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mRNA EXPRESSION ANALYSIS OF MIDBRAIN DOPAMINE NEURONS IN RESPONSE TO NICOTINE INTAKE DURING MATURATION

A Dissertation

Presented to

the Faculty of the Department of Biomedical Engineering

University Of Houston

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

in Biomedical Engineering

by

Pinar Kanlikilicer

December 2013

mRNA EXPRESSION ANALYSIS OF MIDBRAIN DOPAMINE NEURONS IN RESPONSE TO NICOTINE INTAKE DURING MATURATION

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DEDICATION

This dissertation is dedicated to the three most important people in my life. First of all, I dedicate my dissertation to my mother Banu Gurel, to whom I owe everything, for all of her sacrifices, her love and her unwavering support throughout my studies and my whole life. Secondly, to my father; Ahmet Kanlikilicer, for treating me like his princess for 29 years. Last but not least, I dedicate my dissertation to my twin brother Alp Emre Kanlikilicer, whose love and support has been with me for 29 years, 9months and 10 days…

Thank you all for your love, support, and sacrifice throughout my life…

ACKNOWLEDGEMENTS

I would like to express my sincere respect and deepest gratitude to my advisor, Prof. Metin Akay who gave me a great opportunity to mature scientifically. I would like to thank my dissertation committee members Dr. Yasemin Akay, Dr. Andrei Dragomir, Dr. Ahmet Omurtag, and Dr. Cecillia Williams for the time they have denoted and for providing many insightful ideas. I would also like to offer my special thanks to Dr. Yasemin Akay for her guidance, understanding, and most importantly, her kind and warm attitude during my graduate studies at University of Houston.

I would like to express my sincere and profound thanks to Dr. Andrei Dragomir and Dr. Laura Gutierrez for their help and also for their invaluable guidance throughout my PhD study. I would also like to thank Dr. Die Zhang for the training and help with the experiments and manuscripts and Dr. Ting Chen and Allyson Plosko for their guidance as the academic advisors of the department.

I would like to thank Dr. Xiaolian Gao for letting me use her microarray scanner and Anne Katchy for her sincere guidance on microarray experiments.I wish to express my warm thanks to Allison Post and Jessica Diefenderfer for their help with the language of the dissertation.

My sincere thanks go to Didem Yamak for her friendship and support during my first year in Houston. I would also like to thank all my current and former lab mates in Dr. Akay's lab, especially Naze Avci, for their help and support. I give my special thanks to my best friends, Yasemen Gungormez and Ayse Ezgi Akkaya. They never let me

down during my tough times in Houston despite the long distance. Thank you for the sisterhood especially for the past six years.

I devote my deepest gratitude to my family for their unlimited love and support. Finally, I would like to thank Muhlis Unaldi for his never ending encouragement and support for the most stressful times of my Phd and life. Without his patience, love and endless support this dissertation wouldn't have been written.

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An Abstract

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ABSTRACT

Addictive substances including cocaine, nicotine and amphetamine exert their reinforcing properties through the dopamine system. The majority of dopamine (DA) neurons are located in the ventral midbrain comprising of the substantia nigra (SN) and the ventral tegmental area (VTA). Nicotine has been suggested as both neuroprotective and neurotoxic on the central nervous system (CNS). For many neurological disorders, nicotine has been found to be protective against neurodegeneration in Alzheimer's disease, dopaminergic cell death in SN of patients with Parkinson's disease (PD) and neurodegeneration in Huntington's disease. On the other hand, nicotine was shown to be neurotoxic on the developing brain by leading to structural alterations in the brain regions involved in cognition, learning and memory.

Previous studies suggested that the expression of neuroprotective genes was significantly different in the VTA and SN dopamine neurons and nicotine mediated neuroprotection in PD. These studies encouraged us to investigate how nicotine changes the expression of these differentially expressed neuroprotective genes (PACAP, LPL and GRP) in the VTA and the SN at different age stages. Our results suggested that only the LPL gene significantly differentially expressed between the VTA and the SN in the 24-month old rats. In addition, nicotine treatment did not show any up-regulatory effects on the expression of neuroprotective genes in rats after 12-months maturation.

We also investigated the whole-genome expression of midbrain dopaminergic and non-dopaminergic neurons in response to gestational nicotine in newborn rats (PN 7-14). Patch-clamp electrophysiology was used to select and collect DA and non-DA neuron in

newborn rats during the first week of maturation. Then, we use microarray technique to analyze the gene expression of these neurons in response to gestational nicotine.

We identified a set of 135 genes identified as significantly differentially expressed between dopaminergic and non-dopaminergic neurons upon exposure to gestational nicotine. The roles of the genes previously implicated in Alzheimer's Disease, Parkinson's Disease, Huntington's Disease, arachidonic acid and linoleic acid metabolism were identified. We also identified significantly regulated genes by gestational nicotine in DA and non-DA neurons. These specific genes in DA neurons could be paramount in developmental processes.

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CHAPTER 1: INTRODUCTION

1.1 Dopamine System

Dopamine is a catecholamine neurotransmitter that controls many biological functions such as cognition, learning, motor control, motivation, emotion and positive reinforcement (Berridge, 2007; Nieoullon, 2002; Satoh et al. 2003; Wise, 2009). It also plays significant roles in the etiology of many neurodegenerative diseases such as Parkinson's disease (PD), Huntington's disease (HD) and Schizophrenia.

DA release is triggered by drugs such as cocaine, amphetamine and nicotine. Mesocorticolimbic DA systems mediate reinforcing properties of drugs (Volkow et al. 2007; Young et al. 2011). The mesocorticolimbic system consists of dopaminergic neurons of ventral tegmental area (VTA) of the midbrain and their dopaminergic projection areas of the nucleus accumbens (NAcc), prefrontal cortex (PFC) and amygdala. NAcc dopamine release was shown to be affected by drugs such as amphetamine and fetal acute nicotine exposure by several studies (Balfour, 2009; Di Chiara et al. 2004; Kane et al. 2004a; Wonnacott et al. 2005). Long term drug exposure was shown to decrease the size of the VTA neurons and lead to dendritic outgrowth in NAcc (Nestler, 2005; Sklair-Tavron et al. 1996); (Brown & Kolb, 2001; McDonald et al. 2005).

Drugs exert their reinforcing properties through dopamine receptors which mediate physiological functions of the dopamine. The effects of dopamine are terminated by the action of dopamine transporters by reuptaking dopamine into presynaptic nerve

endings. Methylphenidate and cocaine increase DA concentration by blocking DA transporters, whereas amphetamine increases DA release through DA transporters (DAT). (Izenwasser et al. 1 999; Pierce & Kalivas, 1997; Pierce & Kumaresan, 2006; Volkow et al. 2002). DAT activity regulates the extracellular dopamine concentrations (Ciliax et al. 1995). DAT was suggested as a potential target for the treatment of PD symptoms (Nutt et al. 2004). Reduced DAT expression was also reported in chronic schizophrenic patients (Laakso et al. 2001).

1.1.1 Dopamine Receptors

Dopamine receptors belong to the large G protein-coupled receptor family and consist of seven transmembrane domains. The amino acid sequence of seven transmembrane domains are conserved (Jackson & Westlinddanielsson, 1994; Seeman & Vantol, 1993). Five dopamine receptors have been identified (D1–D5) and they can be divided into 2 types (D_1, D_2) (Table 1.1). D_3, D_4, D_5/D_{1b} are the subtypes of D_1 and D_2. $D_1$and D_5/D_{1b} receptors are classified as D_1-like, likewise, D_2, D_3, and D_4 receptor subtypes classified as D2-like based on their ligand-binding characteristics and genetic homology (Gingrich & Caron, 1993; Sokoloff & Schwartz, 1995). D1-like receptors activate adenylate cyclase, whereas inhibition of adenylate cyclase is the dominant characteristic of D2 like receptors (Missale et al. 1998). Dopamine receptor functions are complex in nature since, in addition to five dopamine receptor genes, there are also multiple splice variants of D_2-like family (Monsma et al. 1989).

Different dopamine receptor types are localized in different brain regions. D1 receptors are concentrated in cortical regions, whereas D2 receptors have a higher distribution in

striatum. D3 and D4 receptors are found mostly in the limbic system, whereas D5 receptors density is higher in hippocampal region (Remington, 2008).

There has been a special interest on D2-like receptors since the D2 receptor blockade is involved in antipsychotic activity (Guillin et al. 2007; Kapur & Remington, 2001). D_1 and D_2 receptors have been shown to enhance arachidonic acid release through signaling cascades (Piomelli et al. 1991). The D_2-like receptors, with the exception of D3, also mediate the inhibition of calcium currents and ultimately alter intracellular Ca^{2+} levels (Seabrook et al. 1994). D1 receptors are linked to memory and cognition, and they are thought to be involved in negative cognitive symptoms of schizophrenia (Abi-Dargham, 2003; El-Ghundi et al. 2007; Weinberger, 1987). D1-like receptors are thought to be involved in reward-related learning since evidence suggests the activation of adenylate cyclase mediates the dopamine related learning process (Konradi et al. 1994; Kotter, 1994). It was also shown that cognitive training in human alters cortical D1-like receptor binding (McNab et al. 2009).

Table 1.1 Dopamine receptor subtypes and their properties (Strange, 2000).

Property	D1-like		D2-like		
	D1	**D5**	**D2**	**D3**	**D4**
Physiological Functions	-Motor function, -Cardiovascular function		-Motor function -Behavior -Control of prolactin -αMSH secretion from pituitary -Cardiovascular function		
Homology (%) with D1 with D2	100 44	82 49	44 100	44 76	42 54
Localization	-Caudate nucleus -Putamen -Nucleus accumbens -Olfactory tubercle -Hypothalamus -Thalamus - Frontal cortex	-Hippocampus -Thalamus -Lateral mammillary -Nucleus -Striatum -Cerebral cortex	-Caudate nucleus -Putamen -Nucleus accumbens -Olfactory tubercle -Cerebral cortex	-Nucleus accumbens -Olfactory tubercle -Cerebral cortex -Islands of calleja putamen	-Frontal cortex -Midbrain -Amygdale -Hippocampus -Hypothalamus -Medulla -Retina
Response	Adenylyl cyclase ↑	Adenylyl cyclase ↑	Adenylyl cyclase ↓	Adenylyl cyclase ↓	Adenylyl cyclase ↓

1.1.2 Dopamine Populations

Dopamine populations in the mammalian brain are located in three main regions. The majority of dopaminergic neurons are located in the ventral midbrain (mesencephalon) comprising substantia nigra pars compacta (Snc), VTA and retrorubral field (RrF) (Figure 1.4). There are almost 45,000 neuronal cells in rats, whereas human brain contains approximately 590,000 midbrain dopaminergic neurons (Vernier et al. 2004). Besides the midbrain dopaminergic neurons, there are some small dopamine populations present in diencephalon and telencephalon.

Dopamine neurons also have characteristic projection areas as well as having stereotyping positions in the brain (Figure 1.1). (Dahlstroem & Fuxe, 1964; Fallon, 1981). SNc dopaminergic neurons project to the dorsolateral striatum and caudate putamen which is called the nigrostriatal pathway. VTA neurons project to the nucleus accumbens, amygdala and olfactory tubercle which is called the mesolimbic pathway, and they also project to the prefrontal cortex which is the mesocortical pathway. Each dopamine population together with their projection areas are involved in the modulation of different brain functions. SNc dopaminergic projections to striatum are involved in voluntary movement and habit learning, whereas VTA dopaminergic projections to the mesocortical and mesolimbic systems are involved in the modulation and control of cognitive, emotional and rewarding behaviors (Prakash & Wurst, 2006). Mesolimbic and nigrostriatal dopamine projections are crucial to sensorimotor function and therefore are involved in reward (Koob & Le Moal; 2001; Wise, 1998). Nigrostriatal pathway is primarily associated with Parkinson's disease. Striatum contains both excitatory and inhibitory dopamine receptors (Ohno et al. 1987). While the inhibitory receptors are located in both pre- and postsynaptic, the excitatory receptors are postsynaptic. These receptors mediate the regulation of functions related to control and initiation of motor plans. Mesolimbic and the mesocortical pathways have been linked to reward, motivation, addiction and schizophrenia.

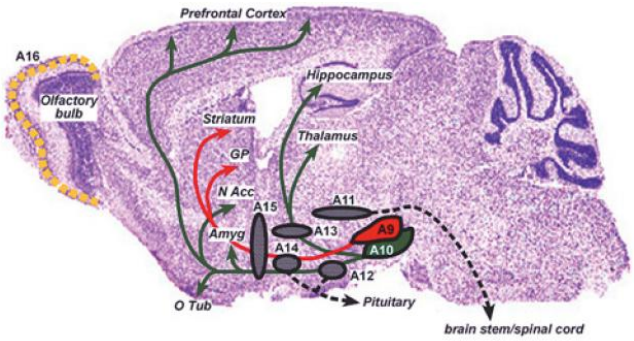

Figure 1.1 Stereotyping positions and projection areas of dopaminergic neuron populations in brain (Prakash & Wurst, 2006).

1.2. Dopamine and Neurodegenerative Diseases

1.2.1 Alzheimer's Disease

The worldwide estimated spread of dementia has an increasing trend starting from 35.6 million people in 2010, 65.7 million in 2030, and 115.4 million people in 2050 (Table 1.2). Alzheimer's disease (AD) is one form of dementia, and it is one of the most common neurodegenerative diseases that deteriorates cognitive functions and affects the learning and memory associated brain regions, such as the neocortex and the hippocampus.

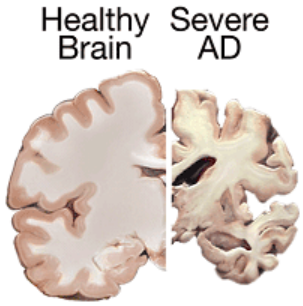

Figure 1.2 Neuronal cell loss leading to shrinkage in the brain of patients with Alzheimer's disease (right).

Cholinergic and the dopaminergic system impairments are the main outcomes of pathological changes seen in AD (DeKosky, 2002; Kemppainen et al. 2000). Dysfunction of the neurons from cholinergic system is the major feature characterizing AD. The treatment of AD is based on the drugs supporting and enhancing acetylcholine neurotransmission. On the other hand, the dopaminergic system, which has strong synaptic interaction with acetylcholine in different brain areas, may also play an important role in processes related to learning and memory (Blokland, 1995; Brooks, 2006; Cao et al. 2005; Di Cara et al. 2007; Millan et al. 2007). More than 30% of AD patients exhibit dopamine system deficiency with 40% to 70% decline in striatal dopamine (DA) neurons with lewy bodies (Joyce et al. 1998; Tyrrell et al. 1990; Walker et al. 2002). Dementia with lewy bodies is one form of dementia that has common characteristics with Alzheimer's disease. AD affects many neurotransmitter systems because many different brain areas such as limbic structures, subcortical nuclei, and cortical regions are all affected.

Extrapyramidal signs such as akinesia and akathisia are often associated with AD and leads to faster cognitive decline and higher mortality (Rosen & Zubenko, 1991). In

AD brains, impaired dopamine transport was observed in the nucleus accumbens (Murray et al. 1995). Experimental studies revealed that hippocampal dopamine 2 (D2) receptor activities has an important role in memory performance (Kemppainen et al. 2003; Umegaki et al. 2001; Wilkerson & Levin, 1999).

Pathological changes of Alzheimer's disease include β-amyloid and senile plaque deposits as well as alterations of several neurotransmitter systems (Selkoe, 1989; Sisodia & Price, 1995). The serotonergic and noradrenergic systems seem to be strongly altered as well as cholinergic systems (Aletrino et al. 1992; Chen et al. 2000; Kovacs et al. 2003; Langlais et al. 1993; Reinikainen et al. 1988; Zaborszky & Cullinan, 1996). In addition, the dopaminergic system is also implicated in AD (Allard et al. 1994; McNeill et al. 1984). Reduced level of dopamine transporters in NAcc and altered dopamine receptors indicate the dopamine content deficits in dopaminergic system as the pathology of AD patients (Kumar & Patel, 2007; McNeill et al. 1984). Different dopaminergic neurons also have different patterns of damage for AD. It has been previously shown that dopaminergic cells of the VTA are severely affected in Alzheimer's disease, whereas those of SN are much less damaged (Mann et al. 1987).

1.2.2 Parkinson's Disease

Age-related PD is one of the most common movement disorder affecting millions over the age of 55. It is characterized by rigidity, tremor, bradykinesia and possibly dementia (Olanow & Tatton, 1999) which are caused by the degeneration of dopaminergic neurons within the substantia nigra pars compacta (Figure 1.3). Parkinson's disease is the most common neurodegenerative disorder after AD. The prevalence of PD

is associated with age, with a greater than 40-fold increase between the ages of 55 and 85 (Fahn & Sulzer, 2004).

PD is characterized by the death of DA neurons of SN. It was demonstrated that there is an 80% loss of neuromelanin pigmented DA neurons in the SN compared to neighboring ventral tegmental neuromelanin pigmented DA neurons (German et al. 1989; Hirsch et al. 1988).

The symptoms of PD can be treated by the L -DOPA which is a DA precursor. L-DOPA is used to increase dopamine concentrations in the synaptic vesicles of DAergic terminals to increase the quantal size of dopamine (Sulzer & Pothos, 2000). Once L-DOPA has entered the central nervous system, it is converted to dopamine by the aromatic L-amino acid decarboxylase and compensate for the innervation. L-DOPA is the most effective drug for the treatment of the motor symptoms of Parkinson disease (Fahn, 2008). Another agent which imitates the clinical effect of dopamine on motor symptoms of PD is the dopamine agonists which stimulate dopamine receptors (Goetz, 1990). The most common dopamine agonists are pramipexole, ropinirole, rotigotine, and apomorphine. However, dopamine agonists are less effective than L-DOPA.

Previous studies have shown that lesions of substantia nigra (SN) induced by 6-hydroxydopamine (6-OHDA) which is a chemical selectively destroy dopaminergic neurons, resulted in memory deficits and increased brain oxidative stress in an animal model of Parkinson's disease (Hritcu et al. 2008). Epidemiologic studies have suggested

Table 1.2 Estimated number of people with dementi and proportionate increases by Global burden of disease world region (Wimo & Prince, 2010)

GBD Region	Over 60 populations	Number of people with dementia			Proportionate increases (%)	
	2010	**2010**	**2030**	**2050**	**2010-2030**	**2010-2050**
ASIA	406.55	15.94	33.04	60.92	107	282
EUROPE	160.18	9.95	13.95	18.65	40	87
THE AMERICAS	120.74	7.82	14.78	27.08	89	246
AFRICA	71.07	1.86	3.92	8.74	111	370
WORLD	758.54	35.56	65.69	115.38	85	225

that cigarette smoking is a strong negative risk factor for the development of PD (Dorn, 1959; Fratiglioni & Wang, 2000; Gorell et al. 1999; Morens et al. 1996). A study showing the effects of low-dose nicotine treatment in rat model of PD indicated that there are cognitive and oxidative stress improvements in response to nicotine (Ciobica et al. 2012).

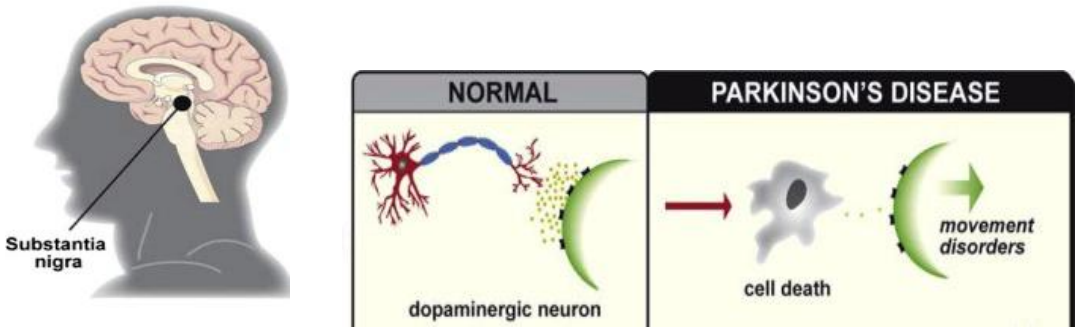

Figure 1.3 Loss of dopaminergic neurons in substantia nigra of a Parkinson's patient (modified from Ruiperez et al. 2010).

1.2.3 Huntington Disease

Huntington's disease (HD) is a neurodegenerative genetic disorder which is characterized by chorea, motor impairment, psychiatric manifestations, and dementia. Huntingtin (HTT) was the first HD-associated gene linked to a polymorphic DNA marker that maps to human chromosome 4 (Gusella et al. 1983).

Abnormal Ca+2 signaling and selective and progressive neuronal loss of the GABAergic medium spiny neurons in the striatum are the main pathology of the HD (Bezprozvanny & Hayden, 2004; Vonsattel & DiFiglia, 1998). Atrophy of the neostriatum is the main morphologic sign of HD and is used to grade neuropathological severity of the disease (Vonsattel et al. 1985). Dopaminergic signaling also plays an important role in HD. The dopamine signaling pathway acting together with glutamate signaling was shown to mediate Ca+2 signaling and induce apoptosis of HD medium spiny neuron (MSNs), and dopamine inhibitors were demonstrated as protectors of HD MSNs from cell death (Tang et al. 2007). L-DOPA has been used for diagnostic testing and symptomatic treatment in Huntington's disease. L-DOPA treatment has been shown to improve the psychiatric and neurological symptoms in patient with Huntington's chroe (Loeb et al. 1976).

Dopamine receptor D2 gene was also shown to be reduced in basal ganglia by PET scanning at the onset of the disease (Andrews et al. 1999; Glass et al. 2000). Animal models for HD also exhibited dopamine receptor 2 expression reductions early in the progression of HD pathology (Cha et al. 1999; Luthi-Carter et al. 2000).

1.2.4 Schizophrenia

Schizophrenia is a chronic and severe mental disorder detected during late adolescents and early adulthood. Economic burden of schizophrenia was reported as $62.7 billion in which $22.7 billion is the excess direct health care cost, $32.4 billion is the total indirect excess cost and 7.6 billion is the total direct non-health care excess costs (Wu et al. 2005).

Negative, positive and cognitive symptoms are associated with schizophrenia. Cognitive symptoms such as deficits in attention and memory are distinctive marks of the illness. Positive symptoms include delusions, hallucinations and disorganized thoughts whereas avolition, alogia (poverty of speech) and anhedonia are considered as the negative symptoms (Blanchard & Cohen, 2006; Kirkpatrick & Fischer, 2006).

The original dopamine hypothesis explains schizophrenia with the excess of dopamine in the brain of schizophrenics. In 1988, the new dopamine hypothesis introduces “dopamine deficiency” in the cortex while the excess of dopamine hypothesis was still valid for the subcortical regions. The positive symptoms are associated with excess of dopamine, whereas the negative and cognitive symptoms of schizophrenia are related to dysfunction of the dorsolateral prefrontal cortex (DLPFC) (Figure 1.4). The mesolimbic pathway is most closely associated with the positive symptoms. Projection of the dopaminergic neurons of the mesocortical pathway plays role in the cognitive, as well as negative, marks of schizophrenia.

In the 1950s, usage of chlorpromazines (CPZ) radically improved the knowledge of and curability of schizophrenia. It is the first compelling cure for the illness

and pointed out that schizophrenia was biologically mediated (Deniker, 1989; Deniker, 1990). CPZ was shown to mediate its antipsychotic activity by acting on D2 receptors and blocking them (Carlsson & Lindqvist, 1963; Seeman et al. 1976). Evidence from functional brain imaging studies exhibited that the negative and cognitive symptoms might arise from altered prefrontal cortex (PFC) functions (Karlsson et al. 2002; Okubo et al. 1997). The presence of D1 receptors in cortical regions prompts scientist to investigate their association with the cognitive and negative symptoms of schizophrenia (Abi-Dargham, 2003; El-Ghundi et al. 2007; Weinberger, 1987). Insufficient D1 receptor signaling in DLPFC negatively affects the relationship between dopamine function and the integrity of working memory. Stimulants that increase the D1 receptor functions are able to restore the cognitive function in schizophrenia (Goldman-Rakic et al. 2004). Central DA receptor blockade by chlorpromazine and haloperidol was the mechanism of their antipsychotic action(Carlsson & Lindqvist, 1963).

Dopamine neuron deficiency in the cortex results in dysfunctionality of the D1 receptors in the cortex. D1 receptors are the most conspicuous dopamine receptors, which are much denser than D2 receptors in the cortex. This deficiency in D1 functions causes negative symptoms and cognitive deterioration. Contrarily, positive symptoms result from the over stimulation of sub- cortical superfluous dopamine neurons on D2 receptors. D2 elevation in schizophrenia and D2 blockade in relation to antipsychotic activity has also been extensively studied and it has been asserted that D2 blockade is essential for antipsychotic activity (Gingrich & Caron, 1993; Guillin et al. 2007; Kapur & Remington, 2001; Seeman, 1987; Seeman et al. 1976; Sokoloff & Schwartz, 1995).

The dopamine hypothesis has been questioned since, even though remedial doses of most antipsychotics employ 60% to 80% of the D2 receptors in patients, with the exceptions of Clozapine and quetiapine, which has clinical capability with only 10% to 45% occupation of D2 receptors (Seeman & Tallerico, 1999).

There are some suggestions about enlarging the dopamine hypothesis of schizophrenia into a serotonin-dopamine hypothesis which states that the enhanced neurotransmission in sub-cortical areas in schizophrenia that leads to positive symptoms are dopaminergic and serotonergic neurotransmission rather than dopaminergic only. Likewise, decreased dopaminergic and serotonergic neurotransmission in the prefrontal cortex together lead to negative symptoms.

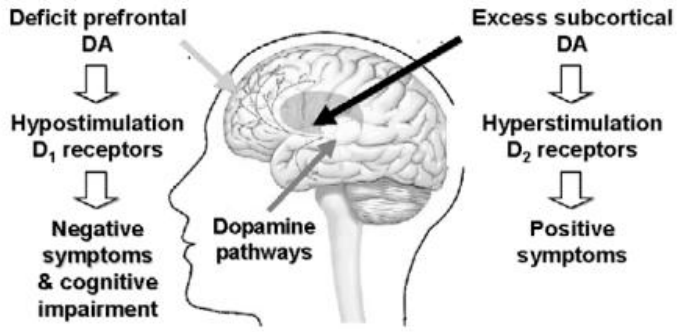

Figure 1.4 Dopaminergic imbalance in schizophrenia (Abi-Dargham, 2002).

1.3 Nicotine and Nicotine Related Neurodegenerative Diseases

1.3.1 Nicotine

Smoking affects a large proportion of the population from the very early to late period of life. In the United States, 20.1% of adults ages 18–24 years, 22.0% of adults ages 25–44 years, 21.1% of adults ages 45–64 years and 9.5% of people ages 65 years and older were reported as current smokers (Centers for Disease Control and Prevention, 2011). In addition, 19.5% of high school students and 5.2% of middle school students smoke cigarettes (Eaton et al. 2010). Approximately 3,800 young people under the age of 18 start smoking and over 1,000 of them become daily cigarette smokers (Substance Abuse and Mental Health Services Administration, 2010). Smoking rates among persons with psychiatric or mental disorders are about two times greater than other persons (Lasser et al. 2000).

Table 1.3 Schizophrenia, dopamine, symptoms and side effects (Remington, 2008)

Dopamine pathway	Symptom domains/ side effects	Clinical features	Receptors	Dopamine
Mesolimbic	Positive	Thought disorder, e.g. disorganization Thought content, e.g. paranoia Perceptual disturbances, e.g. hallucinations	D_2, D_3	↑
	Negative	Amotivation, apathy Anhedonia		↓
Mesocortical	Cognitive	Neuro, e.g. attention, concentration, memory Executive function Social, e.g. face/emotion recognition Decision making	D_1	↓
	Negative	Amotivation, apathy Anhedonia	D_1, D_2	↓
Nigrostriatal	Motor/negative/ cognitive	Antipsychotic-related extrapyramidal symptoms (EPS) Secondary negative and cognitive symptoms	D_2	Postsynaptic receptor blockade
Tuberoinfundibular	Endocrine	Antipsychotic-related hyperprolactinemia	D_2	Postsynaptic receptor blockade

Nicotine is the major psychoactive and addictive component in tobacco which mediates the release of neurotransmitter dopamine and effects the cholinergic nicotinic receptors in both the central and peripheral nervous system. Systemic nicotine has been shown to enhance dopamine release in the nucleus accumbens by stimulation of VTA dopamine neurons (Benwell & Balfour, 1997; Imperato et al. 1986; Mereu et al. 1987) but substantially less in SNc.

Evidence from human and animal models suggests that all drug abuse acts on brain's limbic system (Di Chiara et al. 2004; Dichiara & Imperato, 1988; Pierce & Kumaresan, 2006). The VTA is one of the most important brain regions with regard to the reinforcing effects of nicotine (Nestler, 2005).

Animal and human in vivo studies showed that nicotinic acetylcholine receptors (nAChRs), which are ligand-gated ion channels consisting of α and β subunits, are altered as a result of nicotine exposure (Sabbagh et al. 2002). Upon nicotine administration, decrease in oxidase A and B activity levels in basal ganglia was observed as well as decreased α4β2 nicotinic acetylcholine receptor levels in thalamus and putamen. It is likely that nAChR might play a role in the pathogenesis of some neurological diseases. α6 subtype is suggested to be involved in pathophysiology of Parkinson's disease (Perez et al. 2010) whereas α4 and α7 nAChR expressions altered in AD patients (Burghaus et al. 2000; Guan et al. 2000).

Several studies have linked the association of smoking with Parkinson's, Alzheimer's Disease (AD) and dementia (Checkoway et al. 2002; Lee, 1994; Vanduijn et al. 1994). Nicotine improves several cognitive functions such as attention, working memory and executive functions, indicating that nicotine might be used as medicinal

purposes for CNS disorders. The most commonly reported cognitive effects of cigarette smoking are mediated through enhanced neurotransmission between cortico- basal ganglia- thalamic circuits either by nAChRs or dopamine release stimulation or monoamine oxidase (MAO) inhibition or combination of all of these factors (Brody et al. 2006; Sharma & Brody, 2009). However, there are many outcomes related with nicotine such as cardiovascular and pulmonary diseases, developmental neurotoxicity and hyperphosphorylation of tau protein (Figure 1.5).

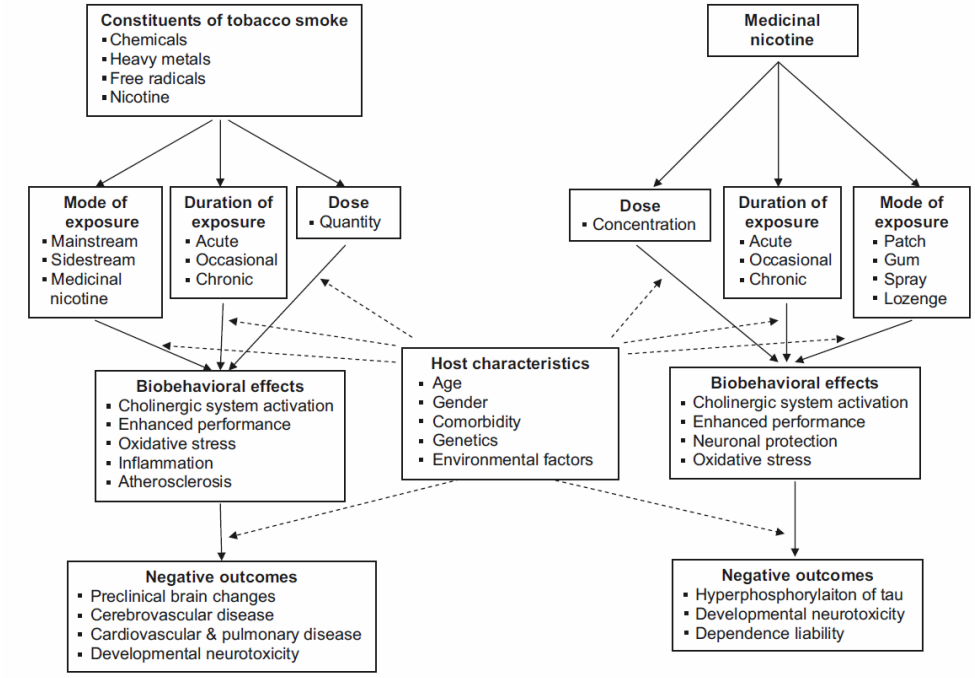

Figure 1.5 The pathways through which smoking and nicotine itself affect neurological outcomes negatively or positively (Swan & Lessov-Schlaggar, 2007).

1.3.2 Nicotine and Neurodegenerative Diseases

1.3.2.1 Nicotine and PD

Midbrain dopamine neurons are associated with one of the most prominent neurodegenerative disorders, Parkinson's disease. This is characterized by the selective degeneration of dopaminergic neurons in SN. Ventral tegmental area (VTA) dopamine neurons are much less affected in PD, which is an observation that is consistent for both humans and animal models of the Parkinson's Disease (Dawson et al. 2002; Greene, 2006). Currently, L-DOPA is widely prescribed as the best treatment for PD, although it doesn't provide long-term protection and has several side-effects (Yacoubian & Standaert, 2009).

Several epidemiological studies showed that there is a negative association between cigarette smoking and PD (Dorn, 1959; Fratiglioni & Wang, 2000; Gorell et al. 1999; Morens et al. 1996). PD patients were shown to be 50% less likely to have smoked cigarettes during their lifetime than of age- and gender- matched controls. This may suggests that cigarette smoking protects against neurodegeneration. An experiment was conducted with rotenone induced toxicity in mice showed that the striatum and substantia nigra neurons were protected from the toxicity by nicotine (Takeuchi et al. 2009). Neuroprotective effect of nicotine depends on the dose and the activation nAChRs of upon Parkinsonian-like damage in vivo. (Ryan et al. 2001). There have been several attempts to characterize the differences in nAChR expression in VTA and SN (Keath et al. 2007; Klink et al. 2001; Wooltorton et al. 2003). It is thought that the activation of

signaling pathways by nAChR leads to neuroprotection through inhibition of apoptosis and induced up-regulation of neurotrophic factors (Mudo et al. 2007).

The mechanism how nicotine protects against PD is speculative since it is still not known whether nicotine just relieve the symptoms of the disease or provide true neuroprotection. Chronic high doses of nicotine, smoking, nicotine patches and nicotine gum were shown to improve motor scores and symptoms such as tremor, disorganized thinking, bradykinesia and increased energy in patients with PD (Fagerstrom et al. 1994; Villafane et al. 2007). However, there are also many opposite results in terms of the effect of nicotine therapy on motor and cognitive deficits in PD (Ebersbach et al. 1999; Jacobsen et al. 2006; Kelton et al. 2000; Lemay et al. 2004; Vieregge et al. 2001).

1.3.2.2 Nicotine and AD

Cholinergic neuron loss in the basal forebrain and cholinergic innervations of the cerebral cortex were reported in Alzheimer's disease (AD) patients (Francis et al. 1999; Perry et al. 1994). nAChRs has been studied with regard to AD (Toyohara & Hashimoto, 2010). The reduced amount of high-affinity nAChRs (mostly α4β2) are associated with the AD progress (Sugaya et al. 1990). Neuroprotection mediated by drugs interacting with neuronal nAChR such as nicotine is thought to be the possible mechanism for memory, learning and attention deficit improvements (O'Neill et al. 2002). Smoking is suggested to protect against AD based on the fact that there is an inverse relationship between smoking and early onset AD (Vanduijn & Hofman, 1991). Evidence suggested that nicotine administration improves prospective memory and cognition following nicotine administration (Newhouse et al. 2004; Rusted & Trawley, 2006). A meta-analysis study restricted to people aged 65 and over, revealed that smoking increases the

risk of AD and probably other dementias and cognitive decline (Peters et al. 2008). Despite of nicotine's neuroprotective effect, the role of nicotinic acetylcholine receptors in the pathophysiology of AD has been speculated (Wu et al. 2010). Better understanding on the role of nAChRs and neuroprotective function of nicotine is required to treat AD.

1.3.2.3 Nicotine and Schizophrenia

In the United States, nicotine-dependent and psychiatrically ill individuals consume around 70% of all cigarettes smoked in whole populations (Grant et al. 2004). A study of schizophrenic patients showed that they spent $142.50 per month on cigarettes which corresponds to 27.36% of their total income of the schizophrenic participants (Steinberg et al. 2004). Several studies examined the association between cigarette smoking and schizophrenia symptoms, in order to elucidate the reason behind the high prevalence of cigarettee smoking among schizophrenic patients (Dervaux & Laqueille, 2008; Patkar et al. 2002).

Evidence suggested that nicotine may help to reduce negative symptoms which is defined by the hypodomanergic state, by increasing dopamine in the brain (Glassman, 1993). Moreover, reduced smoking rates of schizophrenic patients using clozapine, which decrease the negative symptoms, was reported (George et al. 1995). On the other hand, nicotine's action on dopamine system was shown to worsen positive symptoms of schizophrenia (Lohr & Flynn, 1992). There is also other evidence supporting the positive association between smoking and the negative and cognitive symptoms of schizophrenia and, but positive symptoms (Patkar et al. 2002). Sensory gating deficits seen in schizophrenia patients may also result from the interaction between altered expression

and function of the α7 nicotinic cholinergic receptor, with dopamine and glutamate systems (Adler et al. 1998).

1.3.2.4 Gestational Nicotine Exposure

Maternal smoking during pregnancy is associated with low birth weight, increased risk of stillbirth, conduct disorder, attention-deficit/hyperactivity disorder (ADHD) and neurocognitive deficits. (Fried et al. 1992; Hill et al. 2000; Kahn et al. 2003; Kurtoglu et al. 2007; Neuman et al. 2007; Salihu et al. 2008; Thapar et al. 2006; Wakschlag et al. 1997; Weissman et al. 1999). Maternal smoking during pregnancy is also associated with reduced growth of the fetal head with human studies both for full-term and preterm infants (Ekblad et al. 2010; Roza et al. 2007). Nicotine can cross the placenta into the fetal serum and brain. Offspring are not only affected by nicotine during pregnancy, but also affected after the birth through breast feeding.

The nicotine concentration in milk was found to be more than double that of the corresponding serum concentration (Luck & Nau, 1984). Prenatal nicotine exposure was shown to cause structural and morphological alterations in the brain regions involved in cognition, learning, and memory (Roy & Sabherwal; 1998; Roy et al. 2002).

Evidence suggested there are neurotoxic effects on a developing brain from both maternal smoking and second-hand smoke (Swan & Lessov-Schlaggar, 2007). Prenatal nicotine exposure was shown to increase liability of drug abuse in the offspring by altering the development of neural systems that regulates motivation (Buka et al. 2003; Cornelius & Day, 2009).

Osmotic mini pumps releasing nicotine in pregnant rats decreased dopamine release in the cerebral cortex of the pups (Navarro et al. 1988). Whereas, forebrain dopamine release was shown to increase in PN15 days old pups exposed to nicotine from gestational days 12 through 18 to 19 (Ribary & Lichtensteiger, 1989). Another study showed that gestational nicotine exposure decreased dopamine content in nucleas accumbens and striatum in PN 22 day male rats (Richardson & Tizabi, 1994). Gestational nicotine exposure was also shown to lead reduced NAcc dopamine release, while the base line extracellular and total dopamine contents remain the same (Kane et al. 2004a). These results suggest that gestational nicotine exposure reduces the sensitivity of mesolimbic dopaminergic system and therefore affects the different regions of the developing brain.

Nicotine changes the brain chemistry in developing brain. nAChR expression is altered in gestational nicotine exposed adolescent. In VTA, gestational nicotine exposure was found to significantly decrease α3, α4, α5 and β4 subunit nAChR which indicates nicotine induced changes in nAChr expression (Chen et al. 2005). Third trimester equivalent of human gestation was indicated as the most sensitive period of brain development at which brain biochemistry changes by the regulation of cholinergic biomarker expressions (Nunes-Freitas et al. 2011).

1.4 Nicotine Mediated Neuroprotection and Neurotrophic Factors

Nicotine has been suggested as neuroprotective for many neurological disorders. It protects against neurotoxicity in AD, dopaminergic cell death in PD and neurodegeneration in Huntington's disease (Liu et al. 2007; Quik et al. 2012; Tariq et al. 2005).

Nicotinic acetylcholine receptors (nAChRs) are considered a promising therapeutic agent for the management of various neurodegenerative diseases. Either up-regulation of nicotine receptors that are deficient in AD patients or αβ-induced neurotoxicity have been indicated as the marks of nicotine's protective effect in AD (Hellstrom-Lindahl et al. 2004; Kihara & Shimohama, 2004; Nordberg et al. 2002; Zamani et al. 1997). Nicotine prevents activation of NF-κB , which regulates many apoptosis-related gens, and c-Myc that is also associated with apoptosis, by inhibiting the activation of MAP kinases (Figure 1.9). Nicotine decreases αβ protein accumulation via the activation of α7-nAChRs through MAPK, NF-κB, and c-myc pathways. Nicotine also inhibits apoptosis and cell cycle progression (Liu et al. 2007).

The negative association between tobacco use and PD also suggests that nicotine, the main effective and addictive substance in cigarette smoking, protects against neurodegeneration; thus, reducing the risks of PD (Quik, 2004; Quik et al. 2012). The mechanism through which nicotine is thought to exhibit neuroprotection involves nicotinic acetylcholine receptor (nAChR) activation of signaling pathways that inhibit apoptosis and induce up-regulation of neurotrophic factors (Hill-Burns et al. 2012).

Neurotrophic factors have been proposed as candidates in neuroprotective therapies since they can impede apoptotic and necrotic cell death. They can also save the damaged neurons that were subject to toxic or mechanical damage (Dunnett & Bjorklund, 1999; Mudo et al. 2007; Seidl & Potashkin, 2011). Pituitary adenylate cyclase-activating polypeptide (PACAP), Lipoprotein lipase (LPL) and gastrin releasing peptide (GRP) proteins which are known as neuroprotective genes are shown to be highly differentially

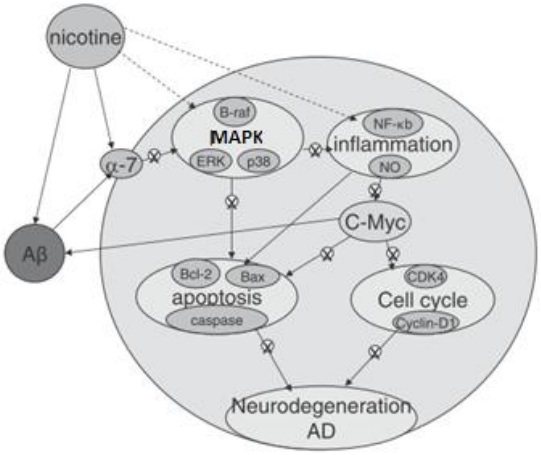

Figure 1.6 Proposed Inhibition mechanism of neurodegeneration in AD by nicotine (Liu et al. 2007).

expressed in VTA neurons compared to SN neurons in three large-scale microarray experiments (Chung et al. 2005a; Greene et al. 2005b; Grimm et al. 2004a).

PACAP gene is one of the well-studied genes which functions as a neurotrophic factor to induce neurito-genesis, to prevent apoptosis and to promote cell growth (Ravni et al. 2006). In a rat model of PD study, tyrosine-hydroxylase immunohistochemistry results revealed that PACAP-treated animals had only approximately 50% loss of dopaminergic cells while control animals had more than 95% loss of the dopaminergic cells in the ipsilateral substantia nigra, which exhibits the neuroprotective effect of PACAP in 6-OHDA-induced lesion of SN (Reglodi et al. 2004). The relevance of PACAP and nAChR signaling was investigated in PC12 cells differentiated with nerve growth factors (Tominaga et al. 2008). They found out that nicotine increases PACAP's mRNA level in a time and dose dependent manner.

LPL is another neuroprotective protein which is a key enzyme involved in the metabolism of lipoproteins, providing tissues like adipose tissue or skeletal muscle with

fatty acids (Santamarinafojo & Brewer, 1994). LPL is also expressed in the brain, however, not much is known about its functions there. It was previously demonstrated that LPL expression in neurons stimulates the extension of neurites and protects the neurons against oxidized lipoproteins toxicity (Paradis et al. 2003a). Thus, LPL could potentially act as a neurotrophic factor for neuronal survival and differentiation.

GRP, a neuropeptide originally isolated from the porcine stomach, is a 27-amino acid peptide (Spindel et al. 1984). GRPR-mediated signal transduction plays an important role in the central nervous system (CNS) (Roesler & Schwartsmann, 2012). Roesler et al proposed that dysfunctions in GRPR expression and signaling might play a role in CNS disorders such as anxiety, autism, memory dysfunction associated with neurodegenerative disorders. The function of the gastrin-releasing peptide in dopaminergic neurons of SN has not been deciphered well. A microarray experimental data indicated that GRP might be protective to DA neurons and the reduction of toxic responses of both PC12-aSyn cells and primary mesencephalic DA neurons against MPP+ by GRP was shown. GRP was suggested to contribute on the reduced susceptibility of VTA DA neurons in PD (Chung et al. 2005a).

1.5 Specific Aims and Hypotheses

The work presented in this dissertation was based on the formation of four specific aims:

Specific Aim 1: Investigate whether the differential expression of PACAP, LPL and GRP neuroprotective genes which were reported to be area-specifically higher expressed in VTA neurons compared to SN, are associated with ageing, and whether the area-specific

difference on genes expressions, which may contribute to the difference in DA neurons survival rate, still exist in aged experiment subjects.

Hypothesis 1:

The expressions of PACAP, LPL and GRP neuroprotective genes will decrease as the age increases.

Hypothesis 2:

The high expression levels of PACAP, LPL and GRP neuroprotective genes in VTA compared to SN will increase as the age increases.

Specific Aim 2: Investigate whether and how nicotine area-specifically regulates the expressions of PACAP, LPL and GRP genes, associated with ageing.

Hypothesis 3:

If nicotine provides neuroprotection to SN dopaminergic neurons via PACAP, LPL and GRP neuroprotective genes which are expressed higher in VTA than SN in normal conditions, nicotine will up-regulate these genes in SN.

Hypothesis 4:

If neurodenegeration of dopamine neurons in SN is an age dependent process and nicotine mediated neuroprotection occurs via PACAP, LPL and GRP, nicotine will differently regulate the expression of PACAP, LPL and GRP neuroprotective genes in adolescents, adult and old age groups.

Specific Aim 3: Investigate genetic alterations in both DA and non-DA midbrain neurons upon gestational nicotine exposure to be able to explain the possible cell-specific pathological mechanisms for the diseases induced by gestational smoking.

Hypothesis 5:

The influence of gestational nicotine on cells of different types, even located in close proximity within the same brain region, will be different.

Specific Aim 4: Investigate the common collective roles of differentially expressed genes between DA and non-DA midbrain neurons to elucidate how gestational nicotine regulates the effected pathways.

Hypothesis 6:

If DA and non-DA neurons have different molecular and functional properties, there will be differentially expressed genes involved specifically in DA-neuron related diseases during the development as a result of gestational nicotine exposure.

CHAPTER 2: MATERIALS AND METHODS

2.1 Animal Treatment Methods

All procedures were in compliance with the Guidelines for the Care and Use of Laboratory Animals. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Houston.

2.1.1 Subcutaneous Injection

Male Sprague-Dawley (SD) rats (Charles River), with an initial age of 3 months (n=3), 12 months (n=3) and 24 months (n=3), defined as adolescent, adult and old group, respectively, were maintained on a 12 h light/12 h dark schedule at a temperature of 22 ± 2°C and 65% humidity. Access to standard food and water was adlibitum. Rats were acclimated to the animal facility for 3 days before any procedures were performed. At day 4th, the animals were then injected subcutaneously twice a day for 14 days with either nicotine hydrogen tartrate (Sigma) at a dose of 0.6mg/kg/day or an equal volume of saline vehicle for the control.

2.1.2 Osmotic Minipumps

Osmotic pumps relasing 0.6mg/kg/day nicotine was implanted subcutaneously into the pregnant female (E5) Sprague-Dawley (SD) rats (n=4) from Charles River Laboratories (Wil-mington, MA, USA). Newborns were sacrificed at intervals from PN7 to 14 days (n= 5 to 8) from each pregnant rat.

2.2 Tissue Collection Methods

2.2.1 Adult, Adolescents and Old Animals

Animals were anesthetized with isoflurane and decapitated using a guillotine 12 hours after the last injection. After decapitation, the brain was removed rapidly from the skull and rinsed with cold artificial cerebro-spinal fluid (ASCF) solution (in nM, 126 NaCl, 1.6 KCl, 1.2 NaH2PO4, 1.2 MgCl2, 2.5 CaCl2, 18 NaHCO3 and 11 glucose). One mm brain slices containing the VTA and SN were dissected using a vibratome (Vibratom) Vibratome cooling tray was filled with ice and buffer tray was filled with ice-cold saline. Small drop of crazy glue was added to the center of buffer tray in proper orientation. VTA and SN are defined according to brain map atlas (Paxinos & Watson, 1986). Target brain areas were visualized under dissecting scope using appropriate lighting. After slicing, slices were kept in RNA-later. Punches from the VTA (Figure 2.1) and SN were subsequently obtained bilaterally from the slices with a 0.5-mm-diameter biopsy punch. Punching was performed according to Palkovits Fresh Punches protocol (Palkovits & Brownsteins, 1983). Tissue was expelled into an eppendorf tube which containing RNA later (QIAGEN) at 4C° for 24 hours.

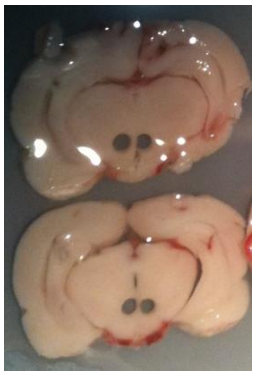

Figure 2.1 Tissue punches of VTA. The ventral tegmental area was sampled from 0.25-mm-thick slices using a 0.5-mm diameter punch.

2.2.2 Infants

The brain was removed from the skull in less than 2 minutes and the 250 μl midbrain slices were cut using a vibratome 1000 (Vibratom, St. Louis, MO). Slices were put into ice-cold artificial cerebrospinal fluid (ASCF) solution and incubated for one hour in the holding chamber at room temperature while they were continuously being perfused with 95% O 2 and 5% CO 2 in ASCF solution until the whole-cell patch clamp recordings performed.

2.3 Electrophysiology Methods

2.3.1 Whole-cell Patch Clamp

Conventional whole-cell recordings were made using a patch clamp amplifier (Multiclamp 700B, Axon Instruments) under infrared-DIC microscopy (Axioskop2 FS Plus, Zeiss). Data acquisition and analysis were performed using a digitizer (DigiData 1440A, Axon Instruments) and the analysis software pClamp 10.2 (Axon Instruments).

Borosilicate glass capillaries were pulled with Flaming-Brown puller (Sutter Instruments) to 2-4mV resistance. Positive pressure was applied to pipette to eliminate any contaminant that may clog the tip of the pipette. Once the pipette lowered onto the nerve cell surface, it positioned in the middle of the nerve. Following 0.2mV increase in resistance, suction was applied to form a gigaseal. After gigaseal formation between the pipette and the cell membrane the holding potential was set to − 70 mV in order to compensate the fast capacitance. The whole-cell configuration was achieved by subjecting the patch membrane to a slight suction. Dopamine cells were then differentiated according to the Ih curves. Dopaminergic VTA neurons have a hyperpolarization-activated cationic inward rectifier current called Ih (Mueller and Brodie, 1989). The family of hyperpolarizing voltage steps (duration 1 s) from a holding potential of -60 to -150 mV (in 10 mV increments, 9step) was used to evoke Ih. The pipette solution containing the cell was transferred to a 0.2 ml pcr tube by gently breaking the tip of the pipette and applying positive pressure with the help of a needle attached to 1mL syringe.

2.4 Molecular Methods

2.4.1 RNA Isolation

Total RNA was extracted using the RNeasy lipid tissue mini kit (Qiagen) and DNase 1 (Qiagen) to eliminate genomic DNA contamination. Plastic pestles were used to grind and distrupt the tissue and QIAzol Lysis Reagent was used for the homogenization. Homogenate was kept at room temperature for 2–3 min before continuing the procedure when the foaming occurred. Homogenate was incubated for 5 minutes as the first step of

RNA isolation protocol. 200µl chloroform was added to the homogenate, and vigorously shaken for 15 s and was kept at room temperature for 2-3 minutes. Following incubation, sample was centrifuged at 12,000x g for 15 min at 4°C. Aqueous phase was transferred to a new tube and 1x volume of ethanol was added and mixed thoroughly by vortexing. 700 µl of the sample was transferred to an RNeasy Mini spin column placed in a 2 ml collection tube and centrifuged for 15 s at 8000 x g (10,000 rpm) at room temperature (15–25°C). Centrifuge was repeated with the remaining of the sample.

Buffer RW1 was added (700 µl) to the RNeasy spin column and centrifuged for 15 s at 8000 x g (10,000 rpm) to wash the membrane. Following centrifugation, 500µl RPE Buffer was added to the RNeasy spin column and centrifuged for 15 s at 8000 x g (10,000 rpm) to wash the membrane. 500µl RPE Buffer was added as a second time to the RNeasy spin column and centrifuged for 2 min at 8000 x g (10,000 rpm) to wash the membrane. RNeasy spin column was transferred to a new 2 ml collection tube and centrifuged at full speed for 1 min. Then, RNeasy spin column was placed in a new 1.5 ml collection tube and 30–50 µl RNase-free water was added directly to the spin column membrane. It was centrifuged for 1 min at 8000 x g (10,000 rpm) to elute the RNA. Elution was repeated using the elute from the previous step in order to get high RNA concentration. The RNA in the elute was then quantitated using Nanodrop spectrometer (Thermofisher).

2.4.2 cDNA Synthesis

Seventyfive nanogram RNA from all samples was reverse transcribed using qScript cDNA SuperMix (Quanta Biosciences), according to the manufacturer's protocol. The reverse transcription reactions were carried out in a reaction volume of 20 µl containing 4 µl of qScript cDNA SuperMix (5X) reaction buffer containing optimized concentrations of MgCls, dNTPs (dATP, dCTP, dGTP, dTTP), recombinant RNase inhibitor protein, qScript Reverse transcriptase, random primers, OligodT primer and stabilizers, 75ng RNA template and the buffer, RNA template bring to volume with RNAse free water. The reaction was consecutively incubated at 25°C for 5 min, 42C for 30minutes and 85C for 5 minutes in thermocylcer. Each ul of cDNA template correlates to 7.5 ng RNA input.

2.4.3 qRT-PCR

Real time PCR amplification was performed using a MX-3005P (Agilent Technologies) cycler and PerfeCta SYBR Green SuperMix (Quanta Biosciences) in a 25ul volume. All samples were analyzed in duplicate or triplicate, and non-template controls were included to ascertain any level of contamination. The reaction mixture consisted of supermix containing optimized concentrations of MgCl2 , dNTPs , AccuStart Taq DNA Polymerase, SYBR Green I dye, ROX Reference Dye and stabilizers and 600nM of each primers, nuclease free water and 2µl of cDNA template corresponding to 15ng RNA input was added to each reaction. The RT-qPCR thermal profile was obtained using the following procedure: 95°C for 2 min, 40 cycles of 95°C

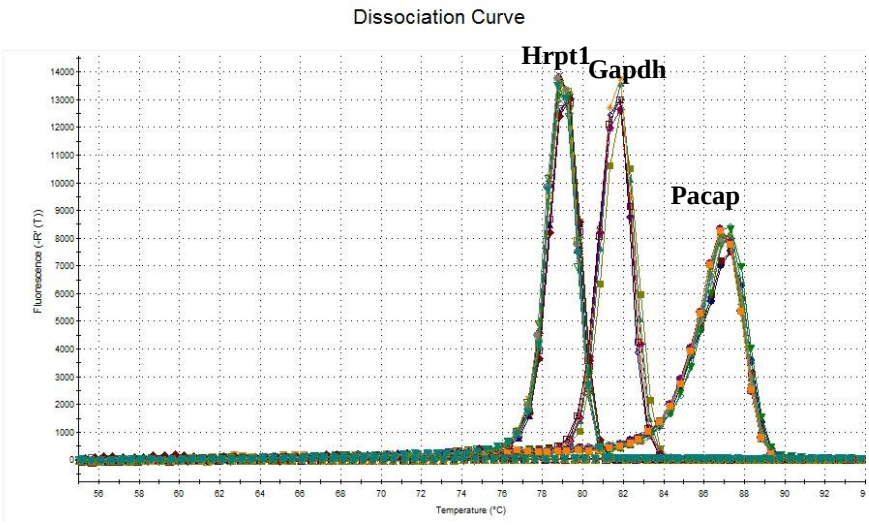

Figure 2.2 Dissociation curve for Hprt, Gapdh and Pacap genes.

for 15 sec, 58°C for 45 sec and 72°C for 3 0 sec, followed by 72°C for 5 min. Amplicon specificity was verified by first-derivative melting curve analysis (Figure 2.2). The primer sequences are listed in Table 2.1. All the RT-qPCR reactions were performed in either duplicate or triplicate to capture intra-assay variability.

Quantitation and normalization of relative expression was calculated using relative quantification model with kinetic PCR efficiency correction. The Ct of each gene was normalized against GAPDH and HPRT housekeeping genes by efficiency correction method (Pfaffl, 2001). The corresponding real-time qPCR efficiencies were calculated according to the equation $E= 10^{[-1/slope]}$ using the ten-fold serial dilutions of cDNA for each gene of interest, as well as housekeeping genes. The relative expression ratio of a target gene was calculated based on E and the Ct deviation of an unknown sample versus a control, and expressed in comparison to a reference gene (Pfaffl, 2001). Expression values smaller than one imply the down-regulation of the genes.

2.4.4 Primer Design

Primers (Sigma Aldrich) used in the qRT-PCR assay were designed using Primer3(Rozen & Skaletsky, 2000) (Table 1). The products ranged from 100 to 200 bp. In order to minimize the possibility of amplifying genomic DNA, each pair of primers was designed to target a short (90–120 bp) segment of the mRNA sequence expanding adjacent exons. To this end, the genomic sequence of each gene was obtained from the ENSEMBL genome database, and the mRNA sequence of the corresponding gene was obtained from GenBank. The sequences of two consecutive exons were used in Primer3 for primer selection.

2.4.5 Gradient PCR

The assay-specific optimum annealing temperature was evaluated by performing a conventional gradient PCR (annealing temperature gradient: 54–66°C). Each PCR amplification (20 μl of final volume) contained 0.6 μM of forward and reverse transcript-

Table 2.1 Primer sequences for gene of interests and housekeeping genes

GENE	Gene Bank ID	Sequence		Size
		Forward Primer	Reverse Primer	
PACAP	NM_016989.2	AGGGGCATGGGCGAGAACCT	TTCCCTAGCACGGCCGCCAA	149
Lipoprotein Lipase	NM_012598.2	CTTCAACCACAGCAGCAAAA	GGCCCGATACAACCAGTCTA	148
Grp	NM_133570.5	GGCAGTCTCCAGCCTACTTG	AGTTCCTTCCTTCCCCTTGA	111
Hprt1*	NM_012583	CAGGCCAGACTTTGTTGGAT	TCCACTTTCGCTGATGACAC	115
Gapdh*	NM_017008.4	TTCTTCCACCTTTGATGCTG	ATGTAGGCCATGAGGTCCAC	119
Hsp90ab	NM_001004082.3	CGCATGAAGGAGACACAGAA	CTCTTGCCATCGAACTCCTT	173
Hrnpab	NM_031330.2	CAGTGGTGGAGGTCAGGGTA	ACATGGGACAGGCCATAAAA	192
Vcan	NM_001170560.1	TCAACAGGCTTGTTTGGACA	CTTCCCCATCATGTCTCCAT	169
Hsp90aa1	NM_175761.2	TACACATCTGCTTCTGGGGAT	ACTTCTAAGCCATGCTTTCGG	158

*Housekeeping gene

specific primers, 0.4mM deoxynucleotides (dNTP), 10x PCR buffer, 2mM MgCl2, 1U of GoTaq Flexi DNA Polymerase (Promega), and 2µl of cDNA template. PCR was initiated with a denaturation step at 95 °C (for 1 min) followed by 30 cycles at 95 °C (for 30 s), 54-66 °C for 40 s (annealing), and 72 °C for 1m (elongation). The PCR-amplified products (10 µl) were analyzed using standard 2% agarose electrophoresis and visualized with ethidium bromide staining (0.5µg/ml). Images were digitally acquired using Alpha Innotech FluorChem SP Imaging System (Protein Simple). Product sizes were identified with a 100bp DNA ladder (Promega).

2.4.6 Agarose Gel Electrophoresis

In order to separate the DNA molecules by size, agarose gel electrophoresis was used. Agarose powder was dissolved in TBE Buffer to a final concentration of 1 per cent (w/v) by heating. 3µl Ethidium bromide was added to the solution to visualize the DNA under UV light after electrophoresis. When the agarose solution has cooled down, it was poured into a casting tray containing a sample comb and allowed to solidify at room temperature. Then, the comb was removed and the gel was placed into electrophoresis tank filled with 0.5X TBE Buffer. The DNA samples were mixed with loading dye, loaded into the wells and run at 100 V for 40-50minutes. The DNA fragments were visualized under UV light**.**

2.5 Expression Array

2.5.1 Amino-allyl aRNA Amplification

The most common *in vitro* transcription (IVT) strategy is the ‘Antisense RNA’ (aRNA) method described by Eberwine et al in 1990. The protocol used in this study is

the Ambion Amino Allyl MessageAmp™ II aRNA Amplification Kit and all reagents were from this kit unless otherwise stated.

First Round

Harvested cells collected in 0.2ml pcr tube were centrifuged at x3000g for 3minutes. The liquid is discarded and the pellet is dissolved in 10ul RNase- free water and placed on ice. 1 µL Nuclease-free Water, 1 µL T7 Oligo(dT) Primer, 2 µL 10X First Strand Buffer, 4 µL dNTP Mix,1 µL RNase Inhibitor and1 µL ArrayScript (Invitrogen) were added. The reaction mixture was mixed thoroughly by pipetting up and down and spun to collect the reaction in the bottom of the tube and incubated at 42°C for 2 hours. Following incubation, reaction mix chilled on ice briefly and mixture for the second strand DNA synthesis was prepared by adding the 63µl of Nuclease-free water, 10µl of 10X Second Strand Buffer, 4µl of dNTP Mix, 2µl of DNA Polymerase and 1µl of Rnase H on ice. The reaction mixture mixed gently before placing them in thermocycler. The samples then incubated at 16°C for 2 hours. No primer used in the second strand cDNA synthesis. RNase H simultaneously degrades the RNA and second-strand cDNA is synthesized using DNA Polymerase.

Following, second strand DNA synthesis, cDNA purification was performed. 250µl of cDNA Binding Buffer was added to each sample, and mixed thoroughly and spun briefly in mini centrifuge to collect the reaction in the bottom of the tube. The samples were loaded into a Filter Cartridge and centrifuge for 1 minute at 10,000xg. The flow-through was discarded. 500µl of wash buffer (Ambion kit) was added to the cDNA filter cartridge and centrifuged for 1 minute at 10,000xg. The flow through was

discarded. The cDNA Filter Cartridge was spun for an additional minute to remove trace amounts of washing buffer. 18µl of nuclease-free water (pre-heated to 55°C) was added to the centre of the filter in the cDNA Filter Cartridge for the elution,and left at room temperature for 2 minutes and then centrifuged for 1 minute at 10,000xg. The elute contains the purified cDNA.

Following cDNA purification, In Vitro Transcription was performed to synthesize amino allyl-modified aRNA. Since three rounds of amplification was performed, this first two round transcription was performed with unmodified UTP. 12µl of ATP, CTP, GTP mix (25mM), 6µl of UTP Solution (50mM), 4µl of T7 10X Reaction Buffer and 4µl of T7 Enzyme Mix were added to the double-stranded cDNA at room temperature and mixed well. The reaction mixture was spun briefly and incubated for 14-16 hours at 37°C. No primers were used for the in vitro transcription. T7 RNA Polymerase was used on the cDNA template bearing a T7 promoter to amplify RNA.

At the end of the in vitro transcription reaction 58µl of nuclease-free water (room temperature) was added. 350µl of aRNA Binding Buffer and 250µl of 100% ethanol was added to each aRNA sample and mixed by pipetting the mixture up and down a few times. The sample was transferred to the center of the filter in an aRNA filter Cartridge and centrifuged for 1minute at 10,000xg. The flow through was discarded. 650µl of wash Buffer was added to each aRNA Filter Cartridge and centrifuged for 1 minute at 10,000xg to wash the filter. The flow-through was discarded and the aRNA Filter Cartridge spun for an additional 1 minute to remove trace amounts of wash buffer. 100µl of nuclease-free water (preheated to 55°C) was added to the center of the filter. The

samples in the filter cartridge incubated in the 55°C heat block for 10 min and then centrifuged for 1 minute at 10,000xg. The aRNA in the elute was then quantitated.

Last Round

For the second round amplification, the entire aRNA sample (200µl) from the first round amplification reaction was dried using the vacuum concentrator (Thermo Fisher) until 10 µl remains in the tube and used as starting material for the second round amplification.

Second round primers were added and then vortexed and centrifuged briefly to collect the reaction in the bottom of the tube and incubate for 10 minutes at 70°C in an equilibrated thermocycler. Samples were placed on ice briefly before starting reverse transcription reaction. 2 µL 10X First Strand Buffer, 4 µL dNTP Mix,1 µL RNase Inhibitor and 1 µL ArrayScript were added to the samples and mixed well and briefly spun and incubated at 42°C for 2 hours. 1 µL RNase H was added to the reaction and incubated for 30 min at 37°C in a thermal cycler. RNase H specifically degrades the remaining aRNA in the sample so that the second strand synthesis reaction will be primed exclusively by the T7 Oligo(dT) Primer. Before the second strand cDNA reaction, 5 µL T7 Oligo(dT) Primer was added to cDNA sample and incubated for 10minutes at 70°C. Following incubation, samples were placed on ice briefly. On ice, 58 µL Nuclease-Free Water, 10 µL 10X Second Strand Buffer, 4 µL dNTP Mix and 2 µL DNA Polymerase was added to the samples , mixed well and centrifuged briefly. The reactions placed in an pre-cooled thermocycler for 2hours at 16°C. After the 2 hr incubation at 16°C, the reactions were placed on ice and continue with the cDNA purification as described above

(first round amplification). Following cDNA purification, 3µl of aaUTP (50mM), 12µl of ATP, CTP, GTP mix (25mM), 3µl of UTP Solution (50mM), 4µl of T7 10X Reaction Buffer and 4µl of T7 Enzyme Mix were added to the double-stranded cDNA at room temperature and mixed gently. The reaction mixture was spun briefly and incubated for 16 hours at 37°C. After the in vitro transcription, aRNA was purified as described in the first round amplification and then quantitated.

2.5.2 Dye Coupling Amino Allyl-Modified aRNA

The aRNA obtained from the amplification step was placed in a nuclease-free microcentrifuge tube and vacuum dry without heating until no liquid remains. Cydyes (CyDye Post-Labelling Reactive Dye Packs, Amersham) were left at room temperature for 20minutes before preparing them for coupling procedure. Dyes were prepared to coupling reaction by adding 11 µL of DMSO to one tube of Cy3 or Cy5 reactive dye, and vortexing .The resuspended CyDye was added to the aRNA in coupling buffer, mixed and incubated at room temperature in the dark for 30 minutes. 4.5ul of 4M hydroxylamine was added to the reaction mixture and incubated at room temperature for 15 minutes. 5.5 µL Nuclease-free Water was added to each sample to bring the volume to 30µL.

For the purification of dye couples aRNA, 105 µL of aRNA Binding Buffer was added to each aRNA sample. 75 µL of 100% ethanol was then added to each labeled aRNA sample,and mixed well by pipetting up and down 3 times. Following ethanol addition, sample was immediately transferred to the center of the filter in the Labeled aRNA Filter Cartridge and spun for 1 min at 10,000xg. The liquid is discarded and the

filter washed with 500µl Wash Buffer by spinning for 1min at 10,000xg. The flow through was discarded. The aRNA Filter Cartridge was spun for an additional minute to remove trace amounts of washing buffer. The Labeled aRNA Filter Cartridge was then transferred to a new collection tube and 10 µL Nuclease-free Water (pre-heated to 55°C) was added to the center of the cartridge. The column was incubated at room temperature for 2 minutes then spun at 10000xg for 1.5 minutes. Another 10µl of pre-heated Nuclease-free Water was added to the column and incubated at room temperature for 2 minutes. The column was spun at 10000gx for 1.5 minutes and the labeled aRNA was then quantified and the samples were pooled before the fragmentation.

2.5.3 Fragmentation

The labeled aRNA was fragmented before hybridization. RNA fragmentation reagents (Ambion) which fragment labeled RNA to sizes between 60–200 nucleotides were used for this procedure. 2–20 µg of RNA was fragmented in 10 µL reaction volume. Varying amounts of Nuclease-free Water was added to the labeled aRNA samples to bring the volume to 9µL. 1 µL of the 10X Fragmentation Buffer was added to the sample and mixed, spun briefly and incubated at 70°C for 15 min in thermocycler. 1 µL of the Stop Solution was finally added to place on ice until hybridization.

2.5.4 Hybridization of Labelled aRNA to Microarrays

Pre-hybridization solution was pre-warmed in water bath at 42°C for 30 minutes. Slides were placed into the warm pre-hybridization solution and incubated in water bath at 42°C for 30 minutes. Pooled, fragmented samples were vacuum-dried using a speed-vacuum (Thermo-Fisher) to reduce the volumes to less than 13.6 µl. The volume of the

sample mix was adjusted to 13.6 µl using Nuclease-Free water. 16.3µl of 20 × SSC (5X), 32.5µl of 100% Formamide (50%) 0.65µl of 10% SDS (0.1%) 1µl of Cot-1 rat DNA (0.2µg/µl), 1µl of tRNA (0.1µg/µl) were added to the aRNAs to make the hybridization mixture. Slides were removed from pre-hybridization solution. Slides were placed with the active side up, into the hybridization chamber (Cornell) 10µl water was added to the humidity wells in the hybridization chambers. The coverslip was then placed onto the slide with the spacer arms sides downwards. The hybridisation mixture was incubated 3 minutes at 95°C and 2 minutes on ice, centrifuged briefly. Labeled aRNA samples were applied under the coverslip, gently from the barcoded end of the slide. The hybridization chamber was then closed and placed in water bath at 42°C. Slides were incubated for 16-18 hours.

2.5.5 Post-hybridization Washes

Wash buffer was pre-warmed to 42°C. Slide was placed into wash buffer 1(see materials) and coverslips were removed. The slide was washed at room temperature for 5 minutes with gentle shaking at 60rpm. The slides was then transferred to wash buffer 2 and washed at room temperature for 5 minutes with gentle agitation at 60rpm. The slides was then transferred into the third tough containing wash buffer 3 and washed at room temperature for 1 minute with vigorous shaking. This step was repeated additional four times with clean wash buffer 3. After a final rinse in fresh wash buffer 3, the slides were quickly transferred to a centrifuge and spun for 1-2 minutes to dry the slides.

2.5.6 Scanning

Microarrays were scanned using the GenePix 4000B Scanner (Molecular Devices). After scanning, images were analyzed using AlphaScan microarray scanner (Alpha Innotech), to generate raw data in the form of asr files.

2.5.7 qRT-PCR Validation

Real time PCR amplification was performed as described in Methods 2.4.6. Quantitation and normalization of relative expression was calculated using delta-delta-Ct method (Livak & Schmittgen, 2001). The Ct of each gene was normalized against either GAPDH alone or the geometric mean of GAPDH and HPRT1 housekeeping genes.

2.6 Materials

Osmotic minipump solutions:

Nicotine Solution:

6mg/kg/day (based on the animal weight): Nicotine hydrogen tartrate salt

MilliQ water

Saline Solution:

6mg/kg/day (based on the animal weight): Sodium Chloride

MilliQ water

Patch Clamp Solutions:

Artificial cerebro-spinal fluid (ASCF) solution:

126nM NaCl

1.6nM KCl

1.2nM NaH_2PO_4

1.2nM $MgCl_2$

2.5nM $CaCl_2$

18nM $NaHCO_3$

11nM Glucose

Pipette Solution:

10mM HEPES

0.2mM EGTA

120mM KCl

2mM $MgCl_2$

0.5 µL RNAse Inhibitor

Agarose Gel :

TBE Buffer:

54 g of Tris base
27.5 g of boric acid
20 ml of 0.5 M EDTA

Agarose gel:

0.5 g Agarose
50 ml 1X TBE

Microarray Solutions:

Wash Buffer 1:

	For 1000ml	*Final Concentration*
MilliQ water	890ml	
20XSSC	100ml	2xSSC
10%SDS	10ml	0.1%SDS

Wash Buffer 2:

	For 1000ml	*Final Concentration*
MilliQ water	985ml	
20XSSC	5 ml	0.1 xSSC
10%SDS	10ml	0.1%SDS

Wash Buffer 3:

	For 1000ml	*Final Concentration*
MilliQ water	995ml	
20XSSC	5ml	0.1 xSSC

Pre-hybridisation solution

	For 100ml	*Final Concentration*
BSA	1g	0.1mg/ml
MilliQ water	74ml	
20XSSC	25ml	5xSSC
10%SDS	1ml	0.1%SDS

aRNA hybridisation Buffer:

	Final Concentration
100% Formamide	50%

20X SSC	5X
10% SDS	0.1%
tRNA	0.1µg/µl
BSA	0.1mg/ml
Cot-1 DNA	0.2 µg/µl

2.7 Data Analysis

All data were analyzed using MATLAB (Mathworks). Results were reported as the mean±SEM. In order to determine the effect of nicotine on the expression of the target genes, separate analyses of variance (ANOVA) were conducted on fold change values for each brain region across all age groups, followed by Tukey post-hoc multiple comparisons tests. One sample t-tests were used to indicate the significant difference of relative expression ratios. In all statistical analyses differences were considered significant when $p < 0.05$.

Microarray data were globally (lowess) normalized to eliminate intensity dependent bias, followed by across slide normalization (Yang et al. 2002). Differentially expressed genes were identified based on filtering the gene expression values (≤ -1 for downregulation, ≥ 1 for upregulations on the log2 expression ratios). We created the sets of down- and up-regulated genes using by keeping only those genes with expression ratios above and below the filtering threshold consistently in all our samples (separately for dopamine and non-dopamine). In a second analysis, we inspected which genes were differentially expressed between the two groups in our data: DA vs non-DA neurons. We conducted a series of t-tests (*p*-value cutoff = 0.05) to identify significant changes in

expression values between the dopamine and non-dopamine samples. False discovery rate correction was then applied (q-value cutoff = 0.05) to detect the set of genes with expression values significantly different between dopamine and non-dopamine samples. The gene lists thus obtained were further analyzed to assess for enrichment of Gene Ontology annotations using the web-based resources DAVID as well as pathway analysis using Webgestalt (Huang et al. 2009a; Huang et al. 2009b; Wang et al. 2013; Zhang et al. 2005).

CHAPTER 3: THE EFFECT OF AGE AND NICOTINE ON THE EXPRESSION OF NEUROPROTECTIVE GENES

3.1 Introduction

Substantia Nigra dopaminergic cell loss is one of the hallmarks of Parkinson's disease. However, the underlying cause of selective neurodegeneration of SN dopamine neurons is poorly understood. Age related molecular and cellular changes interact with genes and environmental factors to determine which cells age successfully and which succumb to neurodegeneration. Accumulated evidence has shown that there are age-related genetic changes in the SN due to the deletions of mitochondrial DNA associated with the functional impairment of neurons (Vanitallie, 2008).

Neurotrophic factors have been proposed as candidates in neuroprotective therapies since they can impede apoptotic and necrotic cell death, as well as they can save the damaged neurons which were subject to toxic or mechanical damage (Dunnett & Bjorklund, 1999; Mudo et al. 2007; Seidl & Potashkin, 2011). Pituitary adenylate cyclase-activating polypeptide (PACAP), lipoprotein lipase (LPL) and gastrin releasing peptide (GRP) proteins are well known neuroprotective genes which were reported to be area-specifically higher expressed in the VTA neurons compared to SN neurons in three large-scale microarray experiments (Chung et al. 2005b; Greene et al. 2005a; Grimm et al. 2004b). One of our present study goals is to investigate whether these changes in genes' expressions are associated with aging, and whether there is an area-specific difference between them, which may contribute to the difference on DA neurons' survival rate, still exist on aged experiment subjects.

Nicotine may offer protective effects against dopaminergic cell damage induced by various neurotoxins. The underlying mechanism of nicotine mediated neuroprotection is still not known. It is thought that the activation of signalling pathways by nAChR leads to neuroprotection through inhibition of apoptosis and induced up-regulation of neurotrophic factors (Mudo et al. 2007). Therefore, another aim of our study is to investigate whether and how area-specific nicotine regulates the expressions of PACAP, LPL and GRP genes, associated with aging.

3.2 Methods

3.2.1 Animal Treatment

Male Sprague-Dawley (SD) rats (Charles River) with an initial age of 3, 12 and 24 months (n=3/each group) were treated either with nicotine hydrogen tartrate (Sigma) at a dose of 0.5mg/kg or an equal volume of saline as described previously (Methods 2.1.1).

3.2.2 Tissue Extraction and RNA isolation

Brains were quickly removed, and 1-mm brain slices containing the VTA and SN were dissected using a vibratome (Vibratom), and then stored in RNA later (QIAGEN) at 4C° for 24 hours. Punches of the VTA and the SN were subsequently obtained bilaterally from these slices with a 0.5-mm-diameter biopsy punch. RNA isolation was performed as described previously (Methods 2.4.1).

Total RNA concentration of each sample was measured by 260nm optical density on a spectrophotometer (Thermo scientific). 260/280 ratios for all the samples were

found to be close to 2 for all the samples. 75ng input RNA were used for the cDNA synthesis reactions for all the samples.

Quantitation and normalization of relative expression was calculated using relative quantification model with kinetic PCR efficiency correction. The cycle threshold (Ct) of each gene was normalized against the geometric mean of the expression levels of Hprt and Gapdh housekeeping genes which were previously shown to have stable expression in nicotine. Their expressions were shown to be stable in any age group for any treatment in VTA or SN (Figure 3.1,Figure 3.2).

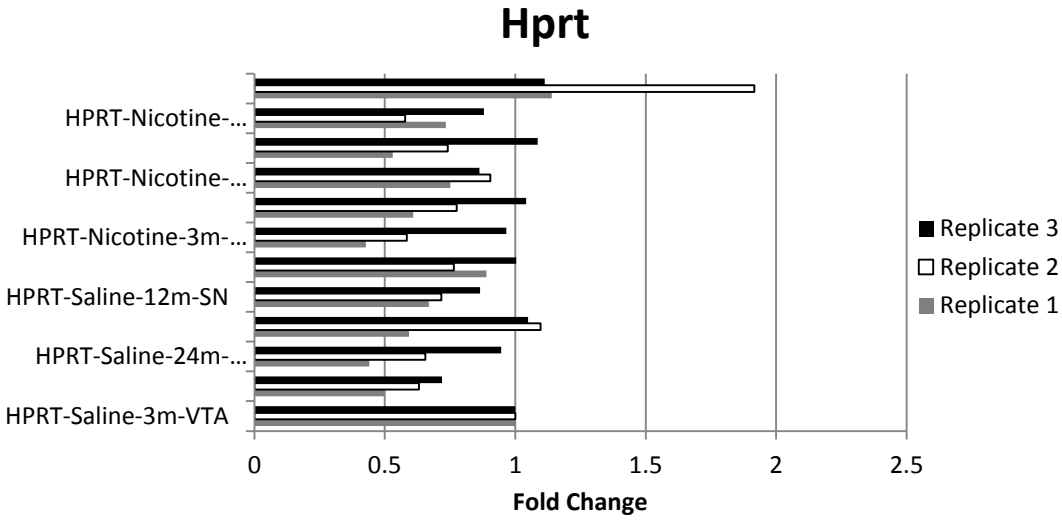

Figure 3.1 Fold Changes for HPRT housekeeping genes for different runs for the same amoung of RNA input. All data were normalized with respect to Saline-3m-VTA group.

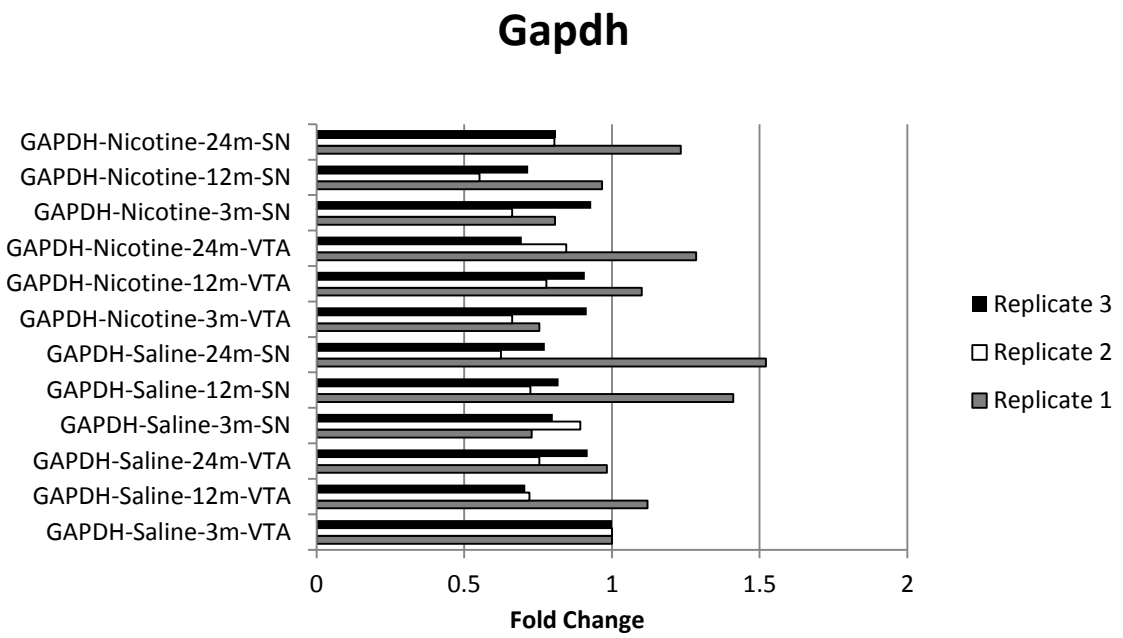

Figure 3.2 Fold Changes for HPRT housekeeping genes for different runs for the same amount of RNA input.All data were normalized with respect to Saline-3m-VTA group.

3.2.4 Statistical Analysis

All data were analyzed using MATLAB (Mathworks). Values are reported as the mean±SEM. In order to determine the effect of nicotine on the expression of the target genes, separate analyses of variance (ANOVA) were conducted on fold change values for each brain region across all age groups followed by Tukey post-hoc multiple comparisons tests. One sample t-tests were used to indicate the significant difference of relative expression ratios. In all statistical analyses differences were considered significant when $p< 0.05$.

3.3 Results

The effect of nicotine on the mRNA expression levels of three neuroprotective genes (PACAP, LPL and GRP) was studied by qRT-PCR in three different age groups and two different midbrain regions (VTA and SN) since one of the pathological hallmarks of PD is the loss of DA neurons in the substantia nigra, while neighboring DA neurons of the VTA are relatively spared (Davie, 2008; Maingay et al. 2006; Phani et al. 2010; Phani et al. 2013).

Each experiment was carried out using three biological and three technical replicates. Data are presented as mean ± SEM (Table 3.1). Statistical analyses were performed using MATLAB and Excel. Statistical significance was determined by Student t-test and ANOVA.

3.3.1 Individual Gene Analysis

3.3.1.1 Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP)

As shown in Figure 3.3, changes in PACAP expressions associated with aging were found within both the VTA and the SN. Comparing that with adolescents, the expression level for VTA was 1.81±0.17 fold increased in adult, and 2.20±0.36 fold increased in old group rats at age 24 months. Similarly, there was a 1.47±0.20 fold increase in adult and a 1.51±0.05 fold increase in 24 month old rats for the SN. However, these changes were not significant ($p > 0.05$). Significance were calculated as the difference between fold changes and 1 (# $p < 0.05$), VTA and SN ($p > 0.05$).

Our results also indicated that nicotine had similar regulation on PACAP expression patterns for both the VTA and the SN. It slightly increased the expressions in the adolescents group (1.31±0.12 fold in VTA, $p > 0.05$; and 1.45±0.12 fold in SN, $p > 0.05$), but greatly inhibited the expressions in both adult (0.76±0.05 fold in VTA, $p < 0.05$; and 0.69±0.06 fold in SN, $p < 0.05$) and old groups (0.41±0.03 fold in VTA, $p < 0.05$; and 0.45±0.04 fold in SN, $p < 0.05$) (Table 3.1). An ANOVA analysis suggested statistical significant differences were only between adolescents and other age groups, but not between adult and old groups, or between brain areas (Figure 3.4). Significance was calculated as the difference between fold changes and 1 (# $p < 0.05$), VTA and SN (* $p < 0.05$) and 12-/24-month and 3-month (ǂ $p < 0.05$).

Table 3.1 Efficiency corrected fold-differences of PACAP, LPL and GRP genes in VTA or SN upon nicotine exposure

Gene ID	VTA			SN		
	3-months	**12-months**	**24-months**	**3-months**	**12-months**	**24-months**
PACAP	1.31±0.12	0.76±0.05	0.41±0.05	1.45±0.12	0.69±0.06	0.45±0.05
LPL	1.15±0.17	0.64±0.05	0.60±0.07	0.33±0.05	0.56±0.08	0.97±0.13
GRP	0.78±0.04	0.95±0.04	1.00±0.09	0.91±0.01	0.84±0.03	1.02±0.02

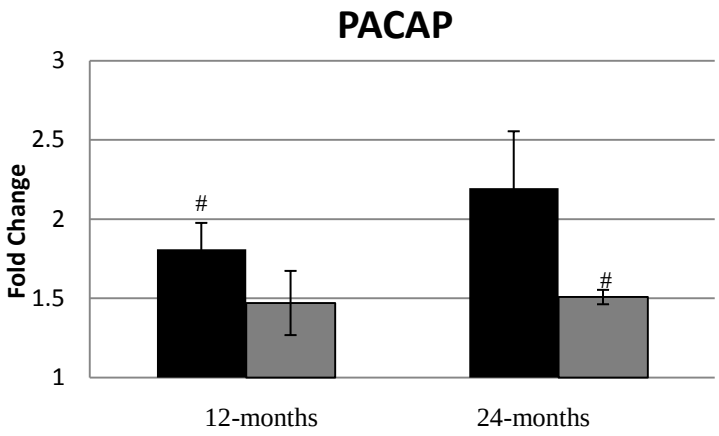

Figure 3.3 Age-related expression changes of PACAP gene in VTA (black) and SN (gray) for 12-month and 24-month control groups, compared to 3-month control group (# $p < 0.05$, t-test). Data normalized against geometric mean of Hprt1 and Gapdh housekeeping genes. Three biological and technical replicates were used.

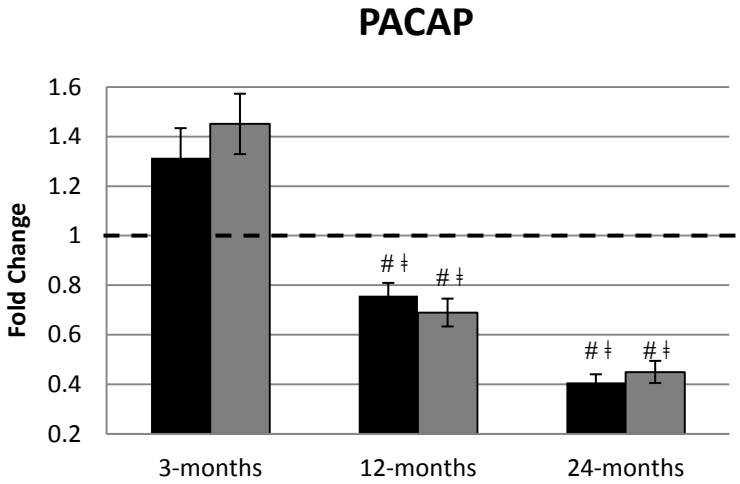

Figure 3.4 Age-related expression changes of PACAP gene in VTA (black) and SN (gray) upon nicotine treatment. Results were expressed as a fold difference (#, ǂ, ; $p < 0.05$, t-test, ANOVA). Data normalized against geometric mean of Hprt1 and Gapdh housekeeping genes. Three biological and technical replicates were used.

3.3.1.2 Lipoprotein Lipase (LPL)

Unlike PACAP, LPL gene expression showed age-related changes only in the VTA (Figure 3.5). Significance were calculated as the difference between fold changes and 1 (#$p < 0.05$), VTA and SN (*$p < 0.05$). Comparing the old rats and the adult rats with adolescents, LPL expressions were 1.66±0.25 fold increased for adult ($p>0.06$) and 1.63±0.12 fold increased for old groups ($p<0.05$) in VTA. However, there were no significant changes found on LPL expression within SN (1.12±0.17 fold for adult and 0.93±0.18 fold for old, $p > 0.05$ compared with adolescents).

Remarkably, nicotine treatments showed totally different regulation on LPL expressions in these two brain areas in different age groups (Figure 3.6). Significance was calculated as the difference between fold changes and 1 (# $p < 0.05$), VTA and SN (* $p < 0.05$) and 12-/24-month and 3-month (ǂ $p <0.05$). In the VTA, nicotine had no effect on gene expression for adolescents, but greatly down-regulated the expression to 0.64±0.05 fold for adult ($p<0.05$) and 0.60±0.07 fold for old groups ($p<0.05$) (Table 3.1). Conversely, in the SN, nicotine treatments significantly inhibited LPL expressions for adolescents (0.33±0.05 fold, $p<0.05$) and adult groups (0.56±0.08 fold, $p<0.05$), but induced no change for old groups (0.97±0.13 fold, $p>0.05$) (Table 3.1).

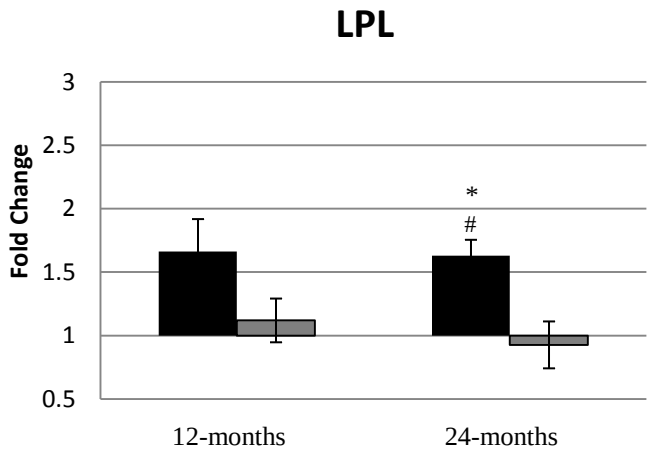

Figure 3.5 Age-related expression changes of LPL gene in VTA (black) and SN (gray) for 12-month and 24-month control groups, compared to 3-month control group (*,# $p < 0.05$, t-test). Data normalized against geometric mean of Hprt1 and Gapdh housekeeping genes. Three biological and technical replicates were used.

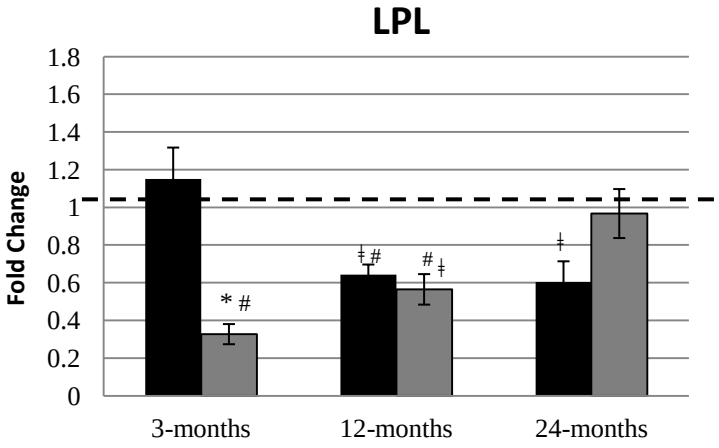

Figure 3.6 Age-related expression changes of LPL gene in VTA (black) and SN (white) upon nicotine treatment. Results were expressed as a fold difference compared with saline control group (#,*, ǂ; $p < 0.05$, t-test, ANOVA). Data normalized against geometric mean of Hprt1 and Gapdh housekeeping genes. Three biological and technical replicates were used.

3.3.1.3 Gastrin-Releasing Peptide (GRP)

GRP expression levels were found to significantly decreased for adult groups (0.75±0.03 fold, p<0.05) and old groups (0.80±0.03 fold, p<0.05). in VTA, compared with adolescents; however, GRP expressions were temporarily increased to 1.35±0.02 fold for adult groups (p<0.05) in the SN, and then lowered back to the normal level for old groups (0.86±0.05 fold, p>0.05) (Figure 3.7). Significance was calculated as the difference between fold changes and 1 (#$p < 0.05$), VTA and SN (*$p < 0.05$).

Our results indicate that nicotine inhibited GRP expression for the adolescent group in both the VTA (0.78±0.04 fold, $p < 0.05$) and the SN (0.91±0.01 fold, p<0.05), but for the adult group, this decrease was only found in the SN (0.84±0.03 fold, p<0.05).

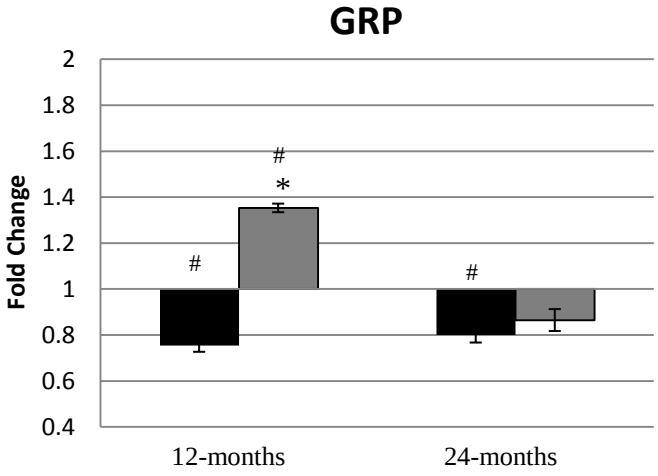

Figure 3.7 Age-related expression changes of GRP gene in VTA (black) and SN (white) expressed as a relative fold change for A. 12-month group and B. 24-month group, compared to 3-month group (*,# $p < 0.05$, t-test). Data normalized against geometric mean of Hprt1 and Gapdh housekeeping genes. Three biological and technical replicates were used.

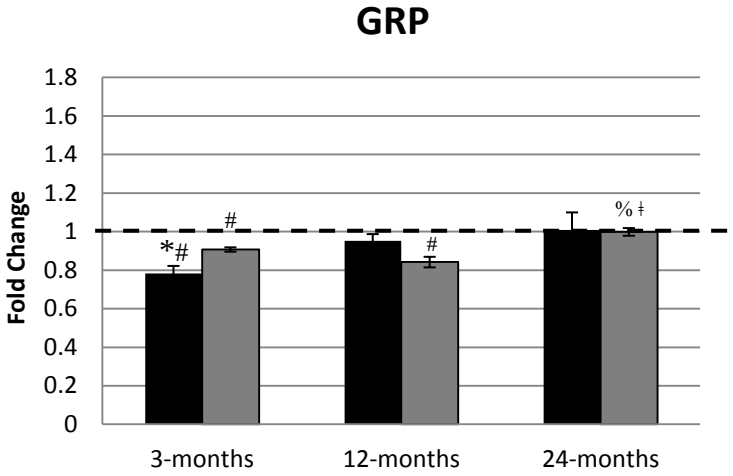

Figure 3.8 Age-related expression changes of GRP gene in VTA and SN upon nicotine treatment. Results were expressed as a fold difference compared to saline control group (#,*, ǂ, %; $p < 0.05$, t-test, ANOVA). Data normalized against geometric mean of Hprt1 and Gapdh housekeeping genes. Three biological and technical replicates were used.

(Table 3.1, Figure 3.8). Significance was calculated as the difference between fold changes and 1 (# $p < 0.05$), VTA and SN (* $p < 0.05$), 12-/24-month and 3-month (ǂ $p < 0.05$), 12-month and 24-month (% $p < 0.05$).

3.4 Discussion

The motor symptoms of PD result from the death of DA neurons in the SN (Davie, 2008). Although it was reported that the VTA DA neurons were also lost in PD patients and/or PD model animals, the degeneration of the SN DA neurons was severe, whereas the VTA neurons were relatively spared (Maingay et al. 2006; Phani et al. 2010). The cause of this cell death is unknown and it is still unclear why some DA cell groups are more susceptible than others. PACAP, LPL and GRP genes are well known for playing important roles against degeneration (Paradis et al. 2003b; Reglodi et al. 2011; Seki et al. 2006; Shioda et al. 2006). To our knowledge, there is still no related report

about how these genes expression changes are associated with aging. This information would provide us with valuable information on whether any/all of these genes contribute to PD development or a subgroup of DA neurons survival during PD.

LPL is a key enzyme involved in the metabolism of circulating triglyceride-rich lipoproteins, which is expressed mainly in adipose tissues and skeletal muscles, providing fatty acids to these tissues for β-oxidation or storage (Santamarina-Fojo & Dugi, 1994). High LPL expression has also been detected in the central nervous system (Ben-Zeev et al. 1990; Lorent et al. 1995; Yacoub et al. 1990); however, presently, only limited information is available regarding the role of LPL in the brain. Previous studies suggest that LPL may protect cells from damage caused by oxidized lipoproteins and that mutant LPL increase the risk of developing Alzheimer's disease (Baum et al. 2000). Recently, LPL was reported as area-specific with higher expression in VTA than the SN (Chung et al. 2005b; Greene et al. 2005a; Grimm et al. 2004b) and our present study also showed that LPL expressions were increasingly associated with aging only in the VTA, but not in the SN, where its expression was maintained at a normal level. This difference on LPL expression regulation between the VTA and the SN was statistically significant in the old group. All of this evidence suggests that the increased LPL expressions within the VTA may provide local neurons more protection and increase their tolerance to harm, especially in old age, helping survival during PD development that was not observed in the SN.

PACAP is a member of the vasointestinal polypeptide gene family and exhibited important neurotrophic activities comparable to those of the classical neurotrophic factors

(Kidane et al. 2008; Vaudry et al. 2000). Previous studies have shown that PACAP promotes the survival of cholinergic neurons in vitro and after injury in vivo, suggesting that PACAP acts as a neurotrophic factor, influencing the development and maintenance of these neurons (Takei et al. 2000; Yuhara et al. 2001). PACAP was also reported to have neurotrophic action on DA neurons and partially protect them against neurotoxicity induced by 6-OHDA, which is widely used to build PD animal models, and improves the behavioral deficits in a rat model of Parkinson's disease (Takei et al. 1998). Our results indicate that PACAP expression increases in association with aging in both the VTA and the SN as well as show no difference between the two brain areas, suggesting that 1) local neurons in these two areas may require and receive additional neuroprotection from increased PACAP expression; 2) consistent increases on PACAP expression in both VTA and SN may not contribute to the difference on survival ability of DA neurons in PD development.

GRP expresses gastrin-releasing peptide receptor (GRPR), which is a member of bombesin (BB)-like peptide receptor subfamily of G-protein coupled receptors (Corjay et al. 1991). Although there is no direct evidence shows GRPR is involved with neuroprotection, it affects a range of cellular and neuroendocrine functions, including cell proliferation, differentiation, regulating synaptic plasticity and memory formation (Mattson et al. 2002). Our results show GRP being held at a down-regulated level for both adult and old groups in the VTA, but greatly increased for adult groups in the SN and then back to the normal level for the old group. We have no explanation why GRP expressions were regulated differently for adults within the two areas; however, one recent study reported that the BB-like peptide was not significantly altered in PD patients,

for both the VTA and the SN, comparing with normal groups (Olincy et al. 1999), suggesting that GRP may not play important role in neuroprotection during PD and therefore induce the difference in survival rate of DA neurons between the VTA and the SN.

As mentioned before, epidemiological and experimental evidence indicates that nicotine is a significant negative factor for the risk of developing PD and protective vulnerable dopamine neurons in PD (Quik, 2004; Quik et al. 2012), but the underlying mechanism of this effect remains unclear. A large amount of research is focused on the contribution of gene expression regulated by nicotine to neuroprotection against degeneration (Greenbaum et al. 2013). Therefore, one of our hypotheses was nicotine will up-regulate these three genes, which have neuroprotective potentials, in SN to help reduce PD risks. However, our results did not confirm this hypothesis. Nicotine treatments did not up-regulate any of these genes expression for adult or old groups, suggesting that the protective effects of nicotine on susceptible DA neurons may not be enforced by increasing expression of LPL, PACAP or GRP genes. Recent studies also suggest that chronic nicotine does not specifically protect against degeneration, but rather modifies the DA receptor dynamics (Garcia-Montes et al. 2012), or excites DA neurons activities gated by cytoplasmic Ca^{2+} (Toulorge et al. 2011).

Genomic regulation plays a very important role in neuroprotection. Dysfunctions in proper gene expressions may induce and/or accelerate cell death progress, resulting in several neural degeneration diseases. Therefore many investigators now focus on neurogenomics in PD. We were assaying for three genes, PACAP, LPL and GRP, which

possess such effects to rescue the degeneration and increase the neuron survival rate. Our results suggest the significant difference on LPL gene expression, but not PACAP or GRP, in the senior population may contribute to the different survival rate for DA neurons within the VTA and the SN, providing additional neuroprotection to VTA DA neurons against damages. Furthermore, we did not find up-regulation effects of nicotine on any of these genes expression for adult and old groups, suggesting that these genes may not be related with the neuroprotection provided by nicotine which reduced the risks of PD.

Here, we have investigated only the transcriptional level changes in Pacap, Lpl and Grp expressions. Post translational modifications may induce additional changes to the gene products. In order to gain a complete understanding of how nicotine induce the transcription of this genes and their products, both levels needs to be analyzed (Hatzimanikatis et al. 1999).

CHAPTER 4: WHOLE-GENOME EXPRESSION ANALYSIS OF MIDBRAIN NEURONS UPON GESTATIONAL NICOTINE EXPOSURE

4.1 Introduction

Maternal smoking during pregnancy is associated with low birth weight, increased risk of stillbirth, conduct disorder, attention-deficit/hyperactivity disorder (ADHD) and neurocognitive deficits. (Fried et al. 1992; Kurtoglu et al. 2007; Neuman et al. 2007; Salihu et al. 2008; Thapar et al. 2006; Wakschlag et al. 1997; Weissman et al. 1999). Prenatal nicotine exposure was shown to cause structural and morphological alterations in the brain regions involved in cognition, learning, and memory (Roy & Sabherwal; 1998; Roy et al. 2002). Moreover, exposure to nicotine in utero is a common risk factor for ADHD (Hill et al. 2000; Kahn et al. 2003). Ample evidence has confirmed the neurotoxic effects of maternal smoking or second-hand smoke on developing brain, (Swan & Lessov-Schlaggar, 2007) including the increased liability of drug abuse in the offspring by altering development of neural systems that regulates motivation (Buka et al. 2003; Cornelius & Day, 2009); the alterations on brain DNA content, morphology and proportion of the cells in the brain (Roy et al. 2002; Slotkin et al. 1987) ; and dopamine release in several brain regions (Navarro et al. 1988; Nisell et al. 1997; Ribary & Lichtensteiger, 1989; Richardson & Tizabi, 1994).

There are several studies examining the effects of gestational nicotine on dopamine system by using midbrain regions tissues as a whole with molecular methods (Harrod et al. 2011; Kane et al. 2004b). However, with the consideration to cell-to-cell differences in molecular and functional properties of neurons (Ding et al. 2011), we

hypothesized that the influence of gestational nicotine on cells of different types, even located in close proximity within the same brain region, may be different. On average, only 50-60% of neurons in the VTA are DA (Alcaro et al. 2007), there are also considerable numbers of Gamma-Aminobutyric Acid (GABAergic) neuron populations and abundance of glial cells which could be also affected by nicotine (Lim et al. 2000; Lopez-Hidalgo et al. 2012). Thus, performing detailed studies on separate types of neurons, but not whole brain region tissues, is a critical step ahead for further and better understanding the impacts of gestational nicotine and related diseases.

In this study, large-scale genetic expressions of DA and non-DA neurons were investigated at single-cell level. Since DA neurons are more prone to actions of drugs of abuse, genetic alterations identified specifically in these neurons will help us to better understand molecular mechanisms leading to nicotine mediated diseases or deficiencies occurring during development. In addition, since DA and non-DA neurons have different molecular and functional properties, differentially expressed genes will help us to elucidate the molecular mechanism for the malfunctions of DA or non-DA neurons by drugs of abuse. To this end, we used patch clamp methods to identify and harvest DA and non-DA neurons on VTA slices. Oligonucleotide arrays and single-cell analysis were used to elucidate the alterations in gene expressions of the rat midbrain upon gestational nicotine exposure. Subsequently, the gene lists retrieved from microarray analysis were checked for Gene Ontology (GO) term enrichment in order to uncover their common functional background. Pathway enrichment analysis was also done to understand the common collective role of the genes found to be differentially expressed between DA and non-DA neurons.

4.2 Methods

4.2.1 Brain Slice Preparation

Nicotine or saline releasing osmotic pumps (Alzet, USA) were implanted subcutaneously into the pregnant (E5) Sprague-Dawley (SD) rats (n=4) for 4 weeks (Charles River, USA). 7-14 days old pups from nicotine/saline treated rats (n= 5 to 8/each pregnant rat) were anesthetized before decapitation, then horizontal slices at 250 μm thickness containing VTA were cut in ice-cold, oxygenated cutting solution containing (in mM) 120 NaCl, 3.3 KCl, 1.2 NaH_2PO_4 , 2.4$MgSO_4$, 1.2 $CaCl_2$, 25 $NaHCO_3$ and 10 glucose, and then were transferred into chamber for incubation at room temperature (24 °C) for 45 minutes and kept for use during a period of less than 6 hours.

4.2.2 Neuron Harvesting

Slices were transferred to a recording chamber and continuously perfused with ACSF at a flow rate of 1.5~2 ml/min. Hyperpolarization-activated cationic inward rectifier currents (Ih) were recorded as described previously (Methods 2.3.1) to distinguish dopamine neurons. Cells expressing Ih > 25 pA at -120 mV were considered as Ih-positive. Presence of this current has been used to identify DA neurons in the SNc and VTA (Figure 4.1) (Keath et al. 2007; Mercuri et al. 1995). Cell cytoplasm was collected into the recording pipette by applying negative pressure at the end of whole-cell recordings under visual control, while the input resistance was monitored during the aspiration procedure. Only cells without a large change in input resistance (< 20%) were accepted. Pipette inserted into 0.2ml PCR tube containing RNase inhibitor. 12 sample tubes each containing 20 DA or 20 non-DA neurons were collected from both saline and nicotine treated animals

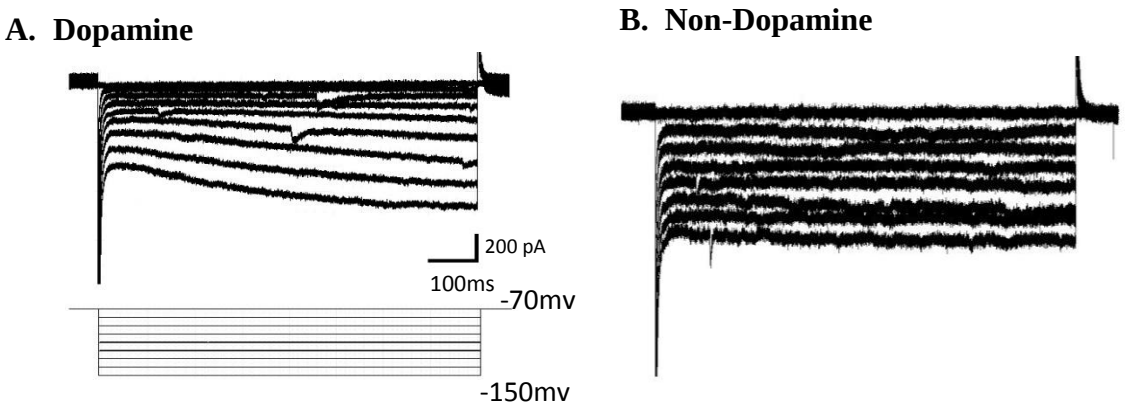

Figure 4.1 Pattern of hyperpolarization-activated cationic inward rectifier current (Ih) in A. Dopamine neurons, B. Non-dopamine neurons.

4.2.3 Single Cell aRNA Amplification

The PCR tubes containing the harvested neurons were further processed for cDNA synthesis using the MessageAmp™ II amino allyl aRNA amplification kit (Ambion) as described previously (Methods 2.5.1). The RT and cDNA synthesis was undertaken in the same tube used for harvesting on a T100 thermal cycler (Biorad). For microarray hybridization, the last round IVT was performed using modified nucleotide, 5-(3-aminoallyl)-UTP (aaUTP) to incorporate it into the aRNA to enable labeling of the aRNA. Following third round amplification, the concentration of aRNA sample (200 µl) was measured between 230 to 1400 ng/ µl by using Nanodrop spectrometer (Table 4.1). Samples were stored at 80°C until labeling process.

Table 4.1 RNA concentrations after the aRNA amplification

Treatment	Sample	Concentration(ng/μl)	260/280 ratio
Saline	Dopamine#1	1338.5	2.01
	Dopamine#2	1236.3	2.01
	Dopamine#3	1227.0	2.01
	Non-Dopamine#1	1241.0	2.02
	Non-Dopamine#2	232.3	1.99
	Non-Dopamine#3	1226.0	2.04
Nicotine	Dopamine#1	1274.7	1.96
	Dopamine#2	727.5	2.03
	Dopamine#3	1209.3	1.99
	Non-Dopamine#1	488.8	1.99
	Non-Dopamine#2	410.2	2.00
	Non-Dopamine#3	1097.5	2.00

4.2.4 Hybridization

Cy3 and Cy5 reactive dyes (Amersham) were prepared by resuspending with DMSO. All 200 μl aRNA samples were vacuum dried to 5-10μl volume. Cy3 and Cy5 ester mono functional dyes were coupled to the Amino allyl aRNA together with coupling buffer. Saline treated samples were labeled with Cy5, nicotine samples were labeled with Cy3. Following dye labeled aRNA purification and spectrophotometric analysis of dye incorporation, aRNA were prepared for hybridization by fragmentation with RNA Fragmentation Reagent (Ambion). 2-5μg labeled-aRNA from control and treatment samples were pooled and hybridized on chips under a coverslip (Thermofisher) at 42°C for 16–18 h. Hybridization was performed for 3 DA and 3 non-DA arrays. Following hybridization, the unbound material was washed from the microarrays and chips were scanned using GenePix (Molecular Devices).

4.2.5 Data Analysis

Microarrays were scanned using the GenePix 4000B Scanner (Molecular Devices). After scanning, images were analyzed using AlphaScan microarray scanner (Alpha Innotech), to generate raw data in the form of asr files. Data were globally (Lowess) normalized to eliminate intensity dependent bias, followed by across slide normalization (Yang et al. 2002). Sets of down- and up-regulated genes in DA and non-DA neurons were created. Differentially expressed genes between DA vs non-DA neurons were also identified as described previously (Methods 2.7). False discovery rate correction was then applied (*q*-value cutoff = 0.05) to detect the set of genes with expression values significantly different between DA and non-DA samples.

4.2.6 qRT-PCR Validation

Four genes (Vcan, Hsp90aa1, Hsp90ab1 and Hrnrpab) from the up and down regulated gene list were further validated by qRT-PCR experiments. Two biological replicates and three technical replicates were used for each experiment. 2µl cDNA sample corresponding to 15ng aRNA input were used for each qRT-PCR experiment which was performed as described previously (Methods 2.5.7). Since we had limited biological replicates left for qRT-PCR experiments, in order to do the statistical test, data were pooled from the samples that are regulated in the same direction from different experiments (Strand et al. 2002).

4.3 Results

Microarray data analysis identified significantly differentially expressed genes between DA and non-DA neurons as well as up and down regulated genes in midbrain neurons upon gestational-nicotine treatment. Two types of analysis were performed on identified gene lists; 1) GO- term enrichment of the up/down regulated gene list 2) Pathway analysis and functional annotation clustering of differentially expressed genes on the differentially expressed gene list.

4.3.1 mRNA Profiling of DA and Non-DA Neurons in Offspring

Thirtynine genes either up or down regulated as a result of nicotine exposure across all the chips. We identified 14 up-regulated and 15 down-regulated genes in DA neurons, of the 14 up-regulated genes, 7 genes are known and unique and of 15 down-regulated genes 11 of the genes are unique and known (Table 4.1), likewise, 5 up-regulated and 5 down-regulated genes were identified in non-DA neurons, of the 5 down-regulated non-DA genes, 4 genes are known and unique (Table 4.2). Besides the up and down regulated gene list, 135 genes were identified as significantly differentially expressed between DA and non-DA neurons upon nicotine treatment.

4.3.2 Up- and Down- Regulated Genes as a Result of Gestational Nicotine Exposure in The DA Neurons

In DA cells, 15 genes were identified as the significantly down-regulated genes upon gestational nicotine exposure ($p \leq 0.01$) (Table 4.2). Within the genes that are down-regulated by gestational nicotine, Cartpt gene encoding Cocaine And

Amphetamine Regulated Transcript Protein is the most striking one since it plays important roles in the positive regulation of catecholamine secretion, G-protein coupled receptor signaling and behavior. Genes encoding chemokine (C-X-C motif) ligand 16 and GABA A receptor beta 1 are the other down-regulated genes that are involved in G-protein coupled receptor protein signaling pathway (Table 4.2).

In addition, our study indicated that gestational nicotine exposure also significantly reduced the expression of Sergef, RGD1359310, Dlx1, Pbx2 and Hsp90ab1genes. The proteins that the genes encode regulates developmental processes as diverse as signal transduction, embryonic, telencephalon, hippocampus, forebrain, regulation of nervous system, appendage and placenta development and cell growth. Ribosomal protein S21 and similar to ribosomal protein s12 are the two other down-regulated genes involved in protein biosynthesis related processes. Biological regulation, metabolic process, response to stimuli, multicellular organismal process, developmental process and localization are the enriched biological process terms within the down-regulated genes (Figure 4.2).

Table 4.2 Genes with expression either decreased by ≤0.5-fold or increased by 2≥ fold after nicotine treatment compared to saline treated DA neurons

Gene ID	Gene Name	Description	Associated KEGG Pathway
Down-Regulated			
ENSRNOG00000006325	Rps21	ribosomal protein S21	Ribosome
ENSRNOG00000001520	Dlx1	distal-less homeobox 1	
ENSRNOG00000019834	Hsp90ab1	heat shock protein 90 alpha (cytosolic), class B member 1	-Antigen processing and presentation -NOD-like receptor signaling pathway -Prostate cancer -Progesterone-mediated oocyte maturation -Protein processing in endoplasmic reticulum
ENSRNOG00000017712	Cartpt	CART prepropeptide	-
ENSRNOG00000007756	RGD1359310	similar to RIKEN cDNA 9430023L20	-
ENSRNOG00000026647	Cxcl16	chemokine (C-X-C motif) ligand 16	-Chemokine signaling pathway -Cytokine-cytokine receptor interaction
ENSRNOG00000029067	RGD1562542	similar to ribosomal protein S12	-
ENSRNOG00000000440	Pbx2	pre-B-cell leukemia homeobox 2	-
ENSRNOG00000011488	Sergef	secretion regulating guanine nucleotide exchange factor	-
ENSRNOG00000002327	Gabrb1	gamma-aminobutyric acid (GABA) A receptor, beta 1	Neuroactive ligand-receptor interaction
ENSRNOG00000017012	Coq7	coenzyme Q7 homolog, ubiquinone (yeast)	Ubiquinone and other terpenoid-quinone biosynthesis
Up-Regulated			
ENSRNOG00000020006	Wdr90	WD repeat domain 90	-
ENSRNOG00000013111	Mettl3	methyltransferase-like 3	-
ENSRNOG00000013009	Ldha	lactate dehydrogenase A	-
ENSRNOG00000001185	RGD1311899	similar to RIKEN cDNA 2210016L21 gene	-
ENSRNOG00000003645	Hnrnpab	heterogeneous nuclear ribonucleoprotein A/B	-
ENSRNOG00000017163	Pfkp	phosphofructokinase, platelet	-
ENSRNOG00000030680	Ddx5	DEAD (Asp-Glu-Ala-Asp) box helicase 5	-

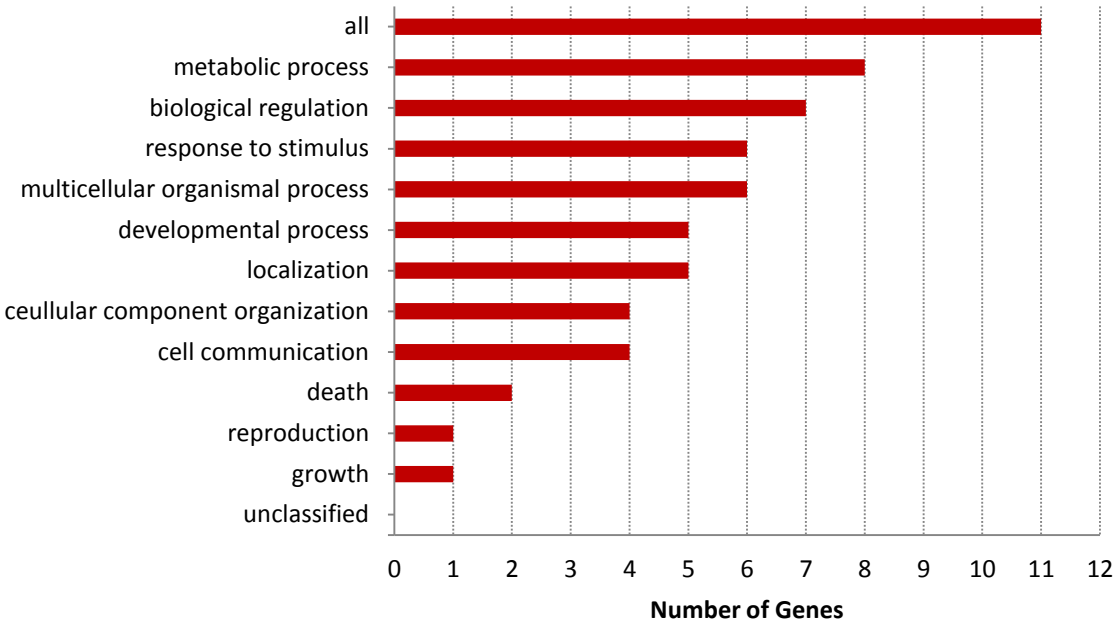

Figure 4.2 Biological processes classifications of DA down-regulated genes.

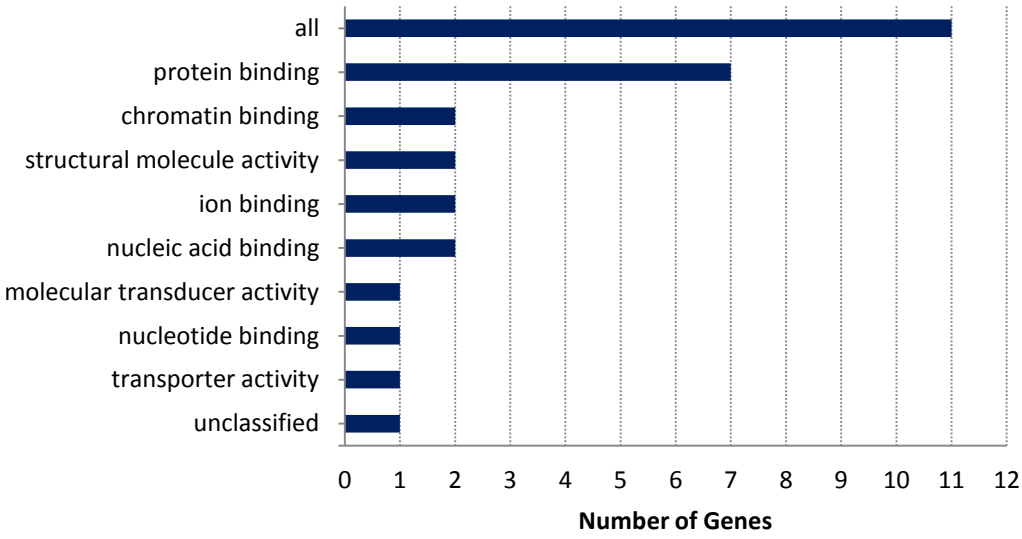

Figure 4.3 Molecular function classifications of DA down-regulated genes.

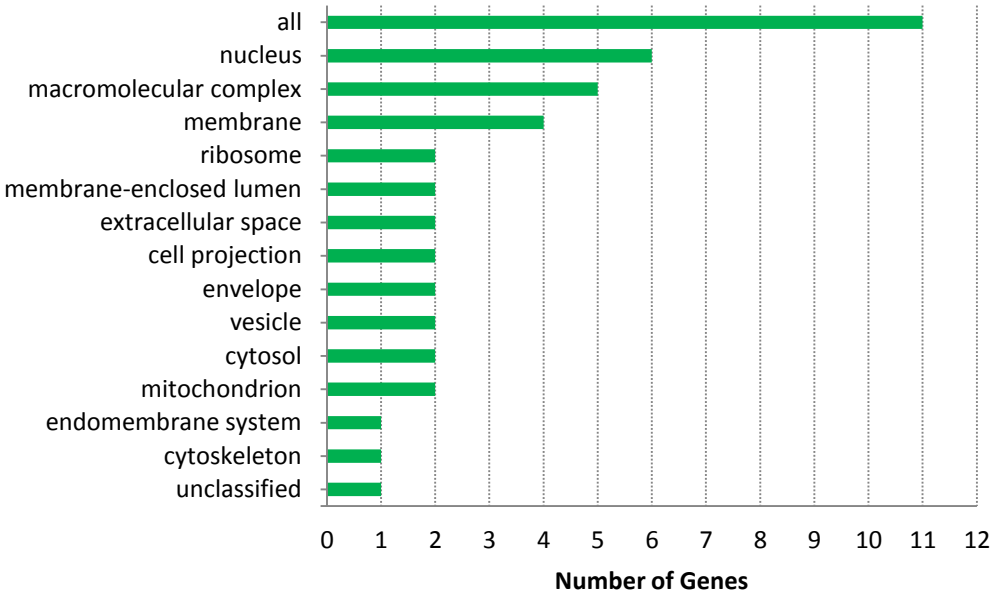

Figure 4.4 Cellular component classifications of DA down-regulated genes.

Among molecular functions the most predominant term is protein binding (Figure 4.3). Among cellular components the most significantly represented terms are nucleus, macromolecular complex and membrane (Figure 4.4).

4.3.3 Up- and Down- Regulated Genes as a Result of Gestational Nicotine Exposure in Non-DA Neurons

In nonDA neurons, 5 genes (Plk3, Rps6kb1, Gfod, Brwd1 and Hsp90aa1) were found to be down-regulated by gestational nicotine exposure ($p \leq 0.01$) (Table 4.3). Polo-like kinase 3 encoded by Plk3 gene regulates the neurological system processes, synaptic transmission, synaptic plasticity and system processes. Ribosomal protein s6 kinase encoded by Rps6kb1 gene regulates many important biological processes such as learning and memory, long term memory, behavior, cognition, muscle system process, neurological system process and many developmental processes such as regulation of

muscle development and developmental growth. Bromodomain and WD repeat containing protein 1 encoded by Brwd1 is involved in regulation of transcription and cytosolic heat shock protein 90, alpha encoded by Hsp90aa1 gene is involved in protein folding and mitochondrion organization. Gfod gene encodes the glucose-fructose oxidoreductase domain containing 1 in which takes place in oxidation-reduction process.

According to GO term analysis of down-regulated non-dopaminergic genes, the most represented biological process terms are metabolic process, response to stimulus, death, multicellular organismal process, and biological regulation. Among molecular functions, nucleotide binding, protein binding and ion binding are the most predominant terms. Cell protection, membrane and nucleus terms are present among the most prominent cellular component classification. Arg2 (arginase), Pol1c (polymerase I polypeptide C), Pot1 (protection of tolemeras 1), Rgs2 (homolog regulator of G-protein signaling 2 polymerase I polypeptide C) were identified as significantly up-regulated genes upon gestational nicotine exposure ($p \leq 0.01$) (Table 4.3). G-protein signaling 2 protein includes negative regulatory biological processes such as negative regulation of G-protein coupled receptor protein signaling pathway and signaling pathway. Pot1 and Pol1c genes are involved in DNA metabolic processes and transcription, respectively. GO term analysis showed that within the up-regulated non-DA neurons metabolic process, biological regulation, reproduction and developmental process, are the most represented GO-terms in biological processes. Table 4.4 shows the M-values of non-DA

Table 4.3 Genes with expression either decreased by ≤0.5-fold or increased by 2 ≥ fold after nicotine treatment compared to saline treated non-DA neurons

Gene ID	Gene Name	Description	Associated KEGG Pathway
Down-Regulated			
ENSRNOG00000014007	Gfod1	glucose-fructose oxidoreductase domain containing 1	-
ENSRNOG00000001632	Brwd1	Bromodomain and WD repeat domain containing 1 (Predicted)	-
ENSRNOG00000003919	Rps6kb1	ribosomal protein S6 kinase, polypeptide 1	-
ENSRNOG00000007219	Hsp90aa1	heat shock protein 90, alpha (cytosolic), class A member 1	-
ENSRNOG00000018484	Plk3	polo-like kinase 3	-
Up-Regulated			
ENSRNOG00000011139	Arg2	arginase type II	-
ENSRNOG00000019079	Polr1c	polymerase (RNA) I polypeptide C	-
ENSRNOG00000019986	Pot1	protection of telomeres 1 homolog	-
ENSRNOG00000003687	Rgs2	regulator of G-protein signaling 2	-
ENSRNOG00000029212	Vcan	Versican	-

up- and down- regulated genes in three different chips, p-values and q-values computed using Benjamini-Hochberg procedure.

4.3.4 Functional Enrichment Analysis

Functional enrichment analysis was performed for the interpretation of significantly differentially expressed genes between DA and non-DA neurons. To this end, DAVID, which is a web-based toolset for functional profiling of gene lists from large-scale experiments, was used (Huang et al. 2009a; Huang et al. 2009b). Among

Table 4.4 M-values, p-values and q-values of up and down regulated genes in non-DA neurons

Gene ID	M-value #1	M-value #2	M-value #3	*p*-value	q-value
Down- regulated					
Brwd1	-3.13227	-2.31683	-3.79012	0.006884	0.0267
Rps6kb1	-1.23616	-1.11275	-1.25601	0.010681	0.0267
Hsp90aa1	-1.03116	-1.48309	-1.95897	0.046354	0.0464
Gfod1	-1.50718	-2.79529	-2.87793	0.018533	0.0304
Plk3	-2.42094	-2.87677	-1.5793	0.024334	0.0304
Up- regulated					
Pot1	4.587096	4.755581	3.185766	0.006884	0.0267
Arg2	4.848534	1.927006	3.481804	0.010681	0.0267
Polr1c	5.50704	1.532578	3.659527	0.046354	0.0464
Vcan	4.233002	2.587008	4.394943	0.018533	0.0304
Rgs2	5.836161	4.722886	4.488495	0.024334	0.0304

cellular components the most significantly represented terms were cytosolic small ribosomal subunit, small ribosomal subunit and cytosolic ribosome (all $p < 0.001$) (Table 4.3). Among biological processes, the most predominant term is translational elongation ($p < 0.01$) (Table 4.5).

rRNA processing and translation were also significant biological processes with a relatively lower p-value ($p < 0.05$). Among molecular functions, structural constituent of ribosome is the most significant go-term represented in dataset ($p < 0.01$) (Table 4.5). Inorganic cation transmembrane transporter activity and nucleobase, nucleoside, nucleotide and nucleic acid transmembrane transporter activity were also represented ($p \leq 0.05$).

4.3.5 Identification of KEGG Pathways and Functional Annotation Clusters

Pathway enrichment analysis was carried out on the differentially expressed gene list to elucidate perturbed pathways specifically in DA neurons upon nicotine exposure.

To determine significantly enriched pathways within the differentially expressed genes between dopamine and non-dopamine neurons, KEGG Pathway Enrichment Analysis was performed using the WebGestalt web-based program (Wang et al. 2013; Zhang et al. 2005).

Ten pathways were found to be significantly enriched in the differentially expressed genes between dopamine and non-dopamine cells (Figure 4.5). 27% of the genes in pathway analysis were found to play role in several metabolic pathways. 17% of the genes involved in olfactory transduction since there are several olfactory transduction receptors identified as differentially expressed between dopamine and non-dopamine genes (Table 4.6). The ribosome pathway was also found to be significantly overrepresented within the differentially expressed genes since there are many ribosomal proteins found in the gene list (Table 4.6).

Disease pathways are the other notable pathways found in the differentially expressed genes between DA and non-DA neurons. 10% of the genes are involved in arachiodonic and linoleic acid metabolism, and 4% of the genes were found to take place in protein processing in endoplasmic reticulum. All of these pathways have p-values lower than 0.001, except linoleic acid ($p < 0.011$) and protein processing in endoplasmic reticulum pathways ($p < 0.004$).

Table 4.5 GO terms enrichment analysis of the significantly differentially expressed genes between DA and non-DA neurons

Category	GO Term	Count	*p*-Value
Biological Processs	Translational elongation	5	0.0023
	rRNA processing	3	0.05
	Translation	7	0.039
Cellular Component	Cytosolic small ribosomal subunit	6	3.5E-06
	Small ribosomal subunit	6	0.000013
	Cytosolic ribosome	6	0.000017
	Ribosomal subunit	6	0.00018
	Cytosolic part	6	0.00065
	Ribonucleoprotein complex	10	0.0049
	Ribosome	7	0.019
Molecular Function	Structural constituent of ribosome	7	0.009

Functional annotation clustering analysis was also performed on the differentially expressed gene list. Functional annotation clustering provides extended annotation compared to GO term analysis since it includes protein-protein interactions, protein functional domains, disease associations, bio-pathways, sequence general features, homologies, gene functional summaries, gene tissue expressions in addition to GO terms. Functional annotation clustering for the 135 genes differentially expressed between DA and non-DA neurons using DAVID identified 27 clusters. The highest enrichment score was found as 2.87 for the annotation cluster 1 which includes genes related to cytosolic ribosome and cytosolic subunit, ribosome, ribosomal protein and subunit, ribonucleoprotein, translation and cytosol (Table 4.7). The annotation cluster group 2

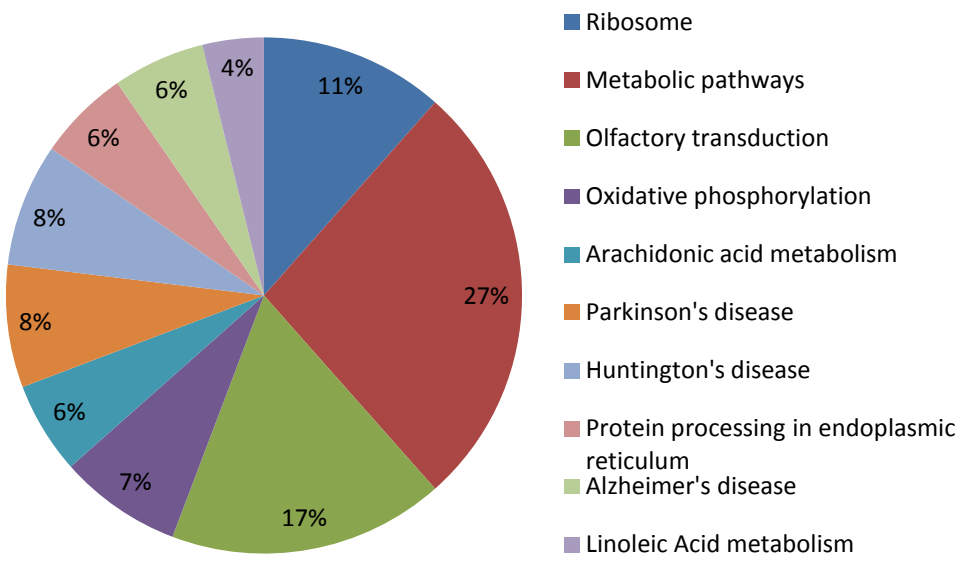

Figure 4.5 Kegg Pathway Analysis of 135 significantly differentially expressed genes between DA and non-DA neurons. Each slice in the pie represents one of the significantly enriched KEGG pathway associated with the genes within the list.

Table 4.6 List of the genes identified in KEGG pathway analysis

KEGG Pathway	Gene IDs
Ribosome	Rpl14, Rps13, Rps20, Rps12, Rps21
Metabolic pathways	Hsd17b1, Ndufb5, Chpf, Dad1, Ndufb4, Cyp2c22, Cyp4f6, Atp5b, Pla2g3, Atp6v0e2, Pon3, Pip5k1c, Prdx6, Nanp
Olfactory transduction	Olr1589, Olr221, Olr1072, Olr741, Olr818, Olr812, Olr1347, Olr1614, Olr1620
Oxidative phosphorylation	Atp5b, Ndufb4, Ndufb5, Atp6v0e2
Arachidonic acid metabolism	Cyp2c22, Cyp4f6, Pla2g3
Parkinson's disease	Atp5b , Ndufb4, Ndufb5, Slc25a4
Huntington's disease	Atp5b, Ndufb4, Ndufb5, Slc25a4
Protein processing in endoplasmic reticulum	Dad1, Nsfl1c, Rnf5
Alzheimer's disease	Atp5b, Ndufb4, Ndufb5
Linoleic Acid metabolism	Cyp2c22, Pla2g3

with an enrichment score 1.04 contains Parkinson's and Huntington's disease related genes. The annotation cluster group 7 with relatively low enrichment score (0.68) contains genes related to developmental processes such as reproductive developmental process and development of primary sexual characteristics. As can be noticed, functional annotation cluster analysis revealed the neurodegenerative disease pathway, developmental process and ribosome related genes.

Table 4.7 Functional Annotation Clustering using DAVID for the 135 differentially expressed genes between DA and non-DA neurons

Annotation Cluster 1	**Enrichment Score: 2.87**	**Count**	**p-Value**	**Benjamini**
GOTERM_CC_FAT	Cytosolic small ribosomal subunit	6	3.5E-06	0.00053
GOTERM_CC_FAT	Small ribosomal subunit	6	0.000013	0.00098
GOTERM_CC_FAT	Cytosolic ribosome	6	0.000017	0.00089
SP_PIR_KEYWORDS	Ribosome	6	0.00017	0.024
GOTERM_CC_FAT	Ribosomal subunit	6	0.00018	0.007
SP_PIR_KEYWORDS	Ribosomal protein	7	0.00026	0.019
KEGG_PATHWAY	Ribosome	6	0.00035	0.018
GOTERM_CC_FAT	Cytosolic part	6	0.00065	0.02
GOTERM_BP_FAT	Translational elongation	5	0.0023	0.81
SP_PIR_KEYWORDS	Ribonucleoprotein	6	0.0042	0.18
GOTERM_MF_FAT	Structural constituent of ribosome	7	0.009	0.86
SP_PIR_KEYWORDS	Protein biosynthesis	5	0.018	0.48
GOTERM_CC_FAT	Ribosome	7	0.019	0.35
GOTERM_BP_FAT	Translation	7	0.039	1
Annotation Cluster 2	**Enrichment Score: 1.04**	**Count**	**p-Value**	**Benjamini**
KEGG_PATHWAY	Parkinson's disease	6	0.0039	0.097
KEGG_PATHWAY	Huntington's disease	6	0.013	0.21

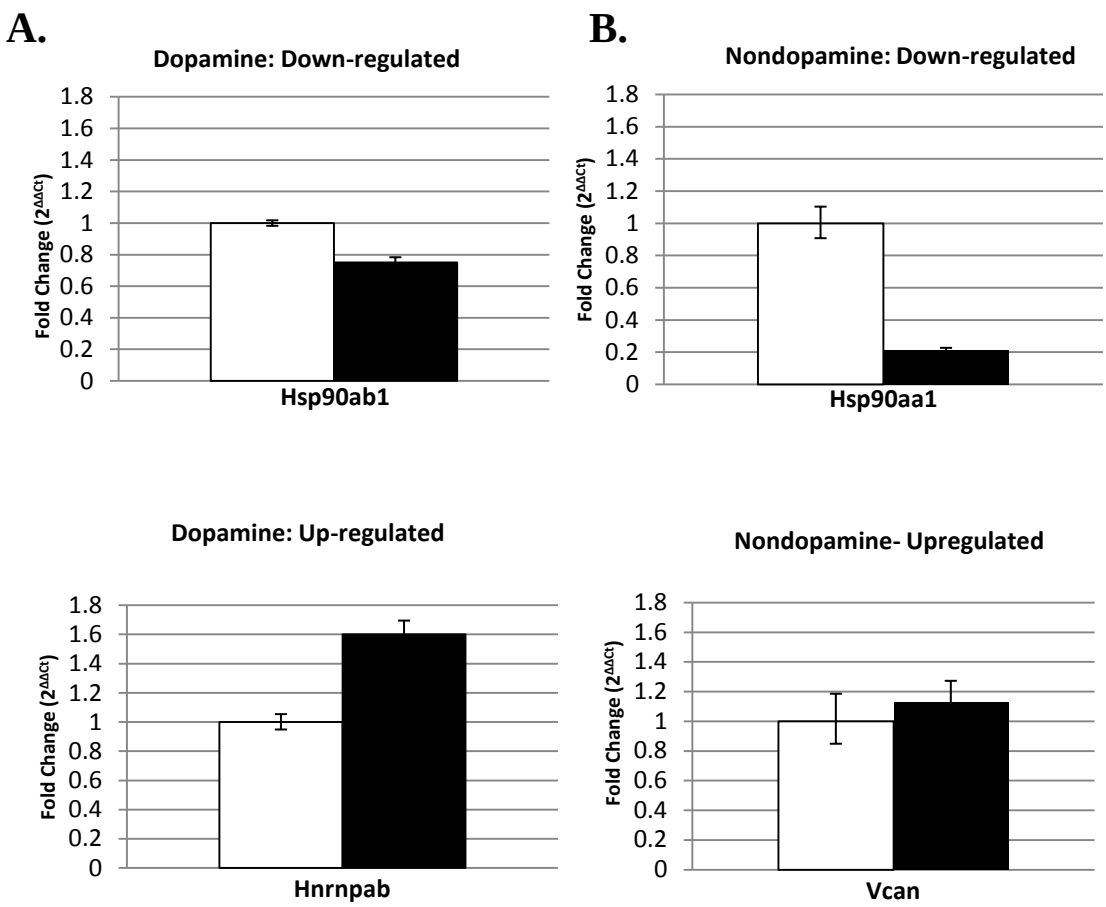

Figure 4.6 Validation of microarray results by RT-qPCR. Hsp90ab1, Hsp90aa1, Hrnrpab and Vcan were selected between the genes identified as the top-ranked up/down regulated genes either in dopamine (A) or non-dopamine groups (B) Genes from nicotine treatment groups is shown in black, genes expressed in control (saline) conditions are shown in white. Statistical significance was calculated by pooling across the genes.

4.3.6 Quantitative Real Time PCR Validation of Microarray Results

Hsp90aa1, Hsp90ab1, Hrnpab and Vcan were chosen for the qRT-PCR validation since these genes were found by microarray experiments to have the most significant alterations in their expression in DA and non-DA samples (Figure 4.6). Owing to limited replication, in order to increase the number of degrees of freedom available to analyze each small experiment, we pool data for the genes that are regulated in the same

direction. Consistent with the array data, Hsp90aa1 and Hsp90ab1 genes were found during the qRT-PCR validation to be significantly down-regulated in DA and non-DA neurons, respectively ($p<0.05$). Likewise, Hrnpab and Vcan genes were found to be significantly up-regulated in DA and non-DA.

4.4 Discussion

Over 25% of women have exposed their babies to prenatal nicotine by smoking during their pregnancy (Bardy et al. 1993). Gestational nicotine exposure via maternal smoking during pregnancy (MSDP) has been associated with many deficits, including ability to self-regulate, ADHD, conduct disorder (CD), decreased in-utero brain growth and cognitive deficits in the offspring (Cornelius & Day, 2009; Herrmann et al. 2008; Pauly & Slotkin, 2008).

In the current work, single cell microarray analysis was employed on dopaminergic cells of nicotine exposed offspring to identify the nicotine responsive mRNAs in dopaminergic neurons of the developing brain. The main purpose of this study was to investigate how gestational nicotine exposure alters the expression levels of the genes expressed in dopaminergic and non-dopaminergic neurons at a whole-genome level. We hypothesized that the influence of gestational nicotine on cells of different types, even located in close proximity within the same brain region, may be different. This is the first study examining the effects of gestational nicotine exposure on single dopaminergic cells of the rat offspring by high-throughput microarray analysis to the best of our knowledge.

Using microarray analysis, we identified both up- and down- regulated genes in each cell types. The up- and down- regulated genes are completely different for dopamine and non-dopamine group. We also identified 135 significantly differentially expressed genes between DA and non-DA cells upon nicotine exposure which confirms our hypothesis that there will be differentially expressed genes as a result of gestational nicotine exposure, if DA and non-DA neurons have different molecular and functional properties. Differentially expressed genes were linked to a series of pathways such as olfactory transduction, ribosome pathway, oxidative phosphorylation, AD, PD, HD, arachidonic and linoleic acid metabolism pathways. Many pathways affected by nicotine were found to be directly related to dopaminergic system which confirms our second hypothesis that there are many differentially expressed genes involved specifically in dopamine related diseases as a result of gestational nicotine treatment.

Taking a closer look at the differentially expressed genes' list, as well as at the genes we found to be up and down-regulated in dopaminergic and non-dopaminergic cells, we were able to uncover the extent to which the molecular alterations brought by gestational nicotine exposure affect dopamine-related pathways and biological processes involved in development

Neurodegenerative disease pathways

Atp5b (ATP synthase H+ transporting mitochondrial F1 complex beta polypeptide),Ndufb4 (NADH dehydrogenase (ubiquinone) 1 beta subcomplex 4) and Ndufb5 (NADH dehydrogenase (ubiquinone) 1 beta subcomplex 5) are the genes found as significantly differentially expressed by our microarray experiments and sharing the

common features as taking parts in the AD, PD, HD and oxidative phosphorylation pathways. Adenine nucleotide translocator (Slc25a4) is another gene that is revealed by the microarray experiments and plays important role both in PD and HD. In addition, Atp6v0e2 is another gene found as significant and playing crucial role in oxidative phosphorylation which is mediated in mitochondria.

Mitochondria have been linked to both necrotic and apoptotic cell death and evidence suggest that mitochondria play a crucial role in PD (Parker & Swerdlow, 1998). Complex I (NADH dehydrogenase) is involved in ATP generation in mitochondria and its inhibition by mitochondrial toxins causes Parkinsonism in humans and animal models of rat (Betarbet et al. 2000; Langston et al. 1983). Evidence demonstrated the reduced expression of β-subunit of ATP synthase (Fukuyama et al. 1996; Parker et al. 1994) and ND4, a subunit of complex I of oxidative phosphorylation in mitochondria, in AD patients, (Chandrasekaran et al. 1997). Adenylate nucleotide translocator gene (Slc25a4) was also found to be down-regulated in preferentially vulnerable neurons in mitochondria in patients with HD (Kim et al. 2010).

Nicotine was reported to protect neurons against complex I inhibitor neurotoxins such as N-methyl-4-phenylpyridinium ion (MPP) and 6-hydroxydopamine (3-NP) (6-OHDA), which may induce Parkinsonism and the neurotoxin 3-nitropropionic acid, which results in HD (Brouillet et al. 1999; Sershen et al. 1986; Soto-Otero et al. 2002). According to our study, Ndufb4, Ndufb5, Slc25a4 and Atp6v0e2 genes are significantly differentially expressed between DA and non-DA neurons upon nicotine exposure. These genes are all down-regulated as a result of nicotine treatment in DA neurons whereas, they are all up-regulated in other neurons. Our results suggest that nicotine may exert a

neuroprotective effect on non-DA neurons through the interaction with mitochondria. Neurons other than dopaminergic ones might express higher level of enzymes of oxidative phosphorylation than DA neurons during development. Genetic expressions in DA producing cells might be more vulnerable to nicotine during development.

Synaptic formation, maintenance and regulation

Dad1 (Defender Against Cell Death 1), Nsfl1c (NSFL1 (p97) cofactor (p47)) and Rnf5 (Ring finger protein 5, E3 ubiquitin protein ligase) are another set of genes which we found as differentially expressed between DA and non-DA neurons They are involved in the protein processing function of endoplasmic reticulum. Many proteins are translocated into endoplasmic reticulum (ER) after translation and post-translational modifications. Misfolded proteins cause neurodegenerative diseases (Kopito & Ron, 2000; Selkoe, 2003). Some neurotoxins have been suggested to cause Parkinsonism by aggregation and deposition of misfolded proteins and ER stress in DA neurons (Kheradpezhouh et al. 2003).

p47 is an UBX domain containing protein (Buchberger et al. 2001) andp97, belonging to the family of AAA ATPases (White & Lauring, 2007). p97-adapter complex facilitates the ER-associated degradation process by binding to the ubiquitylated substrates and deliver ubiquitylated protein to the proteasomes (Meyer, 2012; Schuberth & Buchberger, 2008). Ubiquitination plays an important role in synapse formation, protein degradation, signal transduction, membrane trafficking, synapse maintenance, regulation and brain disorders (DiAntonio & Hicke, 2004; Haglund & Dikic, 2005; Yi & Ehlers, 2007)

Ubiquitin/proteasome system (UPS) regulates the expression of nicotinic receptors (Christianson & Green, 2004; Gaimarri et al. 2007). In our study, Nsfl1c (p97 cofactor 47) expression was down-regulated in DA neurons. Nicotine was reported to lead to accumulation of ubiquitinated synaptic proteins by reducing the proteasomal activity (Rezvani et al. 2007) The significant down-regulation of p97 cofactor p47 gene (Nsfl1c) in DA neurons may be the mechanism of reduced proteasomal activity by nicotine and it might play an important role in the synaptic function of DA neurons. In addition, Rnf5 gene was found to be differentially expressed between DA and non-DA neurons, although the gene is up-regulated in both cell types. Here, our result suggests that the ubiquitinated protein accumulation upon nicotine treatment may also be mediated through the Rnf5 gene up-regulation by nicotine. Since UPS was reported to participate in nicotine dependent synaptic plasticity (Rezvani et al. 2007), nicotine may modulate synapse formation, regulations and signal transduction through the effect of Ring finger protein 5 on ubiquitination process during development.

The dopamine transporter (DAT) is 12-transmembrane glycoprotein with three N-glycosylation sites (Giros & Caron, 1993) responsible for DA uptake. Its activity was determined by its glycosylation status in which more DA is transported compared to non-glycosylated DAT (Li et al. 2004). DAT gene deficiency was suggested to exhibit several key characteristic features of ADHD in animal models (Carpenter et al. 2012; Gainetdinov & Caron, 2000). Many different genes have been reported to regulate DAT function (Gizer et al. 2009; Yamamoto & Novotney, 1998; Zhang et al. 2009). Evidence also showed that nicotine (0.8 mg/kg) decreased DAT cell surface expression in striatum but not in PFC (Zhu et al. 2009). On the other hand, prenatal nicotine exposure was

reported not to change DAT gene expression either in the striatum or the prefrontal cortex neurons (Schneider et al. 2011). In our study, we found out that the expression of Slc6a3 (sodium-dependent dopamine transporter) gene did not change upon gestational nicotine exposure in DA neurons, whereas the expression decreased in non-DA neurons in VTA. Dad1 gene which is related to DAT with regard to being a subunit of oligosaccharyltransferase (OST) that initiates N-linked glycosylation, was found to be significantly differentially expressed between DA and non-DA neurons, however the expression of this gene did not also change in DA cell populations. Therefore, we suggest that nicotine may regulate DAT function beyond expression level probably via regulating DAT protein decoration, or pinocytosis.

Ribosome

Long-term plasticity at synapses is a protein synthesis driven process. Synaptic plasticity requires local translation and it is coupled with synaptic transmission and plasticity (Cajigas et al. 2010; Sutton & Schuman, 2006). In our study, we indicated that many ribosomal subunits geneswere, DA neurons specifically, down-regulated by gestational nicotine treatments. Down regulation of ribosomal subunit genes suggests down-regulation in translation activity and a general down-regulation of protein synthesis in the developing brain upon gestational nicotine exposure. Protein synthesis is also required for several forms of synaptic plasticity such as late phase long-term potentiation (L-LTP), long-term facilitation (LTF) and lon-term depression (LTD) (Klann et al. 2004). In an animal model of ADHD, it was demonstrated that dopamine related protein synthesis was decreased (Li et al. 2007). Hence, the down-regulation of ribosomal

subunit genes by gestational nicotine in DA neurons may also underlie the molecular mechanism of ADHD.

Arachidonic/Linoleic acid metabolism

Brain lipids contain high proportions of the polyunsaturated fatty acids (PUFA) and arachidonic acid (Sastry, 1985). Arachidonic acid is derived from the linoleic acid which is dietary essential fatty acid (EFA) (Sprecher et al. 1995). Arachidonic acid was suggested to be involved in the expression of long-term potentiation which is associated with memory and learning (Nishizaki et al. 1999).

It was shown that linoleic acid deficient diet results in significant decrease in frontal cortex arachidonic acid level and the frontal cortex DA release (Owens & Innis, 1999), resulting in learning memory and habituation impairments (Bourre et al. 1989; Enslen et al. 1991; Frances et al. 1996). Cyp2c22 (Cytochrome P450, family 2, subfamily c, polypeptide 22), Cyp4f6 (cytochrome P450, family 4, subfamily f, polypeptide 6) and Pla2g3(phospholipase A2, group III) are the genes related with Arachidonic/Linoleic acid metabolism pathways and found as significantly differentially expressed between DA and non-DA neurons upon gestational nicotine treatment in our microarray study. Cytochrome P450 (CYP2c) enzymes convert arachidonic acid into its metabolites, thus modulate homeostasis and have anti-inflammatory effect. Phospholipase A2 (Pla2) gene was reported to mediate arachidonic acid release. Evidence showed that nicotine reduced brain arachidonic acid signaling (Garrido et al. 2001).In our study, nicotine greatly down-regulated the expressions of Cyp2c22 and Pla2g3 genes in DA neurons. We suggested that down regulation of arachidonic acid pathway related genes in DA neurons by

nicotine may be involved in long term potentiation impairments and ultimately affect the learning and memory of the offspring during the development.

G-protein coupled receptor signaling pathway

The up- and down- regulated gene lists contained several genes related to the G-protein coupled receptor pathway. These genes are known to play major roles in neural differentiation, apoptosis and cell survival. Cart peptides are found in brain regions associated with drug addiction (Hubert et al. 2008; Jaworski & Jones, 2006; Kimmel et al. 2000). Cocaine- and amphetamine-regulated transcript (CART) is one of the significantly down-regulated genes in DA neurons upon gestational nicotine exposure. Our results showed that nicotine down-regulates the CART expression upon gestational nicotine exposure in DA neurons which suggested that the expression of this gene may also be modulated by gestational nicotine, CART were also suggested contributing to the migration of the DA neurons of the substantia nigra (Brischoux et al. 2001), and had neuroprotective effects, as well as promoting neuronal differentiation and development (Louis, 1996) . Thus, the down regulation of the CART expressions in VTA DA neurons during brain development period may significantly affect the differentiation and development of the young DA neurons, which maybe a possible reason for the DA system dysfunction diseases found in clinical studies.

In addition, CXCL16 is another important gene found being significantly down-regulated in DA neurons. It has been indicated to activate the signaling pathways related to cell survival and apoptosis such as ERK pathway (Vlahakis et al. 2002; Ward & Westwick, 1998). CXCL16 was also known to promote the secretion of neuroprotective

factors by the activation of astrocyte (Lauro et al. 2008; le Blanc et al. 2006; Rosito et al. 2012). The reduced neuroprotection on DA neurons, possibly from the down-regulated of CXCL16 expression, could be another factor that contributed to the pathological mechanism induced by gestational smoking.

CHAPTER 5: CONCLUSION AND FUTURE STUDIES

In the first part of this dissertation, age-area specific regulations of PACAP, LPL and GRP neuroprotective genes by nicotine were investigated in three different age groups; 3-month, 12-month and 24-month old rats and two different brain regions; VTA and SN. One of the main purpose of the study was to investigate whether the differential expression of PACAP, LPL and GRP neuroprotective genes which were reported to be area-specifically higher expressed in VTA neurons compared to SN, are associated with ageing, and whether the area-specific difference on genes expressions, which may contribute to the difference on DA neurons survival rate, still exist on aged experiment subjects. Our results suggest that the LPL gene, not PACAP or GRP, is significantly differentially expressed between VTA and SN in the senior population. Differential expression of LPL gene in senior population may contribute to the different survival rate for DA neurons within the VTA and SN.

Another purpose of the study was to investigate whether and how nicotine area-specifically regulates the expressions of PACAP, LPL and GRP genes, associated with ageing. Nicotine treatment did not show any up-regulatory effects on the expression of neuroprotective genes in adult and old groups. Down-regulation of the gene of interests by nicotine suggests that these genes may not be related with the neuroprotection provided by nicotine that reduces the risks of PD.

Here, we investigated the transcription level changes in Pacap, Lpl and Grp expressions. It would be ideal to integrate our results with the protein level analysis to

explore the relationship between transcripts and corresponding protein levels. Protein level analysis of the Pacap, Lpl and Grp gene can be performed as a future study.

The next set of the experiments in this dissertation is the first study in this field to examine the effects of gestational nicotine on single DA neurons of the offspring by high-throughput microarray analysis. The purpose of the study was to identify nicotine responsive and differentially expressed genes between DA and non-DA neurons upon gestational nicotine exposure. Better understanding of the impacts of gestational nicotine and related diseases in dopaminergic cell specific manner is required since it has been well accepted that DA neurons are more prone to actions of drugs of abuse.

To elucidate the alterations in gene expressions of the rat brain upon gestational nicotine exposure oligonucleotide arrays were used. Microarray analysis revealed up or down regulated genes upon nicotine exposure in DA neurons. A set of 135 genes were also identified as significantly differentially expressed between DA and non-DA neurons upon exposure to nicotine during pregnancy and breastfeeding. Groups of genes related to oxidative phosphorylation, protein synthesis, olfactory transduction, AD, PD, HD, arachidonic acid and linoleic acid metabolism and ribosome pathways were identified.

Our results suggest that gestational nicotine influence mitochondria related neurological diseases unfavorably in DA neurons by reducing the expression of two types of NADH dehydrogenase and one β-subunit ATP synthase mitochondrial gene. NADH dehydrogenases are involved in ATP generation and its inhibition induces Parkinsonism (Betarbet et al. 2000; Langston et al. 1983). In addition, the up-regulation of these genes in non-DA neurons indicate that nicotine may exert neuroprotective effect on non-DA

neurons through the interaction with mitochondria and possibly through the expression of higher level of oxidative phosphorylation enzymes during development.

Our results also suggest that gestational nicotine may affect the synaptic function and plasticity in developing brain through the regulation of genes encoding ER proteins involved in ubiquitin/proteasome system. In addition, stable expression of dopamine transporter (DAT) gene in dopaminergic cells suggests that nicotine does not directly affects DAT expression in these neurons. We suggested that the regulation of nicotine on DAT may be above expression level.

Down regulation of many ribosomal subunit genes suggest a decrease in translation activity, synaptic plasticity and a general down-regulation of protein synthesis in the developing brain upon gestational nicotine exposure. Impaired memory and learning abilities in the offspring is expected, since our study revealed several down-regulated genes in arachidonic acid pathway which is related to locomotor activity.

Significantly up/down regulated genes in DA neurons are mostly related to G-protein coupled protein receptor signaling and developmental processes. The significant down regulation of the genes involved in G-protein coupled protein receptor signaling is involved in processes such as differentiation and development of the neurons and neuroprotection. The deficiencies in these processes may be a possible reason for the DA system dysfunctional diseases found in clinical and they may be the pathological mechanism induced by gestational smoking in DA neurons.

These findings uncover genetic alterations specific to DA neurons induced by gestational nicotine which help us to better understand cell-specific pathological

mechanisms for the diseases induced by gestational nicotine during development. The different set of up or down- regulated genes in DA and non-DA neurons confirms our hypothesis that the influence of gestational nicotine on cells of different types, even located in close proximity within the same brain region, are different.

There were many challenges in this single cell microarray study regarding to the amount of the initial RNA concentrations obtained from single cells. This little amount is difficult to manipulate experimentally. Even after the amplification, since microarray experiments require microgram amounts of input RNA, we were unable to save additional samples for further anaylses such as qRT-PCR. Therefore, the number of replicates decreased and the statistical analysis become difficult with the limited number of replicates. We had to pool our data in order to increase the degree of freedom for the statistical analysis in qRT-PCR experiments.

Another challenge regarding our experiment was to harvest neurons one by one using patch-clamp system. Patch-clamp experiments are technically challenging, and require skill and patience. Forming high-quality tight seals between the glass pipette and the target cell, patch-clamping without damaging the cells, available number of alive neurons on the slice and limited number of dopamine neurons are just some of them. It takes days to collect one sample with enough cell numbers.

The lack of dye-swap pairs in microarray experiment may leave an experiment prone to a systematic color bias since the Cy3 and Cy5 dyes has systematic differences in the red and green intensities which require correction. Therefore, extra attention was given to data normalization to eliminate this problem. Another problem was the low

amount of available RNA to perform validation of the results by qRT-PCR. Although using amplified aRNA was previously reported to be appropriate for the validation of the results (Morris et al. 2011), it may also introduce some questions such as whether it control for the effects that RNA amplification may have on the relative levels.

As future work, the set of up/down regulated and significantly differentially expressed genes identified in this microarray study can be further analyzed. Genes belonging to each pathway identified in this study can be further examined using samples exposed to different nicotine concentrations or different drugs. The effect of different concentration of nicotine on the offspring can also be tested on the whole genome level.

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