Research Article

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Investigation of Gene Expression Levels of Some Proteins Related to the Pathogenesis of Parkinson's Disease in Rats Exposed To Prenatal Stress

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ABSTRACT

This study was carried out in order to better understand how prenatal stress (PS) may affect the future onset of Parkinson's disease (PD). A dexamethasone-induced PS model was established in rats for the study. Changes in the expression levels of tyrosine hydroxylase (TH), α-synuclein (SNCA), dopamine transporter (SLC6A3), and parkin (PRKN) proteins, which play role in PD pathogenesis, were demonstrated by real-time PCR in the cerebral cortex of male rats exposed to PS. From GD 14 to 21, pregnant rats were injected daily with Dex or saline (100 µg/kg and 1 ml/kg). 3 months after birth, male rats underwent decapitation (n=5), cerebral cortex dissection was performed. Total RNA was isolated from cortexes and used for cDNA synthesis. Gene expression analyzes were performed according to the ΔΔCT method. The statistical differences between groups were analyzed by the Mann-Whitney test. Statistics were deemed significant at a level of 0.05. Dex exposure throughout pregnancy significantly increased mRNA levels of TH and SLC6A3. No significant differences were found in the mRNA levels of PRKN and SNCA between experimental groups. In conclusion, offspring exposed to PS may be more susceptible to PD in adulthood through changes in the cortical mRNA levels of TH and SLC6A3.

Keywords: Prenatal stress, Parkinson's Disease, Tyrosine hydroxylase, Dopamine transporter

1. Introduction

The prenatal period covers the period from the beginning of pregnancy until the birth of the baby [1]. The neural system development in this period follows a very complex process regulated by special programming. In this process, basic stages such as cell proliferation, cell migration, and differentiation ensure the formation of normal brain structure and functions. When this critical period is evaluated, it has been concluded that the brain is particularly vulnerable to changes in the prenatal period [2].

Stress is generally a reaction to situations in which a person feels that the demands placed on him/her exceed his/her adaptive limits [3]. Pregnant women may also experience stress during pregnancy for various reasons. In this case, prenatal stress activates the HPA (hypothalamic pituitary adrenal) axis in the expectant mother, resulting in the secretion of various corticosteroids, especially cortisol, the main glucocorticoid in humans, and corticosterone in rodents [4,5]. Although it is known that the HPA axis becomes less sensitive towards the end of gestation [6], prenatal endogenous or exogenous glucocorticoid overexposure can affect different parts of the brain, acting as an endocrine disruptor and interfering with neurogenesis and differentiation. This may set the stage for neurological disorders that may be seen in offspring years later [2,4].

Parkinson's Disease was clinically described more than two centuries ago by the English physician James Parkinson. It consists of a neurodegenerative disorder associated with the reduction of dopaminergic nerve cells and the existence of pathological Lewy bodies in the substantia nigra of the brainstem in the lower part of the brain. This process of neurodegeneration is accompanied by motor symptoms including tremors, bradykinesia, muscle stiffness, dyskinesia, in addition non-motor symptoms including depression, anxiety, dysphagia and cognitive disorders. [7-9]. Although the specific cause of the disease is unidentified, genetic and environmental factors, old age, and male gender are thought to participate in the etiology of the PD [9].

When we focus on the genetic elements of PD, we see that only 5-10% of patients are affected by the monogenic/Mendelian form and rare mutations inherited familially. However, the majority of the remaining patients are thought to be affected by a combination of DNA sequence variations, environmental/lifestyle, and epigenetic factors; thus the disease is thought to be multifactorial. Studies on the genetic basis of PD have accelerated with the discovery of Lewy bodies and mutations in α-synucleins, the main component of these bodies. In the last years, studies have been carried out the inheritance of PD, genes and loci affected by the disease, and mutations. Within the framework of these studies and developing genetic applications, autosomal dominant and autosomal recessive inheritance gene mutations, risk loci, and mutations encountered have been defined. Today, 18 specific chromosomal regions and loci have been identified and named PARK. Among these, SNCA, which plays a role in autosomal dominant inheritance, is numbered as PARK1-4, and Parkin, which plays a role in autosomal recessive inheritance, is numbered as PARK2 [10-12].

The SNCA gene encodes the α-Syn protein, which has 140 amino acids and is highly expressed in the presynaptic regions. Although the functions of α-Syn have not yet been elucidated in detail, it is thought to be involved in synaptic plasticity and neurotransmitter release, thereby contributing to neurotransmission [8,16].

The unfolded monomers and α-helically folded tetramers of α-Syn are in a dynamic balance under normal conditions, and α-Syn has a minimal propensity for aggregation in the same conditions. The aggregation process is initiated by a reduction in the tetramer-monomer ratio and an accompanying rise in the amount of α-Syn unfolded monomers. Soluble α-synuclein monomers initially form oligomers, then gradually coalesce to form small protofibrils and finally large, insoluble α-synuclein fibrils, Lewy bodies. The fibrils acquire a β-sheet-rich conformational structure during the aggregation process. With this change, α-Syn molecules can aggregate and damage neurons [16,17].

Mutations, mismatches between α-Syn synthesis and degradation, and environmental factors can modify the aggregation potential of α-Syn. Mutations detected in α-Syn are thought to promote the development of severe forms of parkinsonism such as early-onset PD, PD with dementia, and idiopathic PD. Similarly, recent studies have shown that neuronal and synaptic activity dynamically regulates the physiological release of endogenous α-Syn, thus, high neuronal activity increases α-Syn release [16,17].

Furthermore, α-Syn undergoes various post-translational modifications, including phosphorylation.

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Phosphorylation of α-Syn at the serine 129 residue has been extensively studied as it is considered to be a major pathological hallmark of PD. It is known that 90% of α-Syn is phosphorylated in the brains of patients with PD, whereas only 4% of α-Syn is phosphorylated in healthy brains. However, there is still no clear consensus on whether phosphorylation plays an active role in α-Syn aggregation [16,18].

According to another approach, the decrease in the activities of the lysosomal autophagy system (LAS), where various autophagic processes are carried out, and the ubiquitin-proteasome system (UPS), one of the most important protein degradation systems, with aging can lead to a boost in α-Syn levels. When misfolded, mutated α-Syn accumulations cannot be degraded by these dysfunctional systems, neuronal death may eventually occur [13,17].

Parkin, a protein of 465 amino acids, is expressed from the Park 2 gene, known as the second largest gene in the human genome [12]. Park 2 is the first gene identified as an autosomal recessive inheritance of Idiopathic Parkinson's Disease (IPD) and the second gene identified in association with IPD. Studies in various ethnic groups have identified a large number and wide spectrum of Parkin mutations, including changes in all exons. Homozygous mutations in Parkin have been shown to be the most common cause of a type of PD known as Juvenile Parkinson's Disease (JPD), which starts at age 20 or younger. Mutations in the Park 2 gene and loss of function in the Parkin protein have been detected to make neurons an easier target for cytotoxic effects [14,15].

Mutations in the Parkin gene prevent Parkin from functioning as an E3 ubiquitin ligase that regulates ubiquitination efficiency and controls the recognition of its substrates through posttranslational modifications such as phosphorylation by reactive oxygen species (ROS) and reactive nitrogen species (RNT) or S-nitrosylation (SNO) [19,20]. This can lead to the accumulation of substrates such as aminoacyl tRNA synthetase complex interacting multifunctional protein-2 (AIMP2), far upstream element binding protein-1 (FBP-1) and subsequently to mitochondrial dysfunction and neuronal toxicity [19].

Some autopsy studies have shown variable results in terms of Parkin and Lewy body pathology. In particular, early studies showed the absence of Lewy bodies in patients with PARK2 mutations and questioned their relevance to sporadic PD. However, in later case reports, Lewy bodies were found in homozygous and heterozygous PARK2-PH cases [18]. Consequently, it is conceivable that any change in Parkin levels or activity may cause a significant difference in neuronal integrity. Thanks to these rich neuronal effects, Park 2 has become a gene region worthy of investigation in Parkinson's Disease.

The dopamine transporter (DAT) gene, identified as SLC6A3, plays a critical role in maintaining the integrity of dopaminergic neurons. The contribution of DAT, which basically regulates dopamine signaling and maintains dopamine homeostasis, in the process leading to Parkinson's Disease is still under investigation, but several theories exist. For this, a detailed focus on the functions of DAT is needed. DAT is expressed at high concentrations in dopaminergic neurons in the substantia nigra. Its main function is to allow the reception of dopamine from the synaptic cleft to presynaptic neurons, thereby terminating dopamine transmission in the brain. However, this reception activity causes dopamine to accumulate, and dopamine accumulating in the cytosol is easily oxidizable, thus increasing the susceptibility of dopaminergic neurons to damage. Oxidation of accumulated dopamine can lead to the production of free radical species and neurotoxicity [21]. According to another view, DAT can also receive neurotoxins such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridinium (MPP^+), into neurons. This can damage dopaminergic neurons [21].

In general, studies on the neurotoxic effects of dopamine are available in the literature. It has also been reported that overexpression of the dopamine transporter leads to neuronal loss. However, the process of dopamine reuptake by DAT is the main mechanism for the maintenance of dopamine storage in neurons, and loss of DAT can lead to dopamine depletion. The excitability of presynaptic terminal neurons is also directly regulated by depolarizing currents produced by dopamine reuptake. Therefore, for dopaminergic neurons to function optimally, DAT must be expressed at reasonable levels [22].

The enzyme tyrosine hydroxylase (TH) constitutes the rate-limiting step in the biosynthesis of catecholamines, which function as hormones and neurotransmitters. This enzyme, found in all dopaminergic cells, is composed of a tetrahydrobiopterin-bound protein with non-heme iron [23]. Dopamine biosynthesis

begins with the conversion of L-tyrosine to L-dihydroxyphenylalanine (L-DOPA) via a hydroxylation reaction using iron (Fe^{2+}), tetrahydrobiopterin (BH_4), and oxygen (O_2) as cofactors and substrate. This step involves tyrosine hydroxylase, the rate-limiting enzyme in biosynthesis. L-DOPA is then converted to dopamine by aromatic L-amino acid decarboxylase, which in turn is converted to noradrenaline by dopamine β-hydroxylase. Finally, noradrenaline is converted to adrenaline by the enzyme phenylethanolamine N-methyltransferase (PNMT) [24].

So far, many different types of prenatal stress models have been created to observe the effect of prenatal stress on offspring. However, most of them involve painful and uncomfortable processes for the experimental animals. Recently, the injection of glucocorticoids, which are expected to increase in the blood due to stress, into experimental animals has been used as a new alternative. Corticosterone or its synthetic derivative, dexamethasone injections, are used for this purpose in rats [25-27]. In this study, a prenatal stress model was created on the basis of dexamethasone injection. We examined the differences in gene expression of proteins involved in the pathogenesis of PD in the cerebral cortex in prenatal stress model induced by dexamethasone in rats. For this purpose, alterations in the expression profiles of TH, SNCA, SLC6A3, and Parkin proteins were determined via real-time PCR in the cerebral cortex of male rats exposed to prenatal stress.

2. Material and Methods

2.1. Establishment of Prenatal Stress Model

The Ege University Ethics Committee on Animal Experiments approved this study (05/24/2017; Reference No. 2017-023). The rats were obtained from the Ege University Experimental Animals Application and Research Center and maintained in the experimental animal laboratory of the same center. The rats were kept in plastic cages covered with steel wire bars, where they had easy access to special feed and water. The male/female ratio of the rats in the cages was adjusted to 1/1, and they were allowed to mate among themselves. The pregnancy of the rats was monitored by daily cervical swabs and female rats were separated into two different groups after identifying the first day of gestation. Pregnant rats in the first group (n=3) were given intraperitoneal (i.p.) injection of 1 ml/kg/day saline from the beginning of the third trimester of pregnancy (day 14) until the last day of pregnancy (day 21) (Control Group). Pregnant rats in the second group (n=4) were given 0.1 mg/kg/day of dexamethasone intraperitoneally (i.p.) from the beginning of the third trimester of pregnancy (day 14) until the last day of pregnancy (day 21) (Prenatal Stress Group). The offspring of each pregnant rat were transferred to cages with their littermates on postnatal day 21, and the young rats were allowed to grow. Thus, experimental groups with and without exogenous steroid exposure were obtained [25,27,28,29]. In the present study, five male rats from each group were used. Prenatal dexamethasone-treated and untreated rats were decapitated at the end of the third month after birth. Cerebral cortex samples were dissected on ice and kept at -86°C until the day of the experiment (Figure 1).

2.2. Isolation of Total RNA & Spectrophotometric Detection of Total RNA Integrity and Concentration

A nucleic acid isolation kit (Lucigen, Catalog No. MC85200) was used to isolate total RNA from cerebral cortex samples. For purity control and concentration determination of RNAs, absorbance data were quantified at 260 and 280 nm wavelengths in a microplate reader. RNAs with Abs260/Abs280 ratio between 1.8-2.0 were diluted with Tris-EDTA buffer to an ultimate concentration of 300 ng/µl. The isolated total RNAs were stored in a deep freezer (-86°C) until the day of cDNA synthesis.

2.3. Synthesis of Complementary DNA (cDNA) by Reverse Transcription & Analysis of Gene Expressions by Real Time PCR

RevertAid First Strand cDNA synthesis kit (Thermo Fisher Scientific, Catalog No. K1622) was used for cDNA synthesis. RNA sample (5 µl), random primer (1 µl) and nuclease-free water (6 µl) were mixed in a PCR tube on ice. Incubated at 65°C for 5 minutes (min) to denature proteins. Then 5X reaction buffer (4 µl), ribonuclease inhibitor (1 µl), 10 mM dNTP mixture (2 µl) and reverse transcriptase enzyme (1 µl) were added and mixed. Samples were incubated with the PCR program consisting of 5 min at 25°C, 1 hour (h) at 42°C and 5 min at 70°C. Synthesized cDNAs were stored in a deep freezer (-86°C).

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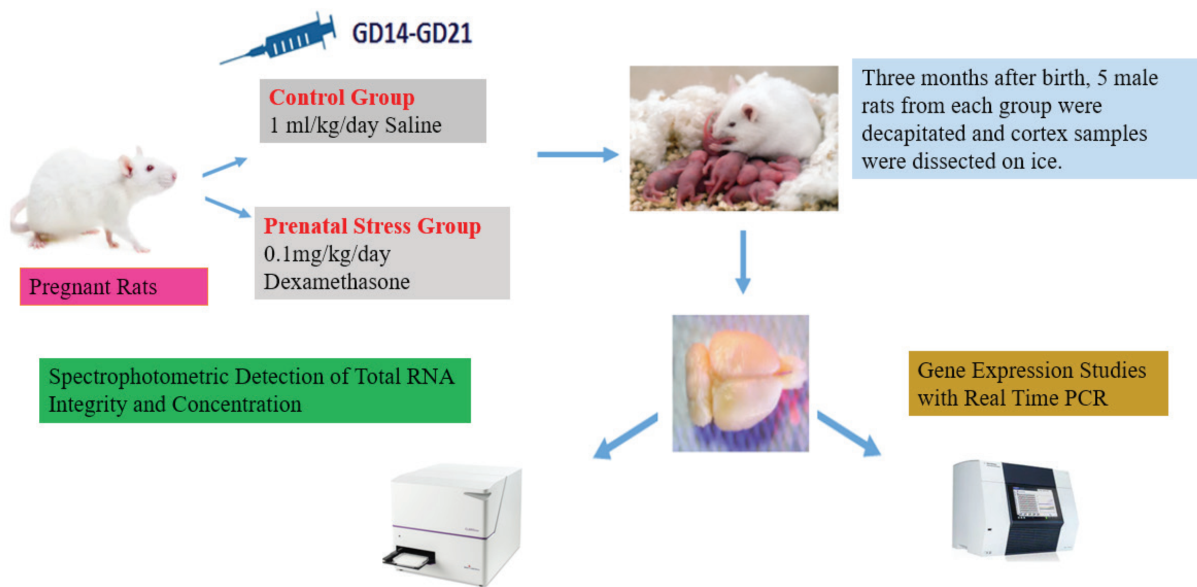

Figure 1. Design of the Experiments

Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies, Cat. No. 600882) was used for the real-time PCR assay. The primers given in Table 1 were used to detect the TH, SLC6A3, SNCA, PRKN, and β-actin genes. Primers were designed by the Primer-BLAST program, and their suitability was verified by an in silico PCR program [30,31].

The cDNA samples were diluted 2/5, and the primers were diluted with nuclease-free water to a final concentration of 25 µM. 1 µl of diluted cDNA, 25 µM forward (F) and reverse (R) primers (1 µl each), and nuclease-free water (7 µl) were mixed. 10 µl SYBR Green solution was added to the mixture without bubbles. Initial denaturation was performed at 95°C for 3 min followed by 40 cycles of 20 seconds (s) at 95°C and 20 s at 60°C. Melting curve analyses were performed by adding a new segment at the end of the 40th cycle for 1 min at 95°C, 30 s at 55°C, and 30 s at 95°C. The temperature required to unfold 50% of a DNA strand is expressed as the melting point (Tm). Specific and non-specific binding between DNA and fluorescent dye during PCR is detected by melting curve analysis [32]. Purity control of gene expression products was performed by melting curve analysis. The comparative ΔΔCT procedure was implemented to determine the expression levels of gene products [33]. β-actin was utilized here as a reference gene, and the control group was utilized for comparison.

2.5. Statistical Analysis

Experiments were performed with five male rats from each experimental group in three replicates. The result data obtained were documented as mean value ± standard error. Statistical contrasts between the two groups were evaluated with the non-parametric Mann-Whitney test with $p<0.05$ being considered significant.

3. Results and Discussion

3.1. Relative TH mRNA Expressions and Melting Curve Analysis

Relative TH mRNA levels were found to be 1.012 ± 0.070 in the control group and 3.954 ± 0.341 in the prenatal stress group (Figure 2). 0,1 mg/kg dexamethasone treatment significantly increased TH expression compared to control ($p<0.01$). The Tm value of the TH gene product was 81.00°C, and the melting curve analysis is given in Figure 3.

3.2. Relative SLC6A3 mRNA Expressions and Melting Curve Analysis

Relative SLC6A3 mRNA levels were 1.009 ± 0.046 in the control group and 1.469 ± 0.083 in the prenatal stress group (Figure 4). 0,1 mg/kg dexamethasone injection significantly increased SLC6A3 expression

Table 1. Primers of PRKN, SNCA, TH, SLC6A3 and β-actin Genes.

Gene:	Gene Bank Numbers:	Primers:	PCR Product:
PRKN	NM_020093.1	F:5'-TGACCAGCTGCGAGTGATT-3' R:5'-CCCCTCCAGAGGCATTTGTT-3'	143 bp
SNCA	NM_019169.3	F:5'-AAGGGTACCCACAAGAGGGA-3' R:5'-AACTGAGCACTTGTACGCCA-3'	178 bp
TH	NM_012740.4	F:5'-CCTTCCAGTACAAGCACGGT-3' R:5'-TGGGTAGCATAGAGGCCCTT-3'	109 bp
SLC6A3	NM_012694.2	F:5'-GTCACCAACGGTGGCATCTA-3' R:5'-AATTGCTGGACGCCGTAGAA-3'	122 bp
β-actin	NM_031144.3	F:5'-GCAGATGTGGATCAGCAAGC-3' R:5'-AAAGGGTGTAAAACGCAGCTC-3'	104 bp

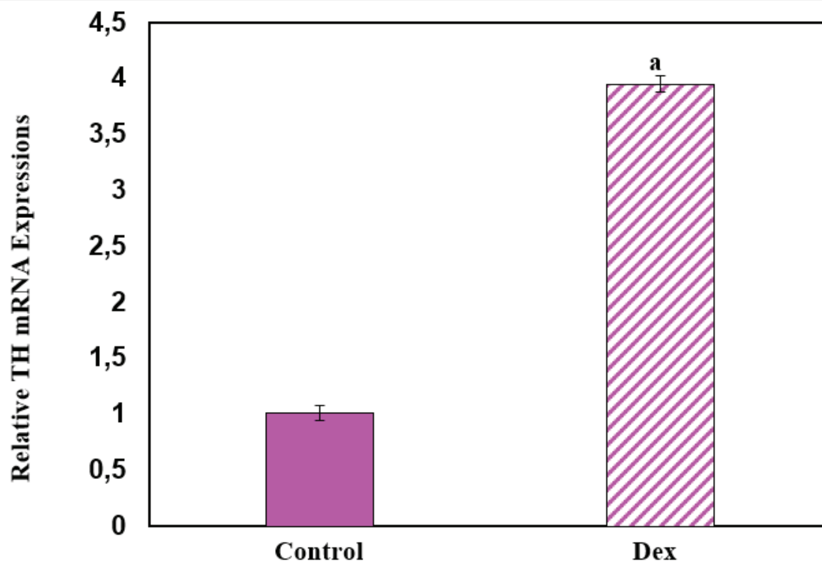

Figure 2. Relative TH mRNA Expression Levels in Cerebral Cortex Samples (Mean ± Std. Error). [a]*p<0.01*; indicates a significant difference between the two groups (n=5)

compared to the control group (p<0.01). The Tm value of the SLC6A3 gene product was 84.50°C, and the melting curve analysis is given in Figure 5.

3.3. Relative SNCA mRNA Expressions and Melting Curve Analysis

Relative SNCA mRNA levels were 1.006 ± 0.054 in the control group and 0.921 ± 0.032 in the prenatal stress group (Figure 6). No remarkable change was found between the two groups. The Tm value of SNCA gene product was 83.50°C, and the melting curve analysis is given in Figure 7.

3.4. Relative PARK2 mRNA Expressions and Melting Curve Analysis

Relative PARK2 mRNA levels were 1.011 ± 0.078 in the control group and 1.335 ± 0.109 in the prenatal stress group (Figure 8). 0.1 mg/kg dexamethasone injection did not cause a significant alteration in PARK2 expression. The Tm value of PARK2 gene product was 82.00°C, and the melting curve analysis is given in Figure 9.

According to Nagatsu and Nagatsu, TH was the discovery of the National Institutes of Health (NIH) in 1964, TH is known to catalyze the primary and rate-limiting step in the biosynthesis of catecholamines,

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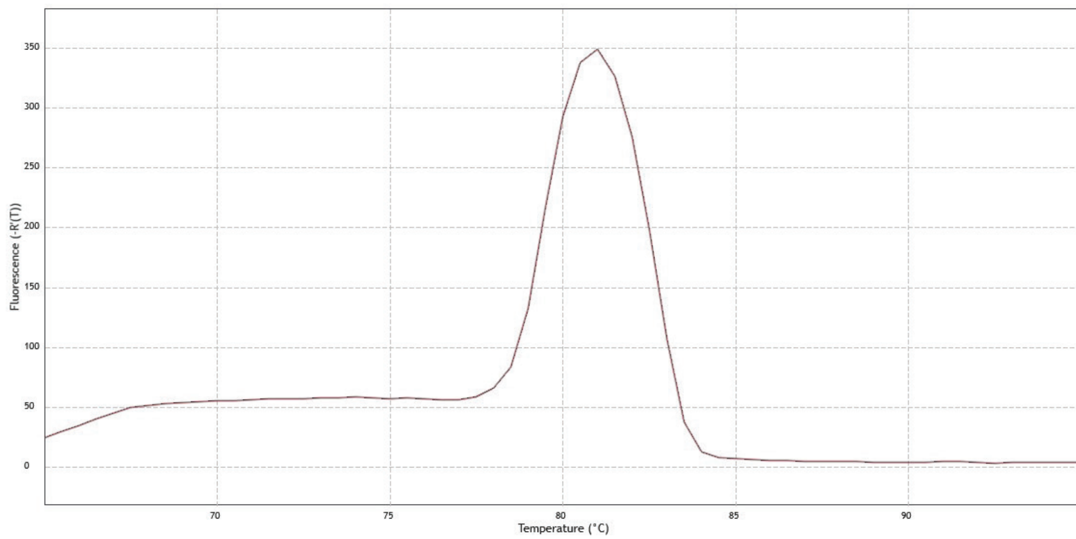

Figure 3. Melting Curve Graph of TH Gene Product (Tm=81.00°C).

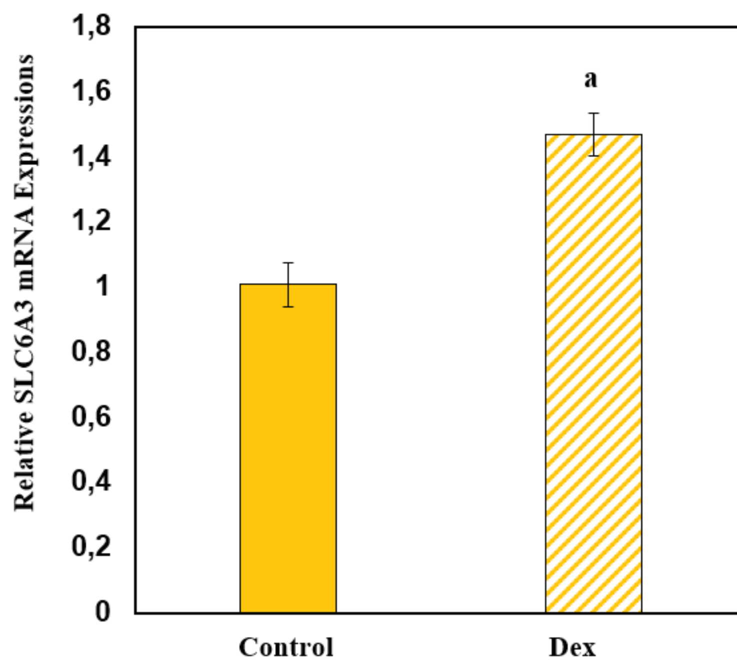

Figure 4. Relative SLC6A3 mRNA Expression Levels in Cerebral Cortex Samples (Mean ± Std. Error). [a]$p<0.01$; indicates a remarkable difference between the two groups (n=5).

including dopamine, adrenaline and noradrenaline. [34]. In a study by Nagatsu and Sawada, significant decreases in TH mRNA expression levels and protein content were detected in the substantia nigra and striatum regions of PD caused by degeneration of dopaminergic neurons [35]. However, according to a review by Tabrez et al., increased phosphorylation of Ser40, one of the three sites regulating TH activity, may increase TH activity and cause high amounts of dopamine production, which may lead to neuronal damage [23]. Therefore, regulation of TH activity is positioned at a very important point in terms of PD.

In a study conducted by Converse et al. on rhesus monkeys, it was stated that daily stress experienced by mothers during pregnancy causes a boost in stri-

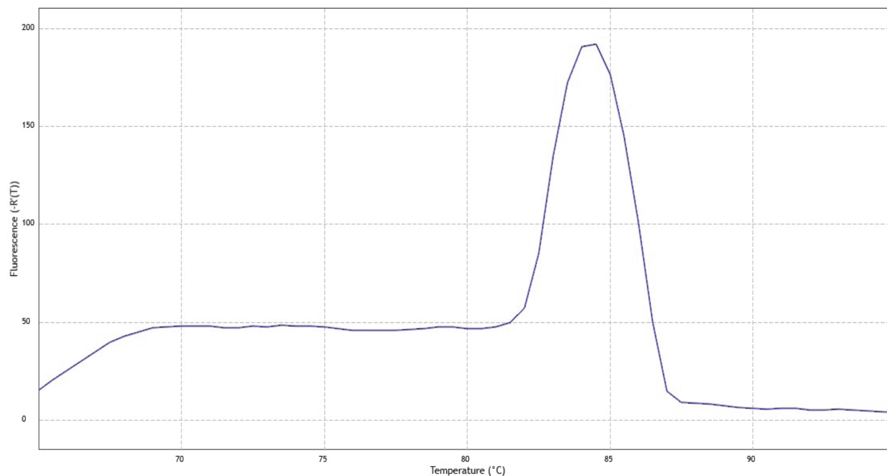

Figure 5. Melting Curve Graph of SLC6A3 Gene Product (Tm=84.50°C).

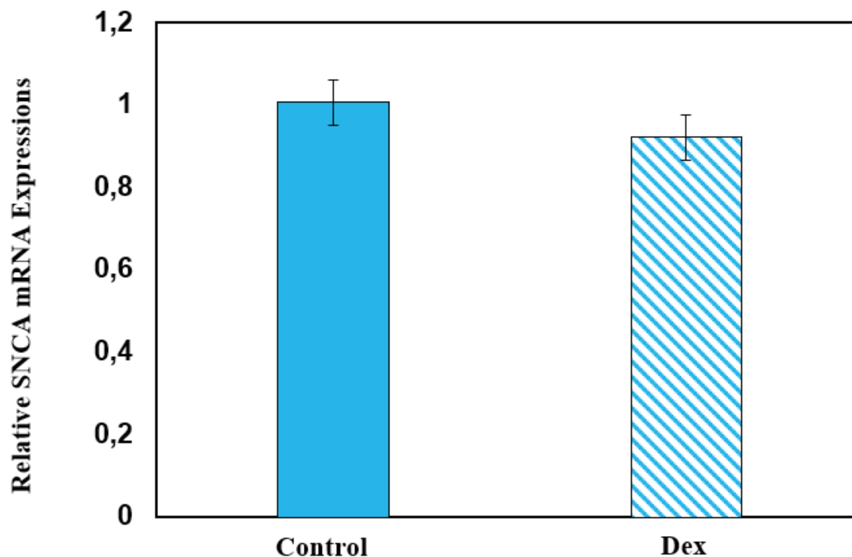

Figure 6. Relative SNCA mRNA Expression Levels in Cerebral Cortex Samples (Mean ± Std. Error, n=5).

atal dopamine transporter connectivity in offspring, which may lead to behavioral abnormalities [36]. There are also studies suggesting that genetic differences in DAT expression levels may explain the selective sensitivity of nigrostriatal dopaminergic neurons observed in PD patients [21]. If the genetic relationship between SLC6A3 and Parkinson's Disease is investigated more extensively, the activity of this gene in PD will be more clearly revealed.

In the experiments conducted within the scope of this study, it was investigated in which direction dexamethasone applications affect the mRNA expression levels of TH, DAT, Parkin, and SNCA proteins involved in the pathogenesis of PD. Although there are no studies directly investigating the relationship of the genes included in the study with prenatal stress or glucocorticoids, according to the results obtained from our study, significant increases were determined in the mRNA expression levels of TH and DAT proteins in the dexamethasone group compared to the control group. Nevertheless, no significant changes were noticed in PARK2 and SNCA genes when compared between the Control and Prenatal Stress groups. Accordingly, TH and SLC6A3 mRNA expression in the cortex of individuals subjected to prenatal stress is higher than that of individuals not subjected to prenatal stress. Due to its chemical na-

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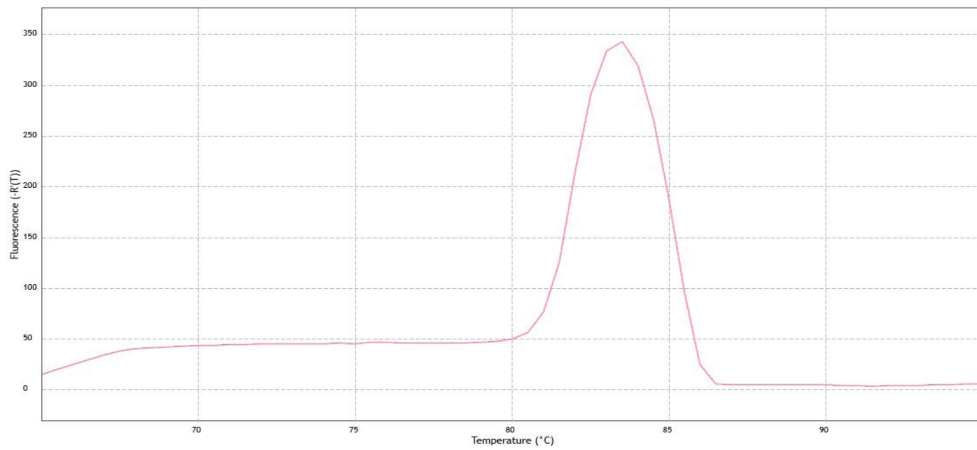

Figure 7. Melting Curve Graph of SNCA Gene Product (Tm=83.50°C).

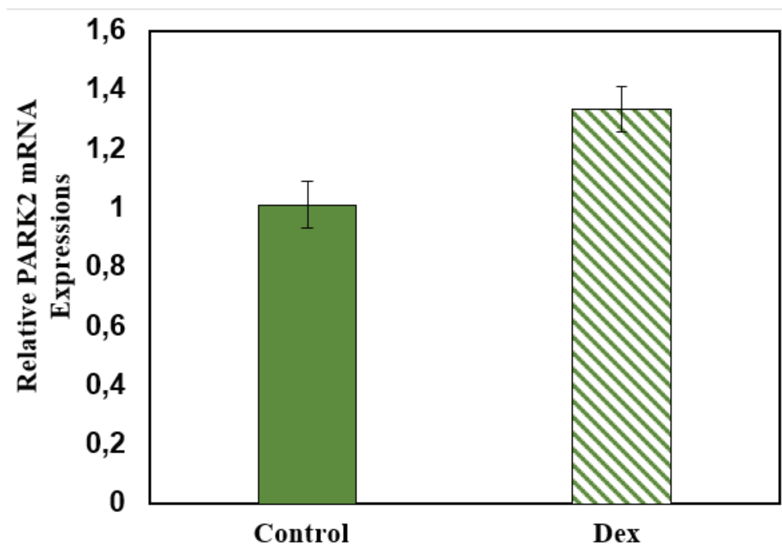

Figure 8. Relative PARK2 mRNA Expression Levels in Cerebral Cortex Samples (Mean ± Std. Error, n=5).

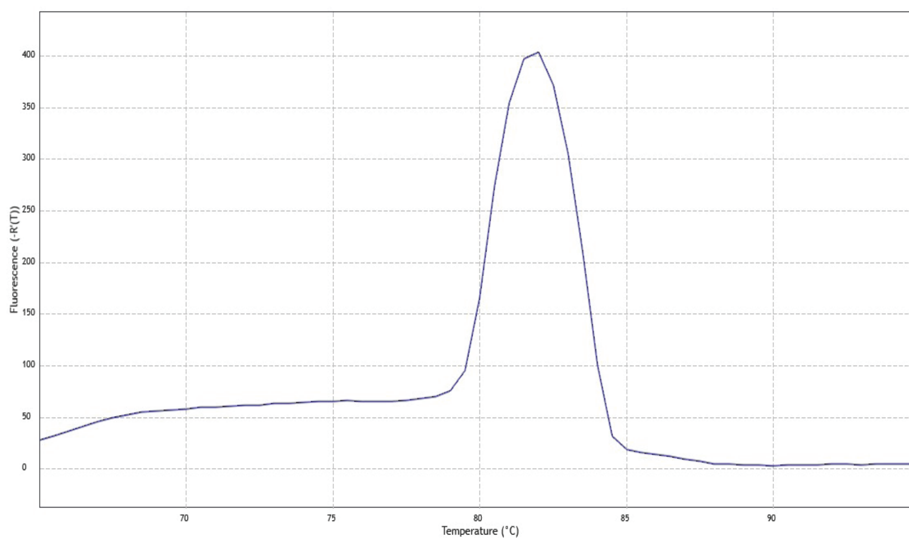

Figure 9. Melting Curve Graph of PARK2 Gene Product (Tm=82.00°C).

ture, dopamine is highly susceptible to mitochondrial dysfunction and oxidative damage, and it is known that TH and DAT proteins have the potential to form an endogenous toxin [21,23], and when these literature data are evaluated together with our study, it can be considered that dopamine levels in neuronal cells may increase due to an increase in mRNA expression levels of TH and DAT proteins more than they should be with prenatal stress, which may expose dopaminergic neurons to irreversible impairment and even cause neuronal death.

4. Conclusion

We assessed whether there is a link between prenatal stress and changes in mRNA expression levels of TH, DAT, Parkin, and SNCA proteins, which are thought to be among the genetic components of Parkinson's Disease. With the noticeable increases in TH and DAT mRNA expression levels in the cerebral cortex of the experimental group that experienced prenatal stress, it can be considered that these individuals will be more likely to develop Parkinson's Disease in the future compared to other individuals.

In the literature, the relationship between PD and both genetic and environmental factors has been emphasized. In this research, the impact of prenatal stress on genes responsible for the pathogenesis of PD was investigated thus developing a new perspective on the subject in terms of the literature. It is predicted that this study will give new ideas and different perspectives to future researchers on PD. In this context, as the effects of prenatal stress and candidate genes associated with PD are investigated, the negative features of these effects can be prevented or eliminated with new treatment plans to be developed and prevented before the disease occurs.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Statement of Contribution of Researchers

Concept – S.E.T.O.; Design – S.E.T.O.; Supervision – S.E.T.O.; Resources – S.E.T.O.; Data Collection and/or Processing – S.E.T.O.; Analysis and/or Interpretation – S.E.T.O.; Literature Search – S.E.T.O., İ.V.; Writing – İ.V.; Critical Reviews – S.E.T.O.

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