. Although data collected from disorders such as stroke, multiple sclerosis, myelopathy, epilepsy, and viral meningitis suggests that Hx plays a role in oxidative tissue injury (Stover et al., 1997; Marklund et al., 2000; Akdemir et al., 2001), the potential role of an Hx-DA interaction has not been exhaustively explored. Recently a number of studies have suggested that elevated Hx is toxic within the basal ganglia (Poulsen et al., 1993; Burke et al., 1999; Bavaresco et al., 2004, 2005). Positive Fluoro-Jade staining has revealed that locally applied exogenous Hx into the striatum of the rat is capable of inducing neurodegeneration of the fiber bundles which course through the striatum (Burke et al., 1999). Consequently a number of markers of oxidative stress were examined in the present study. Western blot analysis did not indicate differences in caspase-3 or in the heat shock proteins HSP-70 and heme oxygenase-1 in either the striatum or substantia nigra. These data do not, however, rule out involvement of any one of the many other proteins known to participate in the highly complex oxidative stress pathways known to be active in mammalian cells. *In vitro* studies with striatal synaptosomes suggest that Hx induces an efflux of DA that can be inhibited by free radical scavengers (Poulsen et al., 1993). Along these lines, oxidative stress induced by

Hx has been suggested to inhibit Na^+, K^+-ATPase activity in striatal synaptic plasma membrane of neonatal rats (Bavaresco et al., 2004). Other studies have demonstrated that oxidative stress inhibits Na^+, K^+-ATPase which maybe the situation with high levels of Hx (Wang et al., 2003; Bavaresco et al., 2004, 2005). Indeed, Hx inhibition of Na^+, K^+-ATPase is prevented by the free radical scavengers glutathione and trolox (Bavaresco et al., 2005). It should be noted however that Na^+, K^+-ATPase is not DA-neuron specific (Wang et al., 2003) and may not fully support a specific Hx-DA interaction.

The present findings of reduced TH-ir positive cells in the substantia nigra support the hypothesis that Hx damages, and may kill, DA neurons. The secondary hypothesis that Hx reduces DA content was not supported by biochemical analysis. However these data do suggest that the homeostatic normal response of the DA system to insult is prevented. Typically when there is a partial loss of DA neurons, such as the present study suggests, the remaining neurons fire at an elevated rate to compensate for the loss (Agid et al., 1973). Elevated (presumably intracellular) DA levels 3 days after Hx treatment without an accompanying elevation in metabolites suggests that there is a relative reduction of DA release. Likewise normal levels of DA 7 days after Hx treatment with an attenuated DOPAC concentration also indicates an imbalance between release and intracellular content further suggesting an inability to maintain homeostasis.

A potential mechanism through which Hx may inhibit the return to homeostasis acts through the GABA receptor complex. Previous studies suggest that Hx at concentrations above normal physiological levels, such as those seen in LND, can compete for the benzodiazepine binding site of the GABA-A receptor (Asno & Spector, 1979; Kish et al., 1985). The similarity between the acute effects of Hx on intracellular DA accumulation (without a corresponding increase in DOPAC) and the acute effects of gamma-hydroxybutyrate (GHB) treatment (Howard & Feigenbaum, 1997) is consistent with a GABA-receptor mediated mechanism of action. A generalized increase in serotonin and its metabolite 5-HIAA (Waldmeier & Fehr, 1978) is also seen after either Hx or GHB treatment. The transient increase in striatal serotonin content and turnover in this study after 3 days of Hx treatment may indicate an attempt to stabilize a system in fluctuation; this would be similar to that seen after 6-OHDA lesions (el Mansari et al., 1994; Molina-Holgado et al., 1994; Reader & Dewar, 1999).

Locomotor activity and stereotypies, considered to be indicative of changes in DA neurotransmission, are both increased by Hx treatment. An increase in locomotor activity is generally associated with an elevated post-synaptic response to DA (Galey et al., 1977; Schwarting & Huston, 1996). Based on the pattern of locomotor activity obtained in the present study, the elevation in locomotor activity coincides with neurochemical reductions in DOPAC after 7 days of Hx treatment, suggesting an elevated post-synaptic response to released DA. Additional evidence for an Hx-DA interaction is the increase in TH protein. These data are similar to hypoactivity of DA transmission induced by resperpine (Labatut et al., 1988) or gamma-butyrolactone (Lew et al., 1999) treatment where there are modest increases in TH protein (Labatut et al., 1988; Lew et al., 1999).

Implications for LND

At birth, LND patients are behaviourally normal, but during early post-natal development (typically within the first year), motor abnormalities develop. Subsequently, the limbic aspect of this disorder (e.g. aggression, impulsivity, and self-mutilatory behaviour) is presented during later developmental ages ranging from 2-8 years of age (Lesch & Nyhan, 1964; Nyhan 1997). Hypoxanthine levels do not abate as patients with LND age (Sweetman, 1968; Rosenbloom et al., 1967); therefore the effects of Hx on the DA system, in theory, should be sustained throughout the life-span. The progression in limbic impairment may be due to the prolonged Hx induced alterations of the DA system, which would alter cortical and subcortical neural networks (Berger-Sweeney & Hohmann, 1997; van Kesteren & Spencer, 2003; Lavin et al., 2005). The age range for the appearance of the limbic disorder aspect of LND is similar to the time frame for the expression of hyperactive and impulsive behaviors seen in ADHD (typically by the age of 7; Sagvolden & Sergeant, 1998). This lends support for a protracted hypoactive DA system and resultant affective circuit dysfunction.

Conclusions

The purpose of this study was to examine the effects of high levels of Hx on the mammalian brain with relevance to LND. Data presented here suggests that Hx induces a loss of DA neurons and causes a transient imbalance of DA and metabolite

concentrations. These changes may have far reaching consequences, especially to the developing post-natal brain and possibly into adulthood. Other animal models of LND examine the developmental consequences of either reduced DA (Breese et al., 1984) or inactive HPRT (Jinnah et al., 1994), but the novel aspect of the current study is the demonstration that high levels of exogenous Hx can damage DA neurons which have already been formed. The neuronal loss in combination with the elevated intracellular DA content may act synergistically to further render the DA neuron susceptible to oxidative stress, potentially by Hx and/or DA itself. The observed changes induced by Hx indicate alterations in a number of target measures. Because it is well established that the timing of insult during development determines adult behavioural and neuroanatomical outcomes (Breese et al., 1984), a developmental model (i.e. elevation of Hx soon after birth) would be an obvious further test of this hypothesis. Another limitation of the present study was the relatively short duration of Hx exposure, as compared to the lifelong elevation of Hx in LN patients. Further, because HPRT is still active in the treated rodents, it is reasonable to think that the exogenous Hx has only a small window of opportunity in which to exert its affects. Even given these limitations, this work provides the first controlled data suggesting that elevated Hx may be related to DA dysfunction in Lesch-Nyhan disease.

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	3d control	7d control	21d control	Overall Average	3d Hx	7d Hx	21d Hx	Overall Average
Striatum								
TH	0.16±0.01	0.15±0.01	0.15±0.02	0.151±0.007	0.18±0.02	0.14±0.01	0.21±0.03	0.163±0.01
DAT	1.34±0.03	1.16±0.09	1.39±0.11	1.274±0.048	1.21±0.03	1.35±0.11	1.38±0.10	1.321±0.061
GAD	N/A	0.32±0.03	0.32±0.02	0.317±0.018	N/A	0.32±0.01	0.24±0.01	0.295±0.013
SERT	N/A	0.98±0.13	0.82±0.09	0.991±0.09	N/A	1.03±0.09	0.96±0.07	0.898±0.067
SN								
TH	0.13±0.01	0.14±0.01	0.14±0.01	0.138±0.005	0.17±0.01	0.16±0.02	0.17±0.02	0.162±0.01
DAT	0.40±0.02	0.48±0.07	0.50±0.02	0.447±0.02	0.39±0.024	0.56±0.08	0.81±0.07	0.565±0.054†
GAD	N/A	0.45±0.06	0.51±0.06	0.474±0.04	N/A	0.56±0.04	0.43±0.05	0.508±0.037
SERT	N/A	1.66±0.11	1.63±0.10	1.651±0.073	N/A	1.78±0.09	1.68±0.13	1.752±0.074

Table 1. TH protein, revealed by immunoautoradiography, reveals a trend for an Hx induced increase in both the SN and striatum (n=18 Hx, n=17 control). The overall average column refers to average values across the three time points. Hypoxanthine induced changes in DAT (fmol/mg) revealed by a non-saturating concentration of [I^{125}]RTI-55 reveals a significant overall increase in the SN (n=17 Hx, n=15 control). Serotonin transporter (fmol/mg; n=13 Hx, n=9 control), revealed by a non-saturating concentration of [I^{125}]RTI-55 and GAD protein (n=13 Hx, n=9 control) was not altered. GAD and TH immunoautoradiography data are presented as average relative optical density (mean±SEM). Autoradiography data are presented as average fmol/mg tissue (mean ±SEM). †*P<0.05*

Figure Captions

Figure 1. Hypoxanthine-induced changes in the substantia nigra pars compacta as revealed by TH-ir cells. TH-ir cells per section are decreased ($p<0.0001$; Hx n=12, control n=11). At 7 days there was a 22% decrease and 21 days by 30%. Data are presented as mean $\pm$SEM.

Figure 2. Neurochemical changes were demonstrated after Hx treatment. Striatal DA concentrations were elevated at 3 days post-surgery (n=6) compared to control (22% n=6) and 7 days (34% n=6) post-surgery groups. DOPAC concentrations were decreased at 7 days post-surgery compared to 3 days post-surgery (26%) and controls (16.7%). Serotonin concentrations were elevated at 3 days post-surgery compared to control (24.7%) and 7 days (27.1%). 5-HIAA concentrations were increased at 3 days post-surgery compared to 7 days post-surgery and controls. Hx did not affect norepinephrine levels. Data are presented as mean$\pm$SEM with DOPAC, HVA, serotonin, 5-HIAA, and norepinephrine levels multiplied by a factor of 10. *$P<0.05$, ** $p<0.01$, ***$p<0.005$.

Figure 3. Hypoxanthine (n=57) induced increases in (a) locomotion ($p<0.01$), (b) stereotypies (15%, $p<0.01$), and (c) movements ($p<0.05$) as compared to control animals (n=38) expressed as mean $\pm$ SEM.

Figure 1

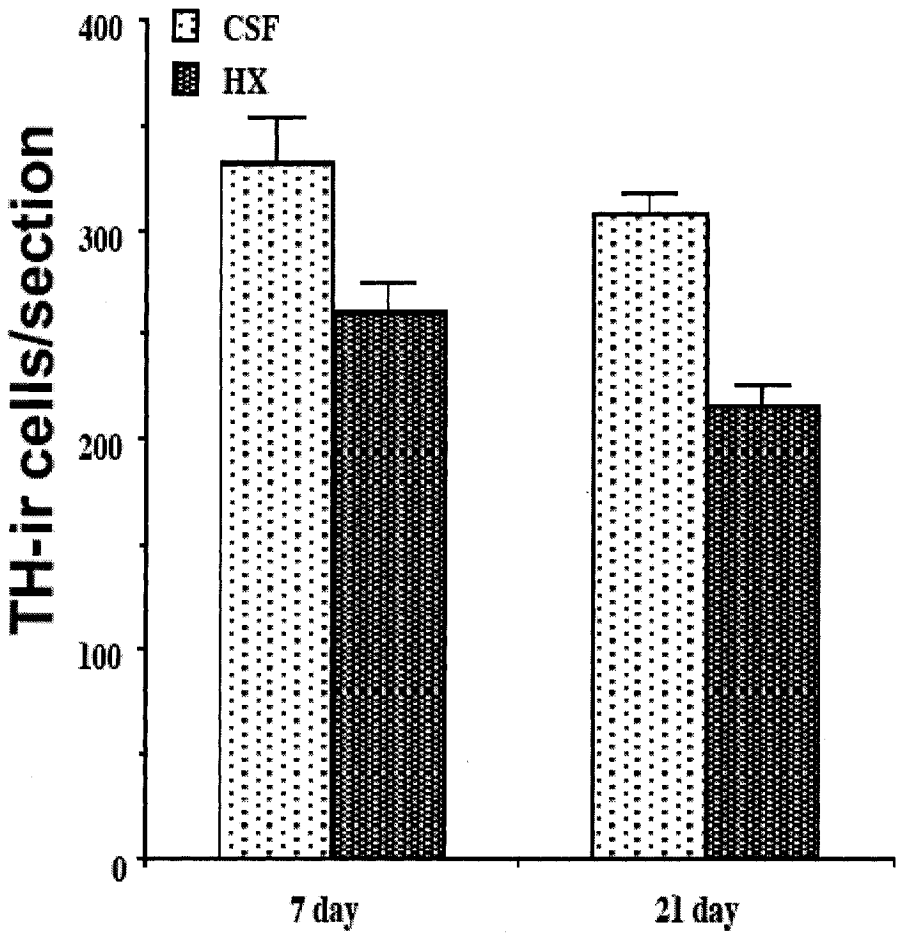


Figure 2

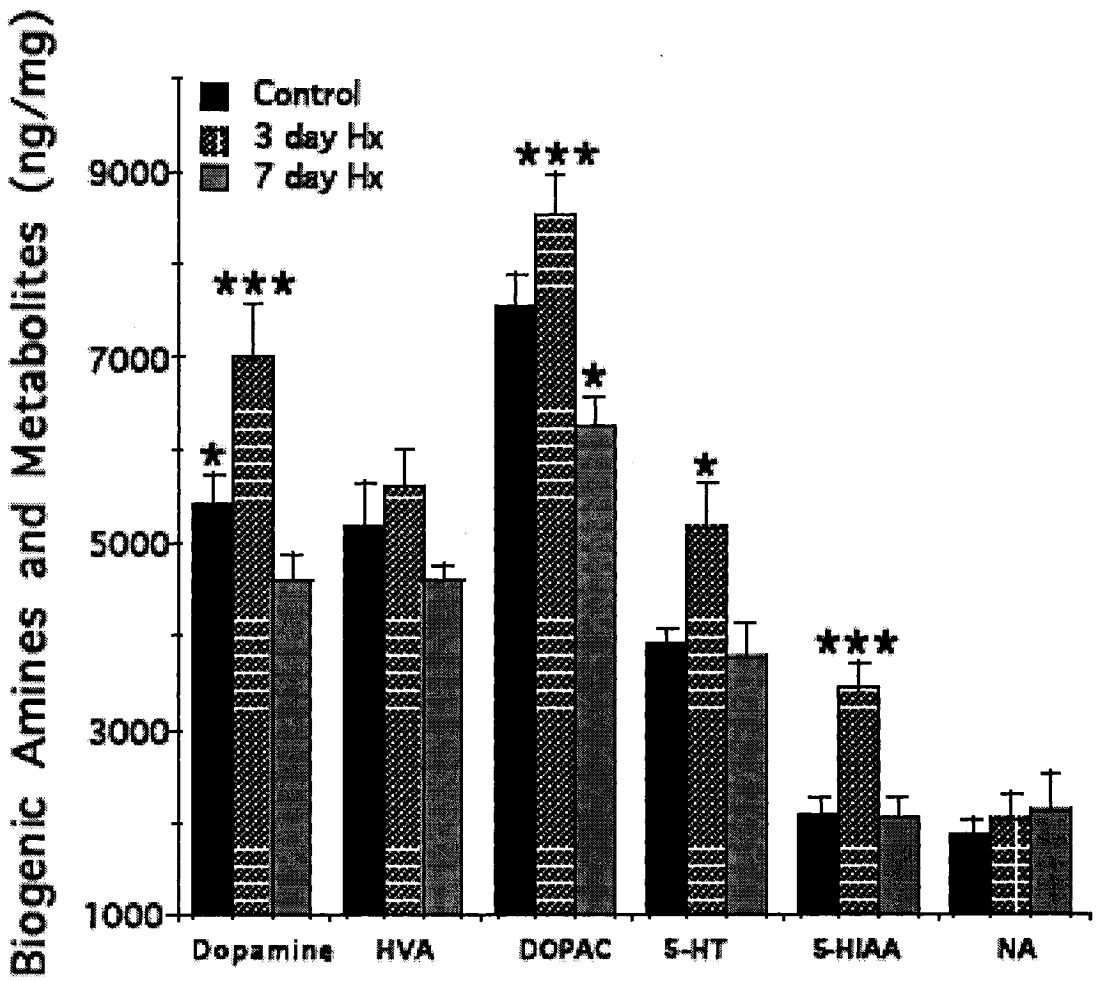
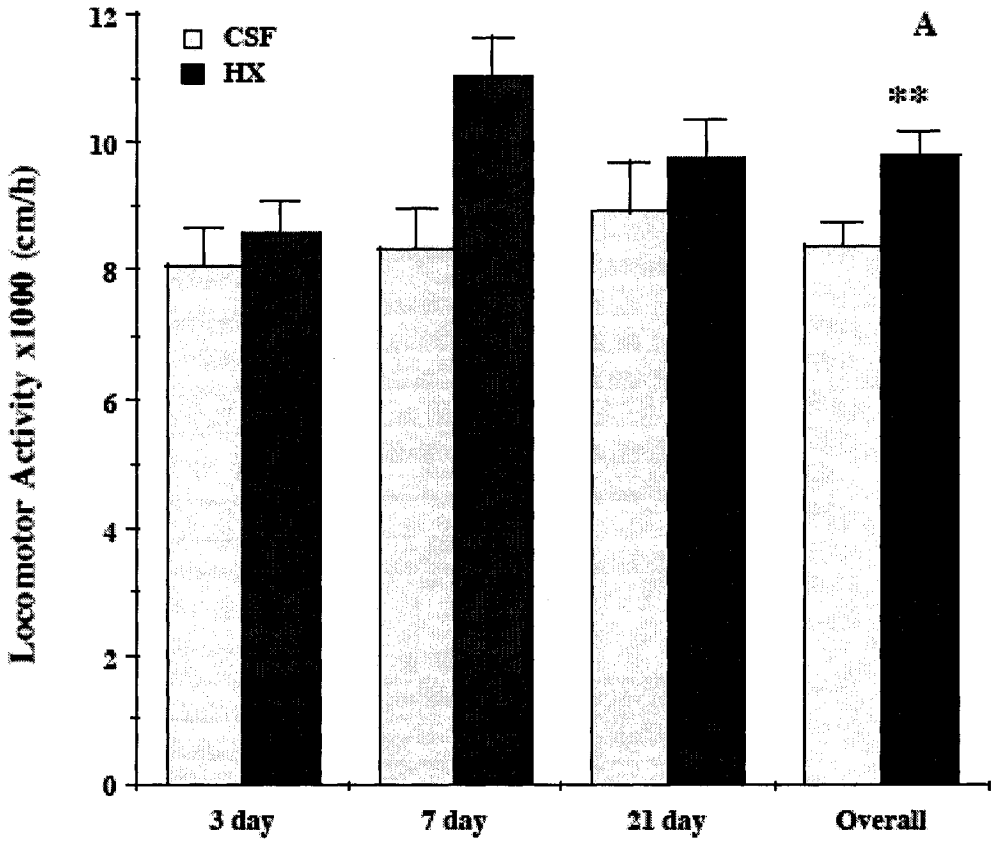
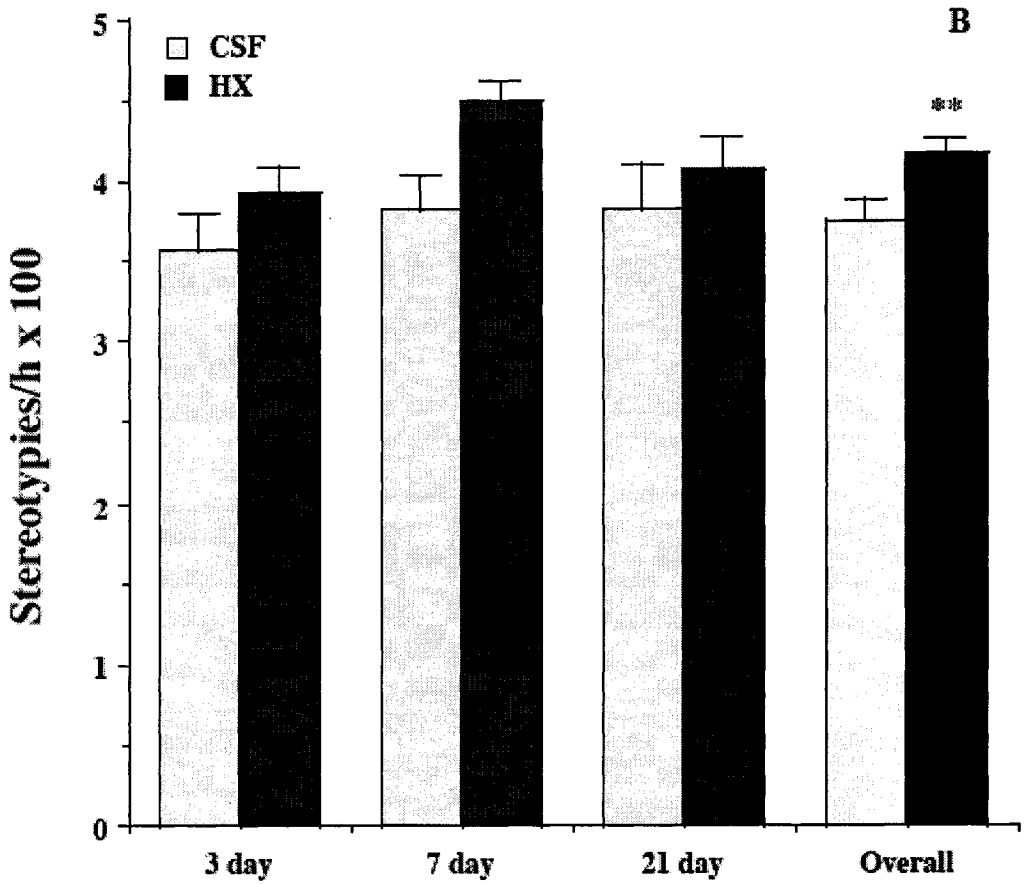
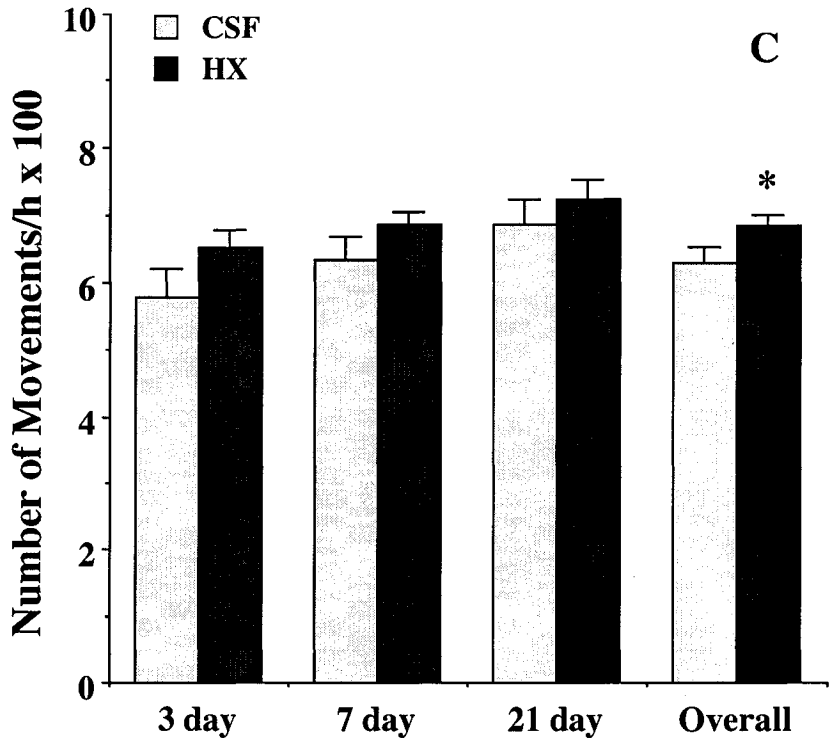

Biogenic Amines and Metabolites (ng/mg)
Control
3 day Hx
7 day Hx
9000
7000
5000
3000
1000
***
*
***
*
*
***
Dopamine
HVA
DOPAC
5-HT
5-HIAA
NA

Figure 3


A
CSF
HX
**
Locomotor Activity x1000 (cm/h)
0
2
4
6
8
10
12
3 day
7 day
21 day
Overall


B
CSF
HX
**
Stereotypies/h x 100
0
1
2
3
4
5
3 day
7 day
21 day
Overall


10
8
6
4
2
0
CSF
HX
C
*
Number of Movements/h x 100
3 day
7 day
21 day
Overall

CHAPTER V

NEUROCHEMICAL PROFILES DISTINGUISH BINGE DRINKING VERVETS FROM HEAVY DRINKERS

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Preface

In the previous chapter it was found that Hx treatment deleteriously affects the nigrostriatal DA system. The experiments in this chapter utilize the DAT and SERT binding assay, detailed in chapter 2, to neurochemically differentiate alcohol preferring vervet monkeys. Like other drug of abuse, alcohol abuse has been linked to both DA and serotonin system (Gessa et al., 1985; Di Chiara & Imperto, 1988; Yoshimoto et al., 1992a,b). Vulnerability to alcohol abuse is widely conceptualized as a deviant reward function resultant from a dysregulated DA reward circuit (Koob, 2003). In our non-human primate model, monkeys freely drink large quantities of alcohol to the point of intoxication (Ervin et al., 1990). Within the striatum of these heavy-drinkers there is a higher concentration of DAT at baseline as compared to non-drinkers (Mash et al., 1996). The current study expands this work in two ways: first, the earlier study was based on sagittal sections with a focus on the DA terminal field in heavy-drinking monkeys. In the present work, we focus on brainstem cell body region. Second, in the previous study, only heavy drinkers and alcohol avoiding animals were studied. However heavy-drinkers are only one type of drinkers observed in our population (Palmour et al., 1997). There is an array of alcohol consumption ranging from abstinent to light-, heavy-, and binge-drinkers, and in the present study these groups are examined. This chapter presents experiments that tests the hypothesis that densities of both DAT and SERT vary in brainstem cell body regions in relationship to four patterns of ethanol consumption in vervet monkeys.

Contribution by authors:

Burke, MW: Co-designed anatomical studies, execution of experiments, analysis of data, and preparation of manuscript.

Young, SN: Biochemical analysis of CSF brain amines.

Louard, E: Execution and supervision of behavioral experiments and collection of samples.

Paliouras, GN: Independent verification and calibration for data analysis.

Ervin, FR: Co-design of study, supervision of experiments, analysis of data and preparation of manuscript.

Palmour, RM: Co-design of study, supervision of experiments, analysis of data and preparation of manuscript.

NEUROCHEMICAL PROFILES DISTINGUISH BINGE DRINKING VERVETS FROM HEAVY DRINKERS

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Running title: Neurochemical profiles in binge and heavy drinking vervets

Abstract

Dysfunctions of dopaminergic and serotonergic neurotransmission are characteristic of abuse of beverage alcohol and other addictive drugs, but there are few consistent clinical observations and considerable evidence of heterogeneity, which may be related to clinical state or to diagnostic subtypes. We have previously reported that alcohol-preferring and alcohol-avoiding vervet monkeys differ with respect to striatal levels of the dopamine transporter (DAT) and several dopamine receptors (Mash et al., 1996; Mash et al., 1994; Haracz et al., 1999). More recent data suggest that both drinking classes can be further subdivided, using operational criteria. In the present study, we measured brainstem levels of DAT and serotonin transporters (SERT) in brains harvested from 32 male vervet monkeys representing four drinking classes: alcohol avoiding (AA), social, heavy and binge drinkers. Mesolimbic DAT levels were significantly higher in heavy drinkers as compared to either AA and binge drinkers, while substantia nigra DAT was lower in binge drinkers than in heavy drinkers. Midbrain tyrosine hydroxylase was positively correlated with levels of DAT in substantia nigra, and cerebrospinal fluid levels of homovanillic acid (the principal metabolite of DA) were positively correlated with DAT in substantia nigra and limbic midbrain regions. SERT levels in both central (substantia nigra) and lateral (substantia nigra pars lateralis) midbrain regions were significantly lower in binge drinkers, as compared to all other groups, and there was a significant reduction in CSF levels of 5-hydroxyindoleacetic acid (the principal metabolite of serotonin) in binge drinkers as compared to AA or heavy drinkers. Regional co-distributions of SERT and DAT in midbrain cell body regions suggest a neuroregulatory basis for the frequently observed positive correlations between HVA and 5-HIAA levels in CSF. These data provide further evidence of biochemical individuation of drinking phenotypes in a population-based non-human primate model of alcohol abuse.

Introduction

Alcohol misuse, currently considered to be the most prevalent psychiatric disorder worldwide (O'Brien, 2003), remains neurobiologically challenging. There is widespread agreement that alcohol dependence is not a unitary disorder, but rather a continuum comprising several orthogonal features (Buchholz et al., 1994; Kendler et al., 1992, 1993, 1994; Kessler, 1995). In humans, distinctions based upon comorbidities with other psychiatric disorders are clinically relevant (Kendler et al., 1997), but only a very few statistically-based phenotypes have been proposed (Cloninger et al., 1981; Cloninger, 1987). Although heuristically important, such classifications either pertain to a relatively small proportion of cases or may be poorly operationalized (Cloninger et al., 1981; Penick et al., 1999; Kovac et al., 2002). Other widely-used phenotypes emphasize family history, polydrug abuse or personality disorders (Hesselbrock et al., 1985; Cadoret et al., 1985, 1995a,b; Jang et al., 1995, 1997).

Efforts to understand the biological basis of alcohol abuse have benefited from the development of animal models in which mechanistic hypotheses can be tested. Inbred strains of mice differ not only in the extent of voluntary ethanol consumption, but also may exhibit variability with respect to the acute or chronic effects of drug abuse (Crabbe and Harris, 1991; Crabbe et al., 1994). Genetically selected paired rodent strains such as Indiana P/NP, Sardinian AP/AA and Finnish AA/ANA are suitable both for investigation of factors which predispose to abuse and mechanisms through which baseline distinctions are modulated by exposure to acute or chronic alcohol (Overstreet et al., 1997; Fadda et al., 1990; Murphy et al., 1998, 1996; Katner & Weiss, 2001), but the characteristics of the preferring members of each pair of strains are not identical. Non-human primate models of alcohol abuse are less well-developed (Ervin et al., 1990; Higley et al., 1996a,b; Vivian et al., 2001), but offer obvious advantages with respect to understanding human alcohol abuse. As is the case with rodent models, it is likely that the different non-human primate models examine different aspects of vulnerability to abuse and may also model different consequences of prolonged exposure to beverage alcohol. To be explicit, the rhesus model has emphasized the role of early life experiences in relation to alcohol abuse (Higley et al., 1996a,b), while the cynomologus model utilizes controlled

introduction to beverage alcohol, followed by an extended period of voluntary access (Vivian et al., 2001). A small proportion of the vervet population drinks heavily or abusively without prior conditioning (Ervin et al., 1990), making it ideal for study of predisposing factors devoid of prolonged exposure to the effects of ethanol. Factor analysis of a large (n >1300 individuals) population of these animals exposed at least twice to a two-week alcohol preference paradigm suggests that there are at least four groups with respect to alcohol consumption (Palmour et al., 1997, and submitted).

There is a robust literature documenting the involvement of the monoamine transmitters dopamine (DA) and serotonin (5-HT) in drug abuse disorders, including alcoholism, and in experimental animal models for these traits (Buydens-Branchey et al., 1989; Cloninger, 1987; Koob & LeMoal, 1997; Wise & Rompre, 1989; Heinz et al., 2004). Like several other drugs of abuse, acute ethanol administration elevates brain levels of both DA (Di Chiara and Imperato, 1988; DiChiara, 1995) and 5-HT (Yoshimoto et al., 1992a,b). In alcoholic patients, recent studies of the 5-HT and DA pathways have relied primarily on in vivo imaging techniques, which despite methodological issues and conflicting reports, tend to suggest both serotonergic and dopaminergic dysfunction (Heinz et al., 1998a,b, 2002; Tiihonen et al., 1995, 1997, 1998; Tupala et al., 2003; Volkow et al., 1996). Although informative, in vivo imaging with human alcoholics presents inherent problems, such as the inability to control for environmental factors influencing alcoholism in any given individual, diagnostic heterogeneity, different lengths of abuse and the difficulty in controlling for varying periods of abstinence.

Because clinically approved radioligands are available for DAT and SERT, these proteins are important targets of opportunity for neuroimaging. These proteins are thought to be the primary regulators of extracellular levels of these amines (Cass & Gerhardt, 1994; Giros et al., 1996; Reith et al., 1997; Chen & Reith, 2000; Horschitz et al., 2001). In a previous study (Mash et al., 1996), we found that DAT density, as measured by quantitative autoradiography with ^{125}I-ßCIT (the same ligand used in human PET studies), was elevated throughout the motor and limbic striata of alcohol-preferring (AP) vervet monkeys, as compared to matched alcohol-avoiding (AA) controls, but levels in chronically drinking monkeys did not differ from those in controls. Receptor autoradiography of sagittal sections showed borderline differences in DAT binding

between AP and AA monkeys, but detailed study of brainstem cell body areas was not performed, and there was no evaluation of SERT binding densities in the Mash et al. (1996) study. The goal of the present investigation was to evaluate levels of both DAT and SERT in brainstem areas of abstinent vervet monkeys from each of the three drinking classes, as compared to abstinent alcohol-avoiding animals.

Methods

Subjects: Brains were harvested from 32 unrelated adult (6-13 years of age) male vervet monkeys, housed in the laboratories of Behavioural Sciences Foundation, St Kitts. Each animal had been screened for voluntary alcohol consumption, as described below, a minimum of two times, but had no exposure to beverage alcohol for at least 6 months (average: 8.5 months; range: 6-13 months) prior to sacrifice. Except for these brief periods of preference screening, animals were group-housed and were fed on Purina chow and fresh local fruit, with water available ad libitum. The experimental protocol was reviewed and approved by an Institutional Review Board established under the auspices of the Canadian Council of Animal Care.

Measurement of voluntary alcohol consumption: Voluntary alcohol consumption was measured in each animal according to a standard procedure (Ervin et al., 1990; Palmour et al., 1998). Briefly, each animal was removed from its home cage under ketamine anesthesia, weighed and examined, and placed in an individual stainless steel cage equipped for quantitative testing of ethanol consumption. On the following day and the four subsequent days, both tap water and 10% ethanol (w/v) in tap water were available ad libitum throughout the 24 h diurnal period. For the next 5 days, the 10% ethanol solution was only available from 9 am to 1 pm (scheduled access). The volumes of alcohol and water consumed during these test periods were recorded to the nearest 5 ml. Each animal was fed on the usual daily schedule with Purina Primate chow and produce. Ethanol consumption (in g/kg/period) and preference ratio (ethanol:total fluid) were computed for each animal in the two conditions.

Biochemical measurements: On the day before each ethanol preference testing period, cisternal cerebrospinal fluid samples were obtained by transcutaneous puncture and were frozen at –80 C until analysis. CSF amines were measured by HPLC with electrochemical detection (Anderson et al., 1979, 1980), as described previously for this species (Young et al., 1989; Palmour et al., 1998). Mean values were computed from

several CSF samples (mean: 3.1, range 2-6) taken from each animal under these same conditions.

Neuroanatomical Studies: Animals were sacrificed by lethal injection with Somnotol®, followed by exsanguination and thoracotomy. Thereafter, brains were rapidly removed, the brainstem with attached cerebellum was dissected out and cerebral hemispheres were separated at the midline. All three regions were immediately frozen by slow immersion in 2-methylbutane prechilled in a dry ice-methanol bath. The brains remained at -80°C until histological sections could be taken. Coronal sections of the rostral brainstem were cut at 20 μm on a Lipshaw cryostat, thaw-mounted on gelatin-coated slides and stored at -80°C.

Transporter Autoradiography: Quantitative measurement of both DAT and SERT binding sites was performed by incubating adjacent (20 μm) sections in a sub-saturating concentration of ^{125}I-RTI-55, a high affinity analogue of cocaine, according to standard protocols (Coulter et al., 1995; Pradhan et al., 2002). Briefly, sections were thawed, and incubated for two hours at room temperature with 10 pM ^{125}I-RTI-55 (NEN/Perkin-Elmer) diluted in a buffer containing 10 mM sodium phosphate, 120 mM sodium chloride and 0.1 M sucrose (pH7.4). Citalopram (50 nM) was used to mask SERT, while 1 μM GBR12935 was used to occlude DAT (Rothman et al., 1998). The sections were washed 3 times in dilution buffer (4°C) containing either citalopram or GBR12935, as appropriate, dipped briefly in double deionized water to remove buffer salts, dried and exposed to BioMax MS film for 3 days along with an ^{125}I radioactive standard (Amersham). Non-specific binding was defined as residual ^{125}I-RTI-55 bound in the presence of either 10 μM GBR12909 for DAT or 100 nM citalopram for SERT (Rothman et al., 1998; Pradhan et al., 2002). All slides were counterstained with haemotoxylin-eosin as soon as film exposure had been verified.

Immunoautoradiography: Serial coronal sections adjacent to those used for DAT and SERT autoradiography were used to determine levels of TH and glutamic acid decarboxylase (GAD) by immunoautoradiography (Izenwasser et al., 1999). Briefly,

sections were fixed in 6% paraformaldehyde, 20% EtOH, 20% ethylene glycol, 10% glycerol, and 0.32M sucrose in PBS for 1 hour at 20°C. The sections were then washed in PBS and 0.3% Tween, blocked (in buffer containing 3% B SA, 3% rabbit serum, 0.05% NaN3) and exposed to either mouse anti-TH (Sigma; 1:10000 dilution) or rabbit anti-GAD 65-67 antibodies (Sigma; 1:10000 dilution) overnight at 4°C. They were then washed and incubated with 0.3 μCi/10ml of either ^{125}I rabbit anti-mouse (for TH; NEN/Perkin-Elmer) or ^{125}I goat anti-rabbit secondary antibody (for GAD; NEN/Perkin-Elmer) plus 1% BSA, 5% rabbit (TH) or goat serum (GAD), and 0.05% NaN_3 for one hour. The sections were then washed 3 times, dried and exposed to BioMax MS film for 6 days along with an ^{125}I radioactive standard (Amersham).

Strategy for Tissue Analysis: The anterior parabrachial nucleus of the ventral tegmental area was chosen to represent the limbic-related midbrain area because this area is readily distinguishable from the substantia nigra pars compacta. The ventral midline and substantia nigra pars compacta were measured together in the present analysis because these areas were not readily distinguishable using the present techniques. Regional measurements for the parabrachial area and substantia nigra for all analyses were defined by DAT autoradiography (figure 1). For the limbic-related parabrachial tegmental (AP+11, Contreras et al., 1981) region, there were on average 10.7 and 4.87 measurements per monkey for DAT and SERT, respectively. The SNc/VTA region spanned from AP+11 to AP+ 6.5 (Contreras et al., 1981) and alternate sections were processed for DAT and SERT to facilitate comparisons. DAT assays were performed in triplicate and SERT in duplicate resulting on average 24.5 and 16.3 measurements for DAT and SERT respectively spanning this entire region. The lateral substantia nigra corresponded to AP+8.5 to AP+6.5 and was operationally defined as the lateral third of the posterior two/thirds of the substantia nigra region (Contreras et al., 1981). On average 7.9 measurements were taken for analysis of the lateral substantia nigra. The midline DA and 5-HT pathways corresponded to AP +7.0 to +6.5 and, on average, 2.5 measurements were analysed for each subject. Each measurement was taken in duplicate, on separate occasions, for each region and each transporter, and any measurement that

failed to meet a 95% coherence criterion was repeated. All measurements were obtained by a trained observer (MB) blind to the drinking status of the subjects.

Image Analysis: Quantification of the autoradiography studies was performed using the public domain NIH Image program (developed at the U.S. National Institute of Health and available on the internet at http://rsb.info.nih.gov/nih-image/). Optical density readings from autoradiographs were transformed into fmol/mg bound using ^{125}I microscale standards (Amersham), which were exposed on each film.

Data Analysis: Data was analyzed by ANOVA (for group contrasts) or linear regression, as appropriate, using StatView V (Systat) on a MacIntosh computer. Pairwise post-hoc tests of significance used Fisher's PSLD.

Results

Patterns of Alcohol Consumption: Animals sacrificed for this study were selected from a population of over 300 adult male vervets on the basis of quantities and patterns of ethanol consumption, using criteria which have been previously described (Mash et al., 1996; Palmour et al., 1998). As noted above, each animal used in this study was tested for ethanol preference on at least two separate occasions. A monkey defined as alcohol-avoiding (AA) never drank more than 2 g/kg ethanol per day (averaged over a 5 day testing period) or more than an average of 1 g/kg in a 4 h period of restricted access. As shown in Table 1, the average values for AA monkeys are well below this threshold. Social drinkers (Soc) consumed more than 2 and less than 5 g ethanol/kg/24 h (averaged over the testing period) on each and every separate 5-day testing occasion. Over the 5-day period of testing, heavy or binge drinkers consumed an average of at least 5 g ethanol/kg/24 h during unrestricted access and at least 3.5 g/kg during restricted access, and again these criteria were applied for each and every drinking occasion. Binge and heavy drinkers were further differentiated from each other based on their pattern of drinking. Every animal defined as a binge drinker in this study had been behaviorally observed to drink to coma on multiple occasions (although not every day) during alcohol preference testing. During the period of scheduled access, this generally occurred within the first 2 h of ethanol availability, but could occur at any time of day during free access. Although heavy drinkers also became intoxicated, they almost never lost consciousness and during continuous access, would drink 0.2 – 0.6 g/kg/h over the course of the entire day. As shown in Table 1, there are significant differences between groups for all measures of ethanol consumption, but no significant differences with respect to body weight.

Each animal in this study was screened for ethanol preference at least two times, a minimum of 6 months apart. The mean number of alcohol preference testing sessions (drinking occasions), as described in Methods, was 3.77 (range, 2 – 11). Although heavy and binge drinkers were tested more frequently (5.25 ± 2.37 and 5.29 ± 1.86 testing periods, respectively) than social drinkers and alcohol avoiding animals (2.4 ± 0.98 and 2.2 ± 0.49, testing periods), this difference did not reach statistical significance ($F_{3,28}$ =

1.38, p = 0.27). Not surprisingly, however, the total ethanol exposure (in g/kg) between groups did differ significantly ($F_{3,28}$ = 3.46, p = 0.03). Mean lifetime exposure levels were 49 ± 18, 128 ± 62, 316 ± 134 and 419 ± 122 g/kg for alcohol avoiding animals, social, heavy and binge drinkers, respectively. As discussed further below, there was no significant relationship between total exposure and any neurohistochemical or biochemical measure reported in this paper.

There were also no group differences with respect to either duration between last ethanol exposure and death ($F_{3,28}$ = 1.59, p = 0.21; AA 8.5 ± 1.1 months, Soc 7.1 ± 1.4 months, Heavy 10.3 ± 3.9 months; Binge 8.2 ± 4.8 months) or estimated age at time of sacrifice ($F_{3,28}$ =1.76, p = 0.18; AA 10 ± 0.5 yr, Soc 8.9 ± 2.1 yr; Heavy 8.2± 2 yr; Binge 10.7 ± 3 yr).

Dopamine Transporter Autoradiography: Each binding assay used in the present experiment (DAT, SERT, TH, and GAD) provided excellent signal-to-noise ratio, as indicated by the fact that non-specific binding was virtually undetectable. DAT estimations were available for 26 animals (7 each AA and heavy drinkers; 6 each social and binge drinkers, except for the caudal linear raphe nucleus for which each group was reduced by one). Measurements of regional densities of DAT were highly consistent within a specific brainstem region in a given individual (coefficient of variation < 5%), but between subject variance (even within a group) was considerably higher. The validity of the experimental protocol with respect to comparative studies and to the discrimination between SERT and DAT has already been presented in detail (Pradhan et al., 2002). Preliminary analysis failed to show any relationship between cumulative ethanol exposure (in g/kg) and levels of DAT, so this variable was excluded from further analyses. Levels of DAT in the parabrachial limbic area varied significantly by drinking group ($F_{3,22}$ = 5.17, p < 0.01), with significant pairwise differences (p<0.05) between heavy and binge drinkers and between heavy and AA (Figure 2). In the SN, DAT levels also differed between group ($F_{3,22}$ = 4.2, p < 0.02), with a significant pairwise difference (p <0.05) between heavy and binge drinkers (Figure 2). There was no significant difference between groups with respect to DAT binding in the caudal linear raphe

nucleus (Figure 2). Examples of pair-wise contrasts between AA, binge-, and heavy-drinkers are shown in Figure 3.

Serotonin Transporter Autoradiography: SERT estimations were available for 26 animals (7 each AA and heavy drinking animals and 6 animals for each of the other groups, except for the caudal linear raphe nucleus, for which only 19 subjects were available 4 each AA and binge, 5 social and 6 heavy drinkers). SERT densities, like DAT densities, did not vary as a function of ethanol exposure but did vary significantly between groups. This effect was most pronounced in lateral midbrain ($F_{3,22} = 4.65$, $p < 0.01$), with pairwise differences ($p<0.05$) between binge and heavy drinkers and between AA animals and binge drinkers (Figure 4). Additionally, central ($F_{3,22} = 3.68$, $p < 0.03$) regions of the substantia nigra, with post hoc pairwise differences ($p < 0.05$) between binge and heavy drinkers and between binge drinkers and AA animals. There were also significant differences in SERT density in the parabrachial nucleus ($F_{3,22} = 3.68$, $p < 0.03$), again with the binge drinkers showing a significantly reduced level of binding relative to AA and heavy drinking animals (Figure 4). In the caudal linear raphe area, binge drinkers also showed a reduction in SERT binding (Figure 3) which did not reach statistical significance ($F_{3,15} = 2.39$, $p = 0.09$), perhaps in part because data was available for only a subset of the animals.

Co-localizations of SERT and DAT within the brainstem: Throughout the rostro-caudal extent of the ventral midbrain there is considerable overlap between DAT and SERT binding. The distribution of DAT and SERT throughout this region is not homogeneous. Interdigitation of high concentrations of DAT and SERT is evident in the lateral and posterior areas (Figure 6).

Immunoautoradiography: Levels of tyrosine hydroxylase (TH), as measured by immunoautoradiography, were significantly correlated with levels of DAT in SN ($F_{1,24}= 6.98$, $p < 0.02$), but were not related to measures of DAT in other midbrain areas. There

was no relationship between midbrain levels of GAD and either DAT or SERT in any midbrain area. Neither TH nor GAD binding varied as a function of drinking class.

Biochemical analysis: Levels of cerebrospinal fluid 5-HIAA, the principal metabolite of serotonin, varied as a function of drinking class (Table 2), with significant pairwise differences between binge and heavy drinkers and between binge drinkers and AA animals. There was also a significant relationship between cerebrospinal fluid levels of HVA (the principal metabolite of DA) and drinking status, with a significant pairwise difference between heavy and binge drinkers and binge drinkers and AA. There was no relationship between levels of MHPG (the principal metabolite of norepinephrine) and drinking status. As has been reported many times previously, there were significant correlations between CSF levels of 5-HIAA and HVA ($r = 0.712$, $p < 0.0001$, in this sample of animals), but no correlation between CSF levels of MHPG and other amine metabolites.

CSF HVA levels were positively correlated with DAT levels in parabrachial tegmental area ($F_{1,24} = 7.08$, $p < 0.02$; $r = 0.48$) and SN ($F_{1,24} = 4.49$, $p < 0.05$; $r = 0.40$). Similarly, CSF 5-HIAA levels tended to show a positive correlation with SERT levels in both the central midbrain area ($F_{1,23} = 3.48$, $p < 0.07$) and the lateral SN ($F_{1,23} = 3.93$, $p < 0.06$). There was no correlation between CSF HVA and DAT levels in the caudal linear raphe nucleus, or between CSF 5-HIAA levels and SERT levels in the dorsal serotonin pathway.

Discussion

The notion that dopaminergic and serotonergic neurotransmission are implicated in alcohol abuse (Heinz et al., 2004) is supported by both clinical and animal studies, but controversy about the timing and specificity of this involvement is considerable. In previous studies of animals from this population, Mash et al. (1996) showed that striatal and accumbens DAT density was elevated in abstinent alcohol-preferring individuals as compared to AA conspecifics, and also reported that DAT binding decreased during chronic alcohol exposure and rebounded upon withdrawal. The extent to which DAT binding in midbrain cell body regions might differ between groups was inadequately explored. The data presented here thus extends the findings of that earlier study by examining brainstem regions, by expanding the range of target molecules and by extending the analysis to additional drinking classes.

An important finding is that binge drinkers and heavy drinkers showed opposite effects with respect to DAT concentrations in parabrachial VTA and SN. DAT levels in parabrachial VTA were elevated in heavy drinkers as compared to either binge drinkers or social drinkers, while SN levels of DAT were significantly reduced in binge drinkers as compared to heavy drinking animals. Positive correlations between DAT binding and midbrain levels of TH, as revealed by immunoautoradiography, and between DAT binding and CSF levels of HVA suggest an acceptable degree of internal consistency in the present findings. It is also noteworthy that there were no significant differences between AA animals and social drinkers, except for ethanol consumption measures.

A novel finding is that SERT binding was reduced in all measured areas of the midbrain for binge drinkers, but heavy, social and avoidant subjects did not differ with respect to SERT. Low SERT binding was particularly obvious throughout the extent of the substantia nigra. Cerebrospinal fluid levels of 5-HIAA were significantly reduced in binge drinkers (as were CSF levels of HVA), and showed a trend toward positive correlation with SERT binding across the entire sample of animals. Low 5-HIAA, but high SERT binding were reported in a macaque model in which aggressive behavior and abusive drinking were associated with peer-rearing (Heinz et al., 1998a,b; Higley et al., 1996a,b).

The present study extends our previous data by including additional groups of experimental subjects (social and binge drinkers) to represent an expanded spectrum of drinking patterns. As discussed in detail in Methods, above, subjects were grouped using defined criteria that took into account both the quantity and patterns of alcohol and water consumption. In a screened population of over 1300 individuals and using the stated criteria, these categorical designations would represent approximately 15% (AA), 70% (social), 12% (heavy) and 3% (binge) of the total (Palmour et al., 1997, and unpublished). There is also good agreement between categorical assignment of drinking class and a global measure of drinking derived from factor analysis of all measures of drinking described above.

There are a number of obvious limitations to the present study. First, the sample size is small for the number of comparisons. Nonetheless, post-hoc estimations of power for many analyses exceeded 0.80. Second, as all animals were exposed to beverage alcohol before sacrifice, it is technically impossible to distinguish whether the findings are a consequence of vulnerability or exposure. Because all animals were abstinent for several months prior to harvesting of the brain, the observed differences are not related to acute ethanol exposure or withdrawal. In addition, no variable reported here was significantly correlated with cumulative exposure to ethanol (g/kg) or with the number of drinking occasions. The extent to which some effects are attributable to chronic alcohol exposure are more difficult to evaluate. For example, if binge drinkers achieve higher peak blood alcohol levels, which seems likely from their relatively higher rate of intake, reduced levels of both DAT and SERT might reflect neuronal damage as a consequence of primary neurotoxicity. The same process could account for the lower levels of CSF 5-HIAA and HVA seen in binge-drinking animals. This question can best be approached in longitudinal, rather than cross-sectional, study. Another possible confound is that many of the animals sacrificed in the present study were feral so that there is no information regarding their life history prior to joining the colony.

In human alcoholics, DAT density may also vary by behavioral subtype. The most consistent finding is a reduction in striatal DAT, as measured by ß-CIT SPECT (single photon emission computerized tomography) imaging, in non-violent (Tiihonen et al., 1995, 1998), late-onset (Repo et al., 1999) or depressive (Laine et al., 1999)

alcoholics, and a trend toward an increase in ß-CIT binding in violent alcoholics (Tiihonen et al., 1995). What is not clear in these publications is the extent of lifetime exposure to ethanol or the relationship between duration of sobriety and ligand binding in the scanned subjects. This is important not only because our previous studies of alcohol preferring monkeys in various states of abuse and withdrawal demonstrate that DAT binding may be influenced by the state of ethanol exposure (Mash et al., 1996), but also because the single human study to use longitudinal measures showed higher levels of DAT in subjects with at least 4 weeks of abstinence (Laine et al., 1999). It is thus clear that DAT binding may exhibit characteristics of both "state" and "trait" markers.

With respect to SPECT studies of SERT, both Tiihonen et al. (1997) and Heinz et al. (1998a) found significantly lower midbrain levels of ß-CIT binding in alcoholics, but in one study low binding was restricted to impulsive-violent (type II) alcoholics (Tiihonen et al., 1997) and in the other, lower SERT was found in type I, but not type II, patients (Heinz et al., 1998a). SPECT has also been used to estimate the level of SERT in alcohol abusing macaque monkeys. In this model, a high level of SERT was negatively correlated with CSF levels of 5-HIAA (Heinz et al., 1998b). A phenotype comprising high SERT and low 5-HIAA was associated with high levels of alcohol consumption and low behavioral response to ethanol (Heinz et al., 2003). In our sample, both SERT binding and 5-HIAA levels are significantly lower, but only in binge drinkers.

Attempts to evaluate possible relationships between the clinical literature and the results presented in this report raise provocative questions. In humans there is limited support for ethanol-induced 5-HT neurotoxicity (Halliday et al., 1995; Baker et al., 1996). MRI results suggest generalized cellular injury of the brainstem in chronic heavy drinkers (Bloomer et al., 2004). In addition, in depressed alcoholics CSF 5-HIAA is low and is related inversely to the severity of depression and related positively to glucose metabolism in various brain regions (Williams et al., 2004). Thus, the low indices of both 5-HT and DA in the binge-drinking vervets may have parallels in human alcoholics and may be related in whole or in part to the neurotoxic effects of alcohol. Future human studies may have greater sensitivity in detecting biogenic amine alterations if they take into account the extent of binge drinking as well as genetically-mediated individual differences in sensitivity to alcohol (Heinz & Goldman, 2000; Heinz et al., 2000).

In heavy steady drinkers the situation is somewhat different. Normal levels of TH are found in association with elevated levels of DAT and HVA. Again, it is not possible from this data to distinguish precursor from consequence, but the findings are consistent with a model in which the primary dysfunction resides in the regulation of DA release, rather than the synthesis of DA or DAT. According to this conceptualization, DAT levels are elevated in order to recapture excess DA released into the synapse, and higher levels of HVA (as compared to binge-drinkers) reflect this increased turnover. This model is also consistent with previous data (Mash et al., 1996) indicating that DAT levels are low, along with those of HVA, in brains of animals sacrificed during chronic exposure to ethanol and are high in brains of acutely withdrawn or abstinent alcohol-preferring monkeys.

GABA interneurons inhibit tonic DA release from neurons originating in the VTA, and may play a regulatory role in phasic release as well (Nestler, 1997; Grace, 2000). Ethanol-induced release of DA may be mediated in part through a reduction of GABA-mediated tonic inhibition. Phasic excitation of DA neurons occurs also under the influence of glutamate, acting most likely through NMDA receptors. DA release is also modulated by opioid peptides, which disinhibit GABA interneurons (Cowen & Lawrence, 1999), but may also act directly in nucleus accumbens terminal areas, especially under the influence of ethanol. Finally, 5-HT_3 receptors on the VTA are facilitatory to DA release (Yoshimoto et al., 1996), as shown by the fact that ondansetron, a somewhat selective 5-HT_3 receptor antagonist, reduces ethanol-stimulated DA release in rodents and voluntary ethanol consumption in human subjects (Sellers et al., 1994; Rodd-Henricks et al., 2003; Kranzler et al., 2003). Accordingly, one might hypothesize that dysfunctions of DA release reside in enhanced sensitivity to glutamate excitation, reduced sensitivity to GABA inhibition, changes in opioid peptide availability or μ receptor activity or anomalies in 5-HT_3 receptor structure or density, to name just a few. Both pharmacological and genetic studies will be useful in resolving these alternate explanations.

Conclusion

Studies of alcohol abuse in both humans and experimental animals implicate multiple neuroregulatory systems including DA, 5HT, GABA, glutamate and endogenous opioids, along with a multitude of second messenger systems. Here we present novel evidence of neuroanatomical and neurochemical differences between two behaviorally-defined types of alcohol preferring monkeys: those that "binge" and those that drink heavily, but steadily throughout the day. These data expand, but are consistent with, data presented previously by Mash et al. (1996) and show that heavy drinkers exhibit elevated levels of DAT in mesolimbic cell body regions as compared to AA, while binge-drinking vervets display significantly lower levels of DAT in both VTA and SN as compared to heavy drinkers. Low levels of midbrain SERT binding were found only in binge drinkers. Quantitative relationships between TH, DAT and HVA in heavy drinkers mandate further evaluation of factors regulating DA release, while the lower levels of both SERT and DAT in binge drinkers justifies longitudinal studies of possible ethanol-induced neurotoxicity. This report reinforces the utility of the vervet model as a tool for the investigation of factors predisposing to ethanol abuse, and underscores the necessity of defining appropriate phenotypes for the study of complex traits.

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Table 1: Drinking Profiles of Study Subjects

CLASS	n	g/kg/24 h	Ratio, 24 h	g/kg/4 h	Ratio, 4 h	Weight (kg)
Alc Avoiding	9	1.58 ± 0.44	0.17 ± 0.07	0.34 ± 0.36	0.15 ± 0.17	5.48 ± 0.48
Social drinker	8	3.58 ± 0.69	0.39 ± 0.12	2.11 ± 0.41	0.37 ± 0.21	5.44 ± 0.36
Heavy drinker	8	6.73 ± 1.26	0.49 ± 0.10	4.09 ± 0.58	0.57 ± 0.12	5.08 ± 0.55
Binge drinker	7	7.94 ± 0.97	0.61 ± 0.10	4.87 ± 0.42	0.76 ± 0.12	5.40 ± 0.39

Alcohol consumption was measured at least twice for each animal in this study, according to the protocol described in detail in Methods. Groups differed significantly on each measure of alcohol consumption, as follows: 24 h quantity $F_{3,28} = 10.8$, $p < 0.0001$; all pairwise comparisons $p < 0.05$ except heavy vs binge; 24 h ratio $F_{3,28} = 6.86$, $p < 0.002$; $p < 0.05$ AA vs social, heavy, binge; social vs binge; 4h quantity $F_{3,28} = 19.2$, $p < 0.0001$; all pairwise comparison $p<.05$, except heavy vs binge; 4 h ratio: $F_{3,28} = 16.77$, $p < 0.0001$; $p < 0.05$ AA vs all other groups, heavy vs binge. There were no significant group differences with respect to body weight ($F_{3,28} = 0.613$, ns).

Table 2. CSF Neurotransmitter Metabolite Levels Vary by Phenotype

CLASS	n	5-HIAA	HVA	MHPG
Alc Avoiding	9	56.3 ± 3.59*	181.1 ± 11.83	22.8 ± 1.75
Social drinker	8	55.5 ± 5.36	188.9 ± 12.23	24.7 ± 1.48
Heavy drinker	8	61.6 ± 3.52¶	224.1 ± 23.14¶	25.0 ± 2.80
Binge drinker	7	43.2 ± 3.62*¶	147.3 ± 12.97*¶	24.3 ± 3.35

Cerebrospinal fluid samples were obtained by cisternal puncture under ketamine anesthesia from all animals, frozen immediately on dry ice and maintained at –80 C until analysis. CSF was drawn for each animal immediately prior to each alcohol testing period, as described further in the text. The minimum interval between replicate tests was 6 months. Amine metabolites were measured by HPLC with electrochemical detection, as described in Methods. The mean of replicate samples (range: 2-6, mean: 3.1) was computed for each individual; group means are expressed in the Table as pmol/ml CSF ± SEM. CSF levels of 5-HIAA ($F_{3,28} = 3.75$, $p < 0.03$) and HVA ($F_{3,28} = 3.53$, $p < 0.03$) varied significantly as a function of drinking class. There was no relation between CSF MHPG and drinking class ($F_{3,28} = 0.23$, $p = 0.88$). Pairwise differences, $p < 0.05$: * alc avoiding vs binge; ¶ heavy vs binge.

Figure Captions

Figure 1. Rostro-Caudal Extent of Measured Brain Sections

These sections represent the rostrocaudal extent of the midbrain region measured in this study. Panels A-F are labeled for DAT and G-L are the corresponding SERT labeled sections. Sections within this cascade (A-F and G-L) extend from Horsley-Clarke AP +11 to +6.5 (Contreras et al., 1981) at 750 micron intervals. A and G are adjacent sections, as are B/H, C/I, etc. A) The DAT labeled parabrachial tegmental area (PTA) is readily visible and was used to define SERT distribution (G) in this area. In sections B and H, the VTA is not readily distinguishable (arrow) from the SN, so these sections were excluded from measurement. Sections C through F show nigral DAT (box in panel C) that was used to define the nigral region for parallel estimation of SERT density (panels I-L; box in panel I). The lateral SN is defined as the lateral third of the posterior SN (arrows J and K); the midline caudal linear raphe nucleus is visible in the posterior region (arrows F and L).

Figure 2. Dopamine Transporter Density in Vervet Monkeys of Four Different Drinking Classes

DAT was labeled with ^{125}I -RTI, in the presence of 50 nM citalopram, as described in the text. The density of labeling was measured in replicate sections taken from each brain, using the operational criteria described in more detail in the text to identify regions of interest (parabrachial limbic, substantia nigra, midline DA pathway). DAT varied significantly across drinking class for parabrachial ($F_{3,22} = 5.37$, $p < 0.01$) and nigral regions ($F_{3,22} = 4.2$, $p < 0.02$) and tended to vary in the caudal linear raphe nucleus (($F_{3,18} = 3.33$, $p = 0.06$). For parabrachial and nigral regions, there were significant post-hoc pairwise differences between heavy drinkers and binge drinkers (*). In the parabrachial region there were significant ($p < 0.05$) pairwise differences between AA animals and heavy drinkers (†).

Figure 3. Pseudocolor Dopamine Transporter Autoradiograms

DAT autoradiography in the parabrachial tegmental area (A-C) and substantia nigra (D-F). The densometer to the right of the sections indicates that within the continuum of colors: purple represents low density and red represents high density. The alcohol-avoiding subject (A and D) illustrates intermediate binding, the binge-drinking subject (B and E) represents low binding, and the heavy-drinking subject (C and F) represents dense binding. These sections represent the median values of each series.

Figure 4. Serotonin Transporter Density in Vervet Monkeys of Four Different Drinking Classes

SERT was labeled with ^{125}I -RTI, in the presence of1 μM SCH12935, as described in the text. The density of labeling was measured in replicate sections taken from each brain, using the operational criteria described in more detail in the text to identify regions of interest (nigral, lateral nigra, midline (caudal linear raphe) serotonin pathway). SERT varied by drinking class in lateral ($F_{3,22} = 4.65$, $p < 0.01$) with post hoc pairwise differences ($p < 0.05$) between binge and heavy drinkers (*) and between binge drinkers and AA animals (‡). There were also significant differences in SERT density in the parabrachial nucleus ($F_{3,22} = 3.68$, $p < 0.03$), again with the binge drinkers showing a significantly reduced level of binding relative to AA (‡) and heavy drinking animals (*). Differences in SERT binding in the caudal linear raphe region did not reach statistical significance ($F_{3,15} = 2.39$, $p = 0.09$).

Figure 5. Pseudocolor Serotonin Transporter Autoradiograms

SERT autoradiography in the substantia nigra. The densometer to the right of the sections indicates that within the continuum of colors green represents low density and blue/purple represents high density. Left panel represents a typical binge-drinking subject represents low binding throughout its mediolateral extent, and in contrast the heavy-drinking subject (right panel) represents dense binding. Illustrated sections are derived from the next animal above the median of each class.

Figure 6. Interdigitation of DAT and SERT in the substantia nigra.

Pseudocolor autoradiograms of A) DAT and B) SERT in the medial substantia nigra of adjacent sections where red represents dense binding and blue represents low densities (based on color scale bar on the left). Overlays of these two sections are represented in panel C where red represents dense SERT binding and blue represents dense DAT binding. Sections D and E represent the posterior substantia nigra, of the same animal, which are approximately 1mm posterior to sections A and B. The posterior substantia nigra demonstrates a similar interdigitation of DAT and SERT as the medial nigra where D) DAT and E) SERT of adjacent sections display a different pattern of dense binding (based on color scale bar on the left). The overlay of these two sections are represented in panel F where red represents dense SERT binding and blue represents dense DAT binding. Color scale bar on the left applies to sections A, B, D, and E.

Figure 1

Rostro-Caudal Extent of Measurements

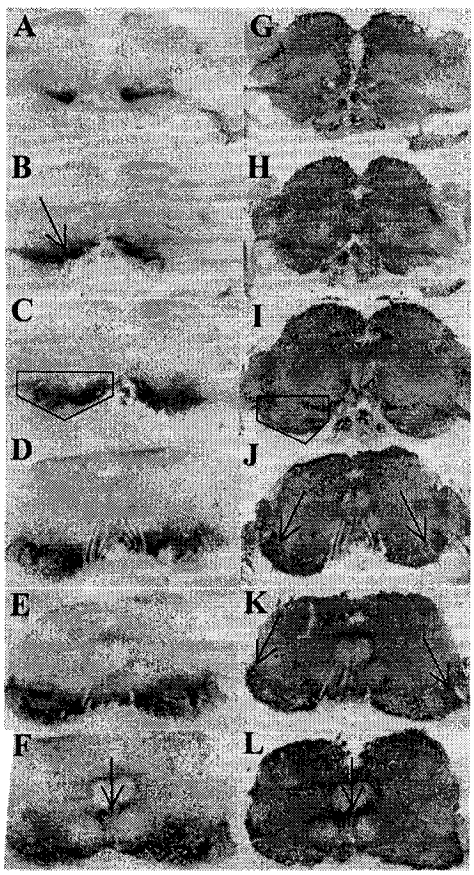

Figure 2

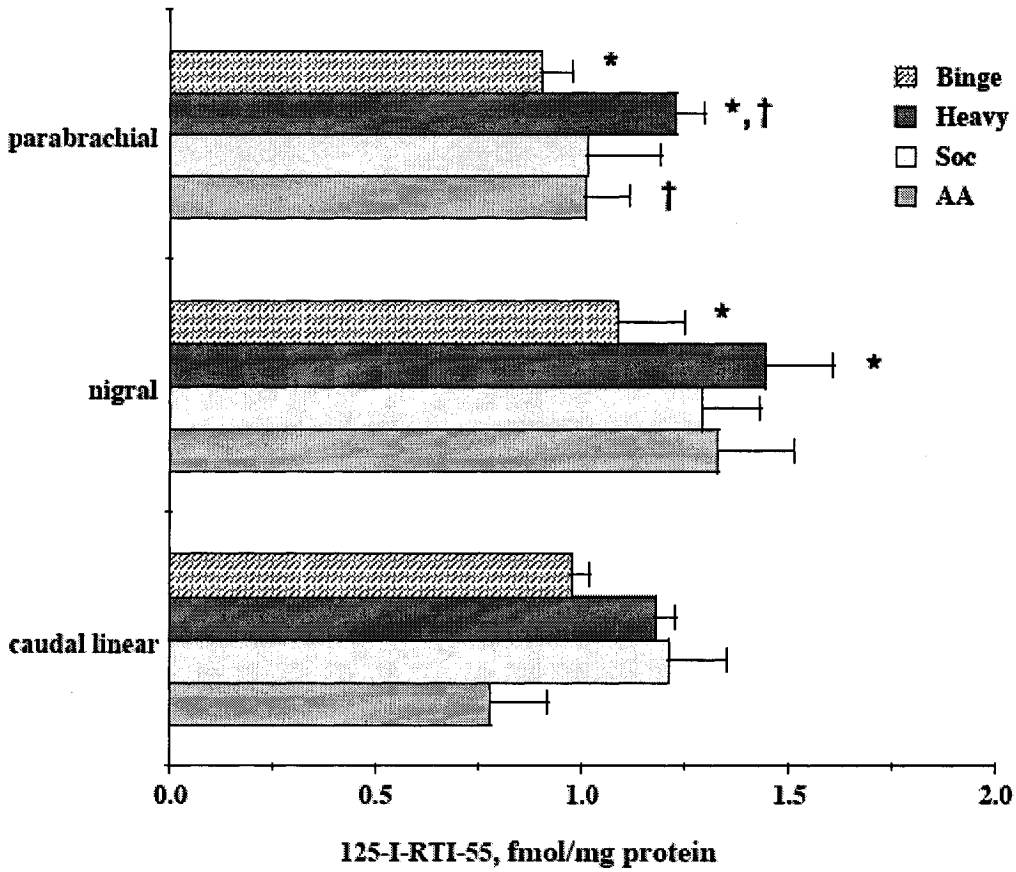
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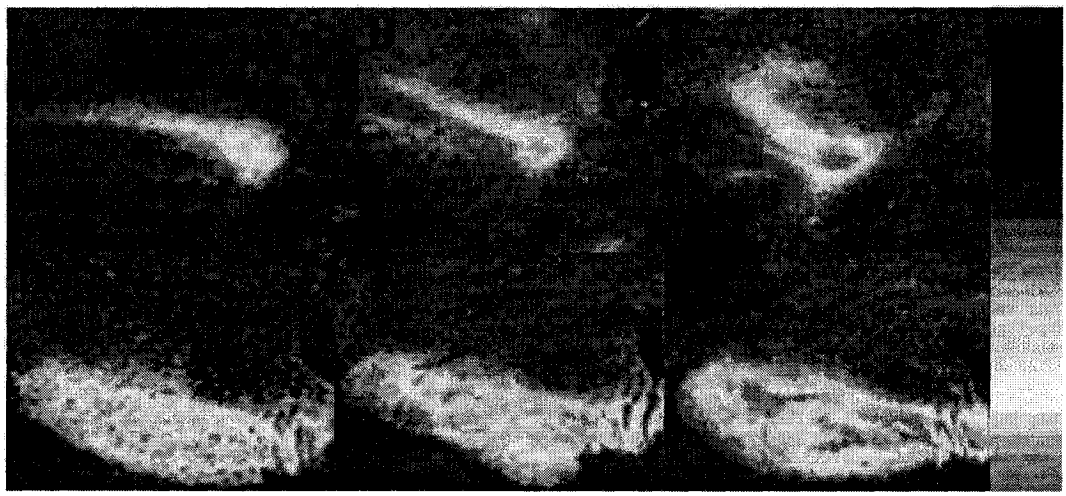
Figure 3



Alcohol-avoiding **Binge drinker** **Heavy drinker**

Figure 4

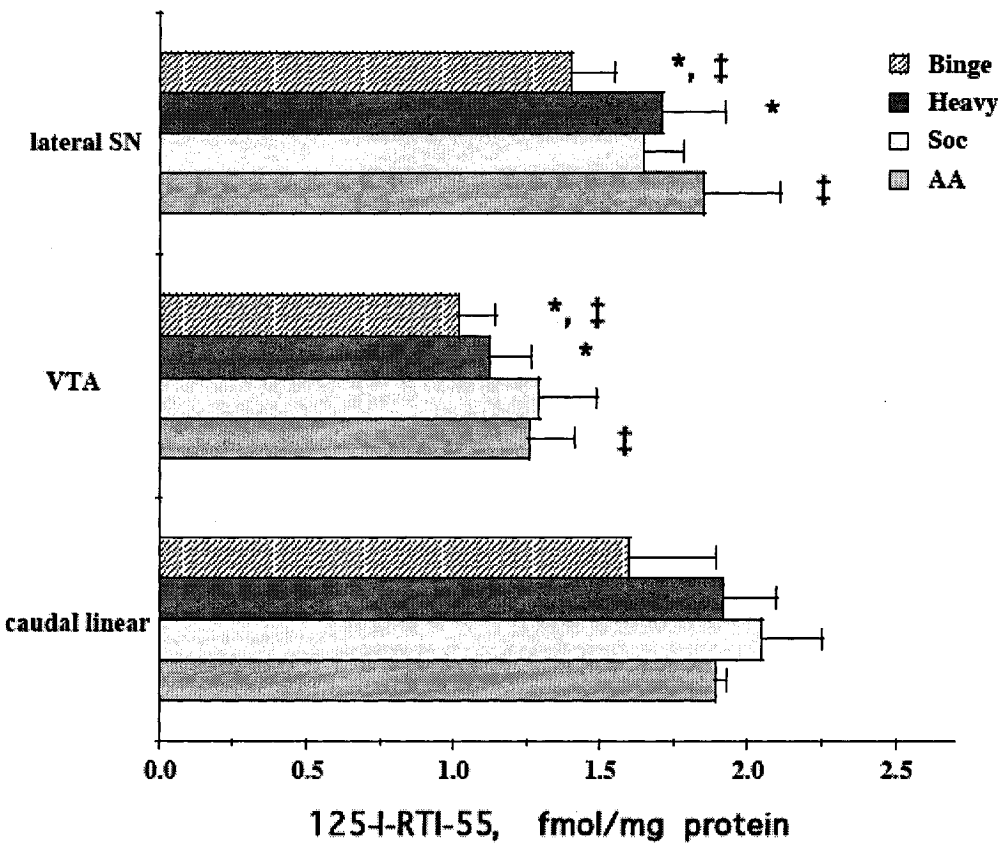

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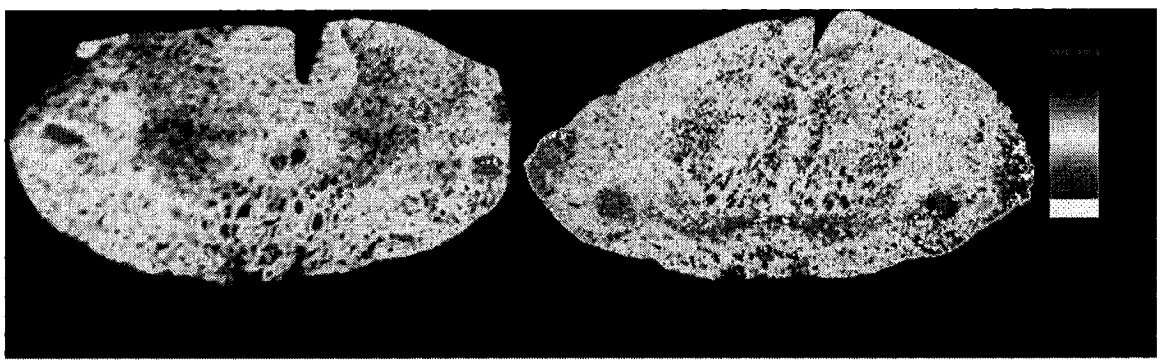
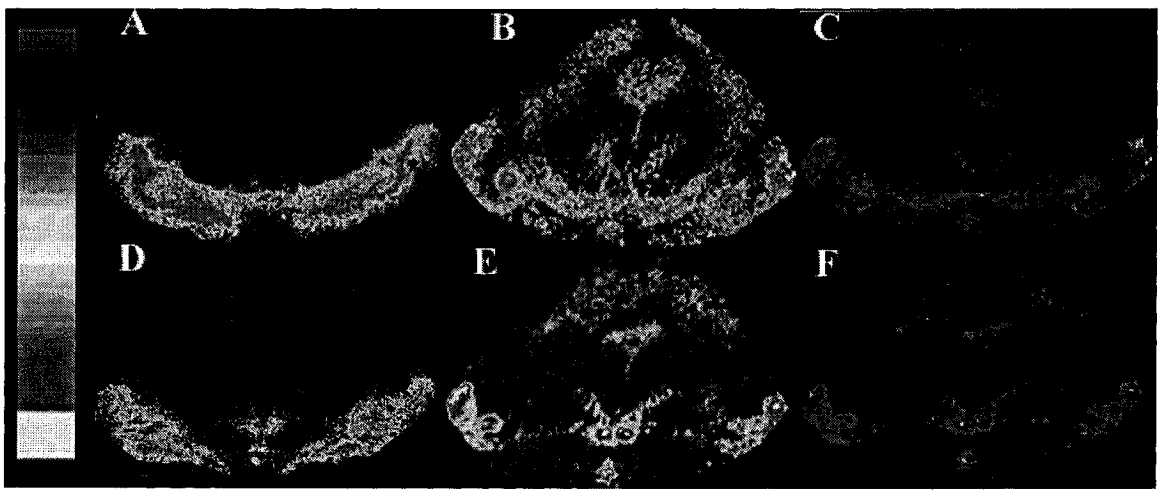
Figure 5



Binge drinker **Heavy drinker**

Figure 6



Dopamine transporter **Serotonin transporter** **Overlay**

CHAPTER VI

SUMMARY AND PERSPECTIVE

Introduction

The general aim of this thesis was to investigate factors that influence the DA neuron through the expression of DAT throughout the DA circuitry; experimental approaches included enzyme inhibition during development, elevated purine levels, and vulnerability to alcohol abuse. In order to complete these studies, we capitalized on clinical information pertaining to three disorders (Brunner's syndrome, LND, and alcohol abuse) exhibiting different levels of determinism and heterogeneity. These clinical situations involve distinct but overlapping regions of the DA pathways, and offer natural experiments into DA dysregulation. The aggressive and impulsive behavior displayed following MAO depletion during development is indicative of an abnormal mesolimbic system (Miczek et al., 2003; Cardinal et al., 2000, 2004; Winstanley et al., 2005). The severe motor impairment in LND suggests a prominent dysfunction within the nigrostriatal motor pathway (Lesch & Nyhan, 1964; Visser et al., 2000), whereas the aggressive and self-mutilatory behavior (Lesch & Nyhan, 1964; Hall et al., 2001; Robey et al., 2003) is indicative of a dysfunctional mesolimbic pathway. Finally, accumulating evidence suggests that some individuals who are vulnerable to alcohol abuse have an array of cognitive deficits, including higher-order executive functions, suggesting a mesocortical abnormality (Drejer et al., 1985; Schaeffer et al., 1984, 1988; Wilson et al., 1988; Tarter et al., 1989a,b). There may also be an independent, but perhaps not unrelated, pre-existing dysfunction in the mesolimbic pathway (Sher et al., 1991; Gabel et al., 1995; Finn et al., 1997, 2000; Caspi, 2000; Soderstrom et al., 2001).

The experimental basis for the first project is rooted in behavioral experiments that suggest that the mesolimbic neural circuitry involving DA and serotonin is disrupted following MAO inhibition during development (Mejia et al., 2002; Miczek et al., 2003; Winstanley et al., 2005). Experimental data from the first study supports the hypothesis that MAO inhibition during development reduces serotonin innervation in various brain regions as revealed by SERT binding densities without affecting DAT densities within the nigrostriatal pathway. Specifically, pervasive reductions in SERT densities were found in the neocortex and raphe, but not within the nigrostriatal pathway following the

combined inhibition of both forms of MAO. DAT densities within the nigrostriatal pathway were unaffected by MAO inhibition.

The second study explores the hypothesis that Hx has detrimental effects on markers of the DA system. Brain levels of Hx were experimentally altered for varying periods of time, and neurochemical markers of the nigrostriatal DA pathway and other basal ganglia systems were measured. The results of this study are consistent with the hypothesis that Hx alters the concentrations of both DA and DA-related neuronal proteins. The focus on the nigrostriatal DA pathway is grounded in extensive evidence showing that patients with LND uniformly have a depletion of DA in the striatal terminal fields (Lloyd et al., 1981; Silverstein et al., 1985; Jankovic et al., 1988; Wong et al., 1996; Earnst et al., 1996; Endres et al., 1997; Saito et al., 1999). However, the mechanism of action leading to the seemingly selective vulnerability of the DA system in LND remains elusive.

The final set of experiments tests the hypothesis that densities of both DAT and SERT vary in brainstem cell body regions in relationship to four patterns of vulnerability to ethanol consumption in abstinent vervet monkeys. Since human alcohol abuse indicates mesolimbic as well as mesocortical dysfunction, the current study examined midbrain DA neurons that project to limbic, cortical, and striatal terminal regions. In contrast to a previous neurochemical study (Mash et al., 1996) which examined whole brain DAT distribution using sagittal plane autoradiography, the current study focuses on the substantia nigra and the midbrain tegmental area using coronal slices through the rostrocaudal extent of the midbrain region. Four groups of animals (alcohol-avoiding, social drinkers, heavy drinkers and binge drinkers) were contrasted. The results of the present study support the hypothesis in that binge- and heavy-drinking subjects differ from one another not only in their patterns of ethanol consumption, but also with respect to expression of DAT and SERT in the midbrain DA region. Specifically, DAT and SERT densities tended to be lower in binge drinking monkeys throughout the midbrain as compared to heavy drinkers. Furthermore, elevated DAT densities were found in the heavy drinking subjects in the parabrachial area as compared to alcohol avoiding and binge drinking subjects.

Methodological Considerations

The choice of species as well as the age of the animal varies as a function of the question being asked and background literature. In the current thesis, three different species were used, as were animals at different stages of development. The species selection for each of the current experiments had been established by prior investigations in our laboratory (Mejia et al., 2002; Palmour et al., 1989; Ervin et al., 1990; Juarez et al., 1993; Mash et al., 1996). However these choices nonetheless warrant justification. The focus of this justification will be placed on developmental versus adult model choice for the MAO and Hx studies as well as species differences between the rodent and primate studies.

Developmental versus Adult Models

Previously our laboratory established a developmental behavioral model of MAO inhibition (Mejia et al., 2002), and also studied the administration of exogenous Hx into the adult striatum (Palmour et al., 1989). There is an abundance of literature concerning the effects of MAO inhibition in the adult (Datla et al., 1991; Kaplan & Sadock, 1998), but the behavioral consequences of MAO inhibition during development are markedly different from the adult experience (Brunner et al., 1993a,b). Interest in the developmental aspect of MAO inhibition arises from human conditions in which there is a complete absence of MAO activity resulting in aggressive and impulsive behavior (Brunner et al., 1993a,b). A complete absence of MAO activity is not necessary for behavioral abnormalities (Whitaker-Azmitia et al., 1994, Mejia et al., 2002), however relatively little is known about the neurochemical effects of partial inhibition of MAO activity during development. Therefore it was necessary to determine the neurochemical consequences of developmental MAO inhibition that might subserve the behavioral abnormalities.

In contrast to what is known about MAO inhibition in adults, relatively little is known about the effects of high levels of Hx in the central nervous system. A number of studies suggest that Hx may play a role in oxidative tissue injury (Poulsen et al., 1993; Stover et al., 1997; Marklund et al., 2000; Akdemir et al., 2001; Bavaresco et al., 2004, 2005). Except for preliminary data from our laboratory (Palmour et al., 1989; Burke et

al., 1999), there has been no detailed *in vivo* investigation of the effects of Hx on the DA system. These preliminary studies from our laboratory specifically targeted the striatum of the adult, which could not be trivially accomplished in a neonate (Palmour et al., 1989, Burke et al., 1999).

Although LND is a developmental disorder, there are two potential time periods in which Hx may affect the DA system, the developmental period and the adult period once DA neurons have formed. In the current study we decided to use an adult as opposed to a newborn animal to investigate the effects of Hx on the DA system. The principle factor that led to this decision was the specific hypothesis being tested which dealt with the effects of Hx on the DA neuron and not if Hx is responsible for the pathogenesis of LND. For that a developmental model would be required.

As a result of the current study interrogating the effects of Hx on the DA neuron, it maybe possible to repeat this experiment in a developmental model. It is possible that Hx has a more profound effect on the developing DA system than what is seen in the adult. However certain methodological issues need to be considered. First, the age in which to introduce Hx must be carefully considered. LND patients show no abnormalities at birth and only begin to show neurobehavioral signs of the disease after six months of age. Typically 6-OHDA neonatal models of LND introduce the toxin within post-natal day 5 (Breese et al., 1984a,b), which is well before the age equivalent of a newborn human (Clancy et al., 2001). A more appropriate exposure age would be between PND15-25 (depending on the species of rodent). Secondly, the duration of exposure to Hx needs to be taken into consideration. Elevated Hx levels are continuous in LND, so a one-time injection or daily injections would not necessarily be appropriate. The necessity for prolonged exposure to Hx has face validity considering the length of time it takes for motor abnormalities to appear in human cases (Lesch-Nyhan, 1964). If a developmental model of elevated Hx is to be considered, a HPRT knockout mouse might be an ideal candidate. Since HPRT knockout mice do not have elevated Hx levels (Jinnah et al., 1994) and do not display spontaneous behavioural abnormalities (Jinnah et al., 1991, 1994), administering exogenous Hx during development may complete the HPRT knockout mouse as a model of LND.

Species Specific Neuroanatomy

Much of our knowledge concerning the regulation and physiology of the DA pathways is derived from rodent work. However there are important neuroanatomical, and therefore possible pharmacological, differences between rodents and primates (human and non-human alike) that must be considered when choosing the species of animal and subsequent hypothesis formulation. Of particular importance is the re-organized mesocortical DA projection of the primate.

In the rat, the mesocortical projections originate primarily from the ventral tegmental, area and terminates within the prefrontal cortex, namely the medial prefrontal and anterior cingulate cortices (Deutch et al., 1988); whereas in the primate midbrain projections to the frontal cortex are expanded to include the motor cortices and the phylogenetically younger dorsolateral prefrontal cortex. Also, within the rat, the parabrachial division of the VTA is more closely associated with the mesocortical pathway while the midline paranigral division is aligned with the mesolimbic pathway (Fallon, 1991; Doherty & Pickel, 2000). In contrast, both the parabrachial and paranigral divisions of the VTA in the primate have substantial projections to the mesocortical and mesolimbic systems (Williams & Goldman-Rakic, 1998). It has also been argued that the rodent does not have a homologous dorsolateral prefrontal cortex, which is involved in executive cognitive function (Preuss & Goldman-Rakic, 1991; Goldman-Rakic, 1999). The use of rats to study complex psychiatric disorders such as alcohol abuse present limitations, in part because the integration of the mesotelencephalic DA pathways cannot be fully represented in the rodent.

Although significant functional neuroanatomical differences exist between rodent and primates, there are similarities between these two species regarding the functioning of the DA system. This is especially true for the relationship between the limbic system and DA as well as for the motor system and DA. For example the medial prefrontal cortex of the rat (a key component of the limbic system) is homologous to that of the primate (Preuss, 1994; Williams & Goldman-Rakic, 1998). The midline DA area, the VTA, has substantial projections to limbic-related cortical (medial prefrontal cortex) and subcortical areas (ventromedial striatum, septum, and amygdala) in both species (Seseck & Pickel, 1992; Fudge & Haber, 1997). Also the ventral tier of the SNc of both species

is involved in the nigrostriatal pathway and the regulation of the extrapyramidal motor system (Fudge & Haber, 1997). Furthermore, the use of the rodent allows the testing of hypotheses concerning factors that influence specific DA pathways such as the nigrostriatal or mesolimbic pathways. Also, from both a practical and an ethical point of view, it is appropriate to use rodents to test initial hypotheses, while reserving the study of non-human primates to final confirmations of hypotheses already well-piloted in rodents, and to situations in which rodents do not provide an adequate degree of social or anatomical complexity.

MAO Inhibition and Development

Despite attempts to identify human beings with complete inactivation of MAO activity (Murphy et al., 1998; Mejia, 2001), there are only a few reported instances where this enzyme is inactivated due to genetic defect (Brunner et al., 1993 a,b; Sims et al., 1989; Lenders et al., 1996). Two such situations are Brunner's syndrome and Norrie's disease. The rare atypical Norrie's disease, in which there is gene deletion in both Norrie gene site and MAO A/B sites, results in mental retardation and sensory anomalies, typically including deafness and blindness (Sims et al., 1989, Donnai et al., 1988; Murphy et al., 1990; Collins et al., 1992; Lenders et al., 1996). Brunner's syndrome, which is described in only one family, is characterized by violent impulsive and aggressive behaviors as well as mild mental retardation (Brunner et al., 1993a,b), but is devoid of major sensory abnormalities. Genetic analysis of affected males demonstrated a point mutation of the monoamine oxidase A (MAO-A) gene (Brunner et al., 1993a,b) in affected family members, resulting in a complete deficiency of enzymatic activity (Brunner et al., 1993b).

The results presented in this thesis, as well as other investigations show that complete inhibition of MAO A/B activity during development is not necessary for the initiation of behavioral and neurochemical alterations (Whitaker-Azmitia, 1994; Vitalis et al., 1998; Mejia et al., 2002; Chen et al., 2004). Whitaker-Azmitia et al. (1994) reported that 60% MAO-A/B inhibition in rats throughout development results in impulsive-like behavior and low SERT binding in the cerebral cortex. Mejia et al. (2002) report that only a 40% inhibition of MAO-A/B enzymatic activity during development in the mouse

is required for spontaneous aggression and impulsive-like behavior. However aggressive behavior can be pharmacologically elicited from mice with developmental inhibition of MAO-A/B activity ranging from 20-30% (Mejia et al., 2002).

The present study shows (1) that MAO inhibition during development is detrimental to serotonin innervation in various brain regions as revealed by SERT binding densities; (2) that alterations in cortex and raphe persist past the period during which MAOI's are now longer present and into adulthood; and (3) that DA innervation (as revealed by DAT levels) is changed only transiently and only during periods when MAOI levels are still high. The most significant finding reported here is that the combined MAO-A/B inhibition significantly and specifically reduced SERT binding in the cortex and raphe nucleus throughout developmental and into adulthood. This effect was not present in the striatum, substantia nigra, or hippocampus, suggesting a relative vulnerability of the cortex and raphe to elevated serotonin levels. The radioligand assay used in the present study precludes the measurement of high affinity DAT sites of the cortex (Boja et al., 1992; Coffey & Reith, 1993). However recent data from *in vivo* electrophysiological recordings also suggest that insult to the cortex during development alters the responsiveness of cortical neurons to VTA stimulation (Lavin et al., 2005). The current study did not find significant differences of DAT binding as a result of MAO inhibition during development, a but this does not rule out the possibility that DA contributes to aggressive and impulsive behavior (Tidey & Miczek, 1996; van Erp & Miczek, 2000; Ferrari et al., 2003; Cardinal et al., 2000, 2004).

During development serotonin and DA play an important role in the formation of target neurons. Serotonin in particular also regulates its own neuronal growth (Whitaker, 2001). Elevated serotonin concentrations would potentially activate 5-HT_{1B}, thereby directly inhibiting serotonin neuronal growth (Whitaker-Azmitia & Azmitia, 1986; Shemer et al., 1991). Additionally, via the activation of S-ß100, DA neurite expansion in the midbrain would be inhibited. This could explain the trend toward a lowered DAT binding in the substantia nigra at PND 14 (Liu & Lauder, 1992). Furthermore data obtained in the present study would be consistent with a scenario in which increased serotonin during development leads to decreased serotonin innervation of the cortex. This decreased innervation of the cortex could underlie the behavioral abnormalities,

such as aggression and impulsivity reported by our laboratory (Mejia et al., 2002). Perhaps the relative vulnerability of the serotonin system, as compared to the DA system, lies in the temporally distinct growth periods of these neuronal systems. Serotonin and DA neurons are both formed early in embryonic development. Both neuronal systems continue to develop during early in post-natal period with the serotonergic system maturing by late post-natal development. Dopamine neurons, in contrast, continue their development during early post-natal period through adulthood (Huttenlocher et al., 1979; Wallace & Lauder, 1983; Lidov & Molliver, 1982a,b; Rakic et al., 1986; Morilak & Ciaranello, 1993; Teicher et al., 1995; Andersen et al., 2000).

The results reported here are congruent with the behavioural data suggesting that the higher level of MAO-A/B inhibition results in persistent neuroanatomical alterations. These data also suggest that the critical level for such alterations lies between 25-40% inhibition of both the forms of MAO during embryonic and early post-natal development. These data should be taken as a note of caution to the administration of any psychoactive drug that increases DA and serotonin during development.

Hypoxanthine and Dopamine

Although the involvement of the DA system in the pathogenesis of LND is generally accepted, the role of elevated Hx in relation to DA dysfunction has received relatively little attention (Palmour et al., 1989). The results obtained in the current model, which delivers Hx into the ventricle, supports the hypothesis that Hx induces changes within the nigrostriatal DA system. This study demonstrates the potential for Hx to destroy DA neurons once formed. The most significant findings of this study are that exposure to elevated Hx levels results in a reduction of TH-ir cells in the substantia nigra pars compacta along with elevated DA levels without the concurrent elevation of its metabolites.

At present, data obtained here suggest that Hx promotes DA cell death and partially inhibits DA release. The time frame of alterations reported here suggest that intracellular DA occurs early after Hx treatment followed by a progressive reduction in DA cell bodies. This raises a number of potential scenarios which would have Hx either inducing neuronal death directly, then preventing compensatory release of DA; or

indirectly by preventing DA release, possibly through the GABA-A receptor (Asno & Spector, 1979; Kish et al., 1985) which would raise intracellular DA and thereby increasing neuronal susceptibility to free radical damage (Offen et al., 1997; Jones et al., 2000; Lotharius & O'Malley, 2000); or a combination of these two possibilities which would have Hx and DA acting synergistically to induce neuronal dysfunction (Offen et al., 1997; Stover et al., 1997; Jones et al., 2000; Marklund et al., 2000; Akdemir et al., 2001; Bavaresco et al., 2004, 2005).

Interest in an Hx-DA interaction arose from the fact that LND patients have elevated Hx and low DA levels; as such the data in the present study may have implications for the pathogenesis of LND. Neonatal 6-OHDA lesions point to the developmental period as being critical factor for the progression of the behavioral abnormalities in LND (Breese et al., 1984a,b). That is, the introduction of insult during development results in an array of behavioral and neurochemical abnormalities that are different from lesions introduced in adulthood (Breese et al., 1984a,b).

The progression in the affective circuit impairment seen in LND may be due to the prolonged Hx induced alterations of the DA system, which would alter cortical and subcortical neural networks. The age range for the appearance of affective disorder aspect of LND is similar time frame for the expression of hyperactive and impulsive behaviors seen in ADHD (typically by the age of 7; Sagvolden & Sergeant, 1998). This lends support for a protracted hypo-active DA system and resultant affective circuit dysfunction. However the effects of Hx on the DA system during early post-natal development would also have a lasting effect on DA neurons and DA terminal fields, which may be related to the pathogenesis of LND (Berger-Sweeney & Hohmann, 1997; Lavin et al., 2005). The DA system has a relatively protracted developmental time period with rapid morphogenesis and synaptogenesis during the prenatal and early post-natal periods (Berger-Sweeney & Hohmann, 1997). The attenuation of DA transmission during development, both early and late, would have deleterious effects on synaptic formation and neurite growth, along with altered terminal projections. Furthermore, a recent publication casts doubt on the commonly held view that cortical neurogenesis in humans cease at birth. It was calculated that between the ages of birth and 72 months that cortical neuronal numbers increase by 60-78% over birth numbers (Shankle et al.,

1999). A prolonged period of cortical neurogenesis and DA neuronal development allows for the possibility for an extended influence of DA/cortical interaction on the establishment of properly functioning neural circuits. The attenuation of DA during the first 72 months of would be detrimental to the formation of neural circuits.

The purpose of the present study was to examine the effects of high levels Hx on the mammalian brain with relevance to LND. This work provides novel insights into the potential role of high concentrations of Hx on the DA system. Such information provides a unique perspective into the investigation of Lesch-Nyhan disease.

Alcohol Abuse and the Basal Ganglia

The biological basis of individual vulnerability to alcohol abuse remains elusive. However DA and serotonin, especially within the mesolimbic reward pathway, have been identified as playing key roles in acute response to ethanol, and may also contribute to alcohol abuse (Imperato & Di Chiara, 1986; Heinz et al., 1998; Tiihonen et al., 1998; Koob, 2000). An important contemporary modality for studying this question in human beings is brain imaging (Tiihonen et al., 1995). *In vivo* imaging with human alcoholics presents inherent problems, such as the inability to control diagnostic heterogeneity, different lengths of abuse, and the difficulty of controlling for varying periods of abstinence (Tiihonen et al., 1997; Tiihonen et al., 1998; Heinz et al., 1998). What is not clear in these publications is the extent of lifetime exposure to ethanol or the relationship between duration of sobriety and ligand binding in the scanned subjects. This is important in part because the single human study to use longitudinal measures showed higher levels of DAT in subjects with at least 4 weeks of abstinence (Laine et al., 1999).

In order to identify vulnerability factors associated with alcohol abuse, the vervet model has been proposed as a tool for the investigation of factors predisposing individuals to alcohol abuse (Ervin et al., 1990; Mash et al., 1996; Palmour et al., 1997). The current set of experiments tests the working hypothesis that densities of both DAT and SERT vary in brainstem cell body regions in relation to patterns of ethanol consumption in abstinent vervet monkeys. An important finding is that binge drinkers and heavy drinkers showed opposite effects with respect to DAT concentrations in

parabrachial VTA and SN. DAT levels in parabrachial VTA were elevated in heavy drinkers as compared to either binge drinkers or alcohol avoiding subjects, while SN levels of DAT were significantly lower in binge drinkers as compared to heavy drinking animals. A novel finding is that SERT binding was low in all measured areas of the brainstem for binge drinkers, but heavy, social and avoidant subjects did not differ with respect to SERT. Low SERT binding was particularly obvious throughout the extent of the substantia nigra.

In previous studies of animals from this population, Mash et al. (1996) showed that striatal and accumbens DAT density was elevated in abstinent alcohol-preferring individuals as compared to AA conspecifics, and also reported that DAT binding decreased during chronic alcohol exposure and rebounded upon withdrawal. Although no significant differences were reported from midbrain DAT measurements, the extent to which DAT binding in the cell body regions might differ between groups was inadequately explored. It should be noted however that there is about a 6-fold difference in the levels of DAT binding in the midbrain reported by Mash et al. (1996) and the present study. This discrepancy is most probably due to the different assay conditions used in the two studies. As mentioned earlier, the radioligand assay used in the present study precludes the ability to measure the high affinity DAT sites (Boja et al., 1992; Coffey & Reith, 1993). However the 6-fold difference cannot be explained solely by the lack of binding to the high affinity DAT sites. The difference in fractional occupancy between these two studies represents the more likely candidate for this discrepancy. At equilibrium the fractional receptor occupancy is a function of ligand concentration (Motulsky, 1995) and since the previous study uses five times the radioligand concentration the discrepancy in reported binding between these studies is reasonable.

Data obtained in the present study suggests possible clues with respect to a potential neural mechanism underlying inherent vulnerability to alcohol abuse. First the elevated levels of DAT in the heavy-drinking individuals could be consistent with a concurrent elevation in DA release. However the HVA concentrations in CSF do not substantiate elevated DA release. The elevated DAT without concurrent HVA elevation might suggest an imbalance of tonic versus phasic firing of DA neurons. In such a scenario, elevated tonic release would promote elevated DAT levels while maintaining

relatively normal levels of HVA. Under this scenario, phasic firing would be reduced, thus rendering post-synaptic receptors hypersensitive to ethanol-induced phasic release (Onn & Grace, 1995; Grace, 1995, 2000). Although such an imbalance has been proposed as a neuroadaptation to prolonged exposure to ethanol (Grace, 2000), it is possible this imbalance is antecedent to alcohol abuse in vulnerable individuals (Marinelli & White, 2000). Within the VTA, tonic and phasic DA firing are under the control of GABA, glutamate (Grace, 1995, 2000), and serotonin (Prisco et al., 1994; Lejeune & Miller, 1998). In the present study levels of 5-HIAA do not suggest that there is an elevation in serotonin transmission and preliminary results do not suggest that there is elevated GABA synthesis as measured by glutamic acid decarboxylase levels (Burke et al., 2001). The next step is to identify receptors that may be involved in a proposed dysregulation of tonic and phasic DA release. Potential candidates known to regulate tonic and phasic DA release in the VTA are the 5-HT_{1A} (Arborelius et al., 1993; Rasmusson et al., 1994; Gobert et al., 1995), 5-HT_{2C} (Di Matteo et al., 1999, 2000), GABA-A, GABA-B (Westerink et al., 1996; Harte & O'Connor, 2005), NMDA, and AMPA receptors (Takahata & Moghaddam, 1998; Westerink et al., 1996, 1998). Given their respective roles in DA transmission, examination of these receptors is warranted in future studies.

By contrast, the binge-drinking subjects display low levels of both DAT and SERT in the VTA and substantia nigra along with lowered HVA and 5-HIAA. These data suggest that both DA and serotonin transmission are attenuated in binge-drinking vervets which raises the possibility that both serotonin and DA neurons are differentially vulnerable to damage in bingers. Low levels of serotonin are also a characteristic of impulsive behavior in human alcoholism (Virkkunen et al., 1995, 1996). A further behavioral characterization of binge-drinking vervets to draw parallels between subtypes of human alcohol abuse is warranted. Furthermore a longitudinal study investigating alterations in CSF HVA and 5-HIAA in binge-dinking vervets would provide evidence for a relative vulnerability of DA and serotonin neurons to damage in bingers.

Alcohol abuse, like other drug abuse disorders, has typically been thought of as a result of a dysregulation of the reward system, mainly the mesolimbic DA pathway (Koob, 2000). The current model suggests that dysregulation in this system at the

midbrain level is associated with the heavy-drinking group because alterations in DAT are predominantly found in the dorsal tier. This is a different pattern than what is seen in the terminal field. At the level of the striatum, all areas of the caudate, nucleus accumbens, and striatum show elevated DAT levels (Mash et al., 1996). The previous study on the striata of heavy-drinking subjects focused on the caudate/putamen decussation, not necessarily the functional heterogeneity of the striatum. In light of this, the relationship between elevated dorsal tier and striatal DAT densities remains elusive. The elevated striatal DAT density may be related to the dorsal tier projections, given that this midbrain area innervates the association as well as the ventromedial striatum. Alternatively, this elevation maybe due to a dysregulation in the spiraling feedback loop of the striatonigrostriatal pathway. In either situation, the current model demonstrates the necessity for further study into the functional areas of the striatum and midbrain region in alcohol abuse subtypes.

Conclusions

In sum, this project demonstrates that many types of factors—developmental, pharmacological, biochemical—differentially affect DA throughout its circuitry. Each of the disorders of interest in the present study (Brunner's syndrome, LND, and alcohol abuse) involve distinct but overlapping areas of the DA pathways. Furthermore, data presented here underscore the utility of examining DAT and SERT to identify alterations in the DA and serotonin neurons respectively. The results of the first study support the hypothesis that MAO inhibition during development is detrimental to serotonin innervation in the cortex and raphe. However, DAT binding did not detect alterations within the nigrostriatal DA pathway. Behaviorally it was previously indicated that the neural circuitry of the mesolimbic system was interrupted by MAO inhibition during development (Mejia et al., 2002). Although the current study demonstrates that serotonin innervation of the cortex is reduced as a result of MAO inhibition, the degree to which a dysregulated interaction between DA and serotonin contributes to the reported aggressive and impulsive-like behavior (Mejia et al., 2002) remains speculative.

Results from the second study support the hypothesis that high levels of exogenous Hx have detrimental effects on the DA system. The primary action of Hx maybe one of neurotoxicity, or inhibition of DA release leading to toxicity, or some interaction leading to a synergism between elevated intracellular DA and extracellular Hx. The extent to which these findings carries over to the developing DA system is worth exploring since the disruptions reported here in the adult would be detrimental to the developing mesotelencephalic DA circuits.

Experimental results from the final study support the hypothesis that densities of both the DAT and SERT densities vary in brainstem cell body regions in relationship to four patterns of ethanol consumption in abstinent vervet monkeys. Data from binge-drinking subjects suggest depressed DA and serotonin within cell body regions of the mesolimbic, mesocortical, and nigrostriatal pathways. Given the abusive pattern of alcohol consumption in the binge-drinking subjects, a longitudinal study examining potential changes in amine metabolites in CSF is warranted in order to determine if ethanol has neurotoxic effects, which would be responsible for the low levels of DAT and SERT reported in this study. Meanwhile, data from heavy-drinking group leads to the assertion that there is an inherent elevation of DAT within the mesolimbic/mesocortical DA cell body area without a corresponding elevation in DA metabolites. These data suggest a potential dysregulation between tonic and phasic DA release (Grace, 2000). Investigations examining receptors known to be involved in the regulation of tonic and phasic release of DA in the midbrain may help to clarify the mechanism underlying this pattern of alcohol consumption. Furthermore, the results from this study suggest that behavioral patterns should be taken into account in human studies. By doing so, this may reduce the heterogeneity seen in typology currently used to classify alcoholics and facilitate studies and treatments for alcohol abuse.

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APPENDIX A

ANIMAL ETHICS FORMS

NOT renewing — project finished.

Guidelines for completing the form are available at www.mcgill.ca/rgo/animal

For office use only
Protocol #: 3933
Investigator #: 932
Approval End Date: June 30, 2004
Facility Committee: MED

McGill University
Animal Use Protocol – Research

Title: Development and validation of an animal model of Lesch-Nyhan disease
(must match the title of the funding source application)

New Application: **Renewal of Protocol: #** 3933 **Pilot**: **Category** *(see section 11)*: C

1. Investigator Data:

Principal Investigator: Roberta Palmour **Phone #:** 398-7303
Department: Psychiatry **Fax#:** 398-4370
Address: 1033 Pine ave W. AMI R&T Bldg Rm326 **Email:** Mc23@musica.mcgill.ca

2. Emergency Contacts: Two people must be designated to handle emergencies.

Name: Roberta Palmour **Work #:** 7303 **Emergency #:** 931-4100
Name: Mark Burke **Work #:** 1501 **Emergency #:** 524-3421

3. Funding Source:

		For Office Use Only:
External:	**Internal**: X	ACTION / DATE
Source (s) :	**Source (s)** : Stairs Foundation	SOE U Jan 6'03
Peer Reviewed: YES NO**	**Peer Reviewed**: XYES NO**	DEAN V July 8'03
Status : Awarded Pending	**Status**: X Awarded Pending	APPROVED
Funding period:	**Fundingperiod**: June7,2000no expiration	

NB V
CO

** All projects that have not been peer reviewed for scientific merit by the funding source require 2 Peer Review Forms to be completed e.g. Projects funded from industrial sources. Peer Review Forms are available at www.mcgill.ca/rgo/animal

Proposed Start Date of Animal Use (d/m/y): **or ongoing:** X

Expected Date of Completion of Animal Use (d/m/y): **or ongoing:** X

Investigator's Statement: The information in this application is exact and complete. I assure that all care and use of animals in this proposal will be in accordance with the guidelines and policies of the Canadian Council on Animal Care and those of McGill University. I shall request the Animal Care Committee's approval prior to any deviations from this protocol as approved. I understand that this approval is valid for one year and must be approved on an annual basis.

Principal Investigator's signature:		**Date:** 2003/5/11

Approved by:

Chair, Facility Animal Care Committee:		**Date:** 3/6/03
University Veterinarian:		**Date:** 6/03/03
Chair, Ethics Subcommittee (as per UACC policy):	—	**Date:** —
Approved Animal Use	**Beginning:** July 1, 2003	**Ending:** June 30, 2004

This protocol has been approved with the modifications noted in Section 13.

2002

RDC 8 Vumpof

Guidelines for completing the form are available at www.mcgill.ca/rgo/animal

McGill University
Animal Use Protocol – Research

For office use only
Protocol #: 4738
Investigator #: 932
Approval End Date: MAY 31, 2005
Facility Committee: MCD

Title: Ethanol Sensitivity in Adolescent Vervet Monkeys
(must match the title of the funding source application)

New Application: **Renewal of Protocol:** # √ 4738 (205021) **Pilot**: **Category** *(see section 11)*: C

1. Investigator Data:

Principal Investigator: Roberta M. Palmour, Ph.D. **Phone #:** 398-7303
Department: Psychiatry **Fax#:** 398-4370
Address: 1033 Pine Avenue West, Suite 326 (H3A 1A1) **Email:** mc23@musica.mcgill.ca

2. Emergency Contacts: Two people must be designated to handle emergencies.

Name:	Amy Beierschmitt, DVM	**Work #:**	869-465-7280	**Emergency #:**	869-465-7807
Name:	Frank Ervin, MD	**Work #:**	869-465-7280	**Emergency #:**	869-465-5698; 514-931-4100

3. Funding Source:

External: Yes	Internal:
Source (s) : : NIH (NIAAA)	**Source (s)** :
Peer Reviewed: √ YES NO**	**Peer Reviewed**: YES NO**
Status : √ Awarded Pending	**Status**: Awarded Pending
Funding period: 5/03 - 04/07	**Funding period**:

For Office Use Only:

ACTION	✓	DATE
[illegible]	√	July 25'04
[illegible]	√	July 28'04
APPROVED		

** All projects that have not been peer reviewed for scientific merit by the funding source require 2 Peer Review Forms to be completed e.g. Projects funded from industrial sources. Peer Review Forms are available at www.mcgill.ca/rgo/animal

Proposed Start Date of Animal Use (d/m/y): 7/01/04 **or ongoing**: started 06/01/03

Expected Date of Completion of Animal Use (d/m/y): 5/31/07 **or ongoing**:

Investigator's Statement: The information in this application is exact and complete. I assure that all care and use of animals in this proposal will be in accordance with the guidelines and policies of the Canadian Council on Animal Care and those of McGill University. I shall request the Animal Care Committee's approval prior to any deviations from this protocol as approved. I understand that this approval is valid for one year and must be approved on an annual basis.

Principal Investigator's signature: [signature] **Date:** 03/06/04

Approved by:

Chair, Facility Animal Care Committee:	[signature]	**Date:** 6/2/04
University Veterinarian:	[signature]	**Date:** June 8, 2004
Chair, Ethics Subcommittee (as per UACC policy):		**Date:** —
Approved Animal Use	**Beginning:** April 1, 2004	**Ending:** MAY 31, 2005

This protocol has been approved with the modifications noted in Section 13.

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Guidelines for completing the form are available at www.mcgill.ca/rgo/animal

McGill University
Animal Use Protocol – Research

For office use only
Protocol #: 4631
Investigator #: 932
Approval End Date: Sept 30, 2004
Facility Committee: MCD

Title: Biobehavioral trajectories to alcohol abuse
(must match the title of the funding source application)

New Application: **Renewal of Protocol:** # 4631-030930 **Pilot:** **Category** *(see section 11)*: C

1. Investigator Data:

Principal Investigator: Roberta M. Palmour, Ph.D. **Phone #:** 398-7303

Department: Psychiatry **Fax#:** 398-4370

Address: 1033 Pine Avenue West, Suite 326 (H3A 1A1) **Email:** mc23@musica.mcgill.ca

2. Emergency Contacts: Two people must be designated to handle emergencies.

Name:	Amy Beierschmitt, DVM	**Work #:**	869-465-7280	**Emergency #:**	869-465-7807
Name:	Frank Ervin, MD	**Work #:**	869-465-7280	**Emergency #:**	869-465-5698; 514-931-4100

3. Funding Source:

External: Yes

Source (s) : : NIH (NIAAA)

Peer Reviewed: √ YES NO**

Status : √ Awarded Pending

Funding period: 10/02 - 09/05

Internal:

Source (s) :

Peer Reviewed: YES NO**

Status: Awarded Pending

Funding period:

For Office Use Only:

ACTION	Y/N	DATE
CCAC	√	Sept 26
DB	CD √	Oct 6/03
APPROVED		

** All projects that have not been peer reviewed for scientific merit by the funding source require 2 Peer Review Forms to be completed e.g. Projects funded from industrial sources. Peer Review Forms are available at www.mcgill.ca/rgo/animal

Proposed Start Date of Animal Use (d/m/y): 10/01/02 **or ongoing**: √

Expected Date of Completion of Animal Use (d/m/y): 09/31/05 **or ongoing**:

Investigator's Statement: The information in this application is exact and complete. I assure that all care and use of animals in this proposal will be in accordance with the guidelines and policies of the Canadian Council on Animal Care and those of McGill University. I shall request the Animal Care Committee's approval prior to any deviations from this protocol as approved. I understand that this approval is valid for one year and must be approved on an annual basis.

Principal Investigator's signature: **Date:** ~~19/09/02~~ 24/08/03

Approved by:

Chair, Facility Animal Care Committee:		**Date:** 9/9/03
University Veterinarian:		**Date:** Sept 18, 2003
Chair, Ethics Subcommittee (as per UACC policy):		**Date:**
Approved Animal Use	**Beginning:** Oct. 1, 2003	**Ending:** Sept 30, 2004

This protocol has been approved with the modifications noted in Section 13.

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SEP 17 2003