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The Underlying Mechanisms of Dystonia

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THE UNDERLYING MECHANISMS OF DYSTONIA

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Abstract

Dystonia is a neurological movement disorder associated with uncontrolled and painful twisting of the body. This study is focused on DYT1 dystonia, which is an inherited form of early onset generalized dystonia. It is caused by a GAG (Guanine, Adenine, Guanine) tri-nucleotide deletion in the DYT1 gene. Although the mechanisms underlying the symptoms associated with dystonia are not known, a number of research studies suggest that impaired signaling via the dopamine neurotransmitter is involved. Since the onset of DYT1 dystonia occurs in childhood, the hypothesis is that changes in dopamine neurotransmission in DYT dystonia occur early in development. To test this hypothesis, a transgenic knock-in (KI) mouse model of DYT1 dystonia was utilized. This mouse model carries one copy of the mutant Dyt1 allele (Dyt1 ΔGAG) recapitulating the genetic background in DYT1 dystonia patients. Wild type (WT) littermate mice were used as controls. The goal was to examine whether the process of dopamine neuron apoptosis that occurs during normal development of the brain is altered in the mouse model, and whether such alterations result in altered number of dopamine neurons in the brain. The focus was on postnatal day 16 (P16), a period when developmental cell death is ongoing, and on substantia nigra (SN) and ventral tegmental area (VTA) of the midbrain, two brain regions that are enriched in dopamine neurons. Dopamine neurons were labeled using tyrosine hydroxylase immunohistochemistry in histological sections of the brain. The volume of the SN and VTA as well as the number of tyrosine hydroxylase labeled cells in these two brain regions were significantly increased in the Dyt1 transgenic mouse. The Dyt1 mutation did not produce significant effect on the numerical density of tyrosine hydroxylase labeled cells. These data offer evidence that the Dyt1 mutation may influence the normal development of the SN and VTA by influencing both the volume and dopamine neuron numbers in these two brain regions at P16, and that impaired dopamine neurotransmission reported in DYT1 and other dystonias may arise due to these and other changes in the development of the dopamine system.

Introduction

Dystonia is among the most common movement disorders in the world ranking third only after Parkinson's disease and essential tremor. In the United States alone, a quarter of a million people are afflicted with dystonia (American Association of Neurological Surgeons). Dystonia is classified as primary (also called isolated dystonia, when dystonia is the only symptom) or secondary (when other co-morbid conditions exist). Primary dystonia may be focal or generalized. Focal dystonia affects one body segment (e.g. limb, neck etc.) whereas generalized dystonia affects the entire body. Some types of generalized dystonia have an early onset, which means that the symptoms such as twisting of the foot or hand, begin in childhood or teenage years and "spread" over time to encompass the whole-body (Bressman et al., 2000).

DYT1 is a form of heritable early onset generalized dystonia. It is caused by a tri-nucleotide (GAG) deletion in the DYT1 gene (Ozelius & Lubarr, 1993). DYT1 codes for a protein called torsinA, which has chaperone functions in the cell and is involved with the packaging and transport of proteins. Consistent with the early onset nature of DYT1 dystonia, torsinA mRNA and protein expression is the highest in early development, and peaks at postnatal day 14 (P14) in the mouse brain (Vasudevan, Breakefield, & Bhide, 2006). TorsinA is ubiquitously distributed in the brain. However, it is enriched in dopaminergic neurons of the midbrain, in Purkinje neurons of the cerebellum, and neurons in the frontal cortex, brain stem and thalamus (Augood et al., 2002). When the GAG deletion is present in the gene, the gene codes for a mutant variant of torsinA, which is believed to interfere with the physiological function of torsinA.

While the exact connection between dopamine and torsinA is not fully known, a number of reports suggest that DYT1 dystonia is associated with an imbalance in dopamine neurotransmission (Augood et al., 2004; Carbon et al., 2009; Wichmann, 2008). Mouse models of DYT1 dystonia are available and have been valuable tools for gaining mechanistic insights into the changes in dopamine neurotransmission (Balcioglu et al., 2007; Zhang, McCarthy, Sharma, & Bhide, 2015). One such model is the Dty1 knock-in (KI) mouse carrying one copy of the GAG deletion in the Dyt1 gene (Dang et al., 2005). In the present study, this mouse model was utilized to examine whether the phenomenon of developmental cell death is altered in midbrain dopamine neurons in DYT1 dystonia. The focus was on developmental cell death because it plays a critical role in establishing the final number of neurons and final architecture of sensory and motor systems in the mature brain. Disorders with a developmental onset, such as DYT1 dystonia, have the potential to disrupt the normal process of cell death during brain development.

In this project, dopamine neuron densities were analyzed in P16 Dyt1 KI mouse model in two brain regions with high density of dopamine neurons, namely the substantia nigra (SN; pars compacta and pars

reticulata) and the ventral tegmental area (VTA). The dopamine neurons were identified in histological sections of the brain using immunohistochemistry for tyrosine hydroxylase, an obligatory enzyme in the dopamine biosynthesis pathway (Molinoff & Axelrod, 1971).

Materials and Methods

Animals

In this study male C57/BL6 heterozygous Dyt1 ΔGAG knock-in (Dyt1 KI) mice were used. Wild type (WT) littermates served as controls. Homozygous Dyt1 KI mice could not be used for this study as these mice perish within 2-3 days of birth and are unsuitable for this study in which we examined mice at postnatal day 16 (Dang et al., 2005). The mice were maintained at the institutional laboratory animal care facility at the Department of Biomedical Sciences, Florida State University (FSU) in a temperature and humidity-controlled room on a 12-hour light dark cycle with free access to food and water. All of the experimental procedures were approved by the FSU Institutional Animal Care and Use Committee and also were in full compliance with the NIH Guide for the Care and Use of Laboratory Animals.

Tissue Collection and Processing

Postnatal day 16 (P16) Dyt1 KI and WT mice were anesthetized and perfused via the heart with 4% paraformaldehyde, their brains were removed, and immersed in 4% paraformaldehyde. The brains were shipped to NeuroScience Associates (Knoxville, TN) for further processing, which included sectioning the brains on a Vibratome and immunohistochemistry for tyrosine hydroxylase (TH) (Healy-Stoffel, Omar Ahmad, Stanford, & Levant, 2014). The fully processed material was received at FSU for microscopic analysis.

Microscopic Analysis

The histological sections (35µm coronal) of the brains received from NeuroScience Associates were examined in a Zeiss Imager M2 Axio microscope. The SN and VTA were identified based on anatomical landmarks using the Allen mouse brain atlas as a guide. I examined every 4th section from a series of consecutive sections through the SN and VTA (Bregma Coordinates -2.48mm to -3.47mm). In each of the sections sampled, the borders of these two areas were drawn at 2.5x magnification and surface area measurements were obtained using StereoInvestigator Software (Microbrightfield INC, Williston, VT) (Figure 1A and B). Volume was calculated by multiplying the area by the final average thickness of the section (7µm). For each brain, 9 sections were examined. In every section, every TH-labeled cell in the SN and VTA was counted at 63x magnification (Figure 1D). Area and cell count measurements were performed on only one hemisphere (Figure 1C). The data for the counts and volume for each brain were calculated by multiplying the data obtained for the 9 sections analyzed by 4 (because every 4th section was used) and then by 2 (to obtain estimates for both the left and right hemispheres of the brain). A total

of 4 Dyt1 KI and 4 WT brains were analyzed. Numerical density of TH-labeled cells was calculated separately for the SN and VTA by dividing the total number of cells by the total volume.

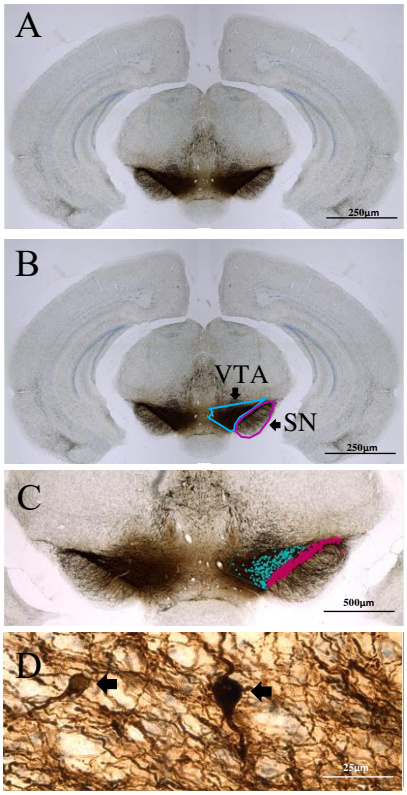


Figure 1. Histological section of a P16 mouse brain in the coronal plane processed for tyrosine hydroxylase (TH) immunohistochemistry. A. Low magnification (2.5x) image showing dense TH-labeling (brown staining) predominantly confined to the ventral midbrain. **B**. The borders of the ventral tegmental area (VTA; blue) and substantia nigra (SN; pink) are superimposed on the section image at 2.5x magnification. **C.** Higher magnification (10x) of the section in which every TH-labeled cell in the VTA (blue) and SN (pink) is marked digitally. Each colored dot represents a single TH-labeled cell. **D.** Higher magnification (63x) showing two TH-labeled cells (arrows).

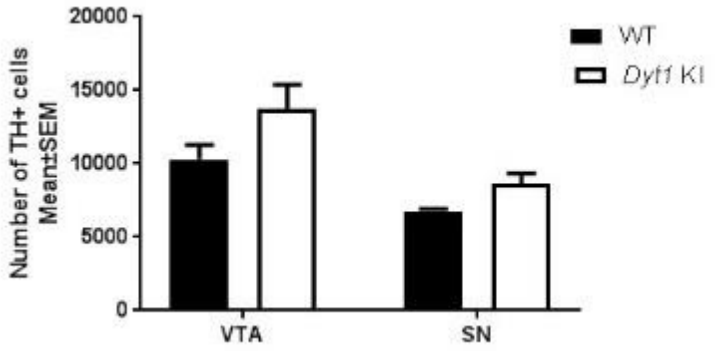
Statistical Analysis

Differences between Dyt1 KI and WT groups in cell numbers, volume and density were analyzed for statistical significance using a two-way analysis of variance (ANOVA) with genotype (WT versus Dyt1 KI) and brain region (VTA versus SN) as the two factors. A Bonferroni's multiple comparison test was used to compare differences between WT and Dyt1 KI within each brain region.

Results

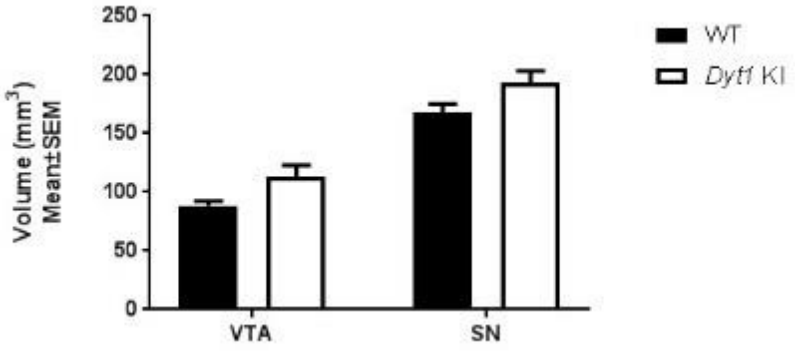
Average Number of TH-positive Cells

There was a significant main effect of genotype ($F_{(1, 12)}$ =6.518, $p<0.05$) and brain region ($F_{(1, 12)}$ =16.52 $p<0.01$). The interaction was not significant ($F_{(1, 12)}$ =0.579, $p>0.05$). Pairwise comparisons did not reveal significant differences between WT and Dyt1 KI groups in the VTA (Mean±SEM: WT = 10,284±1005; Dyt1 KI = 13,704 ±1696; t= 1.735; df =6; $p>0.05$) or the SN (Mean±SEM: WT = 6,800±147; Dyt1 KI = 8,614±736; t=2.418; df =6; $p>0.05$).



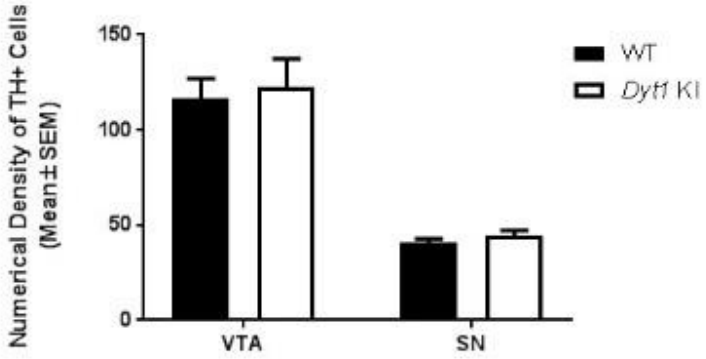
Volume (mm^3)

There was a significant main effect of genotype ($F_{(1, 12)}$ =8.306, $p<0.05$) and brain region ($F_{(1, 12)}$ =87.71, $p<0.0001$). The interaction was not significant ($F_{(1, 12)}$ =0.002, $p>0.05$). Pairwise comparisons did not reveal significant differences between WT and Dyt1 KI in the VTA (Mean±SEM: WT = 88.63±3.84; Dyt1 KI =112.70±10.32; t= 2.188; df =6; $p>0.05$) or the SN (Mean±SEM: WT =167.7±7.34; KI =192.6±10.64; t=1.922; df =6; $p>0.05$).



Numerical Density of TH-positive Cells (# TH+/mm^3).

There was no significant main effect of genotype ($F_{(1,12)}$ =0.31, p>0.05) but there was a significant main effect of brain region ($F_{(1,12)}$=11.34, p<0.01). The interaction was not significant ($F_{(1, 12)}$ =0.018, p>0.05). Pairwise comparisons did not show significant differences between WT and Dyt1 KI groups in the VTA (Mean ±SEM: WT = 116.30±10.62; Dyt1 KI = 122.60±14.68; t =0.3503; df =6; p>0.05) or the SN (Mean ±SEM: WT = 40.78±2.06; Dyt1 KI = 44.66±2.49; t =1.203; df =6; p>0.05).



Discussion

This study examined whether the process of dopamine neuron apoptosis that occurs during normal development of the brain is altered in the Dyt1 KI mouse model, and whether such alterations result in altered number of dopamine neurons in the SN and VTA at P16. The data show significant effects of the Dyt1 mutation on dopamine neurons and regional volumes in both the SN and VTA. Specifically, two-way ANOVA revealed that the volume of the VTA and SN and the total number of TH-labeled cells in these two regions were significantly increased in the Dyt1 KI mouse compared to the WT control. However, the mutation did not produce significant effect on the density of TH-labeled cells in either brain region.

The data suggest that impaired dopamine neurotransmission suggested in DYT1 dystonia may arise due to volumetric changes and changes in dopamine neuron numbers during the development of the mid brain dopamine system. The increase in regional volume may be due to the greater numbers of TH-labeled cells or an increase in glial cell numbers. The greater TH+ cell numbers at P16 suggests decreased developmental cell death, which occurs in dopamine neurons of the ventral midbrain between P4 and P21 (Jackson-Lewis et al., 2000). Other developmental processes such as neurogenesis, neuronal migration or dopamine receptor expression may also be affected by the Dyt1 mutation. In fact, previous studies have shown decreased dopamine receptor expression in the Dyt1 KI mouse brain (Zhang et al., 2015).

The data from the present study are consistent with other studies that examined TH-labeled cells in the rodent VTA and SN (Jackson-Lewis et al., 2000); (Flores et al., 2005); (Chen et al., 2017); (Brandt et al., 2017);(Vadasz et al., 2007). The numbers of TH-labeled cells in the VTA (~10,000) and SN (~ 7000) in the WT control group in the present study are well within the range reported in the literature. Most of the other studies in the literature used stereological methods. While stereology could not be used in the present study due to the relatively thin sections (~7um), a rigorous quantitative approach involving adequate sampling was employed. For example, every 4^{th} section from a series of consecutive sections through the SN and VTA was examined, which is a sampling method that was used in previous studies as well.

During early development, there is a period of natural, programmed cell death, which occurs for dopamine neurons of the mid brain between postnatal days 4 and 21 (Jackson-Lewis et al., 2000). This process is critical for the establishment of adult dopamine neuron numbers. Postnatal day 16, a period when cell death is ongoing, was the focus of the present study. It is possible that changes may occur earlier or later during development in the Dyt1 KI brain. Future studies could focus on other time points in development. Moreover, other mouse models of dystonia such as the Dyt1 knock-out model, where torsinA is knocked out instead of expressing the mutant form may be an alternative approach.

In conclusion, the volume and the number of TH-labeled cells is significantly increased in the SN and VTA of the Dyt1 KI mouse brain at P16. The increase in regional volume may be due to the increased dopamine neurons or due to an increase glial cell numbers. The increase in TH-labeled cell numbers suggests decreased developmental cell death in this population of neurons by P16. These data offer evidence that the Dyt1 mutation influences dopamine neuron development and support the concept that dopamine neurotransmission may be impaired in DYT1 dystonia.

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