THE EXPRESSION OF DOPAMINE-RELATED GENES AND BEHAVIORAL PERFORMANCE IN MICE

A thesis submitted in partial fulfillment of the requirements for the degree of
Master of Science

By

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ABSTRACT

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While the neurotransmitter dopamine has been well-studied for its role in mood regulation and activation of the intrinsic reward pathway, several psychiatric disorders linked to dopamine are also known to cause memory impairment, a phenomenon which has attracted much less attention. In the current study, whole-transcriptome RNA sequencing was performed, and transcript levels of several dopamine-related genes were compared to results of behavioral assays designed to test spatial and emotional memory, as well as anxiety. The results suggest a positive relationship between expression level of Nurr1, a nuclear receptor known to initiate transcription of genes necessary for dopaminergic signaling, and both emotional and spatial memory. However, no correlation was observed between expression of tyrosine hydroxylase, dopamine transporter, or any variant of dopamine receptor, and any of the behavioral results. These results are consistent with previous research findings that Nurr1 plays a role in memory consolidation, and suggests a dopamine-independent regulation.
# TABLE OF CONTENTS

I. Introduction...........................................................................................................1  
  - Purpose...........................................................................................................1  
  - Dopamine......................................................................................................3  
  - NURR1.........................................................................................................10  
  - Dopamine Transporter (Dat1)....................................................................13  
  - Tyrosine Hydroxylase (Th)........................................................................14  
  - Epinephrine and the β-adrenergic receptors............................................16  

II. Hypothesis and Aims.........................................................................................17  

III. Materials and Methods....................................................................................19  
  - Ethics statement........................................................................................19  
  - Responsibilities of the Author.................................................................19  
  - Animals.......................................................................................................19  
  - Chronic Variable Stress.............................................................................20  
  - Elevated Plus Maze...................................................................................20  
  - Morris Water Maze....................................................................................21  
  - Fear conditioning.......................................................................................21  

iv
- Tissue collection and RNA sequencing .................................................. 22
- Bioinformatics ....................................................................................... 22
- Analysis ................................................................................................... 23

- Results ....................................................................................................... 24
  - RNA sequencing ..................................................................................... 24
  - Categorization of groups by mRNA expression ...................................... 24
  - Categorization of groups by task performance ....................................... 24
  - Nurr1 correlations across entire population .......................................... 25
  - Quantitative Trait Loci mapping ........................................................... 25

- Discussion .................................................................................................. 38
  - Nurr1 .................................................................................................. 38
  - Nurr1 targets and dopamine receptors .................................................. 39
  - Final thoughts ....................................................................................... 42

- References .................................................................................................. 44
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Roles of genes examined in the present study.</td>
<td>4</td>
</tr>
<tr>
<td>2. Dopamine receptors act on adenyl cyclase to stimulate or inhibit CREB-mediated gene transcription.</td>
<td>6</td>
</tr>
<tr>
<td>3. The catecholaminergic synthesis pathways.</td>
<td>9</td>
</tr>
<tr>
<td>4. Crystal structure of Nurr1.</td>
<td>12</td>
</tr>
<tr>
<td>5. Graphical representation of target gene mRNA expression.</td>
<td>28</td>
</tr>
<tr>
<td>6. Comparison of task performance between high and low Nurr1 mRNA expression groups.</td>
<td>29</td>
</tr>
<tr>
<td>7. Comparison of task performance between Nurr1 target gene mRNA expression groups.</td>
<td>30</td>
</tr>
<tr>
<td>8. Comparison of task performance between dopamine receptor mRNA expression groups.</td>
<td>31</td>
</tr>
<tr>
<td>9. Comparison of Nurr1 mRNA expression levels between high and low task performances.</td>
<td>32</td>
</tr>
<tr>
<td>10. Comparison of Dat1 mRNA expression levels between high and low task performances.</td>
<td>33</td>
</tr>
</tbody>
</table>
11. Comparison of Th mRNA expression levels between high and low task performances.............................................................................................34

12. Comparison of dopamine receptor mRNA expression levels between high and low task performances.................................................................35

13. Correlation of Nurr1 mRNA expression and latency to platform during Morris Water Maze across the entire subject population..............................36

14. Correlation of Nurr1 mRNA expression and time spent frozen during day three of fear conditioning.................................................................36

15. Quantitative Trait Loci mapping of Nurr1 mRNA expression...............................37

16. The observed ratios of hippocampal dopamine receptor mRNA levels............42
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Summary of stressors and behavioral assays over the experimental period</td>
<td>20</td>
</tr>
<tr>
<td>2. Expression of target gene mRNA expression across the entire studied population</td>
<td>27</td>
</tr>
<tr>
<td>3. P-values of one-tailed t-tests for subjects grouped by mRNA expression</td>
<td>31</td>
</tr>
<tr>
<td>4. P-values of one-tailed t-tests for subjects grouped by task performance</td>
<td>35</td>
</tr>
<tr>
<td>5. Suggestive loci associated with Nurr1 mRNA expression</td>
<td>37</td>
</tr>
</tbody>
</table>
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I. INTRODUCTION

Purpose

According to the National Institute of Mental Health, 18.1% of adults and 46.3% of children suffered from a mental illness in 2014. In the same year, over 42,000 lives were lost to suicide in the United States alone, making it the country’s tenth leading cause of death (Kochanek, Murphy, Xu, & Tejada-Vera., 2016). Furthermore, despite countless advances in psychiatric research in recent years, the suicide rate has climbed from 10.5 to 13 per 100,000 individuals since 1999 (Curtin, Warner, & Hedegaard, 2016). It has been demonstrated that chronic stress can contribute to development of psychiatric disorders, although we are far from fully understanding the biochemical mechanisms by which this occurs. Many psychiatric disorders are believed to involve dysregulation of dopamine signaling, and therefore, there is tremendous interest in the biochemical causes of these disorders. This is a broad field, however, as dopamine signaling is regulated by a diverse range of proteins. These include enzymes involved in catecholamine synthesis, transporters involved in regulation of synaptic neurotransmitter concentrations, and receptors which intercept synaptic dopamine and translate the messenger signal into intracellular cascades which lead to transcription, translation, or post-translational modification which modulate activity of fully-formed proteins.

Many conditions listed in the Diagnostic and Statistical Manual of Mental Disorders, 5th Edition that are associated with dopamine, such as Major Depressive
Disorder and schizophrenia, feature symptoms relating to impaired or altered ability to consolidate or access long-term memory (American Psychiatric Association, 2013).

While dopamine has been highly studied in regards to mood disturbances, less emphasis has been placed on the mechanisms by which the pathophysiology of these disorders affects memory formation and/or retrieval. Because of this, the current study focuses on dopaminergic signaling in the hippocampus, a neural structure which is closely associated with declarative—including emotional and spatial—memory (Duvernoy, 2005). By expanding our knowledge of the causes behind memory-related psychiatric symptoms, the current study may assist in furthering our knowledge of how to treat them.

For this study, critical genes from various aspects of dopaminergic regulation (Fig. 1) were examined: tyrosine hydroxylase (TH, Th), which converts tyrosine into L-DOPA, the precursor to dopamine; the dopamine transporter (DAT, Dat1), which facilitates reuptake of unbound synaptic dopamine; the five subtypes of dopamine receptor (both excitatory and inhibitory); and NURR1 (Nurr1), a nuclear receptor which transcriptionally activates both TH and Dat1. Using RNA sequencing and behavioral assays, the current study sought to determine whether or not mRNA expression levels of these genes in the hippocampus relate to various measures of memory and emotion after exposure to a chronic stress environment. By studying the relationship between dopamine-signaling related gene expression and behaviors related to memory and fear, insight may be gained into the genetic pathologies of diseases that affect these cognitive functions. By narrowing down which genes are most crucial in these pathways, they can
then be further investigated in pharmaceutical and other clinical studies.

**Dopamine**

Dopamine (3,4-Dihydroxyphenethylamine) is an organic biochemical messenger that was first synthesized *in vitro* by George Barger and James Ewens in 1910 (Fahn, 2008). Arvin Carlsson (who would eventually win the 2000 Nobel Prize in Medicine or Physiology for his discoveries) determined that not only is dopamine a precursor to other catecholamines, but is a neurotransmitter in its own right (Fahn, 2008). It has since been discovered that dopamine plays a role in many aspects of neural activity, including intrinsic reward, decision making, behavior, and physical movement (Schultz, 2007).

Upon release of dopamine into the synapse, it accomplishes these functions by binding dedicated receptors on the post-synaptic neuron. There are two distinct classes of dopamine receptor, both of which are G protein-coupled receptors: the D₁-like and the D₂-like. The D₁-like group consists of DRD1 and DRD5, which activate adenyl cyclase by coupling to the Ga₅ protein, while the D₂-like group, comprised of DRD2, DRD3, and DRD4, inhibit adenyl cyclase by way of the Gaᵢ or Gaₒ proteins (Beaulieu & Gainetdinov, 2011). According to Rondou, Haegeman, and Van Craenenbroeck (2010), the affinities of the dopamine receptors, as measured by Kᵢ values, are approximately 2500 nM for DRD1, 500 nM for DRD2, 20-100 nM for DRD3, 43-400 nM for DRD4 (depending on allelic variations), and 225 nM for DRD5. Together, these receptors modulate the conversion of the neurotransmitter signal into intracellular signaling cascades, which can either facilitate or inhibit the phosphorylation of CREB and resulting gene transcription (Fig. 2).
Figure 1: Roles of genes examined in the present study. NURR1, a nuclear receptor, is a transcriptional activator of dopamine-related genes, such as Th and Dat1. TH converts tyrosine to L-DOPA as part of the catecholamine synthesis pathway. DAT regulates synaptic dopamine concentration by facilitating reuptake of DA into the presynaptic neuron. Finally, the five subtypes of dopamine receptor (DRD1-5) are found in the membrane of the postsynaptic neuron and bind DA that has been released into the synapse.
The first aspect of the current study was to explore how subjects' mRNA levels of the studied genes (including dopamine receptors) varied across the population, despite identical handling and stress protocols. At least two studies have demonstrated a decrease in extraneuronal dopamine levels in response to stress (Ahmad, Rasheed, Banu, & Palit, 2010; Rasheed et al., 2010), and there is evidence to suggest that dopamine receptor expression is inversely proportional to synaptic dopamine concentrations. With this in mind, it is important to understand the processes involved in the catecholaminergic synthesis pathway and release of DA into the synapse.

The precursor to all catecholamines is the amino acid tyrosine, which goesthrough a number of enzymatic alterations to reach its final state(s). Tyrosine is first converted to L-DOPA by TH, and then to dopamine by L-aromatic amino acid decarboxylase (AADC). Alternately, a second, minor pathway exists in which tyrosine undergoes decarboxylation and is converted into tyramine (another amino acid) before being hydroxylated to dopamine by CYP2D6 (Meiser, Weindl, & Hiller, 2013; Hiroi, Imaoka, & Funae, 1998). In dopaminergic neurons, this step is the endpoint. However, in (nor)adrenergic neurons, further steps are necessary. Dopamine is converted to norepinephrine by dopamine-β-hydroxylase (DBH; Ciaranello, Wooten, & Axelrod, 1976), and then, as appropriate, to epinephrine by phenylethanolamine N-methyltransferase (PNMT) (Sabban & Kvetňanský, 2001). These pathways are illustrated in Fig. 3.
Figure 2: Dopamine receptors act on adenyl cyclase to stimulate or inhibit CREB-mediated gene transcription. The D1-like receptors (DRD1, DRD5) stimulate adenyl cyclase and downstream cascades which lead to phosphorylation of CREB and resulting gene transcription. Conversely, the D2-like receptors (DRD2, DRD3, DRD4) inhibit adenyl cyclase and prevent these downstream effects. DRs, stimulatory dopamine receptors; DRDs, inhibitory dopamine receptors; Gαs: stimulatory G-protein alpha subunit; Gαi: inhibitory G-protein alpha subunit; cAMP: cyclic adenosine monophosphate; PKA: protein kinase A; EPAC: exchange protein activated by cAMP; DARPP-32: dopamine- and cAMP-regulated neuronal phosphoprotein; RAP1: Ras-related protein 1; B-Raf, serine/threonine-protein kinase B-Raf; PP1: protein phosphatase 1; MEKs: Mitogen-activated protein kinases; ERK: extracellular-signal-regulated kinase; CREB: cAMP response element binding protein; pCREB: phosphorylated CREB. Arrows denote activation, dotted lines denote inhibition.
There is some disagreement in the existing literature as to how catecholaminergic tyrosine hydroxylase expression is affected by chronic stress. However, in a review of existing literature, Sabban and Kvetňanský (2001) concluded that TH mRNA and immunoreactivity levels are increased in the ventral tegmental area (a region containing a relatively high concentration of dopaminergic neurons) as a result of chronic stress, and this is consistent with what is known of stress-related activation of the hypothalamic-pituitary-adrenal (HPA) axis. HPA activation leads to increased production of glucocorticoids, and in binding to their receptors, they increase phosphorylation and activation of the cAMP response element binding protein (CREB), a transcription factor for which a response element is found alongside the NGFI-B response element (NBRE) in the promoter region of TH (Ghee, Baker, Miller, & Ziff, 1998). Therefore, while TH is regulated at the transcriptional level by NURR1, it has also been demonstrated that TH transcription is also inhibited by the downstream effects of increased catecholamine concentration (Meiser, Weindl, & Hiller, 2013). Consequently, dopamine itself also contributes to negative feedback of catecholamine synthesis through decreasing CREB-mediated synthesis of TH.

AADC, on the other hand, is regulated primarily by DA receptor-mediated phosphorylation. Treatment with dopaminergic antagonists such as SCH23390 or haloperidol has been shown to result in increased AADC activity (Hadjiconstantinou & Neff, 2008; Zhu, Juorio, Paterson, & Boulton, 1993). Furthermore, okadaic acid, a protein phosphatase I (PP1) inhibitor, also results in increased AADC activity (Hadjiconstantinou & Neff, 2008). This suggests that antagonism of the D₁-like
excitatory receptors leads to decreased inactivation of PP1, and as a result, greater
dephosphorylation and inactivation of AADC. In this way, AADC is also regulated by
dopaminergic feedback.

In addition to enzymes which regulate dopamine synthesis and further
metabolism, dopamine's concentration is also regulated, specifically in the synapse, by
transport proteins in the cell membrane of the presynaptic axon. The vesicular
monoamine transporter 2 (VMAT2) is an integral membrane protein that, as the name
suggests, facilitates transport of monoamines into vesicles. According to a literature
review by Kvetňanský, Lu, and Ziegler (2013), only VMAT2, not VMAT1, is expressed
in neurons, and VMAT2 has a tendency to colocalize with TH. It has been previously
demonstrated that expression of VMAT2 is downregulated in response to chronic stress in
the nucleus accumbens (Zucker, Weizman, & Rehavi, 2005), and since vesicular transport
is necessary for neurotransmitter release, this further contributes to decreased
extraneuronal dopamine levels. There is also general agreement about the effects of
chronic stress on expression of Dat1. In various neural structures including the nucleus
accumbens (Scheggi et al., 2002), caudate nucleus, and putamen (Isovich, Mijnster,
Flügge, & Fuchs, 2000), increases seen in extraneuronal dopamine levels are generally
attributed to decreased expression of DAT on the presynaptic neuron. Once dopamine is
removed from the synapse, it is broken down by a number of catecholamine degradation
enzymes, including monoamine oxidase (MAO). Kvetňanský et al. (1984) demonstrated
that MAO activity is decreased in the hypothalami of animals subjected to repeated
Figure 3: The catecholaminergic synthesis pathway. TH: tyrosine hydroxylase; TD: tyrosine decarboxylase; AADC: aromatic L-amino-acid decarboxylase; CYP2D6: cytochrome P450 2D6; DBH: dopamine β-hydroxylase; PNMT: phenylethanolamine N-methyltransferase.
stress, which downregulates the process of catecholamine degradation.

To summarize, there are five subtypes of dopamine receptor, which are differentially expressed across various regions of the brain. The D₁-like excitatory receptors stimulate the cAMP pathway and upregulate transcription of CREB target genes, while the D₂-like inhibitory receptors inhibit cAMP. The abundance of dopamine itself is regulated by a number of enzymes—those upstream which facilitate conversion of tyrosine to dopamine, and those downstream which convert dopamine into norepinephrine and epinephrine. Synaptic concentration of dopamine is regulated by transporters which facilitate the reuptake of excess dopamine into the presynaptic neuron, as well as enzymes which degrade the dopamine once it has been reabsorbed. As this network of genes demonstrates, dopaminergic signaling is highly regulated.

**NURR1**

NURR1 (also known as NR4A2) is a member of the nuclear receptor family of proteins which has been implicated in transcription of genes necessary for dopaminergic regulation. Nuclear receptors are a class of proteins which bind to hormone response elements (HREs) in promoter regions of DNA to activate transcription of genes coupled to the specific receptor's RE. Once bound, these receptors initiate transcription through the recruitment of additional complex substituents, such as coactivators and/or additional transcription factors (Wärnmark, Treuter, Wright, & Gustafsson, 2003). Khorasanizadeh and Rastinejad (2001) characterized these proteins as sharing a structure consisting of several features: a degenerate amino terminus, hinge region, and conserved DNA-binding (DBD) and receptor-specific ligand binding (LBD) domains. However, structural studies
have indicated the lack of a ligand-binding pocket within the native conformation of
NURR1, leading to its classification as an orphan receptor (Wang et al., 2003), as
illustrated in Fig. 4.

NURR1 is first expressed in the midbrain at day 10.5 of embryogenesis, but its
presence continues into maturation and adulthood (Torii, Kawarai, Nakamura, &
Kawakami, 1999; Sakurada, Ohshima-Sakurada, Palmer, and Gage, 1999). It is believed
to be transcriptionally activated by the NF-κB and the cyclic AMP response element
binding protein (CREB) factors, based on binding assays involving the Nurr1 promoter
region (McEvoy et al., 2002; Kovalovsky et al., 2002), and according to Liu, Serova,
Kvetňanský, and Sabban (2008), immobilization stress has been demonstrated to induce
transcription of Nurr1 in the adrenal medulla. There are multiple 5'-ATTTA-3' sequences
in the 3' untranslated region of the gene, a sequence which is known to cause mRNA
instability and is suggestive of an immediate early gene, or IEG (Torii et al., 1999).

NURR1 is a member of the Nur77 receptor family, alongside Nur77 and NOR-1,
the three show a large amount of sequence homology, sharing the NBRE consensus
sequence consisting of the sequence 5'-AAAGGTCA-3' (Sacchetti, Mitchell, Granneman,
& Bannon, 2001; T. Kim et al., 2013). Although an orphan receptor, there have been
cases where RXR has been demonstrated to dimerize with NURR1 in a mechanism
which is believed to promote increased expression of target genes (T. Kim et al., 2013),
and at least one study has demonstrated dysfunctional dopamine signaling and
locomotion in an RXR knockout mouse model (Kręzel et al., 1998). However, RXR is
not necessary for expression of all NURR1 targets (Sacchetti et al., 2001).
There is a lack of differentiation of dopaminergic neurons in Nurr1-null mice (Kadkhodaei et al., 2009), and overexpression of the gene induces mesenchymal stem cell differentiation into dopaminergic neurons (Park et al., 2012), suggesting a major role for the gene in neuronal development. However, it is also implicated as a major regulatory component of mature dopaminergic neurons, as it has been demonstrated to act as a transcriptional activator of proteins crucial for dopaminergic signaling, such as tyrosine hydroxylase (Sakurada et al. 1999; T. Kim et al., 2013) and the dopamine transporter (Sacchetti et al., 2001; Bannon et al., 2002). Because of this role, NURR1 has garnered interest as a target for therapeutic interventions aimed at dopamine-related
psychiatric disorders. Polymorphisms and mutations of the gene have been linked to Parkinson's disease (Carmine et al., 2003), bipolar disorder, and schizophrenia (Buervenich et al., 2000), and tissue analyses have found downregulation of NURR1 in both peripheral neurocytes and dopaminergic neurons of PD patients (Kadkhodaei et al., 2009). Furthermore, it has been demonstrated that a deficiency in NURR1 leads to a decrease in dopamine levels, which may be the mechanism by which it leads to Parkinson's (Park et al., 2012). A Nurr1-heterozygous mouse model showed hyperactivity which was reduced by administration of haloperidol, a DRD2 antagonist, as well as an increase in immobility during the Forced Swim Test (indicating depression), consistent with symptoms of schizophrenia (Rojas, Joodmardi, Hong, Perlmann, & Ögren, 2007).

Dopamine Transporter (Dat1)

The dopamine transporter (also known as SLC6A3) is a protein found in the synaptic membrane that facilitates the reuptake of synaptic dopamine into presynaptic terminals (Sacchetti et al., 2001; Felten, Montag, Markett, Walter, & Reuter, 2011). It has been demonstrated that Dat1 is induced by NURR1 independently, without the necessity of RXR binding. Furthermore, it has been demonstrated that binding to the NBRE is not necessary for NURR1 activation of Dat1 transcription, a phenomenon which has not been otherwise observed in the Nur77 family; but which has been demonstrated in the interactions of other nuclear receptors (Sacchetti et al., 2001).

There is a variable number of tandem repeats (VNTR) sequence present in the Dat1 gene, and this polymorphism alters transporter density, resulting in a downstream
effect on synaptic dopamine concentration (Felten et al., 2011). However, the polymorphism showed no significant effect on results of the Affective Neuroscience Personality Scales (Felten et al., 2011), the Schedule for Affective Disorders and Schizophrenia-Lifetime Version, and the Structured Clinical Interview for DSM-IV—Patient Version (Frisch et al., 1999). Furthermore, meta-analyses of existing literature showed that the polymorphism showed no association with schizophrenia (Gamma, Faraone, Glatt, Yeh, & Tsuang, 2005; Joober et al., 2000). Nevertheless, it is believed that DAT pathology may play a role in the same disorders as NURR1, along with disorders involving attention deficits and addiction (Joober et al., 2000). Rowe et al. (1998) presented data to suggest that this VNTR polymorphism may play a role in the pathology of “internalizing disorders:” generalized anxiety, social anxiety, phobias, obsessive-compulsive disorder, and Tourette’s syndrome.

Tyrosine Hydroxylase (TH)

As mentioned previously, tyrosine hydroxylase is the rate-limiting enzyme in catecholamine synthesis (Thibaut et al., 1997), which converts the amino acid tyrosine into L-DOPA. In rodents, it has been demonstrated that TH is necessary for dopaminergic functioning; however, in humans, while it has been confirmed that the NBRE is present in the Th promoter region (Jin et al., 2006), it is not strictly necessary, as the CYP2D6 pathway presents an alternative method of dopamine synthesis, and it has been demonstrated that siRNA downregulation of human NURR1 transfected into an animal model did not affect TH expression (Jin et al., 2006). Further investigation showed that the sequence homology between human and murine Th promoter regions is only 46.4%
(Jin et al., 2006). Despite its biological redundancy in humans, it is included in this study because of its known significance in rodent models.

Despite this redundancy, however, mutations in the human Th gene can still produce a phenotype consistent with certain psychiatric disorders. A perfect ten tetranucleotide repeat located in the first intron of the gene shows a significant correlation with schizophrenia, whereas a 1 base-pair deletion within this sequence is considered wild-type (Thibaut et al., 1997). A postmortem analysis of depressive patients and suicide victims demonstrated an upregulation in TH transcription in the locus coeruleus, a region of the brainstem, which the authors believed to be an adaptive mechanism based on low norepinephrine levels (Zhu et al., 1999). This suggests that conversely, a functional mutation in TH may also cause depressive symptoms, as without it, neurons which lack the necessary enzymes for the secondary dopamine synthesis pathway may present with low norepinephrine levels. Polymorphisms in CYP2D6 have been linked to tardive dyskinesia and other movement disorders in response to second-generation antipsychotic medications, suggesting that it plays an equally vital role in dopamine synthesis (Arranz, Blanco, & Samperiz, 2016). Additionally, another study of TH and symptomology found a significant link between certain allelic variations of the gene and occurrence of bipolar disorder (Serretti et al., 1998).

Despite the evidence that an otherwise healthy individual can overcome abnormally low TH levels by alternate dopamine synthesis pathways, if this abnormality is compounded with abnormalities in sister pathways, it can cause a number of psychiatric symptoms. Therefore, as with DAT, there is a possibility that low TH levels
can be responsible for NURR1-related symptoms in individuals with an irregularity in CYP2D6 or its synthesis.

**Epinephrine and the β-Adrenergic Receptors**

As mentioned previously, epinephrine/adrenaline is the final endpoint of the catecholaminergic synthesis pathway, and is the main neurotransmitter implicated in the physiological manifestations of the “fight-or-flight” response. The adrenergic receptors are grouped into two classes of G-protein coupled receptors by their effects on systemic circulation, α (vasoconstrictive) and β (vasodilating), and of the latter, there are three subtypes (Waarde, Vaalburg, Doze, Bosker, & Elsinga, 2004). All three β-receptors are coupled to the excitatory Gαs protein (Gurdal, Friedman, & Johnson, 1995; Sidhu, 1998), thereby leading to activation of the same adenyl cyclase pathway as the excitatory dopamine receptors and culminating in increased phosphorylation of CREB.

A study by Patki, Atrooz, Alkadhi, Solanki, & Salim (2015) showed increased hippocampal (as well as prefrontal cortical and amygdalar) epinephrine in response to a several-day social defeat paradigm. H. Kim *et al.* (2013) also demonstrated that a control group of animals exposed to chronic cold stress showed a significant upregulation in transcription of phenylethanolamine N-methyltransferase (PNMT), as well as a significant decrease in dopamine and a significant increase in adrenaline levels in the hypothalamus and hippocampus.
II. HYPOTHESIS AND AIMS

Based on what has been previously discovered about the roles dopamine plays in learning, memory, and emotion, we hypothesize that transcription of genes which encode proteins that contribute to the synthesis and signal transduction of dopamine is higher in the hippocampi of animals which display higher levels of spatial and emotional memory. In particular, we focused on the following areas and aims:

- **Aim 1:** To assess the relationship between dopamine receptor gene expression and behavioral performance following chronic stress exposure in mice.
- **Aim 2:** To test whether expression of genes that facilitate dopaminergic signaling in the hippocampus (Nurr1 and Th) is positively associated with behavioral performance.
- **Aim 3:** To test whether expression of a gene that inhibits dopaminergic signaling in the hippocampus (Dat1) is negatively associated with behavioral performance.

In regards to Aim 1, although the D₁-like excitatory and D₂-like inhibitory dopamine receptors serve opposite roles in transmission of the dopaminergic signal, we anticipate an overall increase in mRNA expression across all subtypes in higher-performing animals (in line with Aim 2), rather than a bidirectional change dependent on receptor subtype. In Aim 2, Nurr1 is a nuclear receptor which facilitates transcription of dopamine-regulation genes, and Th is necessary for the synthesis of dopamine from tyrosine. In Aim 3, Dat1 is
responsible for facilitating reuptake of dopamine into the presynaptic neuron, thereby preventing postsynaptic binding. By analyzing a variety of genes involved in different aspects of this system, the goal of the current study is to determine which aspect may be responsible for memory deficits resulting from dopamine dysregulation.
III. MATERIALS AND METHODS

*Ethics Statement*

All procedures involving live animals were performed consistent with the standards set forth by the National Institutes of Health and the National Research Council's *Guide for the Care and Use of Laboratory Animals*. Studies were approved by the Wright-Patterson Air Force Base Institutional Animal Care and Use Committee.

*Responsibilities of the Author*

Although the author of the current study performed the subsequently mentioned animal handling procedures as part of her laboratory responsibilities, all animal handling in the current study was performed prior to her membership in the lab, as described in Carhuatanta, Shea, Herman, and Jankord (2014). RNA sequencing analysis was performed by Dr. Seung Ho Jung. The author of the current study designed the hypothesis and research aims, and was provided the expression data derived from RNA sequencing, as well as raw behavioral data, and performed the statistical analysis.

*Animals*

9 week old male BXD (C57BL/6J and DBA/2J recombinant inbred strain, Jackson Laboratory, Bar Harbor, ME, USA) mice were housed individually and given standard food and water *ad libitum*. Prior to the experimental paradigm, animals were allowed to acclimate to the animal facility for a period of ten days. The facilities were climate controlled under the following parameters: 18-24°C, 30-70% humidity, 12 hour
dark/light cycle. All experimental handling occurred Monday through Friday of each week, during the light cycle.

**Chronic Variable Stress**

All animals were subjected to a four-week Chronic Variable Stress regimen, during which subjects were exposed to up to two of the following stressors each day (5 days per week): 15 minutes of cold (4°C) exposure, 30 minutes of hypoxia, 1 hour of cage placement on a shaker, novel housing overnight, and 30 minutes of open field exposure. The sequence of stressors was randomized to prevent habituation; however, all animals were subjected to an identical sequence. The handling schedule is summarized in Table 1.

![Table 1: Summary of stressors and behavioral assays over the experimental period.](image)

**Elevated Plus Maze**

The Elevated Plus Maze is a behavioral assay which serves to quantify an animal's anxiety level (Hogg, 1996). The maze was elevated 1 meter from the floor and consisted of two pairs of 40 cm x 8 cm platforms arranged in a cross: one in which the arms are open to the air, the other enclosed with 29.5 cm tall walls constructed of gray plexiglass. The natural inclination of rodents is for dark, enclosed spaces, and therefore, lack of anxiety is measured by the percent of the total test time (5 minutes) which is spent exploring the open arms. Video of each trial was recorded and duration spent in each arm
was automatically calculated using EthoVision XT 7.0.418 tracking software (Noldus Information Technology, Leesburg, VT, USA).

**Morris Water Maze**

The Morris Water Maze is a behavioral assay used to examine subjects' spatial memory and learning abilities (Morris, 1984). Briefly, subjects are placed into a pool of opaque water, under the surface of which is a solid platform which when stood upon, enables the majority of the animal's body to remain above water. During a single day, subjects are placed into the pool in varying quadrant positions, and are provided with environmental directional cues (large varying printed symbols positioned above the pool). Various measures of each trial (4 per animal per day) are recorded, including subject position, swim path, speed, and latency to platform. Subjects completed four trials per day, for five days, followed by a one day learning extinction procedure during which the platform was removed from the pool, and on the final day, post-extinction recall was tested. The final two days of the study were performed for parallel studies; the current study examined average latency to platform over 20 trials (4 trials per day across the first five days). All trials were recorded using EthoVision XT.

**Fear Conditioning**

Fear conditioning, as the name suggests, is utilized as an assessment of emotional learning and memory (Phillips & LeDoux, 1992). Subjects were placed into sound dampening chambers which contained cameras and consisted of two metal (side) and two plexiglass (front and back) walls (Med Associates, Inc., St. Albans, VT, USA). The first day of the three-day assay consisted of four 3 kHz tones at 85 dB, lasting 30 seconds
each, which were immediately followed by a 2 second, 0.75 mA shock delivered through the floor of the chamber. Between each tone/shock pairing, there was a thirty second interval. Day 1 served to establish classical conditioning, pairing the unconditioned freezing response to fear with the pre-shock tone. On day 2, subjects were placed into the conditioning chamber once more, in the absence of both tone and shock, and freezing behavior (both instances and percent time frozen) were observed. On day 3, the metal grid on the floor of the chamber was replaced with white plexiglass, and freezing behavior was observed in response to a tone in the absence of a shock. Instances of freezing and time frozen on day 3 were analyzed as a measure of emotional memory.

*Tissue Collection and RNA Sequencing*

The day following the conclusion of all behavioral assays, subjects were euthanized via rapid decapitation, and brain tissue was quickly removed and stored at -80°C. Whole hippocampi were dissected from each sample, and total RNA was isolated via an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the provided directions. Isolated RNA samples were shipped overnight to the Next Generation Sequencing facility at UCLA. Whole transcriptome RNA sequencing was performed using an HiSeq 2500 instrument (Illumina, San Diego, CA, USA), and multiplexed files were downloaded from the UCLA database, demultiplexed, and merged into one file.

*Bioinformatics*

The FASTX-Toolkit (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA) was utilized to remove sequencing adapters (FASTQ Clipper) and remove barcodes and noise (FASTQ trimmer). Quality control of files was validated using FastQC
TopHat (v. 2.0.6, Johns Hopkins University, Baltimore, MD, USA) and Bowtie (v. 0.12.8, Johns Hopkins University) were used to align reads to the *Mus musculus* (NCBI: M37) genome assembly hosted by the University of California, Santa Cruz Genome Browser. Once aligned, HTSeq (v. 0.5.3p9, European Molecular Biology Laboratory, Heidelberg, Germany) was used to quantify reads, and DESeq (European Molecular Biology Laboratory) was utilized to normalize the quantifications and discard genes which were not displayed across all samples. Read values were normalized by dividing the raw read count by a scaling factor which accounted for the assumption that the majority of genes analyzed were not differentially expressed. All bioinformatic analysis was performed by Dr. Seung Ho Jung.

**Analysis**

Normalized read levels for genes of interest from each subject were extracted from processed data. Statistical analysis and generation of graphical data representations were performed using SigmaPlot (v. 13.0, Systat Software, San Jose, CA, USA). Subjects were sorted and grouped by both mRNA expression and individual task performance, and due to the previously mentioned predictions of directional change, the highest and lowest quadrants were compared via one-tailed t-tests. Correlations across the entire population were measured by the Pearson product-moment correlation coefficient (“Pearson's r”). Quantitative Trait Loci (QTL) mapping was performed using GeneNetwork (University of Tennessee, Knoxville, TN, USA), with 1000 permutation tests and 2000 bootstrap tests.
IV. RESULTS

RNA sequencing

The hippocampi of all 25 subjects underwent whole-transcriptome RNA sequencing. All of the genes studied displayed a range of relative expression values, as seen in Table 2. The results are visually summarized as well in Fig. 5.

Categorization of groups by mRNA expression

Subjects were grouped into quartiles (n=6, with the exception of Drd3, which had 10 subjects displaying a transcription level of zero) according to mRNA expression level. Task performances were compared between the highest and lowest quartiles, and p-value was determined by one-tailed t-test (Figs. 6-8; Table 3). The resulting data shows a statistically significant difference in latency to platform during the Morris Water Maze (spatial memory), as well as instances of and time spent displaying freezing behavior during fear conditioning (emotional memory), between high and low Nurr1 mRNA expression groups (Fig. 6; MWM p = 0.0351; FC p = 0.009).

Categorization of groups by task performance

Subjects were grouped into quartiles according to individual task performance. mRNA expression levels were compared between the highest and lowest quartiles, and p-value was determined by one-tailed t-test (Figs. 9-12; Table 4). While we see a similar trend of higher performance (lower latency to platform in MWM, higher time in open arms in EPM, and both lower freezing instances and total time frozen in FC) associated
with higher gene expression, no statistically significant difference was observed. Likewise, no statistically significant change was observed for any task or any other gene studied.

*Nurr1 correlations across entire population*

Given that initial analysis demonstrated a significant change in task performances across *Nurr1* expression groups, the correlations between performance and expression variables were examined across the entire subject population. While not meeting the threshold for statistical significance, weak negative correlations (as measured by Pearson's $r$) were demonstrated for both latency to platform (MWM) and percent time frozen (FC), as shown in Figs. 13 and 14. A Pearson's $r$ value between $|0.3|$ and $|0.5|$ indicates a weak correlation between variables, whereas a value less than $|0.3|$ indicates a negligible correlation, while a positive value represents a positive correlation and negative values represent negative correlations. Given that the Pearson's $r$ values for MWM latency to platform and FC total time frozen are solidly in the “weak” range, while not statistically significant, these results are encouraging, and warrant further investigation.

*Quantitative Trait Loci mapping*

Quantitative traits are polygenic; in other words, there are multiple genes, or regions of genes, which influence the phenotype. To explain simply, a series of associations between genomic regions and phenotype are aggregated to determine which regions are associated with the trait of interest. In the current study, the goal of QTL mapping was to determine what areas of the genome were predictive of hippocampal
Nurr1 mRNA expression. At each region of the subjects' chromosomes, the variant corresponded randomly to either the C57 or DBA strains due to the randomness of genetic recombination, and the higher the peak for a given region of the genome, the higher association there was between a subject's variant at that position and their normalized transcription of Nurr1. While no peaks were observed above the likelihood ratio statistic (LRS) significance threshold of 18.21, chromosomes 4, 6, and 11 demonstrated peaks above 11.37, the suggestive threshold (Fig. 15). Closer analysis identified 20 loci across these three chromosomes which may contribute to variation in expression (Table 5). The chromosomal regions which demonstrate peaks associated with Nurr1 expression, especially on chromosome 11, while not definitively demonstrated to be the cause of the demonstrated behavioral trends, are nevertheless predictive of performance on memory-related tasks. By narrowing down the predictive regions, this paves the way for identification of causative genes and more thorough pathway mapping.
### Table 2: Expression of target gene mRNA expression across the entire studied population

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<th>Subject</th>
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<th>Dat1</th>
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<th>Drd2</th>
<th>Drd3</th>
<th>Drd4</th>
<th>Drd5</th>
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Figure 5: Graphical representation of target gene mRNA expression. Dotted lines represent the divisions between Quartiles 1 and 2, and Quartiles 3 and 4, used for analytical grouping by expression level. Range of Quartile 1 for Drd3 was extended due to identical values of a quantity greater than the standard n=6.
Figure 6: Comparison of task performance between high and low Nurr1 mRNA expression groups. P-values determined by one-tailed t-test, n=6 for both groups. Notice that Morris Water Maze latency to platform, and time frozen during fear conditioning demonstrated statistical significance between expression groups. Error bars denote standard error. * denotes statistical significance (p < 0.05).
Figure 7: Comparison of task performances between Nurr1 target gene mRNA expression groups. No statistically significant differences were observed between any expression group pairs. Error bars denote standard error.
Figure 8: Comparison of task performance between dopamine receptor mRNA expression groups. No statistically significant differences were observed between any expression group pairs. Error bars denote standard error.

Table 3: P-values of one-tailed t-tests for subjects grouped by mRNA expression. MWM, average latency to platform in Morris Water Maze; EPM, percent time spent in open arms in Elevated Plus Maze; FC Count, instances of freezing during day three of fear conditioning; FC Percent, percent of total time spent frozen during fear conditioning. * denotes a p-value less than 0.05.
Figure 9: Comparison of *Nurr1* mRNA expression levels between high and low task performances. No statistically significant changes were observed between groups for any of the behavioral assays performed. Error bars denote standard error.
Figure 10: Comparison of Dat1 mRNA expression levels between high and low task performances. No statistically significant differences were observed between groups for any behavioral assay performed. Error bars denote standard error.
Figure 11: Comparison of *Th* mRNA expression levels between high and low task performances. No statistically significant differences were observed between any performance group pairs. Error bars denote standard error.
Figure 12: Comparison of dopamine receptor mRNA expression levels between high and low task performances. No statistically significant differences were observed between any performance group pairs. Error bars denote standard error.

Table 4: P-values of one-tailed t-tests for subjects grouped by task performance. MWM, average latency to platform in Morris Water Maze; EPM, percent time spent in open arms in Elevated Plus Maze; FC Count, instances of freezing during day three of fear conditioning; FC Percent, percent of total time spent frozen during fear conditioning.
Figure 13: Correlation of Nurr1 mRNA expression and latency to platform during Morris Water Maze across the entire subject population. The Pearson's $r$ value denotes a weak correlation, although the p-value falls outside the range of statistical significance.

Figure 14: Correlation of Nurr1 mRNA expression and time spent frozen during day three of fear conditioning. The Pearson's $r$ value denotes a weak correlation, although the p-value falls outside the range of statistical significance.
Figure 15: Quantitative Trait Loci mapping of \textit{Nurr1} mRNA expression. Notice that regions within chromosomes 4, 6, and 11 fall above the grey line (the LRS threshold for suggestibility).

Table 5: Suggestive loci associated with \textit{Nurr1} mRNA expression (as derived from the QTL mapping illustrated in Fig. 15).
V. DISCUSSION

This study examined the transcription of several genes related to the regulation of dopaminergic signaling. Our results demonstrate that in tests of spatial and emotional memory, there is a positive relationship between transcript abundance of a regulatory element of dopaminergic signaling, Nurr1, and task performance (Figs. 6, 13, 14); however, no other studied genes demonstrated such a relationship. Furthermore, they demonstrate that these genes show a range of variation across our subject population, and that in regards to Nurr1, there are multiple chromosomal regions which may affect mRNA expression by allelic variation. However, this study also raises the question of whether NURR1 affects memory by dopaminergic regulation, or some other mechanism.

Nurr1

Given that both statistically significant changes between expression groups and weak correlations between transcription and task performance were observed, the present study agrees with previous literature that suggest a major role of Nurr1 in the studied behaviors. Colón-Cesario et al. (2006) found that rats treated with antisense DNA corresponding to Nurr1 successfully knocked down protein expression and interfered with discrimination of relevance of spatial information during a spatial memory task. In other words, knockdown of Nurr1 impairs spatial long term memory, thus demonstrating that it plays a vital role. Furthermore, Hawk et al. (2012) refer to several previous studies that provide evidence of a role for Nurr1 in emotional and spatial memory. One such
study demonstrated an increase in *Nurr1* in the CA1 and CA3 regions of the hippocampus following hippocampal learning. Another demonstrated that in a heterozygous *Nurr1* null model, mice show impaired emotional memory (measured through a passive avoidance task). The current and previous studies both demonstrate a role for NURR1 in hippocampal memory formation; specifically, in relevance discrimination during memory consolidation.

One detail of special interest is that while a significant change in task performance was observed between groups when subjects were grouped by *Nurr1* expression, the significance did not hold true when subjects were grouped by task performance. This is likely due to the fact that *Nurr1* influences task performance, but is not the only factor to do so. The observation that mRNA expression can weakly predict task performance, but task performance does not predict *Nurr1* mRNA expression, suggests a relationship confounded by the contributions of other signaling cascades. Furthermore, in two of the behavioral measures (latency to platform in the Morris Water Maze and percent time frozen in Fear Conditioning), a weak correlation was demonstrated between *Nurr1* expression and task performance; however, the p-values exceeded the threshold of significance (p < 0.05).

*Nurr1 targets and dopamine receptors*

Despite our initial model which predicted a significant relationship between task performance and expression of the direct targets of NURR1 which were examined in the present study, no significant group differences or correlations were observed. As previously mentioned, vital pathways such as catecholamine synthesis demonstrate the
existence of alternate mechanisms, and furthermore, many genes contain multiple
response elements within their promoter regions. In addition to NURR1, both the human
and mouse *Th* promoters contain binding elements for HNF-3β, HOXA4, and HOXA5
(Kessler, Yang, Gollomp, Jin, & Iacovitti, 2003). The *Dat1* promoter region likewise
contains response elements for CREB and AP-1, along with several potential response
elements which were identified (but not experimentally verified) by Bannon *et al.* (2001).
While a change was expected, due to the fact that previous literature has demonstrated
that mutations or knockdowns of Nurr1 are associated with dopaminergic dysfunction,
transcription of these genes is regulated by multiple pathways. Therefore, this provides a
credible explanation for the lack of a relationship between Nurr1 expression-related task
performance, and expression of NURR1 target genes.

Like the target genes of NURR1, no significant correlation was observed between
task performances and expression of dopamine receptors. While a relationship was
predicted based upon evidence suggesting that synaptic dopamine concentration regulates
receptor expression, this is consistent with the lack of correlation in *Th* and *Dat1*, as both
these genes play a role in regulation of dopamine's presence in the synapse. Interestingly,
while not statistically significant, there are complimentary trends in the Elevated Plus
Maze and fear conditioning time frozen data for the dopamine receptors. Low *Drd1* and
*Drd2* expression, as well as high *Drd5* expression, are associated with higher time in
EPM open arms, indicative of a lower unconditioned anxiety response, and lower time
frozen during FC, indicative of a less solid conditioned reaction to an unpleasant
stimulus. However, low *Drd3* and high *Drd4* demonstrate a trend toward higher time in
open arms, but lower time spent frozen during fear conditioning. While the similarity in
trends between Drd1 and Drd5, as well as Drd3 and Drd4 are to be expected as they are
grouped together by G-protein response, it is worth noting that Drd2, in this instance,
demonstrates a trend consistent with the D1-like receptors.

Interestingly, our observed relative dopamine receptor mRNA levels conflict with
previous literature. Meador-Woodruff et al. (1994) estimated that hippocampal Drd2,
Drd4, and Drd5 levels were roughly equal, while Drd3 expression was 2- to 5-fold lower,
and Drd1 mRNA levels were 2- to 3-fold lower still. Likewise, Khan et al. (1998) stated
that Drd4 mRNA is the most prevalent inhibitory dopamine receptor type in the
hippocampus. An examination of mean mRNA level across the entire population for each
dopamine receptor subtype in the current study, however, demonstrated that this was not
the case. Our results indicated that Drd2 was the most abundant, followed closely by
Drd1. Drd5 was approximately -1.5-fold lower than Drd1, and by comparison to the other
subtypes, the levels of both Drd3 and Drd4 were negligible (Fig. 16). Although the
current study provides no non-stressed control for comparison, these studies may suggest
that chronic stress changes the relative ratios of hippocampal dopamine receptors.

Animals were restrained for one hour on the morning of the final day, prior to the
collection of tissues following euthanasia. Based on previous studies, such as that
described by Jankord and Herman (2008), we believe that exposure to this novel stressor
led to changes in the mRNA expression profile. Thus, the mRNA expression profiles
described in this study represent an interaction between the previous chronic stress and
this final acute stressor, which immediately preceded tissue collection. Because tissue
samples were collected approximately one hour after the final stressor, changes in immediate early genes (IEGs), like *Nurr1*, were likely due, at least in part, to the acute stress response. While this timeframe likely captured changes in IEGs, we would not necessarily expect to see changes in downstream genes, such as *Th* and *Dat1*, which require additional time for transcriptional changes to filter down the signaling cascades.

![Average mRNA Expression of Dopamine Receptors Across Experimental Population](image.png)

Figure 16: The observed ratios of hippocampal dopamine receptor mRNA levels are not in agreement with previous literature which suggests *Drd4* is the most abundant subtype mRNA. Error bars denote standard error.

**Final Thoughts**

This study demonstrates that hippocampal expression of *Nurr1* predicts behavioral performance in mice. Our results show that *Nurr1* mRNA transcription levels predict performance during tests of spatial and emotional memory. We did not observe a statistically significant relationship between other dopaminergic genes measured (*Dat1*, **42**
and five subtypes of dopamine receptor) and behavioral performance. Overall, this study is consistent with previous literature that suggests a role for NURR1 in memory, and provides evidence for genetic variability in its regulation. However, it also provides preliminary evidence that NURR1 may also modulate memory-related abilities by some mechanism other than regulation of Dat1 and TH transcription, which may be independent of dopaminergic regulation entirely.
V. REFERENCES


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