5-2-2009

Determination of induction of Nur77 (NR4A1), Nor1 (NR4A3), and Nurr1 (NR4A2)

Josiah Wilcots

DETERMINATION OF INDUCTION OF NUR77 (NR4A1), NOR1 (NR4A3), AND NURR1 (NR4A2)

By

Josiah Wilcots

A Thesis
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Master of Science
in Veterinary Medical Science
in the Department of Basic Sciences

Mississippi State, Mississippi

May 2009

DETERMINATION OF INDUCTION OF NUR77 (NR4A1), NOR1 (NR4A-3), AND NURR1 (NR4A2)

By

Josiah Wilcots

College of Veterinary Medicine

Mississippi State University

Approved:

_____	_____
Jeffery B. Eells Associate Professor College of Veterinary Medicine Basic Sciences Department (Major Professor)	Janice E. Chambers Professor College of Veterinary Medicine Basic Sciences Department (Committee Member)
_____	_____
Nikolay Filipov Associate Professor University of Georgia College of Veterinary Medicine Dept. of Physiology and Pharmacology (Committee Member)	Larry A. Hanson Professor College of Veterinary Medicine Basic Sciences Department (VMS Graduate Coordinator)
_____	
Kent H. Hoblet Dean of College of Veterinary Medicine College of Veterinary Medicine	

Name: Josiah Wilcots

Date of Degree: May 2, 2009

Institution: Mississippi State University

Major Field: Veterinary Medical Science (Neuropharmacology)

Major Professor: Dr. Jeffery B. Eells

Title of Study: DETERMINATION OF INDUCTION OF NUR77 (NR4A1), NOR1 (NR4A3), AND NURR1 (NR4A2)

Pages in Study: 61

Candidate for Degree of Master of Science

Previous data have implicated both neuronal activity and dopamine (DA) autoreceptor activation in the modulation of Nur receptor expression in dopamine neurons. Since dopamine receptor antagonists block the autoreceptors but also alter dopamine release and dopamine neuron activity, the mechanism responsible for gene expression is unclear. We hypothesized that blocking the stimulation of dopamine autoreceptors will be the mechanism that causes the induction of the nuclear receptor genes. To test this hypothesis, we treated 78 mice (1hr or 4hr incubation periods) with either a dopamine D_2 receptor agonist (quinpirole), a D_2 receptor antagonist (sulpiride or haloperidol) or γ-butyrolactone (GBL), a drug that inhibits dopamine neuron activity. HPLC and real time pCR was performed on each mouse to measure gene expression and neurochemical levels. We found that D_2 receptor treatments induced Nur receptor expression levels and caused significant differences among neurochemical levels in the striatum and nucleus accumbens.

DEDICATION

First and foremost I would like to thank God for allowing me to start and complete this research, because without him nothing is possible. I would like to dedicate this research to my loving parents Joe and Peggy Wilcots, who have loved and supported me throughout my career as a graduate student and in previous years. I would also like to dedicate this research to all my sisters and brothers who have also shown me much love and support throughout this process. A special dedication is also given to my girlfriend Aneesa Lala who is the sole reason I attended Graduate School and who also has stood by my side throughout this journey. I would like to thank my friends Kelvin Harris and Jerylyn Belle for taking me under their wings as an undergraduate and showing how to succeed as a graduate student.

ACKOWLEDGEMENTS

I would like to acknowledge my advisor Dr. Jeffery Eells for taking a chance on me when no one else wanted to. I would also like to acknowledge my fellow graduate students Talisha Moore and Jessica Carlsen for their support and friendship. I would like to give a special thanks to Timothy Brown who was very instrumental in helping me start my project. I would like to also thank Aimee Belle our present lab coordinator for all of her help and support.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

1.1 Dopamine Function and Importance

Dopamine is an essential neurotransmitter found throughout the brain and concentrated within the basal ganglia (Cooper et al. 2003). Dopamine is the most recent catecholamine neurotransmitter to be discovered and probably one of the most important transmitters to be found due to its relevance in disease states of certain CNS disorders such as Parkinson's disease, schizophrenia and attention deficit hyperactivity disorder (ADHD), which has annually costs of billions of dollars each year for therapeutic treatments. The majority of dopamine in the brain originates from neurons located in substantia nigra pars compacta and ventral tegmental area. The dopamine neurons in the substantia nigra pars compacta innervate the striatum (nigrostriatal dopamine system) while those in the ventral tegmental area innervate the prefrontal cortex (mesocortical dopamine system) and the nucleus accumbens, amygdala and hippocampus (mesolimbic dopamine system).

The nigrostriatal dopamine system is an important component of the basal ganglia, a structure found to be directly involved in motor control, which has been found to be dysfunctional in Parkinson's disease. Parkinson's disease results from 80-90% loss of dopaminergic neurons in the nigrostriatal pathway and results in impaired motor

movements and speech disorders (Lang et al 1998a, and 1998b; Steece-Collier et al. 2002). Dopamine neurotransmission from the neurons in the ventral tegmental area are involved in the regulation of reward and motivation as well as cognitive function. Abnormalities in dopamine neurotransmission have been demonstrated in schizophrenia, specifically elevated subcortical dopamine neurotransmission and impaired mesocortical dopamine neurotransmission (Seeman, P. Synapse, 1987 and Carlson et al. 2001). Dysfunction of mesolimbic dopamine neurons have also been implicated in disorders such as ADHD and addiction (Solanto, M.V. 2002).

Dopamine is produced via a cascade of enzymes working together to create the final product. The basic amino acid tyrosine serves as the precursor to dopamine, which is converted to L-DOPA via tyrosine hydroxylase (TH) the rate limiting enzyme during dopamine production. L-DOPA is then converted to dopamine by DOPA decarboxylase (DDC), the final enzyme necessary for the conversion of tyrosine into dopamine. Additionally, tetrahydrobiopterin (BH_4) is an essential cofactor for TH. BH_4 is synthesized from GTP via three enzymes; the final and rate-limiting enzyme is GTP cyclohydrolase (GTPCH). Once synthesized, dopamine is concentrated into synaptic vesicles via the vesicular monoamine transporter (VMAT2) for release into the synapse. Following synaptic release, dopamine can be actively taken back up into the synaptic terminal by the dopamine transporter (DAT) or metabolized by the enzymes monoamine oxidase and/or catechol-o-methyltransferase to the metabolites 3,4 dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA). Dopamine carries out its function via binding to receptors both postsynaptic and presynaptic. There are two categories of dopamine receptors known as D1 like (D1 and D5) and D2 (D2, D3 and D4) like. A

receptor of major importance is the D2 receptor which is known two have two isoforms, D2S, the presynaptic autoreceptor, and D2L, the postsynaptic receptor (Usiello et al. 2000). These receptors are major targets of many antipsychotic medications such as haloperidol used to treat schizophrenic patients and recent research has shown that these receptors could serve as essential targets for future drug development which can be used to target specific isoforms of a particular receptor ablating non specific binding and the side effects that result.

1.2 Nur Receptor Subfamily Expression

Nurr1 is an immediate early gene that is expressed in dopamine neurons in the substantia nigra and the ventral tegmental area (Zetterstrom et al. 1997; Castillo et al. 1998; Saucedo-Cardenas et al. 1998). Previous research has shown that Nurr1 is essential for the proper differentiation of these dopamine neurons as shown in Nurr1 knockout (-/-) mice. These mice do not survive after birth due to inability to feed and problems congenital hypoventilation (Zetterstrom et al. 1996a, Saucedo-Cardenas et al. 19998, Castillo et al. 1998, Nsegbe et al. 2004). Additionally, the Nurr1 -/- newborn pups have profound deficits of dopamine in the midbrain and striatum along with the absence of most midbrain dopaminergic neuron markers including TH, GTPCH, DAT, VMAT2, and DDC (Zetterstrom et al. 1996a, Saucedo-Cardenas et al. 19998, Castillo et al. 1998). Additional experiments have implicated Nurr1 directly in controlling the expression of these genes necessary for dopamine synthesis and regulation (Zetterstrom et al. 1996a, Saucedo-Cardenas et al. 1998, Castillo et al. 1998). In vitro studies have shown that Nurr1 can bind to the promoters of TH to induce reporter gene expression (Sakurada et al. 1999; Maira et al. 1999). Additionally, Nurr1, when expressed in vitro, can increase

the expression of a reporter gene via the DAT and GTPCH promoters (Bannon et al. 2001; Gil et al. 2007). Previous studies with Nurr1 null heterozygous (+/-) mice have shown that a reduction in Nurr1 causes decreased dopamine levels in the midbrain, nucleus accumbens and prefrontal cortex, along with decreased TH activity in the nucleus accumbens and striatum, however striatal dopamine levels remain normal (Saucedo-Cardenas et al. 1998; Zetterstrom et al. 1997). Additionally, reduced TH and GTPCH mRNA levels have been reported in the Nurr1 +/- mice (Eells et al. 2006). Dopaminergic neurons in Nurr1+/- mice also show greater vulnerability to neurotoxicants and impaired survival *in vitro* as compared dopamine neurons in wild type mice, indicating that Nurr1 possesses a neuroprotective role in mature dopamine neurons (Le et al. 1999; Eells et al. 2002). This genotype allows for a possible application to study dopamine production and neurotransmission during periods of altered Nurr1 expression.

Nurr1 along with two very closely related genes Nur77 and Nor1 are all orphan nuclear receptors of the steroid/thyroid hormone receptor subfamily, categorized as part of the Nur receptor subfamily. Each receptor in this family possesses distinct characteristics of function, structure, and location of expression; however all share very similar DNA binding properties. All are expressed in the CNS. Nur77 expression is concentrated in dopaminoceptive areas such as the striatum and nucleus accumbens (Zetterstrom et al. 1996b; Beaudry et al. 2000). At basal levels, Nur77 mRNA is barely detectable in the dopamine neurons (Maheux et al. 2004). Data from Nur77 -/- mice demonstrates that Nur77 has a role in regulating dopamine neurotransmission. Nur77 -/- mice showed higher locomotor activity when placed in a novel environment as compared to +/+ mice after quinpirole administration (Gilbert et al. 2006). Also, Nurr1 and TH

mRNA expression was altered in the Nur77 -/- mice. Specifically, TH mRNA expression was elevated in dopamine neurons in the substantia nigra pars compacta and Nurr1 mRNA expression was increased in dopamine neurons in the substantia nigra pars compacta and ventral tegmental area. (Gilbert et al. 2006). Gilbert et al. also reports that haloperidol has an effect on DA turnover in the striatum of Nur77 +/+ mice, although there is a greater effect on striatal DOPAC levels in Nur77 -/- mice. Additionally, D2R antagonists have been shown to significantly increase Nur77 mRNA expression in dopamine neurons (Maheux et al 2004). Collectively, these data demonstrate an important involvement of Nur77 in dopamine neuron function and a close relationship with Nurr1 such that these transcription factors may be able to compensate for the down-regulation or improper function of the other.

Nor1 is also found in the dopaminoceptive areas of the striatum and nucleus accumbens along with Nur77, with a wide distribution in the hippocampus, amygdala, and cerebellum, but with no expression in dopamine neurons (Zetterstrom et al. 1996b). Disruption of the Nor1 gene has demonstrated that it is involved in proper neuron development, survival and axon guidance of neurons in the hippocampus (Ponnio and Conneely et al. 2004). Nor1 function and regulation within dopaminergic neurons has yet to be extensively studied in detail, however similar to Nur77, this gene can be induced by antagonism of the D_2 receptor (Maheux et al 2004). As Nur77, Nor1 and Nurr1 all share similar DNA binding properties, expression of Nur77 and Nor1 could produce similar effects on expression of dopamine neuron targets genes as Nurr1.

1.3 Nur Receptor Subfamily Homology

The Nur receptors exemplify the same protein structural moiety of all steroid/thyroid hormone nuclear receptors, which consists of an amino terminal domain, DNA binding domain, and a carboxyl-terminal domain (Freedman and Luisi 1993). Additionally Nurr1, Nur77, and Nor1 also share deeper homology when individually compared to each other (Eells et al. 2000). The greatest structural homology found between the three is among the DNA binding domain, in which there is greater than 90% homology among all three (Milbrandt et al. 1988; Law et al. 1992; Ohkura et al. 1994). As a result of previous research it is known that Nurr1 and Nor1 both show the greatest homology as compared to Nur77 and Nurr1 or Nur77 and Nor1. This could be important in transactivation properties of response elements due to lack of function of another receptor. The three receptors also show similar homology to gene structure, as all of them contain six translated exons with common exon/intron splice sites (Milbrandt et al. 1988; Hazel et al. 1988; Castillo et al. 1997; Ohkura et al. 1996). Nurr1 and Nor1 again show greater similarity among each other by both possessing two 5' untranslated exons as compared to Nur77 which only contains one 5' untranslated exon. There also exists an exon/intron junction between the two zinc finger motif's which is different from the other nuclear receptor superfamily genes, which possess a conserved splice site different from that of Nurr1, Nur77, and Nor1 (Eells et. al 2000).

In contrast to their strong homology, however there exist differences among promoter elements among Nurr1, Nur77, and Nor1. They contain various combinations of GC-rich, AP-1, Sp-1, CRE, CArG, and ETs like sequences (Ryseck et al. 1989; Woronicz et al. 1995; Williams et al. 1993; Yoon and Lau 1993). The promoter regions

contained within these genes have various response elements that can regulate Nurr1, Nur77, and Nor1 by immediate early response pathways or delayed early response pathways (Eells et al. 2000).

Nurr1, Nur77, and Nor1 must be able to bind to DNA to exert their function of regulating mRNA expression so proteins can be produced which will carry out a specific function. These genes bind DNA as monomers to an octomer sequence known as Nur77 binding response element (NBRE: AAAGGTCA) to which Nur77 has the highest affinity followed by Nurr1 then Nor1 (Wilson et al. 1991; Paulsen et al. 1995; Zetterstrom et al. 1996a). Receptor binding to NBRE is a result of the T-box and A-box located at the C-Terminal end of the DNA binding domain. All three Nur receptors have been shown to be able to bind as a homodimer to the Nur77 response element (NurRE), which is an inverted repeat sequence of NBRE (Phillips et al. 1997 35). As a homodimer Nur77 has a higher affinity for NuRE as compared to its monomeric state for NBRE and is able to upregulate in vitro gene expression through this sequence (Phillips et al. 1997). Also, Nurr1 and Nor1 have been shown to bind as homodimers on NurRE and cause transactivation of gene expression though Nur77 homodimers have a greater probability for dimerization than either of the other two homodimers formed (Maira et al. 1999). Nur77 also possesses the ability to form heterodimers with Nurr1 and Nor-1, which has a synergistic effect on reporter gene expression (Maira et al. 1999). Nur77 and Nurr1 are also able to form heterodimers with retinoid X receptor (RXR) (Perlman et al. 1995). Heterodimerization between RXR-Nur77/Nurr1 allows binding to hormonal response elements in close proximity to genes that they regulate (Zetterstrom et al. 1996a). This binding adds yet another complexity to the response elements that nuclear receptors are

responsible for activating, which will in turn control specific genes regulated by these response elements. Nurr1 has the highest affinity to RXR compared to RAR and Nur77 (Perlman et al. 1995). Heterodimerization between RXR and RAR will inhibit any dimerization between Nurr1 or Nur77 and RXR (Perlman et al. 1995). Nor1 is unable to form heterodimers with RXR (Perlman et al. 1995), and has been shown to be able to interfere with Nurr1 and Nur77-RXR heterodimer signaling (Zetterstrom et al. 1996a).

1.4 Rationale

Currently the exact mechanism by which dopamine neurons are lost in Parkinson's disease is unknown, though it is known that individuals with this disorder produce less dopamine in the nigrostriatal pathway. This results in the symptoms of Parkinson's disease such as the inability to carry out movement and inhibiting uncontrolled movement (tremors). Also, schizophrenia is believed to be caused by an over production of dopamine in the mesolimbic pathway and a lack of dopamine in the mesocortical pathway. Nurr1 is known to be a direct contributor to the development of dopaminergic neurons in the midbrain, therefore presenting a possible avenue for the development of therapeutic treatments that directly stimulate dopamine neuron production. Since Nur77 and Nor1 both share similar DNA binding domains to Nurr1, they may present possible targets for future drug development. Dopamine agonist and antagonist both affect dopamine production whether it is directly or indirectly. These drugs offer the ability to study gene regulation as it relates to dopamine neuron function and dopamine production.

1.5 Objectives

The goal of the current study was to 1) determine the effect of antipsychotic treatment on Nurr1, Nor1 and Nur77 expression concurrent with dopamine neurochemistry, dopamine neuron gene expression, 2) determine the mechanism of Nurr1, Nor1 and Nur77 expression in dopamine neurons, and 3) determine effect of reduced Nurr1 expression on Nur77 and Nor1 expression in dopamine neurons.

1.6 Hypothesis

We *hypothesize* that haloperidol should stimulate dopamine release and decrease intracellular dopamine. Dopamine agonist treatment should block dopamine release therefore increasing intracellular dopamine. Due to the blockage of dopamine there should be a downregulation of all genes necessary for dopamine production and metabolism. Since these nuclear receptors are induced by haloperidol and implicated in regulating expression of genes involved in dopamine production, we expect that haloperidol will induce nuclear receptor expression in the substantia nigra and ventral tegmental area and that this will result in elevation of dopamine neurotranmssion gene expression. Since Nur77 and Nor1 share DNA binding properties with Nurr1, induction of these genes is expected to influence expression of dopamine neurotransmission genes regulated by Nurr1. To investigate this we measured expression of Nur77, Nor1 and Nurr1 1 hour after haloperidol treatment and dopamine neuron gene expression 4 hours after haloperidol treatment. Expression of TH, GTPCH, DAT, VMAT2, and DDC were measured along with the major dopamine metabolite of levels (DOPAC and HVA) and the essential cofactor for dopamine synthesis BH_4.

Previous data has implicated both neuronal activity and dopamine autoreceptor activation in the modulation of Nur receptor expression in dopamine neurons. Since dopamine receptor antagonists block the autoreceptors but also alter dopamine release and dopamine neuron activity, the mechanism responsible for gene expression is unclear. We expect that blocking the stimulation of dopamine autoreceptors will be the mechanism that causes the induction of the nuclear receptor genes. To conduct this experiment we treated mice with either a dopamine D_2 receptor agonist, a D_2 receptor antagonist or γ-butyrolactone, a drug that inhibits dopamine neuron activity and subsequent dopamine release and autoreceptor activation and measured Nurr1, Nur77 and Nor1 expression in the ventral tegmental area and substantia nigra pars compacta. Additionally, dopamine and metabolite levels and BH_4 levels were measured.

Due to the similarities in the regulation of expression between these nuclear receptors, we propose that altered expression of Nurr1 will influence the expression of Nur77 and Nor1 in dopamine neurons. To investigate this we measured Nur77 and Nor1 in Nurr1 +/- and +/+ mice. This allowed us to determine the effects Nurr1 down-regulation has on the other members of the Nur77 receptor subfamily. This data should provided information important to determining the exact roles that Nur77 and Nor-1 are responsible for carrying out during periods of Nurr1 arrest in midbrain dopaminergic neurons along with neurotransmitter production.

CHAPTER II

MATERIALS AND METHODS

2.1 Animals

Ninety day old male HSD: ICR Outbred Male Mice purchased from Harlan Bioproducts for Science for the purpose of this study. Animals were housed at Mississippi State University in an AAALAC accredited facility on 12 hour light/dark cycle with food and water available at liberty. A total of 79 animals were used in this study as shown in Table 2.1. Thirty two of the 79 animals were used for systemic haloperidol and saline injections at two different time intervals of 1-hour and 4-hours. Thirty two other animals were subjected to systemic injections of quinpirole, sulpiride, GBL, or saline solution. The final 14 animals were Nurr1 +/+ or +/- mice that were used to perform experiments to determine the differences expressed in the Nur77 receptor subfamily due to genetic differences.

Table 2.1 Number of animals sacrificed in each experiment.

	Saline	Haldol	GBL	Sulpiride	Quinpirole	Non treated
1-Hr Treatment	16	8	9	8	8	
4-Hr Treatment	8	8				
Nurr1 +/+ or +/-						14
Total	23	16	9	8	8	14

2.2 Dosage Regimen

Haloperidol was dissolved in acetic acid and was administered intraperitoneal (IP) at a dose of 1mg/kg. Saline acetic acid solution served as our control treatment. Mice were sacrificed 1 and 4-hours after injection. GBL, quinpirole, and sulpiride, were also administered via IP injections and sacrificed 1-hour after injection.

2.3 Sacrificing Schedule and Brain Harvesting

In each experiment all animals were sacrificed using CO2 asphyxiation. Animals were sacrificed on time intervals of 5-minutes to ensure a fixed incubation time for each individual treatment. Each mouse was decapitated according to IACUC procedure and the brain was removed from the skull. A coronal cut was made approximately 1mm caudal to bregma, which separated the brain into two distinct regions (midbrain and forebrain) from which tissue punches could be obtained. Both sections were immediately frozen on dry ice for future sectioning.

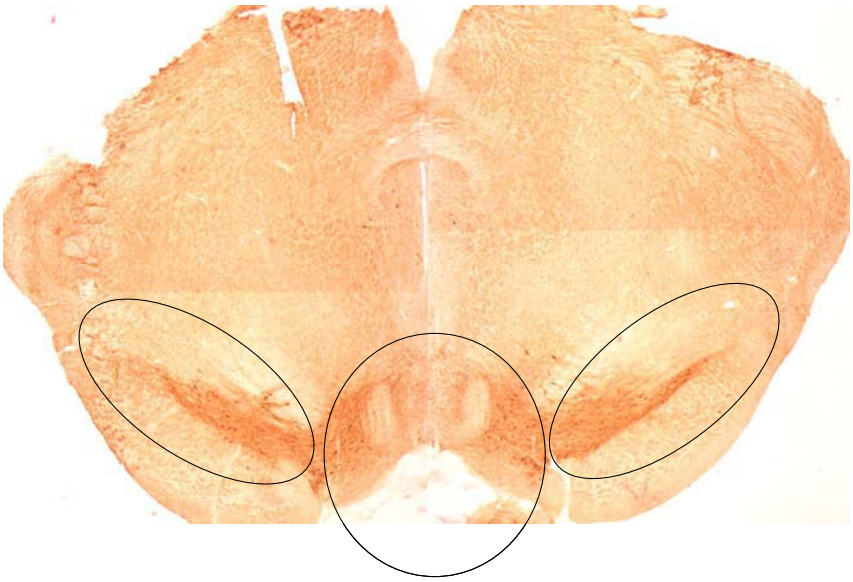

Figure 2.1 The previous schematic shows the regions of the brain which were excised for analysis. The two outside circles represent the substantia nigra pars compacta and the middle circle represents the ventral tegmental area.

2.4 Tissue Collection

Midbrain and forebrain sections were bound on a custom made cold aluminum tissue slicer using Tissue-Tek O.C.T. compound, from which 300 µm sections were taken (Fig 2.2). Bilateral punches were taken from the substantia nigra pars compacta and ventral tegmental area, which were placed immediately in lysis buffer for future extraction of RNA (Figure 2.1). Bilateral punches were also taken from the striatum and nucleus accumbens to measure neurochemical changes using Waters HPLC detection. Right striatum and nucleus accumbens punches were used to measure biopterin levels and left striatum and nucleus accumbens punches were used to analyze catecholamine levels.

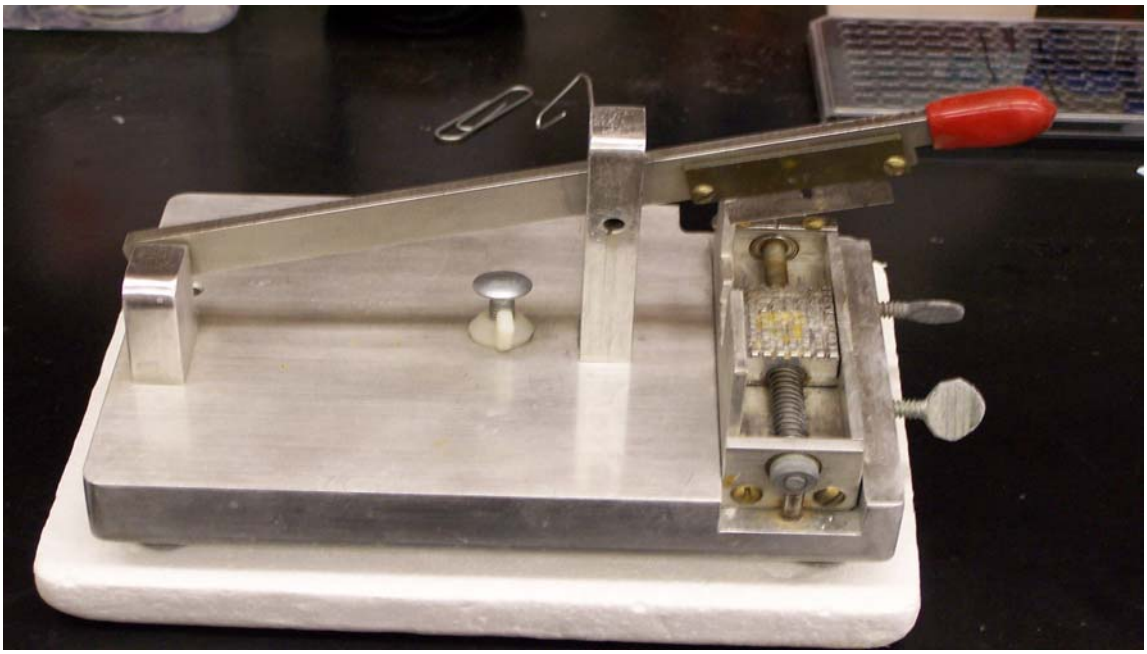

Figure 2.2 Aluminum Tissue Slicer

2.5 RNA Extraction

To isolate RNA from the 1-hour samples an Absolutely RNA Microprep Kit obtained from Stratagene was used in accordance to the directions indicated in the instruction manual. An AllPrep RNA/Protein kit purchased from Qiagen was used to extract RNA and protein from 4-hour haloperidol treated samples. RNA extracted in both procedures was used in subsequent reactions to detect gene expression levels of the Nur receptor genes.

2.6 Reverse Transcription and Quantitative PCR

Isolated RNA was used to make cDNA using an Omniscript RT Kit obtained from Qiagen on the 1-hour haloperidol, 4-hour haloperidol, and GBl, quinpirole, and sulpiride treatment groups. To obtain cDNA for quantitative reactions with the 1-hour dopamine

agonist and antagonist treatment group, random nanomers obtained from Gene link were used in replace of oligo dT's in the Qiagen Omniscript RT Kit. This was done to increase the amount of cDNA obtained in the reaction due to suspected sample quality concern. cDNA was made by reverse transcribing our isolated RNA by the materials provided within the Omniscript RT kit provided by Qiagen. Real time quantitative PCR was used in order to measure and analyze gene expression levels of Nurr1, Nur77, and Nor1 in the substantia nigra pars compacta and ventral tegmental area among different treatment groups. β-actin was also measured, which served as an internal control being that it is a standard housekeeping gene. Lux primers obtained from Invitrogen were designed to provide high specificity and sensitivity to our specific genes of interest (Table 2.2). Systemic 1 hour haloperidol and dopamine agonist and antagonist injections were analyzed using the iCycler iQ real-time PCR detection system from BioRad. The expression levels of each gene were examined based on standard curves obtained from fluorescently amplified PCR products of each gene of interest and normalized to β-actin. To determine the expression of the Nur77 receptor subfamily in the 4 hour haloperidol treatment and Nurr1 +/+ or +/- mice the Strategene Mx3005p QPCR machine was used. Platinum qPCR Supermix-UDG obtained from Invitrogen was used in both quantitative experiments in amounts of 10.8µl per reaction.

2.7 HPLC Electrochemical Detection

Tetrahydrabiopterin, dopamine, HVA, and DOPAC levels were detected using the Waters 2465 Electrochemical Detector system, in which absolute amounts of catecholamines were determined based on the response to known standards. Detection of

these neurochemical levels required to mobile phases: ROMP (pH 3.65), which consisted of Sodium Phosphate Monobasic (Sigma), Sodium Octyl Sulfate (Sigma), EDTA, disodium (Sigma), Methanol (Fisher), H20 (Millipore), Tryethylamine (Fluka) and Biopterin (pH 5.22), which consisted of Sodium Acetate (Sigma), Citric Acid (Sigma), EDTA (Sigma), 1,4 Dithioerythritol (Fluka), and Diethylenetriamine-pentaacetic acid (Fluka). To detect tetrahydrobiopterin levels a biopterin standard was used in conjunction with the biopterin mobile phase and to detect catecholamine levels Mix 9 (Dopamine, Serotonin, DOPAC, HVA, DOPA, MHPG, Norepenephrine, DHBA, 3MT, 5HIAA) obtained from Sigma was used in conjunction with ROMP. Brains were frozen and mounted on a custom tissue slicer (Figure 2.2) which allowed sections to be cut approximately 300μm thick. Tissue punches were then taken from the nucleus accumbens and striatum and prepared for catecholamine and tetrahydrabiopterin analysis. All data were analyzed and processed using Waters HPLC Empower software. One tissue punch was suspended in 0.1 M perchloric acid, homogenized, centrifuged twice to clear the supernatant and injected into a high pressure liquid chromatography (HPLC) system consisting of a Waters 2695 Separation module, a Supleco LC-18-DB column with the Waters 2465 electrochemical detector (ECD). The ECD was set at 20nA and an Ec=+0.67V using a mobile phase of 100mM phosphate, 17.5% methanol, 25 mM EDTA, 1mM octyl sodium sulfate at pH 3.65. These conditions provided good separation and detection of dopamine and its metabolites DOPAC and HVA. The other tissue punch was used to measure BH4. The tissue was suspended in biopterin moile phase (50 mM sodium acetate, 5mM citric acid, 5μM EDTA, 1mg/ml DTE and 1mg/ml DTPA at a pH of 5.22), homogenized, and cleared by centrifugation. The supernatant was injected into

the biopterin mobile phase. The quantity of each comp[ound was determined based on the response of a known amount of standards. A Bradford assay was performed to measure total protein for each tissue punch, which was used to normalize measurements across samples. The Bradford reagent protein assay was performed on pellets formed on the bottom of the microcentrifuge tubes from which the supernatant that was extracted for HPLC analysis. To perform this reaction pellets were solubilized with 100µl of 0.5 M NaOH by incubating at 37oC for 1hr. ddH2O was then added to the samples (200ul to NuAcc samples and 100ul to Striatal samles) according to determined dilution capability of each sample and incubated for an additional thirty minutes. The samples were loaded into a 96-well plate and assayed using the ThermoMax microplate reader with Softmax pro software. Bovine Serum Albumin was used as our standard.

2.8 Chemicals and Reagents

Haloperidol, γ-butyrolactone (GBl), quinpirole, and sulpiride were all obtained from Sigma with 99% purity. 0.1M Acetic acid was used to dissolve haloperidol into solution, which was then further diluted with saline to reach a final concentration 0.1mg/k.

Table 2.2 D-Lux Select Primers (Invitrogen)

Nurr1	*ATTGCTGCCCTGGCTATGGT_FL, GACCATGTGACTTTCAATAATAATGGG_RU*
Nur77	Invitrogen Designed Primers
Nor-1	Invitrogen Designed Primers
β-actin	Invitrogen designed Primers
VMAT2	*caccagAGACAGCTCCTTTCCTGGtG-forward ,GGATGGCTGGAGCACAAAGAG-reverse*
TH	*gacattgCCCAGAGATGCAAGTCCAATGtC_FL, TGTTGGCTGACCGCACATTT_RU*
D2R	*catccaAGTGAACAGGCGGAGAATGGAtG_FL, AGGATGTGGCGATGATCCAG_RU*
DDC	*caccttCCAGTAGGGCCACCAAGGtG_FL, GGGAGGAGTGATCCAGGGAAGT_RU*
DAT	*cacataCCCCTGCTTCCTCCTGTATGtG_FL, AGGATGTGGCGATGATCCAG_RU*

CHAPTER III

RESULTS

Our first goal of interest was to successfully antagonize the D2 receptor to induce the upregulation of the Nur receptor subfamily (Nurr1, Nor1, and Nur77) within the substantia nigra pars compacta and ventral tegmental area and measure their expression levels after a 1 hour incubation period. As described in the introduction Nurr1, Nor1, and Nur77 are all inducible transcription factors, which are thought to play a major role in dopaminergic neuron production and activity. To achieve this goal we used haloperidol to antagonize the D_2 receptor and induce the expression of the three members of the nuclear receptor subfamily. As stated earlier haloperidol is a strong D_2 receptor antagonist, which will bind to both the presynaptic and postsynaptic receptors located on dopaminergic neurons in the midbrain as well as within the dopaminoceptive areas such as the striatum and nucleus accumbens. To conduct this experiment, 16 mice were injected intraperitoneal with a 1mg/kg dose of a haloperidol acetic acid solution or a saline solution. After a 1 hour incubation period each mice was mice was sacrificed and brains were removed and immediately frozen on dry ice. To determine the relative expression of each transcription factor quantitative real time PCR was used and all of our data was normalized to β-Actin, which served as our internal control.

3.1 Nur77 Receptor Subfamily Expression levels and Neurochemical Changes at 1-Hour

The expression levels of Nur77 and Nor1 were shown to be significantly increased in the substantia nigra pars compacta and ventral tegmental area in haloperidol treated animals versus vehicle treated animals after 1h, which is consistent with the results found by Maheux et. Al (Figures 3.1 & 3.2). However, there were no significant changes found among Nurr1 in either the substantia nigra pars compacta nor the ventral tegmental area, which was contradictory to previous research. Nur77 and Nor1 increased expression levels also corresponded to neurochemical and metabolic changes within the dopaminoceptive regions such as the striatum and nucleus accumbens. Tissue level dopamine was found to be lower in haloperidol treated mice as compared to vehicle treated mice in the nucleus accumbens and striatum, which corresponded with increased release of dopamine resulting in an increase in metabolism as shown by an increase in HVA and DOPAC due to the antagonistic effects induced by haloperidol on the D_2 receptor (Figures 3.3 & 3.5). DOPAC levels were shown to be significantly increased in the striatum after haloperidol treatment (Figure 3.3) and was not shown to be significant in the nucleus accumbens (Figure 3.5). HVA levels were shown to be significantly increased after haloperidol treatment groups in both the nucleus accumbens and striatum (Figures 3.3 & 3.5). Dopamine turnover was found to be significantly higher in haloperidol treated mice in both the striatum and nucleus accumbens suggesting that there was an increase in the amount of dopamine being produced and released (Figures 3.4 & 3.6). Tetrahydrabiopterin levels were shown to be significantly higher for haloperidol

treated mice in the nucleus accumbens while, there was no significant difference found in the striatum among treatment groups (Figure 3.7).

haloperidol treated mice in the nucleus accumbens while, there was no significant difference found in the striatum among treatment groups.

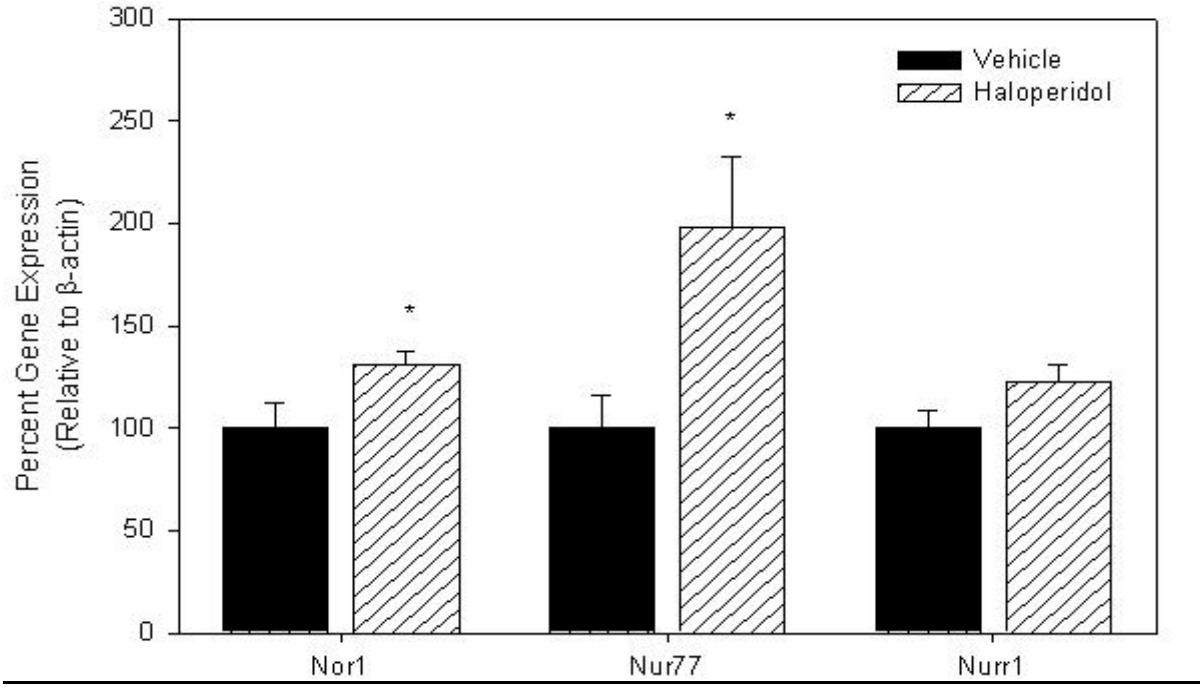

Figure 3.1 Nor1, Nur77, and Nurr1 gene expression levels in the substantia nigra pars compacta after 1h injection with 1mg/kg haloperidol. This figure shows the significant upregulation of Nor1 and Nur77 in the substantia nigra pars compacta after induction with haloperidol compared to vehicle treated animals. Nurr1 gene expression levels in the substantia nigra pars compacta were shown to exhibit a non-significant trend for the upregulation of Nurr1 in the substantia nigra pars compacta after haloperidol compared to vehicle treated animals. All gene expression level data was normalized to β-actin a standard housekeeping gene. (*) Indicates significant differences from the vehicle treated ($p<0.05$).

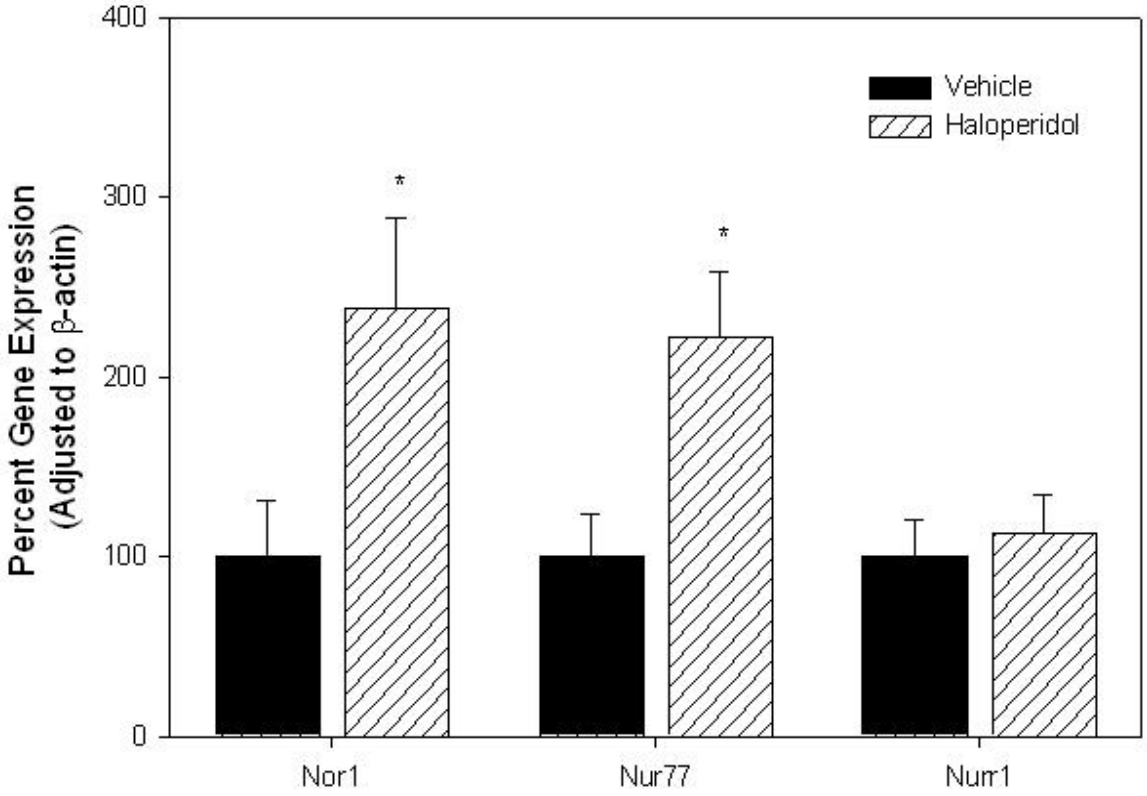

Figure 3.2_ Nor1, Nur77, and Nurr1 gene expression levels in the ventral tegmental area 1h after injection with 1mg/hg haloperidol. This figure shows the significant upregulation of Nor1 and Nur77 in the ventral tegmental area after induction with haloperidol compared to vehicle treated animals. Nurr1 gene expression levels in the ventral tegmental area were shown to exhibit a non-significant trend for the upregulation of Nurr1 in the ventral tegmental area after induction with haloperidol compared to vehicle treated animals. All gene expression level data was normalized to β-Actin a standard housekeeping gene. (*) Indicates significant differences from the vehicle treated ($p<0.05$).

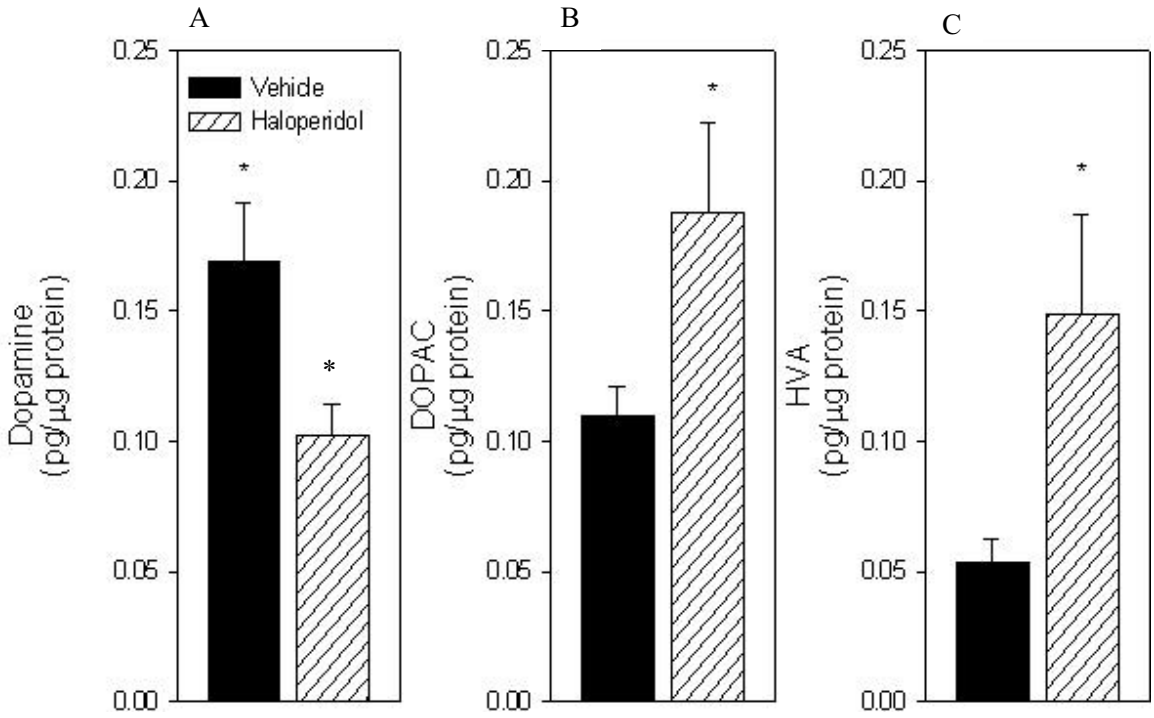

Figure 3.3 Dopamine (A), DOPAC (B), and HVA (C) levels are shown in the striatum after 1h injection with 1mg/kg of haloperidol. A significant decrease in dopamine levels were found in the striatum due to the increased release of dopamine. Also haloperidol produced a significant increase levels of DOPAC and HVA due to the increased release of dopamine. (*) Indicates significant differences from the vehicle groups ($p<0.05$).

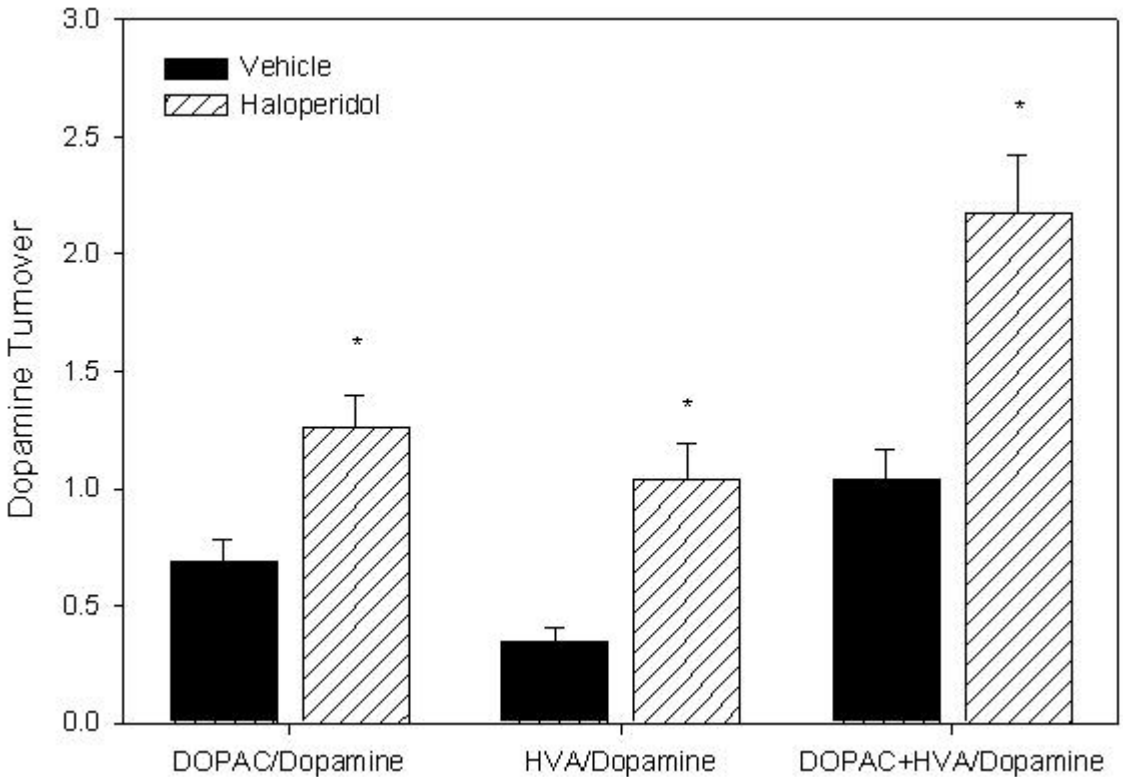

Figure 3.4 Dopamine turnover in the striatum as it relates to the metabolite DOPAC and HVA. There was significantly greater dopamine turnover in the striatum based on ratios of DOPAC/Dopamine, HVA/Dopamine, and DOPAC+HVA/Dopamine. Combined dopamine turnover confirms that there is a greater release and metabolism of dopamine in the striatum, therefore producing an increase in the amount of DOPAC and HVA produced. (*) Indicates significant differences from the vehicle groups ($p<0.05$).

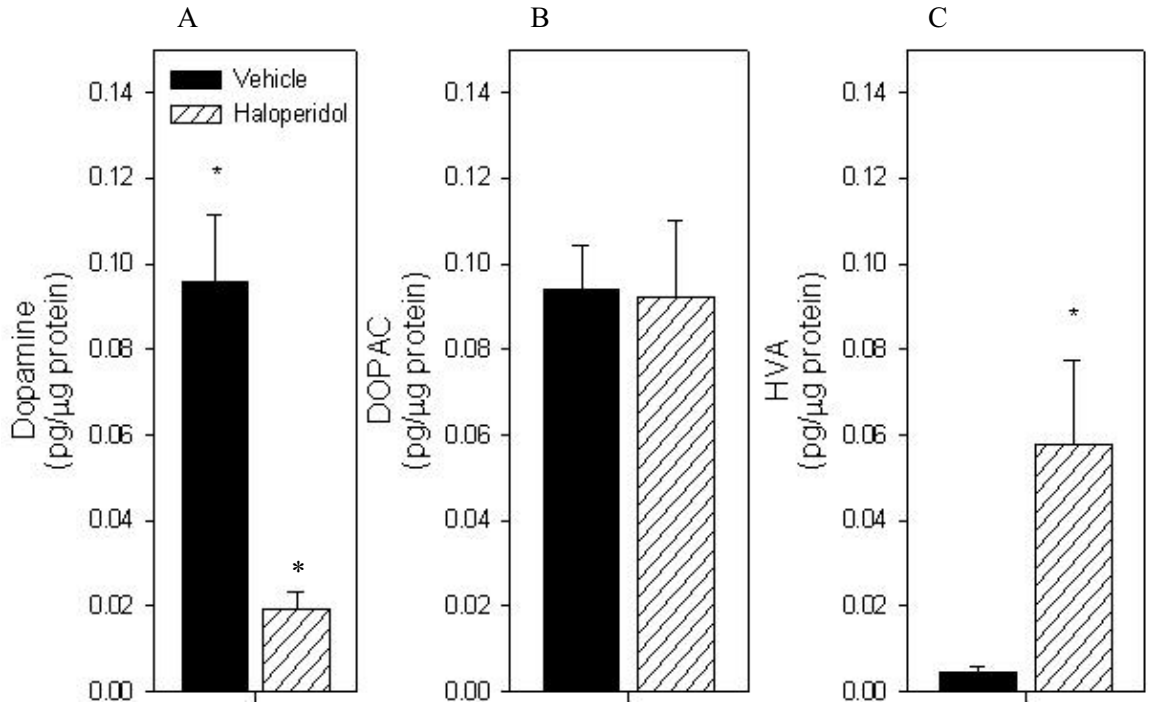

Figure 3.5 Dopamine (A), DOPAC (B), and HVA (C) levels are shown in the nucleus accumbens 1h after injection of 1mg/kg haloperidol. A significant decrease of dopamine levels were found in the nucleus accumbens to the increased release of dopamine. Also haloperidol produced a significant increase of HVA levels in the nucleus accumbens due to the increased release of dopamine. DOPAC expression in the nucleus accumbens after did not show any significant difference between treatment and non-treatment groups. (*) Indicates significant differences from the vehicle treated ($p<0.05$).

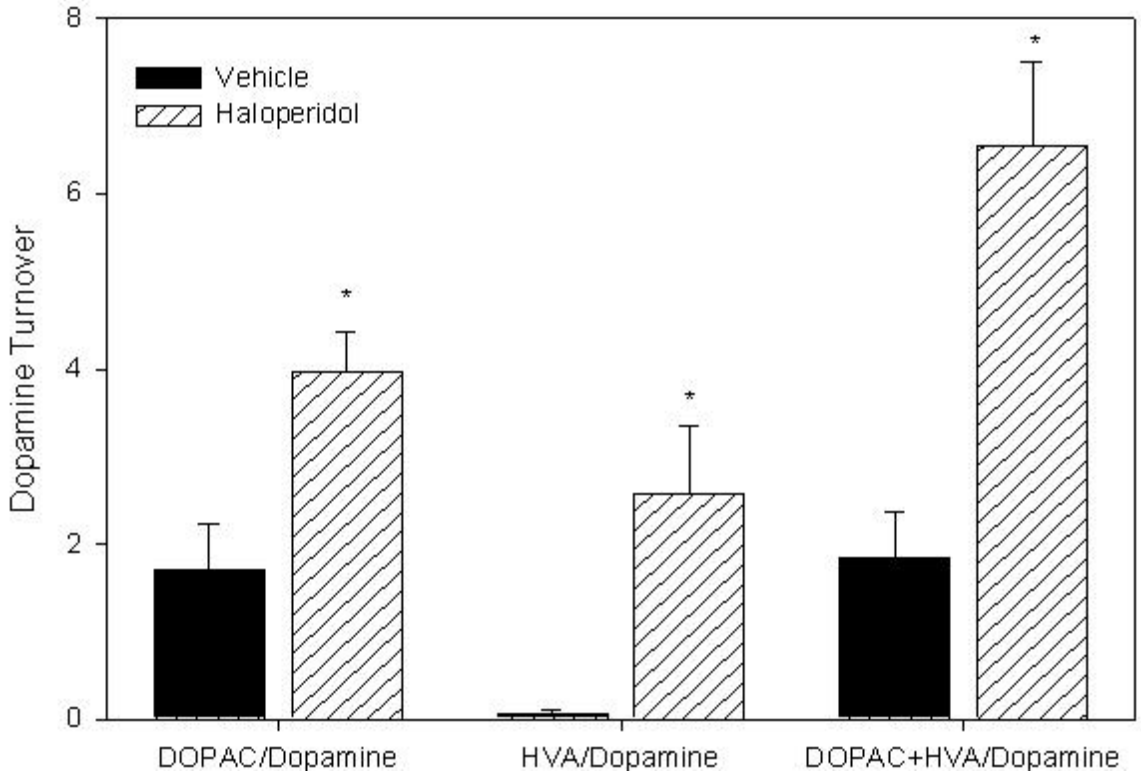

Figure 3.6 Dopamine turnover in the nucleus accumbens as it relates to the metabolite DOPAC and HVA. . There was significantly greater dopamine turnover in the nucleus accumbens based on ratios of DOPAC/Dopamine, HVA/Dopamine, and DOPAC+HVA/Dopamine. Combined dopamine turnover confirms that there is a greater release and metabolism of dopamine in the nucleus accumbens, therefore producing an increase in the amount of DOPAC and HVA produced. (*) Indicates significant differences from the vehicle treated ($p<0.05$).

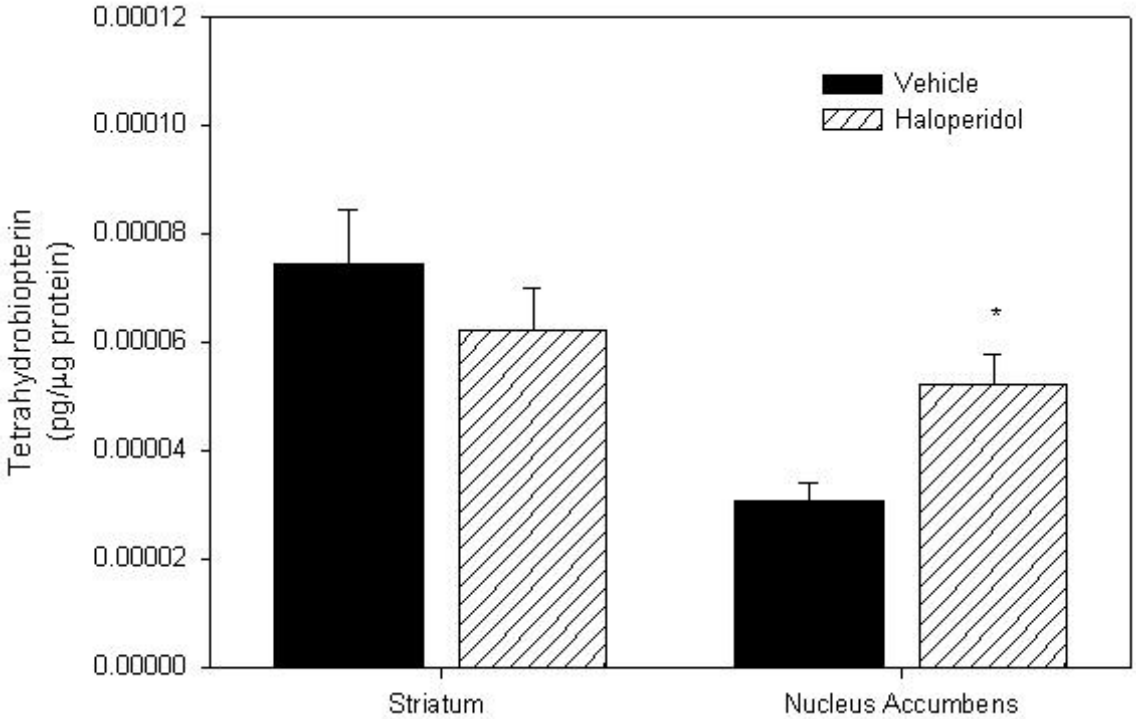

Figure 3.7 Tetrahydrobiopterin levels in the striatum and nucleus accumbens 1h after systemic injection of haloperidol. Tetrahydrobiopterin levels not significantly higher in different treated mice in the striatum compared to haloperidol treated mice indicating that dopamine production is not affected by haloperidol in the nigrostriatal pathway. Although in the nucleus accumbens tetrahydrobiopterin levels were found to be significantly higher in mice that were given 1mg/kg injections of haloperidol indicating that dopamine production may be upregulated in the mesolimbic pathway by increasing tetrahydrobiopterin levels. (*) Indicates significant differences from the vehicle treated ($p<0.05$).

3.2 Regulatory Gene Expression levels and Neurochemical Changes at 4-Hours

Gene expression levels of TH, DAT, DDC, VMAT, GTPCH and D2R were measured 4hrs after haloperidol challenge. The time point of 4h was chosen to provide sufficient time for the transcription factor RNA to be translated into protein and alter expression of target gene RNA. This will give us insight into the regulatory control that these genes could have on dopamine production. Dopamine and dopamine metabolite

levels were also measured to assess the neurochemical changes that correspond to the upregulation of regulatory gene control. Dopamine levels were found to be lower in haloperidol treated groups in the striatum (Figure 3.8). In the nucleus accumbens the levels of dopamine were higher in haloperidol treated mice (Figure 3.10). Dopamine metabolite levels were shown to be significantly increased in both the striatum and nucleus accumbens in mice who received the haloperidol treatment, which corresponded to increased dopamine turnover levels in both regions for haloperidol treated animals (Figures 3.9 &3.11). There were no significant changes in BH_4 levels in the striatum or nucleus accumbens although, biopterin levels did seem to be higher in the striatum in mice treated with haloperidol (Figure 3.12).

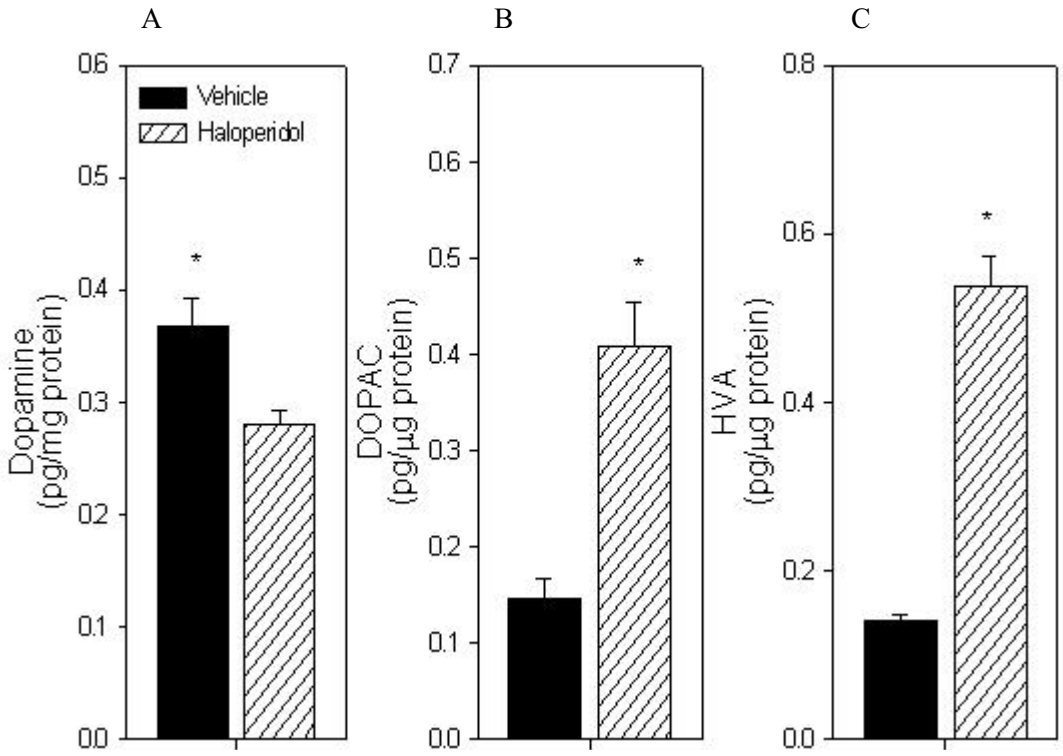

Figure 3.8 Dopamine (A), DOPAC (B), and HVA (C) levels in the striatum are shown 4h after injection with 1mg/kg of haloperidol. A significant decrease in dopamine levels were found in the striatum due to the increased release of dopamine. Also haloperidol produced a significant increase in DOPAC and HVA levels due to the increased release of dopamine. (*) Indicates significant differences from the vehicle treated ($p<0.05$).

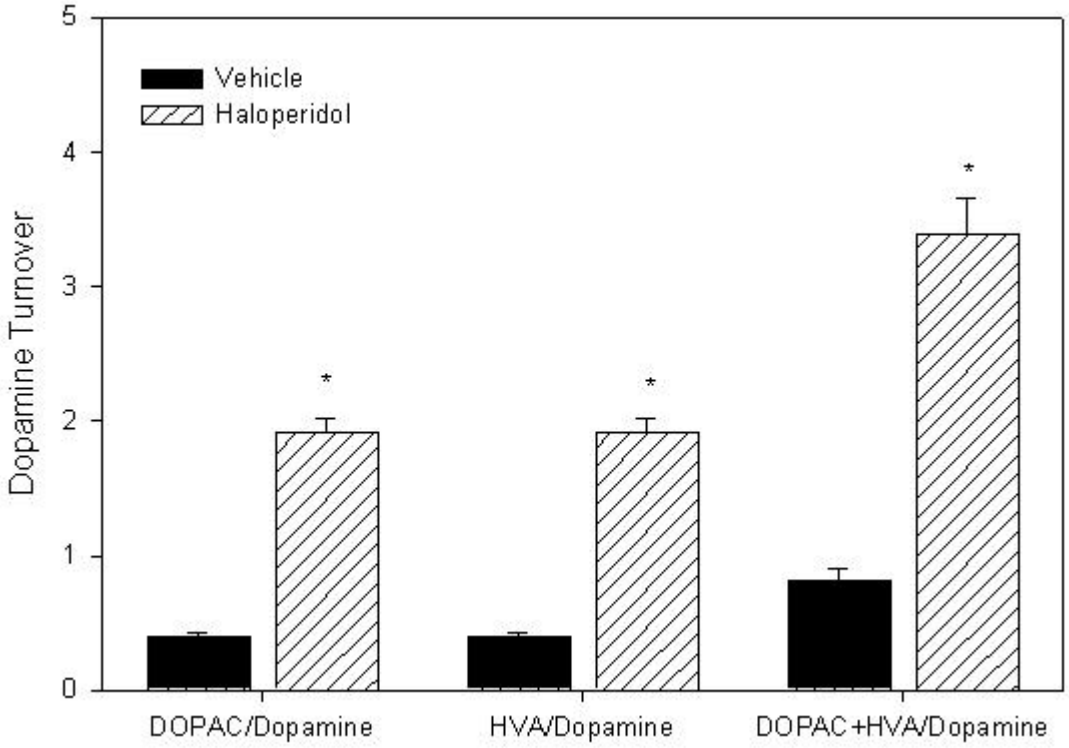

Figure 3.9 Dopamine turnover in the striatum as it relates to the metabolite DOPAC and HVA 4h after a 1mg/kg systemic injection of haloperidol. There was significantly greater dopamine turnover in the striatum based on ratios of DOPAC/Dopamine, HVA/Dopamine, and DOPAC+HVA/Dopamine. Combined dopamine turnover confirms that there is a greater release and metabolism of dopamine in the striatum, therefore producing an increase in the amount of DOPAC and HVA produced. (*) Indicates significant differences from the vehicle treated ($p<0.05$).

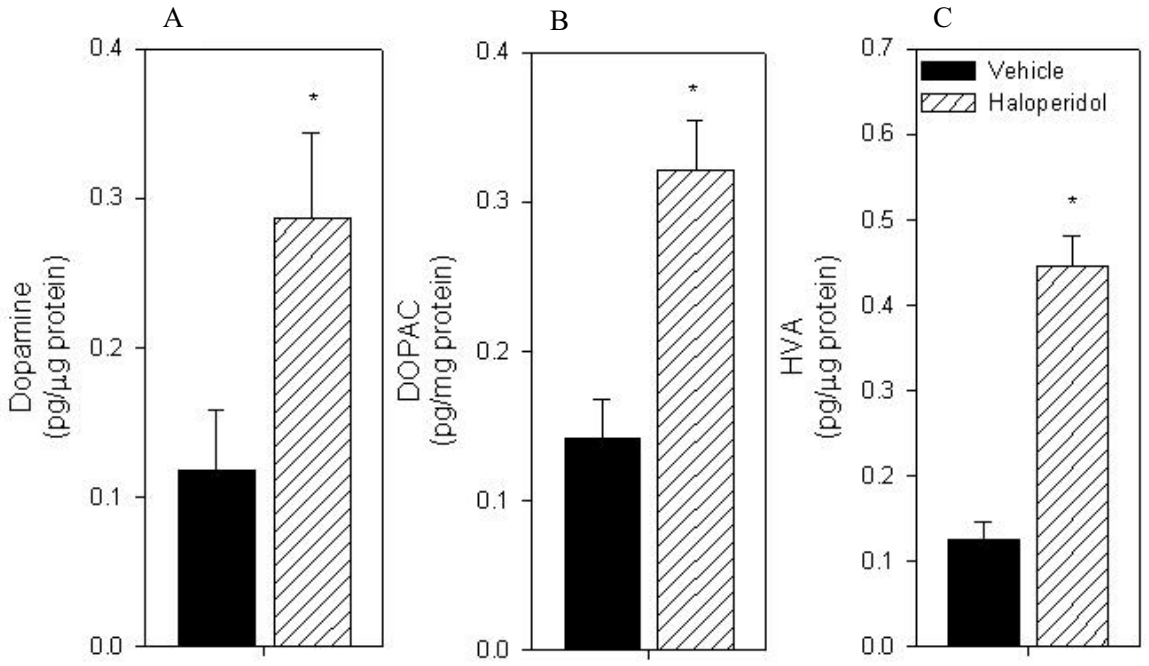

Figure 3.10 Dopamine (A), DOPAC (B), and HVA (C) levels in the nucleus accumbens are shown 4h after a 1mg/kg systemic injection of haloperidol . A significant increase in dopamine levels were found in the nucleus accumbens indicating that increased release of dopamine from haloperidol induction has ceased after a 4hr incubation period. Also haloperidol produced a significant increase in DOPAC and HVA levels due to the initial increased release of dopamine. (*) Indicates significant differences from the vehicle treated ($p<0.05$).

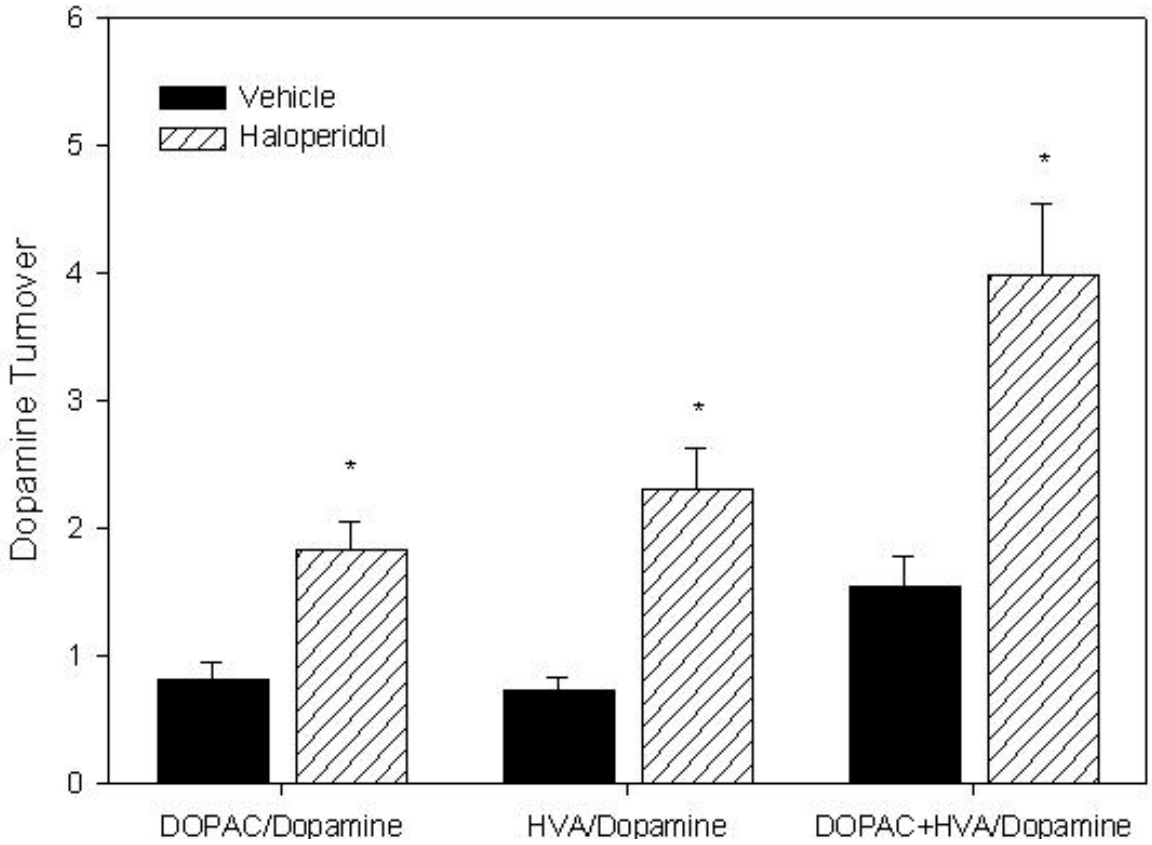

Figure 3.11 Dopamine turnover in the nucleus accumbens as it relates to the metabolite DOPAC and HVA 4h after a 1mg/kg systemic injection of haloperidol. There was significantly greater dopamine turnover in the nucleus accumbens based on ratios of DOPAC/Dopamine, HVA/Dopamine, and DOPAC+HVA/Dopamine indicating that dopamine turnover is still upregulated 4h after treatment. Combined dopamine turnover confirms that there is a greater release and metabolism of dopamine in the nucleus accumbens, therefore producing an increase in the amount of DOPAC and HVA produced. (*) Indicates significant differences from the vehicle treated ($p<0.05$).

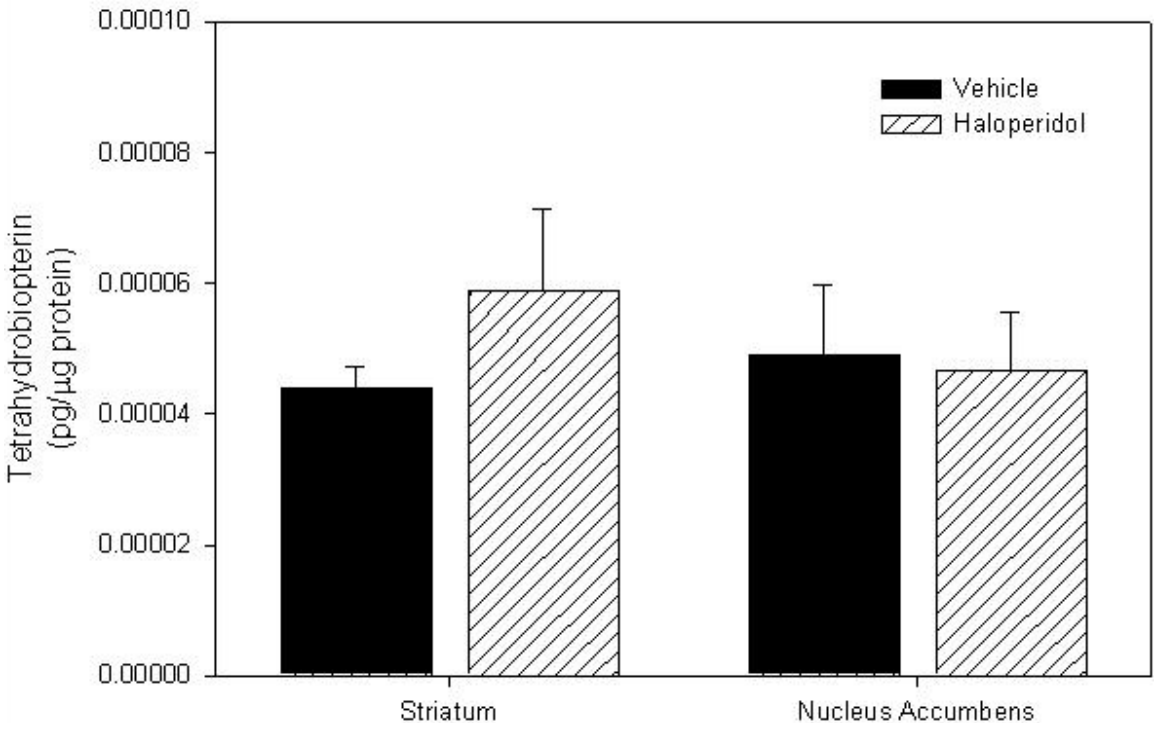

Figure 3.12 Tetrahydrabiopterin levels in the striatum and nucleus accumbens 4h after a 1mg/kg systemic injection of haloperidol. Tetrahydrobiopterinm levels were not significantly different in vehicle tereated mice in the striatum or nucleus accumbens indicating that the need for increased dopamine production has ceased in both the nigrostriatal and mesolimbic pathway. (*) Indicates significant differences from the vehicle treated ($p<0.05$).

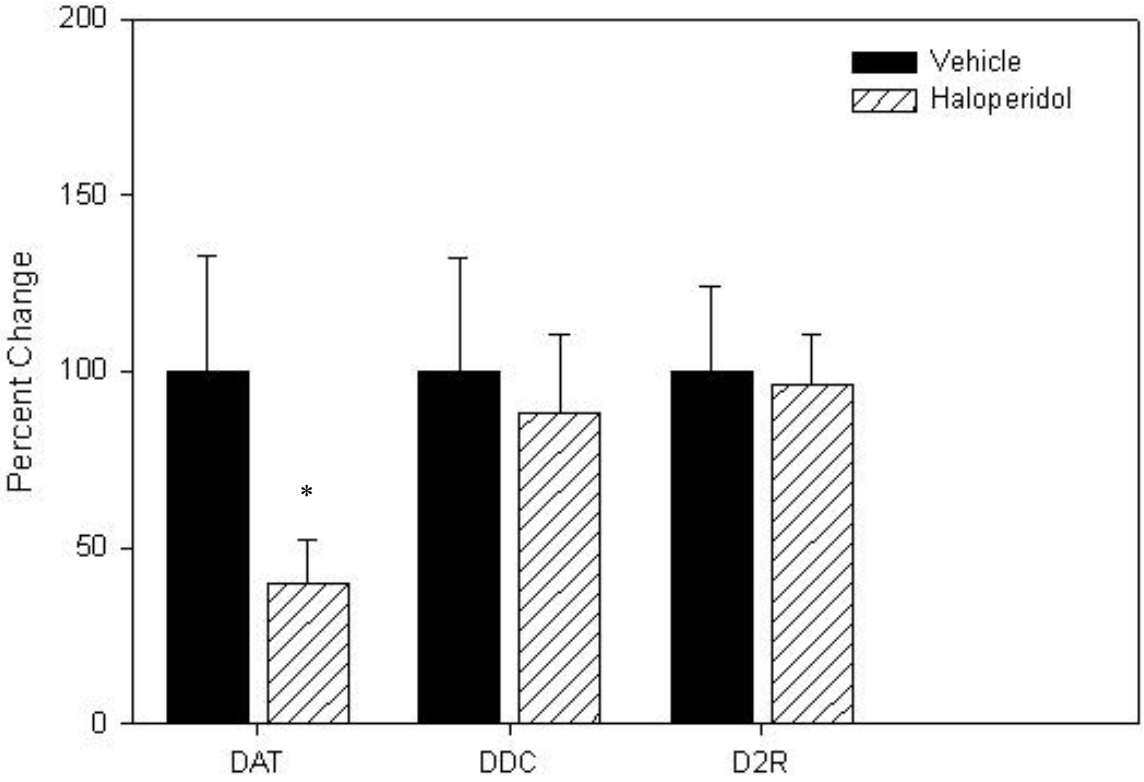

Figure 3.13 Dopamine neuron gene expression in the substantia nigra pars compacta after 4h after a 1mg/kg systemic injection of haloperidol. Significantly reduced expression of dopamine transporter (DAT) was found to be present after haloperidol treatment. Dopamine decarboxylase (DDC), and dopamine D_2 receptor were not shown to be significantly altered. Also tyrosine hydroxylase and VMAT levels were found to be unmeasuarable, which is not reflected in the graph shown. (*) Indicates significant differences from the vehicle treated ($p<0.05$).

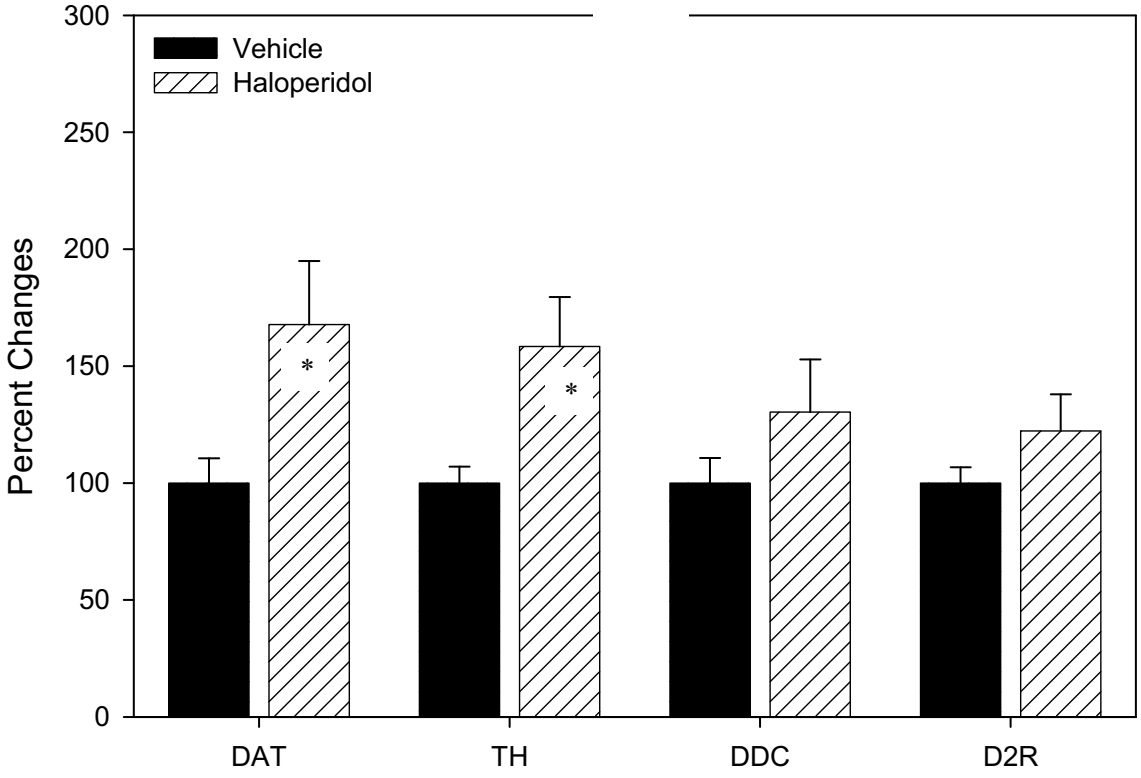

Figure 3.14 Dopamine neuron gene expression in the ventral tegmental area 4h after a 1mg/kg systemic injection of haloperidol. Tyrosine hydroxylase (TH) and dopamine transporter (DAT) shown to be significantly elevated after haloperidol treatment. Dopamine decarboxylase (DDC) and dopamine D_2 receptor were not shown to be significantly altered. (*) Indicates significant differences from the vehicle treated ($p<0.05$).

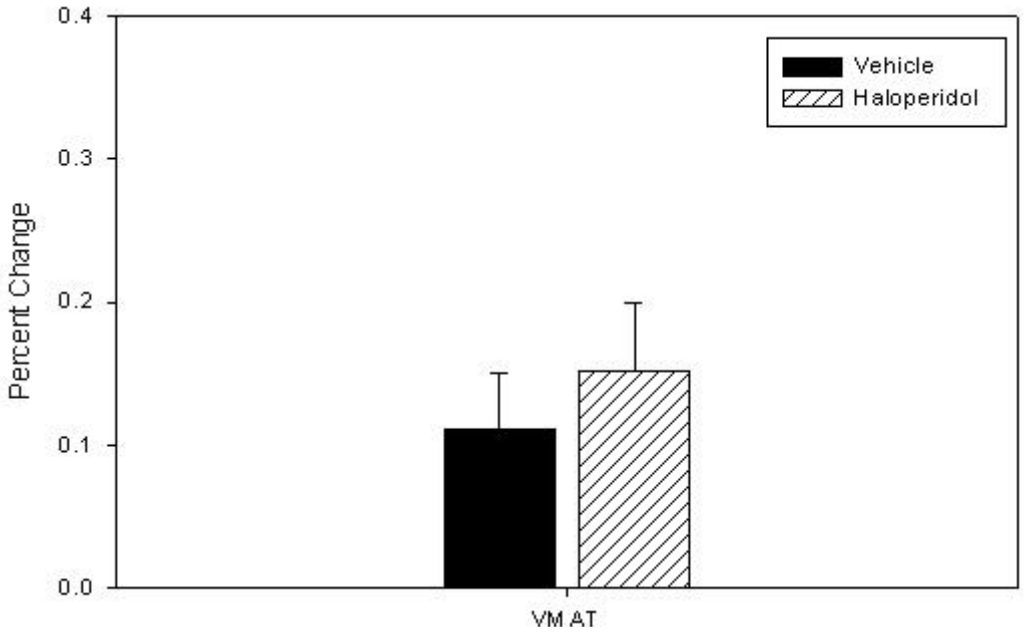

Figure 3.15 Dopamine neuron gene expression in the ventral tegmental area after a 1 mg/kg systemic injection is allowed incubate for 4hrs This graph shows the nonsignificant increase of VMAT in the ventral tegmental area. (*) Indicates significant differences from the vehicle treated ($p<0.05$).

3.3 Nur77 Receptor Subfamily Expression levels after D_2R Agonist/Antagonist Challenge

Nur77 receptor subfamily levels were measured after challenge with quinpirole, sulpiride, or γ-butyrolacotne in order to try and elucidate the mechanisms of gene expression of the Nur receptor subfamily. Due to tainted RNA quality exact measurements of gene expression levels were compromised. Also, there were limited amounts of control samples available for significance comparisons among treatment groups. In order to overcome these issues samples were analyzed across plates and normalized to each other. After analysis we found that quinpirole caused a significant reduction of Nurr1 compared to control samples. Sulpiride and γ-butyrolactone did not cause any significant increases or decreases in expression of any one of the three Nur receptor subfamily genes.

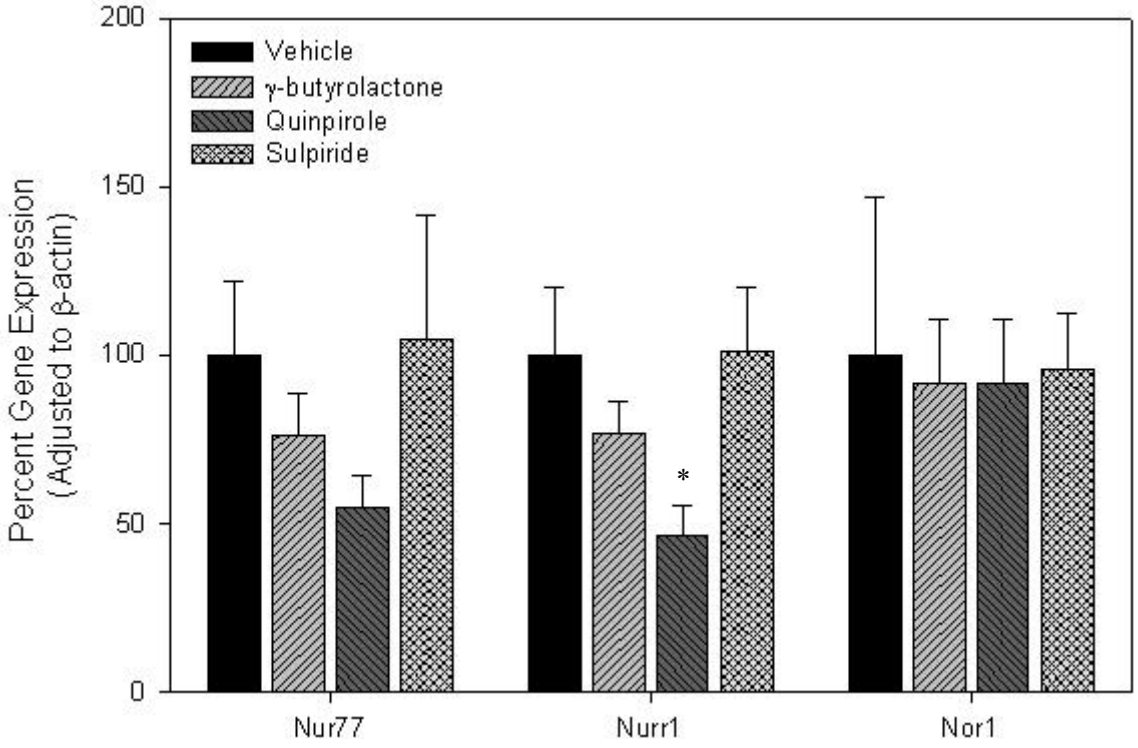

Figure 3.16 The effects of quinpirole, γ-butyrolactone, and sulpiride on Nur receptor subfamily expression. Quinpirole and γ-butyrolactone both show a trend to cause a relative decreased level of expression of Nur77 and Nor-1 when compared to control samples. Only quinpirole was found to produce a significant change in Nur receptor expression levels. It was shown that Nurr1 expression is significantly decreased in mice that received quinpirole treatment versus those that received vehicle treatments. This decrease in Nurr1 expression level could be due to stimulation of the D2 receptor by quinpirole, which acts as an agonist. (*) Indicates significant differences from the vehicle treated ($p<0.05$).

3.4 Neurochemical Effects of Dopamine Agonist and Antagonist Treatment in the Striatum and Nucleus Accumbens

To access the neurochemical changes caused by dopamine altering activation of the D_2 autoreceptor, γ-butyrolactone (GBL), sulpiride, quinpirole, and saline was used to challenge 32 mice. In the striatum it was found that dopamine was significantly increased in mice treated with GBL and quinpirole compared to mice administered the

vehicle treatment (Figure 3.17A). Sulpiride treated mice were shown to have the same levels of dopamine in the striatum as mice treated with the vehicle (Figure 3.17A). Dopac levels were significantly higher in the striatum after GBL and quinpirole treated samples compared to vehicle treatments. Sulpiride did not alter DOPAC or HVA levels in the striatum. Interestingly, HVA levels were significantly lower in the striatum of quinpirole treated mice compared to vehicle treated animals (Figure 3.18). GBL, however had no effect on HVA levels. Dopamine turnover was significantly decreased by both GBL and quinpirole. Sulpiride had no significant effect on dopamine turnover. In the nucleus accumbens, tissue dopamine levels of DOPAC were significantly higher in GBL treated animals compared to vehicle treatments (Figure 3.19). GBL had no effect on HVA levels in the nucleus accumbens. Dopamine and DOPAC levels were also significantly elevated in the nucleus accumbens in quinpirole treated mice compared to vehicle treated mice (Figure 3.19). Sulpiride had no effect on dopamine and HVA levels but did elevate DOPAC levels when compared to vehicle treatements. Dopamine turnover analysis showed indicated that GBL and quipirole significantly reduced dopamine turnover while sulpiride sulpiride increased dopamine turnover in the nucleus accumbens (Figure 3.20).

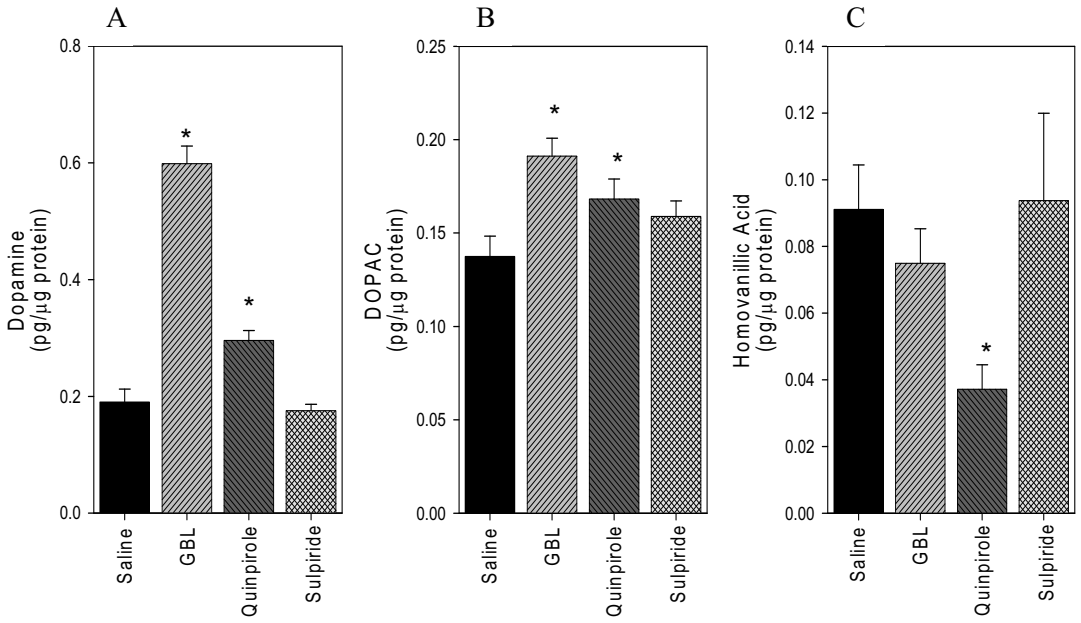

Figure 3.17 Dopamine (A), DOPAC (B), and HVA (C) levels in the striatum 2h after γ-butyrolactone (GBL), quinpirole, or sulpiride treatments. GBL and quinpirole treated groups have a significantly higher amount of tissue dopamine compared to control groups due to the reduced release of dopamine. Sulpiride administered mice did not show any significant changes related to dopamine when compared to mice that administered the control treatment. Interestingly, GBL and quinpirole treated mice produced significant increases in the amount of DOPAC produced from metabolized dopamine when compared to control groups. Sulpiride administered mice did not show any significant changes in DOPAC when compared to mice administered the control treatment. Mice treated with quinpirole have significant decreases in the amount of HVA when compared to control groups. GBL and Sulpiride administered mice did not show any significant changes of HVA levels when compared to mice administered the control treatment. (*) Indicates significant differences from the vehicle treated ($p<0.05$).

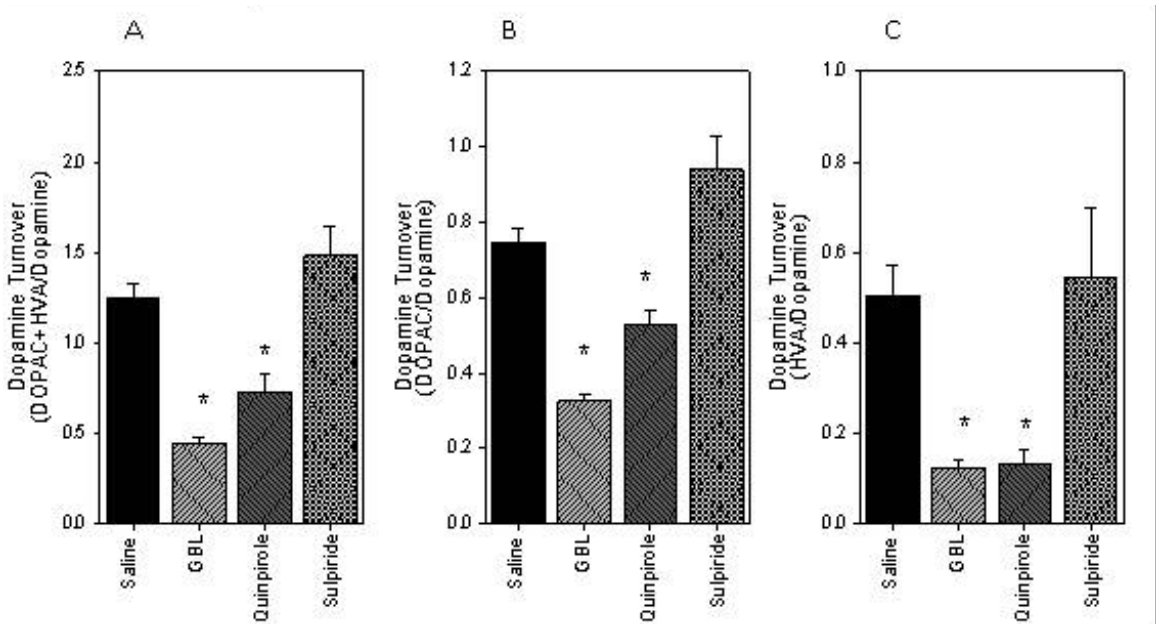

Figure 3.18 Combined dopamine turnover (A), DOPAC/Dopamine (B), and HVA/Dopamine (C) in the striatum 2h after γ-butyrolactone (GBL), quinpirole, or sulpiride treatment. GBL and quinpirole treated groups showed a significant decrease in the amount of dopamine turnover as it relates to combined (HVA +DOPAC) metabolite production and individual production of DOPAC and HVA when compared to vehicle treated groups. Sulpiride administered mice showed a significant increase in the amount of combined dopamine turnover and individual dopamine turnover as it relates to DOPAC when compared to mice administered the control treatment indicating that there is a increased amount of dopamine release and transmission in the nigrostriatal pathway resulting in increased metabolism. (*) Indicates significant differences from the vehicle treated ($p<0.05$).

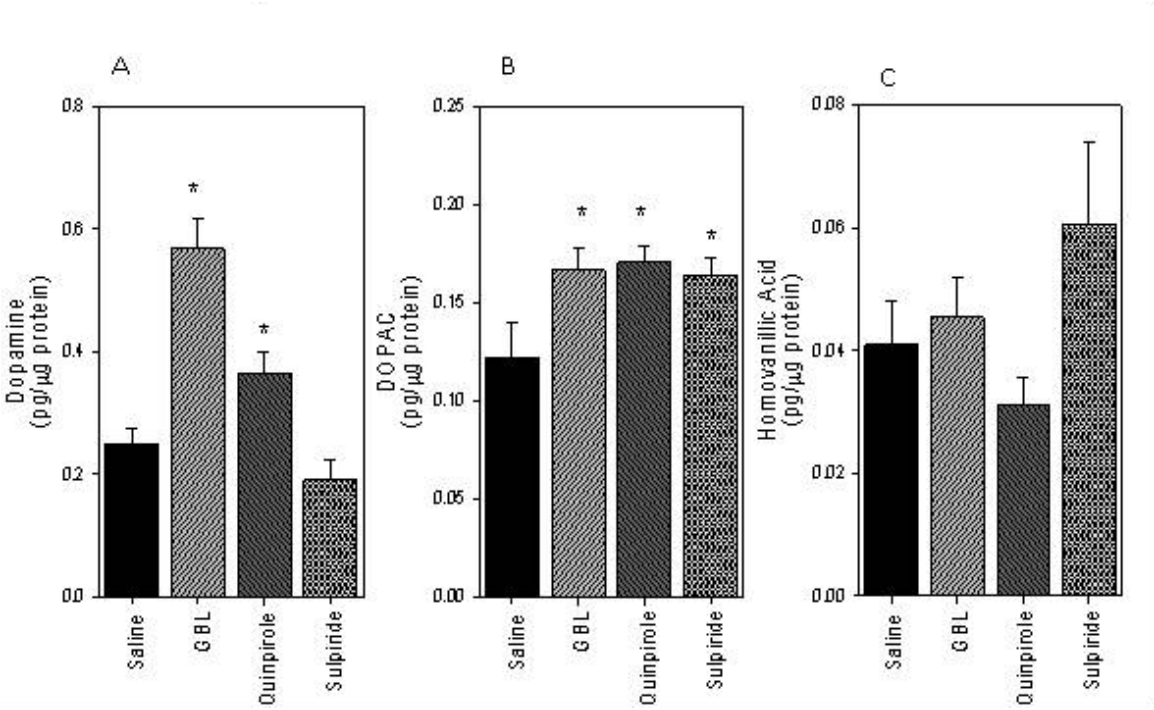

Figure 3.19 Dopamine (A), DOPAC (B), and HVA (C) levels in the nucleus accumbens 2h after γ-butyrolactone (GBL), quinpirole, or sulpiride treatments. GBL and quinpirole treated groups have a significantly higher amount of tissue dopamine compared to vehicle treated groups due to the blocked and reduced release of dopamine. Sulpiride administered mice did not show any significant changes related to dopamine when compared to mice that administered the control treatment only a trend to have less measurable tissue levels of dopamine. Interestingly, GBL, quinpirole, and sulpiride treated groups showed significant increases in the amount of DOPAC produced from metabolized dopamine when compared to vehicle treatments, indicating that dopamine levels have risen due to some stimulus. Treated mice showed no significant increases or decreases in the amount of HVA produced from metabolized dopamine when compared to vehicle treatments. (*) Indicates significant differences from the vehicle treated ($p<0.05$).

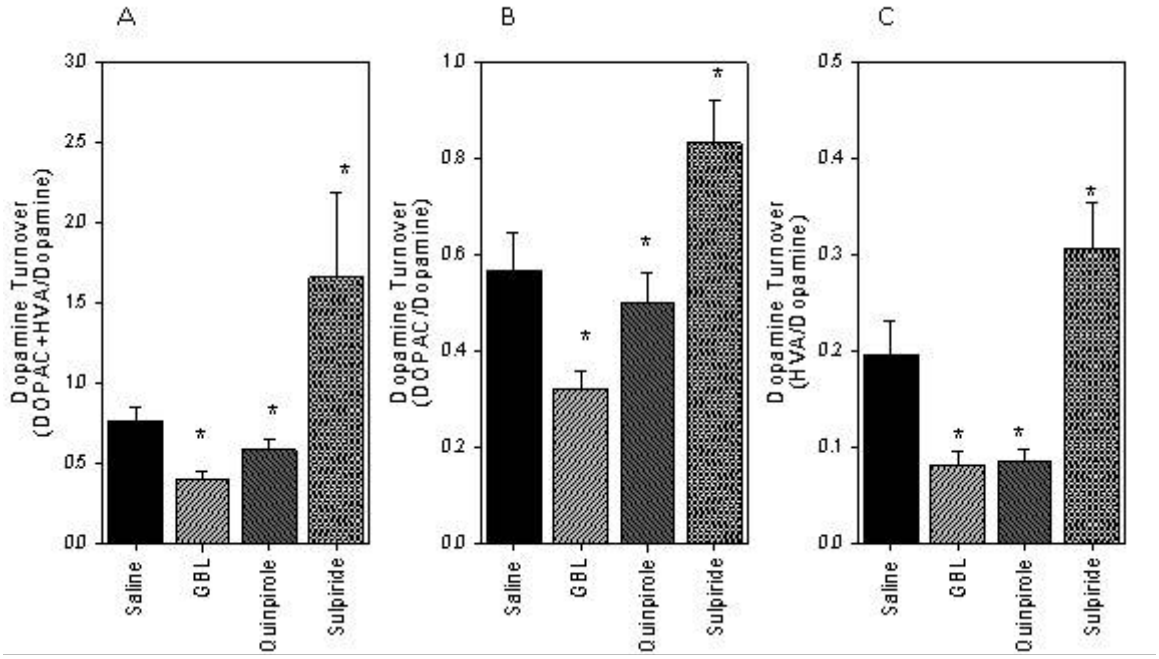

Figure 3.20 Combined dopamine turnover (A), DOPAC/Dopamine (B), and HVA/Dopamine (C) in the nucleus accumbens 2h after γ-butyrolactone (GBL), quinpirole, or sulpiride treatment. GBL and quinpirole treated groups have a significant decrease in the amount of dopamine turnover as it relates to combined (HVA +DOPAC) metabolite production and individual production of DOPAC and HVA when compared to vehicle treatments. Sulpiride administered mice show a significant increase in the amount of combined dopamine turnover and individual dopamine turnover as it relates to DOPAC and HVA when compared to mice administered the vehicle treatment indicating that there is a greater release of dopamine in the mesolimbic pathway resulting in increased metabolism. (*) Indicates significant differences from the vehicle treated ($p<0.05$).

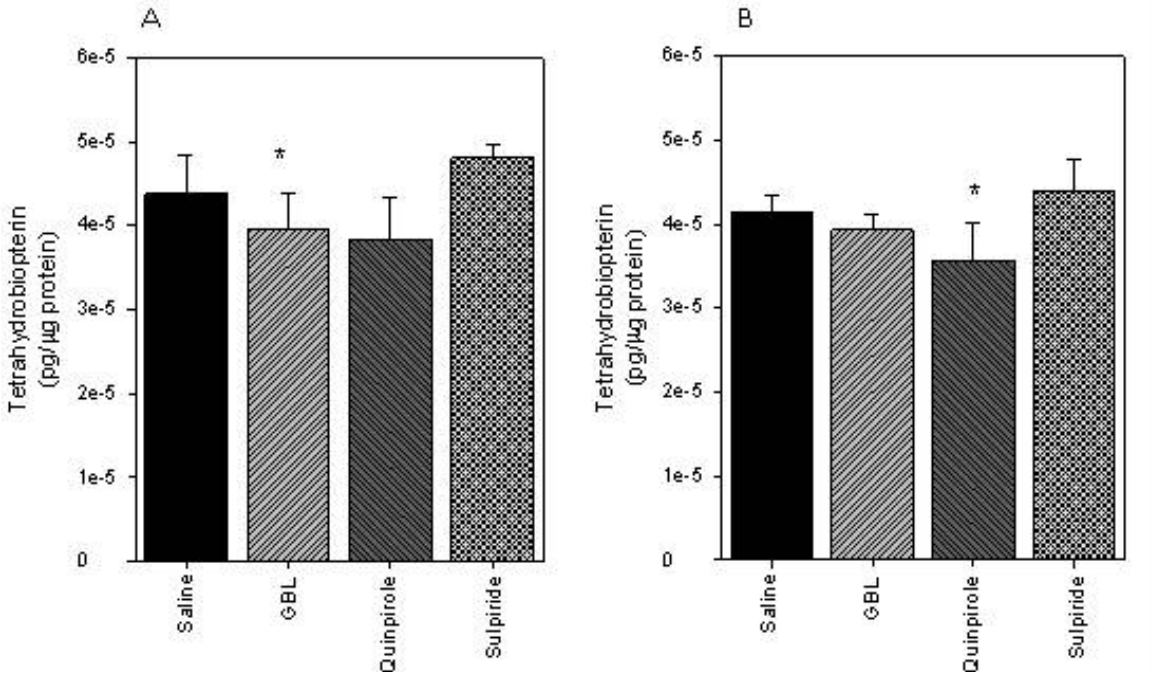

Figure 3.21 Tetrahydrabiopterin levels in the nucleus accumbens and striatum. Tissue punches from the both regions were prepared for HPLC analysis then quantified using waters 2465 electrochemical detector. Significant lower levels of biopterin were shown to be present in striatal punches of GBL samples, indicating that the blocked release of dopamine has not produced an extra need for the cofactor. Quinpirole and sulpiride samples did not show any significant effects when compared to control group animals. However in the nucleus accumbens significant lower levels of biopterin were shown to be present in nucleus accumbens punches of quinpirole samples, indicating that the reduced release of dopamine has not produced an extra need for the cofactor. Sulpiride and GBL samples did not show any significant effects when compared to control group animals. (*) Indicates significant differences from the vehicle treated ($p<0.05$).

3.5 Effects of altered Nurr1 expression on Nur77 and Nor-1 receptor expression levels

It is known that Nurr1 plays a major role in regulation of dopamine neuron production and survival. Whether or not Nur77 and Nor-1 can compensate for Nurr1

malfunction remains an unanswered question. Since Nur77 and Nor1 possess some of the same similar DNA binding domains and transactivation properties it is thought that they may be able to elevate their expression levels in times when Nurr1 levels drop. To investigate this theory we performed experiments using Nurr1 +/+ and +/- mice in order to determine if there were any significant change of expression levels in Nur77 and Nor1. We found that there was no change in the expression levels of Nur77 and Nor1 in either the substantia nigra in Nurr1 +/+ and +/- mice (Figure 3.22). This indicates that even though Nurr1 +/- mice have an incomplete copy of the Nurr1 gene, Nur77 and Nor1 expression levels remain at basal conditions.

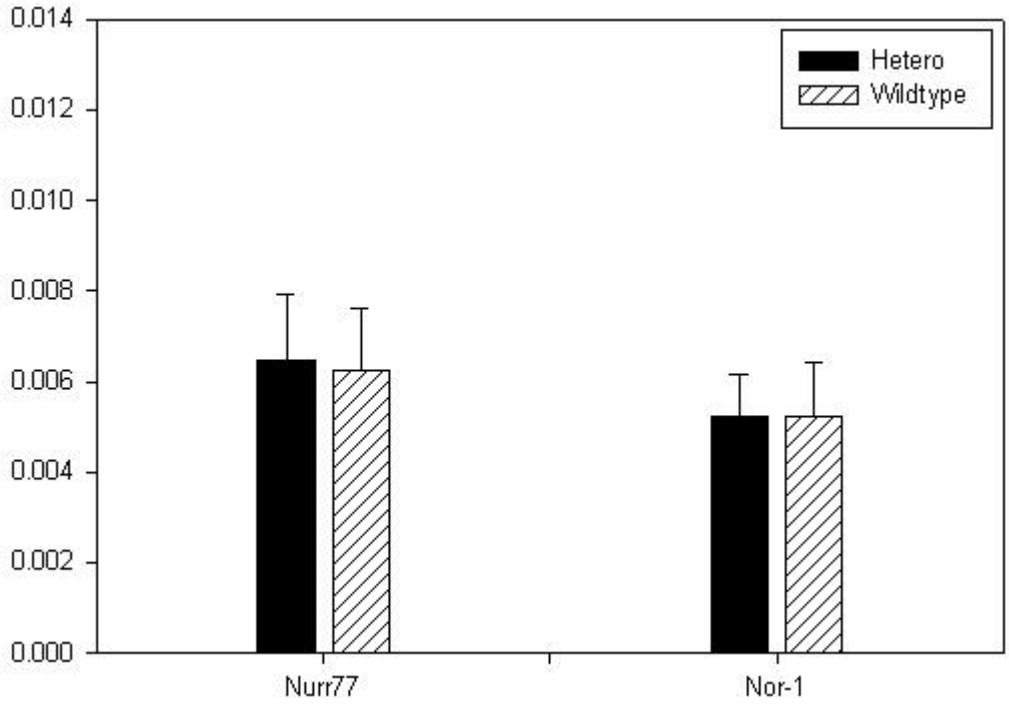

Figure 3.22 Expression levels of Nur77 and Nor1 from the substantia nigra Nurr1 +/+ and +/- mice. Quantitative real time PCR was used in order to quantify expression levels of each gene, which were normalized to internal standard control β-actin. There were no significant differences found in expression of either due to the Nurr1 genotype. (*) Indicates significant differences from the vehicle treated ($p<0.05$).

CHAPTER IV

DISCUSSION AND CONCLUSIONS

4.1 Discussion

Nurr1 is fundamental to the proper development and sustainability of dopamine neurons in the midbrain regions of the substantia nigra and the ventral tegmental area. Therefore, its function and regulation could be important in the pathology of certain neurological disorders such as Parkinson's, schizophrenia, attention deficit disorder, and other diseases which are related to dopamine neuron function and transmission. Nor-1 and Nur77 are both nuclear transcription factors related to Nurr1 but are concentrated within the dopaminoceptive regions (striatum and nucleus accumbens). As all three share structural and functional domains and DNA binding properties (Zetterstrom et al. 1996b; Beaudry et al. 2000; Milbrandt et al. 1988; Law et al. 1992; Ohkura et al. 1994, Wilson et al. 1991; Paulsen et al. 1995; Zetterstrom et al. 1996a) changes in the expression of these genes in dopamine neurons must be considered as regulating similar target genes.

Although the role of these proteins is as transcription factors, little is known about the target genes they regulate. Because Nurr1 is essential for proper differentiation and function of dopamine neurons, it is important to understand the genes and dopamine neuron functions regulated by the Nur-receptors. Nurr1 has been directly related to TH, DAT, VMAT, GTPCH, and DDC which are all specific markers necessary for dopamine

synthesis and regulation (Zetterstrom et al. 1996a, Saucedo-Cardenas et al. 1998, Castillo et al. 1998, Eells et al, 2006, Gil et al., 2007). Many of these relationships, however, are either indirect, based on *in vitro* transfection assays or cell-lines in culture.

In addition to being transcription factors, the Nur-receptors are also considered immediate early genes, meaning that there promoter regions have response elements through which expression can be increased without the need for protein synthesis (Eells et al. 2000). The most notable immediate early gene is c-fos whose activation is dependent on early response element CArG (Rivera et al. 1990). c-Fos has been shown to be directly related to neuronal firing of action potentials. Previous research has shown that Nur77, Nor-1, and c-Fos mRNA is increased in the nucleus accumbens and caudate putamen after acute haloperidol administration (Werme et al. 2000). Also, Maheux et al. (2004) reports that acute administration of antipsychotic drugs, at doses likely to produce extrapyramidal symptoms drastically increases c-Fos mRNA levels in the striatum. Another member of the Fos family, Fos-B, has been shown to have possible regulatory control of TH expression in olfactory dopaminergic neurons via AP-1 and CRE motifs, both of which has been shown to be present in the promoter regions of each member of the Nur receptor subfamily (Liu et al. 1998 and Eells et al. 2000). Currently, there is limited data on the parameters that regulate the expression of the Nur-receptors in dopamine neurons in vivo.

4.1.1 Gene expression after Haloperidol

The current studies examined the expression of the Nur-receptors one hour and 4 hours after haloperidol treatment. Additionally, the expression of potential target genes was also examined. Our findings indicated that there was an elevated expression of

Nur77 and Nor1 in the substantia nigra pars compacta and ventral tegmental area 1h after a systemic injection of haloperidol was allowed to incubate for 1 hour. No significant difference in Nurr1 expression in either the substantia nigra pars compacta of the ventral tegmental area was found in the current study. Previous research has indicated similar findings (increases in Nur77 and Nor1) along with increases in Nurr1 in both regions of the midbrain (Maheux et al. 2004). One possible explanation for differences found among nurr1 expression could be the method used. In the current study, real time PCR was used to detect changes among gene expression in the midbrain which provided us with a measurement of expression from RNA isolated from the dissection of either the substantia nigra or ventral tegmental area. Maheux et al. used in situ hybridization which has better anatomical resolution and gives expression levels for individual neurons. In situ hybridization, however, samples are not normalized to a housekeeping gene the way quantitative PCR is. Additionally, at basal levels, Nur77 and Nor-1 expression is barely detectable; therefore the percent increase will be greater than that of Nurr1, which may help explain why there was no significant increase found for Nurr1 in our analysis. Additionally, Maheux et al., used rats and the current study used mice so that there could be subtle species differences.

Since Nur77 and Nor-1 possess the ability to bind to the same promoter regions as Nurr1, any increases in the receptor molecules should affect the regulation of many of the same genes that are controlled by Nurr1. To test this hypothesis, samples from the 4 hour haloperidol experiment were analyzed to provide data that would show how much if any up-regulation of dopamine neuron genes was occurring. Previous research has demonstrated that TH gene expression can be directly regulated by Nurr1 (Sakudurada et

al. 1999; Cazorla et al. 2000). Nurr1 has been shown to transactivate the promoter activity of the TH gene in different cell line indicating that the TH promoter region contains known sequences of NBRE, which is the DNA binding domain of Nurr1 (Kim et al. 2003). Additional research has indicated that TH and Nurr1 mRNA is elevated in the substantia nigra pars compacta along with Nurr1 mRNA levels being elevated in the ventral tegmental area also after systemic haloperidol treatment in Nur77 -/- mice (Gilbert et al. 2006). This previous research suggests that with a lack of Nur77, Nurr1 expression is upregulated in order to compensate for the loss of Nur77. It also shows that haloperidol is able to induce a need for higher amounts of dopamine during periods of Nur77 dysfunction. However, other research reports no change in TH mRNA after chronic administration of haloperidol, which was probably due to tolerance formed by dopamine D2 receptors from constant stimulation by haloperidol (Cho et al. 1999). Also TH mRNA expression is significantly reduced in vivo in Nurr1 +/- mice with a corresponding decrease in TH activity in the striatum (Eells et al. 2006). Based on the current data, Nur77 and Nor1 could be factors that regulate TH expression. TH expression however, may be regulated differently by the Nur-receptors between nigrostriatal dopamine neurons and mesolimbic dopamine neurons.

The role for the Nur- receptors in DAT regulation is not as straightforward. No differences in DAT expression was found in either the substantia nigra pars compacta or ventral tegmental area of the Nurr1 +/- mice. This, however, does not rule out the possibility that transient induction of the Nur-receptors could induce DAT expression. Bannon et al suggests a role for Nurr1 in DAT regulation of cocaine users who have Nurr1 deficiency's due to chronic use of the drug. Their research found that cocaine

abusers who had deficiencies in Nurr1 also had reduced DAT expression (Bannon et al. 2001). The transcriptional activity of hDAT has been shown to be enhanced by Nurr1 (Sacchetti et al. 1999). Previous research also directly demonstrated that Nur77 and Nurr1 increases expression of the hDAT gene in SN4741 dopamine cell lines although Nurr1 effects are greater than that of Nur77 (Sacchetti et al. 2000). It remains to be determined if Nor1 could share similar properties with Nurr1 involving the regulation of DAT.

The previous research mentioned has shown that TH and DAT both could be regulated by Nurr1 indicating that these genes possess NBRE response elements that can be bound and activated by Nurr1 and quite possibly other members of the Nur receptor subfamily. Interpretation of these results indicates that regulation of dopamine neurotransmission can be inhibited and enhanced at various steps, starting at dopamine neuron maturation, synthesis of dopamine, and finally transmission of the neurotransmitter at its destination.

By comparing gene expression with neurochemical data, our findings indicate that elevated Nur77 and Nor-1 expression corresponds to an increase in dopamine release, turnover and, based on previous reports, dopamine synthesis. The neurochemical effects from 1 hour of systemic haloperidol administration were decreased levels of dopamine in both the striatum and nucleus accumbens. A 4 h systemic haloperidol administration resulted in decreased dopamine levels in the striatum but an increase in dopamine levels in the nucleus accumbens. Our findings suggest that the longer-term response to haloperidol is different between the nigrostriatal and mesolimbic dopamine systems. One possible explanation for this difference could be the function of the D_2R

autoreceptor in various dopamine neurons. Recently Lammel et al. (2008) found differential expression and function of the D_2R autoreceptor between mesolimbic/cortical and nigrostriatal dopamine neurons. Although other differences have also been described, this may explain differential effects of haloperidol between the nigrostriatal and mesocortico limbic systems.

(BH_4) was measured in both experiments of 1-hour and 4-hours after systemic haloperidol administration. Our findings indicate that BH_4 levels are significantly elevated in the nucleus accumbens of haloperidol treated mice when compared to vehicle treated mice 1h after injection. Haloperidol had no effect on BH_4 in the striatum at 1 h or the striatum or nucleus accumbens at 4 hours. This differential regional effect on BH_4 levels after haloperidol may help explain the elevated dopamine levels in the nucleus accumbens 4 h after haloperidol injection. BH_4 is a necessary cofactor for TH for the conversion of tyrosine to DOPA and is normally found at limiting concentrations such that an increase or decrease in BH_4 can either increase or decrease dopamine synthesis, respectively. If the response of synapses in the nucleus accumbens is a rapid (1h) increase in BH_4 after haloperidol, this could cause a further increase in dopamine synthesis resulting in greater dopamine levels 4 h later. In contrast, within the striatum, there was no change in BH_4 levels as a result of haloperidol treatment and a reduction in dopamine levels 4 h after haloperidol. Fluctuations in BH_4 levels in the mesoaccumbens pathway, could, therefore represent an important mechanism to regulate dopamine synthesis in response to acute antipsychotic treatments. This is the only data available, to our knowledge, linking antipsychotics with alterations in BH_4 levels.

4.1.2 The effects of GBL, dopamine agonists and antagonists

Although antagonism of the D2 receptor can elevate expression of Nurr1, Nur77, and Nor1 in dopamine neurons *in vivo*, it is still unclear the precise mechanism for this. To investigate the activation properties of these genes we used a combination of D2 receptor agonist and antagonist treatments to determine the different effects each would have on Nur-receptor expression in the dopaminergic system along with the neurochemical effects. Quinpirole, a D2 receptor agonist, should stimulate presynaptic autoreceptors therefore decreasing the rate of dopamine synthesis, release, and subsequent dopamine turnover. Sulpiride a strong D2 receptor antagonist, should have the same antagonistic effects as haloperidol. It was expected that due to blockage of autoreceptor function, dopamine synthesis should be elevated along with increased release followed by elevated dopamine turnover. GBL acts essentially as a dopamine autoreceptor antagonist but does this by blocking dopamine release. Although both GBL and sulpride inhibit the stimulation of the autorecpetor, they have opposite effects on dopamine release.

Our findings indicate that there was a significant decrease in Nurr1 expression in quinpirole treated mice when compared to vehicle treated mice. This decease could be due to presynaptic D2 receptor stimulation which would inhibit any intracellular signaling for increased dopamine synthesis, consequently down regulating genes necessary for dopamine synthesis or could be the result of accumulation of dopamine or alterations in electrical activity in dopamine neurons due to stimulating the D2 receptor. No effect on Nurr77 or Nor1 was observed. As stated earlier, Nur77 and Nor1 expression is already barely detectable in the midbrain at basal conditions. Treatment with

quinpirole is expected to decrease any expression that is present, making it that more difficult to detect expression signals by the methods that we have chosen. The effects of quinpirole on dopamine neurochemistry included higher levels of dopamine in the striatum and nucleus accumbens due to reduced release of dopamine from presynaptic neurons, i.e. reduced dopamine turnover. This results in a significant increase of intracellular dopamine similar to what was previously reported for the striatum and nucleus accumbens of Nurr1 +/+ and +/- mice after quinpirole treatment (Eells et al., 2006)

In this experiment, sulpiride failed to elevate expression of any of the Nur-receptor genes as haloperidol did in the previous experiment. The explanation may be differences in receptor affinity and/or dose used. Since haloperidol has a higher affinity for the D2 receptor, it may have a greater effect on Nur-receptor expression as well. Based on neurochemical data, haloperidol had significant impact on dopamine neurochemistry, increasing dopamine release as indicated by reduced tissue dopamine and increased metabolites. Sulpiride, however, had no significant effect on dopamine neurochemistry. It seems that sulpiride is not a strong enough antagonist of the D2 receptor or at a high enough dose to cause the excess stimulated release of dopamine as haloperidol. These data suggest that a certain level of impact on D2 receptors and subsequent dopamine neurochemistry is necessary to induce expression of the Nur-receptor genes.

As expected, GBL treatment significantly elevated DOPAC, as would be expected as DOPAC is the primary intracellular metabolite of dopamine, and elevated dopamine levels. Overall this significantly reduced dopamine turnover similarly in the striatum and

nucleus accumbens. No Nur-receptor expression differences were found after GBL treatment although these significant neurochemical changes were observed. Therefore, if antagonism of the D2 receptor alone is the direct mechanism for induction of Nur-receptor genes, then GBL should have caused elevated Nur-receptor subfamily expression similar to haloperidol. Because it did not suggests that other mechanisms such as intracellular dopamine levels and/or electrical activity of dopamine neurons may also contribute to regulation of Nur receptor gene expression.

In addition to determining levels of dopamine and metabolites, BH_4 levels were also measured after these treatments to give an indication of a key component of dopamine synthesis. Our data show that in the striatum GBL treated mice contained significantly decreased levels of BH_4 compared to vehicle samples. However, in the nucleus accumbens only quipirole treated mice were shown to have significantly lower amounts of BH_4, which is opposite of what we expected to happen since dopamine synthesis should be decreased when a D2 receptor agonist is administered. However, again there is significant activity present in the mesolimbic pathway as opposed to the nigrostriatal pathway.

4.1.3 Relationship between Nurr1 expression and Nur77 and Nor1

Complete abolishment of Nurr1 (Nurr1-/-) inhibits terminal dopaminergic neuron differentiation and results in the absence of midbrain dopamine (Eells 2003). Since Nurr77 and NOR-1 share similar DNA binding domain regions as Nurr1, it is often questioned whether Nurr77 and NOR-1 can compensate for an incomplete Nurr1 gene. Experiments with Nurr1 +/+ and Nurr1 +/- mice were done in order to access whether or not Nur77 and Nor-1 expression is altered due to a chronic reduction in Nurr1 caused by

the Nurr1 +/- genotype. We found no significant differences in Nur77 and NOR-1 expression between Nurr1 +/- and Nurr1 +/+ mice in either the substantia nigra or the ventral tegmental area. This indicates that Nur77 and NOR-1 expression remains unchanged even after one copy of Nurr1 has been removed. Therefore, although the Nur-receptors have similar DNA binding properties and functions, there does not appear to be regulation of Nurr1 at the promoter regions of Nur77 or Nor-1. Similarly, since the Nurr1 +/- genotype also alters a number of parameters of dopamine neuron function (Eells et al., 2006 and Moore et al., 2008) these changes are also insufficient to affect Nur77, Nor-1 or Nurr1 expression.

4.2 Conclusion

The Nur-receptors can be altered in dopamine neurons in vivo as the result of D2 receptor antagonism and stimulation. Whether this is directly related to actions at the receptor or the neurochemical and/or electrophysiological consequences of modifying the activity of the receptors remains to be determined. The current data suggests that the neurochemical changes may be involved as GBL, which reduced dopamine release and therefore autoreceptor activation, did not alter Nur receptor expression when direct antagonism with of the D2R elevated Nur77 and Nor1 expression. The longer term consequences of an increase in Nur receptor expression could include regulation of dopamine neurotransmission genes as TH and DAT were elevated in a manner consistent with previous elevation in Nur77 and Nor1. By studying Nur77 and Nor-1 expression in Nurr1 +/- mice we found no evidence of cross regulation of Nurr1 effecting Nur77 and Nor-1 expression. Further study on the direct mechanism responsible for inducing Nur-

receptor expression and the consequences on gene expression and dopamine neuron function continue.

REFERENCES

Bannon MJ, Michelhaugh SK, Wang J, Sacchetti P. "The human dopamine transporter gene: gene organization, transcriptional regulation, and potential involvement in neuropsychiatric disorders." Eur Neuropsychopharmacol, 11, 2001, pp 449-55.

Beaudry G, Langlois M-C, Wepe I, Rouillard C, and Levesque D. "Contrasting patterns and cellular specificity of transcriptional regulation of the nuclear receptor nerve growth factor-inducible B by haloperidol and clozapine in the rat forebrain." J. Neurochem, 75, 2000, pp 1694.

Castillo SO, Xiao Q, Lyu MS, Kozak CA, and Nikodem VM. "Organization, sequence, chromosomal localization, and promoter identification of the mouse orphan nuclear receptor Nurr1 gene." Genomics, 41, 1997, pp 250.

Castillo, SO, Baffi, JS, Palkovits, M, Goldstein, DS, Kopin, IJ, Witta, J, Magnuson, MA, and Nikodem, VM. "Dopamine biosynthesis is selectively abolished in substantia nigra/ventral tegmental area but not in hypothalamic neurons in mice with targeted disruption of the Nurr1 gene." Mol. Cell. Neurosci., 11, 1998, pp 36.

Cazorla P, Smidt MP, O'Malley KL, Burbach JP. "A response element for the homeodomain transcription factor Ptx3 in the tyrosine hydroxylase gene promoter." J Neurochem.,74, 2000, pp 1829-37.

Cho S, Duchemin A.M, Neff N, Hadjiconstantinou M. "Tyrosine hydroxylase, aromatic L-amino acid decarboxylase and dopamine metabolism after chronic treatment with dopaminergic drugs." Brain Res., 830, 1999, pp 237-245.

Cooper J, Bloom F, and Roth R." The Biochemical Basis of Neuroparm." 2003.

Eells J.B. "The control of dopamine neuron development, function, and survival: Insights from transgenic mice and the relevance to human disease." Current Med Chem., 10, 2003, pp 857-870.

Eells, JB, Witta, J, Otridge, JB, Zuffova, E, and Nikodem, VM. “Structure and Function of the Nur77 Receptor Subfamily, a Unique Class of Hormone Nuclear Receptors.” Curr. Genom, 1, 2000, pp 135.

Eells JB, Yeung SK, and Nikodem VM. “Dopamine neurons heterozygous for the Nurr1-Null allele have reduced survival in vitro.” Neurosci. Res. Comm., 30, 2002, pp 173-178.

Eells JB, Misler JA, and Nikodem VM. “Reduced tyrosine hydorxylase and GTP cyclohydorlase mRNA expression, tyrosine hydorxylase activity, and neurochemical alterations in Nurr1-null heterozygous mice.” J Brain Research Bulletin, 70, 2006, pp 186-195.

Freedman L.P. and Luisi B.F. “On the mechanism of DNA binding by nuclear receptors: a structural and functional perspective.” J. Cell. Biochem., 51, 1993, pp 140.

Gil M, McKinney C, Lee M, Eells J, Phyillaier M, and Nikodem V. “Regulation of GTP cyclohydrolase 1 expression by orphan receptor Nurr1 in cell culture and in vivo.” J Neurochem, 101, 2007, pp 142-150.

Gilbert F, Morissette M, St-Hilaire M, Paquer B, Rouillard C, Paolo TD, and Levesque D. “Nur77 gene knockout alters dopamine neuron biochemical activity and dopamine turnover.” Biol. Psy., 60, 2006, pp 538-547.

Hazel T.G, Nathans D, and Lau L.F. “A gene inducible by serum growth factors encodes a member of the steroid and thyroid hormone receptor superfamily.” Proc. Natl. Acad. Sci. USA., 85, 1988, pp 8444-8448.

Kim KS, Kim CH, Hwang DY, Seo H, Chung S, Hong SJ, Lim JK, Anderson T, Isacson O. “Orphan nuclear receptor Nurr1 directly transactivates the promoter activity of the tyrosine hydroxylase gene in a cell-specific manner.” J Neurochem., 85, 2003, pp 622-34.

Lammel S, Hetzel A, Häckel O, Jones I, Liss B, Roeper J. “Unique properties of mesoprefrontal neurons within a dual mesocorticolimbic dopamine system.” Neuron., 13, 2008, pp 760-73.

Law, SW, Conneely, OM, DeMayo, FJ, and O’Malley, BW. “Identification of a new brain specific transcription factor, Nurr1.” Mol. Endo., 6, 1992, pp 2129.

Le W, Conneely O.M, Zou L, He Y, Saucedo-Cardenas O, Jankovic J, Mosier D.R, and Appel S.H. “Selective agenesis of mesencephalic dopaminergic neurons in Nurr1-deficient mice.’ Exp. Neurol., 159, 1999, pp 451-458.

Liu N, Cigola E, Tiniti C, Jin B.K, Conti B, Volpe B.T, and Baker H. “Unique regulation of immediate early gene tyrosine hydorxylase expression in the odor-deprived Mouse olfactory bulb.” J. Bio Chem., 274, 1998, pp 3042-3047.

Maheux J, Ethier I, Rouillard C, and Levesque D. “Induction patterns of transcription factors of the Nur Family (Nurr1, Nur77, and Nor1) by typical and atypical antipsychotics in the mouse brain: implication for their mechanism of action.” J. Pharm. Exp. Ther., 313, 2004, pp 460.

Maira M, Martens C, Phillips A, and Drouin J. “Heterodimerization between Members of the Nur Subfamily of Orphan Nuclear Receptors as a Novel Mechanism for Gene Activation. Mol. Cell.” Bio, 19, 1999, pp 7549.

Milbrandt J. “Nerve growth factor induces a gene homologous to the glucorticoid receptor gene.” Neuron., 1, 1988, pp 183-188.

Moore TM, Brown T, Cade M, Eells JB. “Alterations in amphetamine-stimulated dopamine overflow due to the Nurr1-null heterozygous genotype and postweaning isolation.” Synapse., 62, 2008, pp 764-74.

Nsegbe E, Wallén-Mackenzie A, Dauger S, Roux JC, Shvarev Y, Lagercrantz H, Perlmann T, Herlenius E. “Congenital hypoventilation and impaired hypoxic response in Nurr1 mutant mice.” J Physiol. 556, 2004, pp 43-59.

Ohkura N, Hijikuro M, Yamamoto A, and Miki K. “Molecular cloning of a novel thyroid/steroid receptor superfamily gene from cultured rat neuronal cells.’ Biochem. Biophys. Res. Commun., 205, 1994, pp 1959-1965.

Ohkura N, Ito M, Tsukada T, Sasaki K, Yamaguchi K, and Miki K. “Structure mapping and expression of a human NOR-1 gene the third member of the Nur77/NGFI-B family.” Biochim. Biophys. Acta., 1308, 1996, pp 205-214.

Paulsen R.F, Granas K, Johnsen H, Rolseth V, and Sterri S. “Three related brain nuclear receptors, NGFI-B, Nurr1, and NOR-1, as transcriptional activators. J. Mol. Neurosci., 6, 1995, pp 249-255.

Perlman T. and Jansson, L. “A novel pathway for vitamin A signaling mediated by RXR heterodimerization with NGFI-B and Nurr1.” Genes Dev., 9, 1995, pp 769-782.

Philips A, Lesage S, Gingras R, Maira M.H, Gauthier Y, Hugo P, and Drouin J. “Novel dimeric Nur77 signaling mechanism in endocrine and lymphoid cells.” Mol. Cell. Bio, 17, 1997, pp 5946-5941.

Ponnio T, and Conneely O. “Nor1 Regulates Hippocampal axon Guidance, Pyrimidal Cell Survival, and Seizure Susceptibility.” Mol and Cell Bio, 24, pp 2004 9070.

Rivera VM, Sheng M, Greenberg ME. “The inner core of the serum response element mediates both the rapid induction and subsequent repression of c-fos transcription following serum stimulation.” Genes Dev., 4, 1990, pp 255-68.

Ryseck R.P, Macdonald-Bravo H, Mattei M.G, Ruppert S, and Bravo R. “Structural mapping and expression of a growth factor inducible gene encoding a putative nuclear hormonal binding receptor.” EMBO. J., 8, 1989, pp 3327-3335.

Sacchetti P, Brownschidle LA, Granneman JG, and Bannon MJ. “Characterization of the 5’-flanking region of the human dopamine transporter gene.” Mol Brain Res, 74, 1999, pp 167-174.

Sacchetti P, Mitchell T, Granneman J, and Bannon M. “Nurr1 enhances transcription of the human dopamine transporter gene through a novel mechanism.” J Neurochem, 76, 2000, pp 1565-1572.

Sakurada K, Ohshima-Sakurada M, Palmer T, and Gage F. ”Nurr1, an orphan nuclear receptor, is a transcriptional activator of endogenous tyrosine hydorxylase in neural progenitor cells derived from the adult brain.” Development., 126, 1999, pp 4017-4026.

Saucedo-Cardenas O, Quintana-Hau JD, Le WD, Smidt MP, Cox JJ, De Mayo F, Burbach JP, and Conneely OM. “Nurr1 is essential for the induction of the dopaminergic phenotype and the survival of ventral mesencephalic late dopaminergic precursor neurons.” Proc. Natl. Acad Sci. USA., 95, 1998, pp 4013.

Seeman P. “Dopamine receptors and the dopamine hypothesis of schizophrenia.” Synapse. 1(2):1987, pp 133-52.

Solanto MV. “Dopamine dysfunction in AD/HD: integrating clinical and basic neuroscience research.” Behav Brain Res. 10, pp 65-71.

Steece-Collier K, Maries E, Kordower JH. “Etiology of Parkinson's disease: Genetics and environment revisited.” Proc Natl Acad Sci U S A. 99, 2002, pp 13972-4

Usiello A, Baik Ja-H, Rouge-Pont F, Picetti R, Dierich A, LeMeur M, Piazza P, and Borelli E. “Distinct functions of the two isoforms of dopamine D2 receptors.” Nature., 408, 2000, pp 199-202.

Werme M, Ringholm A, Olson L, and Bren s. “Differential patterns of induction of NGFI-B, Nor1 and c-fos mRNAs in striatal subregions by haloperidol and clozapine.” Brain Res. 863, 2000, pp 112-119.

Williams G.T and Lau L.F. “Activation of the inducible orphan receptor gene Nur77 by serum growth factors: dissociation of immediate-early and delayed-early responses.” Mol. Cell. Biol., 13, 1993, pp 6124-6136.

Wilson T.E, Fahrner T, Johnston M, and Milbrandt J. “Identification of the DNA binding site for NGFI-B by genetic selection in yeast.” Science, 252, 1999, pp 1296-1300.

Woronicz J.D, Lina A, Calnan B.J, Szchowski S, Cheng L, and Winoto A. “Regulation of the Nur77 orphan steroid receptor in activation-induced apoptosis.” Mol. Cell. Biol., 15, 1995, pp 6364-6376.

Yoon J.K, and Lau L.F. “Transcriptional activation of the inducible nuclear receptor gene Nur77 by nerve growth factor and membrane depolarization in PC12 cells.” J. Biol. Chem., 268, 1993, pp 9148-9155.

Zettrstrom RH, Solomin L, Mitisiadis T, Olson L, and Perlman T. “Retinoid X receptor heterodimerization and developmental expression distinguish the orphan nuclear receptors NGFI-B, Nurr1, and Nor1.”Mol. Endo., 10, 1996a, pp 1656.

Zetterstrom R, Williams R, Perlmann T, and Olson L. “Cellular expression of the immediate early transcription factors Nurr1 and NGFI-B suggests a gene regulatory role in several brain regions including the Nigrostraial dopamine system.” Mol Brain Research, 41, 1996b, pp 111-120.

Zetterstrom RH, Solomin L, Jansson L, Hoffer BJ, Olson L, and Perlmann T. “Dopamine neuron agenesis in Nurr1-deficient mice.” Science., 276, 1997, pp 248.