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Influence of fluoride on neuronal differentiation in dopaminergic differentiation SH-SY5Y cells

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ABSTRACT

Fluoride is widely used in dentistry to prevent dental caries by increasing the fluoride content in saliva and aiding enamel remineralization. Excessive fluoride in the blood can cause adverse health effects such as fluorosis, which alter cerebral function. The influence of fluoride on dopaminergic neurons however, remains largely unclear. The present study examined the effect of sodium fluoride toxicity on dopaminergic neurons in retinoic acid-induced differentiation in SH-SY5Y cells. Cell viability was reduced by fluoride in both time- and concentration-dependent manners. Moreover, immunoblot analysis showed that fluoride decreased neuronal marker microtubule-associated protein-2 expression and levels of tyrosine hydroxylase, a rate-determining step enzyme in dopamine synthesis, even at a nonlethal dose. These results suggest that fluorosis may adversely affect dopaminergic neurons and may have harmful effects in individuals with degenerative dopaminergic neuron conditions, such as Parkinson's disease.

Keywords: dopaminergic; fluoride; tyrosine hydroxylase

1. INTRODUCTION

Fluoride is abundantly found in nature. Topical fluoride is frequently applied in dentistry as a means of caries prevention. Application of fluoride varnish has been proven to greatly reduce the occurrence of dental caries (Marinho et al., 2013). Fluoride can also be ingested systemically in the form of fluoridated water. Upon imbibition, fluoride is rapidly absorbed into the blood-stream via the stomach and intestines (Whitford, 1994). Once the blood fluoride level rises, the fluoride level in secreted saliva also correspondingly increases (Ingram et al., 2005). Salivary flow brings fluoride ions in contact with tooth surfaces, where the ions incorporate into enamel and promote remineralization. Accordingly, water is commonly fluoridated worldwide and supplied to approximately 400 million people in 25 regions, as reported by the World Health Organization (WHO) (O'Mullane et al., 2016).

Although the benefits of fluoride in dentistry are significant, studies have linked fluoride with several health issues. Excessive systemic fluoride, termed fluorosis, can accumulate in hard tissues such as bone and teeth, the latter of which is easily visualized and diagnosed by the dentist during routine examinations (Nakornchai et al., 2016). Fluorosis can also affect soft tissues, including blood cells and vessels, as well as organs such as the kidneys, lungs, and brain (Kurdi, 2016). A meta-analysis linked high fluoride levels in drinking water with a decrease in children's intelligence quotient (IQ), revealing

the detrimental effect of fluoride on cerebral function (Xu et al., 2020). Despite the separation of the brain from the rest of the body by the blood brain barrier, studies have shown that fluoride can be carried to the brain by active transport (Hu and Wu, 1988). Studies, both *in vivo* and *in vitro* show evidence of the neurotoxicity of high fluoride exposure in the brain, leading to disruption of the glutaminergic, γ-aminobutyric acid (GABA) and cholinergic neurotransmitter systems. Acetylcholine, glutamate, and GABA are important neurotransmitters for memory consolidation in the hippocampus. Several studies have linked fluoride suppression of neurotransmitter release and receptor expression with memory impairment (Dec et al., 2017). Because of the adverse health effects of fluorosis, WHO guidelines limit fluoride content in drinking water to no more than 1.5 mg per liter. However, incidences of fluorosis have still been reported as a result of overconsumption of fluoride from other sources (McGrady et al., 2012; Nakornchai et al., 2016).

A few studies reported the influence of chronic fluoride exposure on the cerebral dopamine system. Dopamine is a monoamine neurotransmitter, responsible for motivation, reward, and motor control (Bernheimer et al., 1973; Brydon et al., 2008). A decrease in dopamine levels in certain parts of the brain is associated with the development of depression, addiction, and Parkinson's disease.

The present study aimed to investigate the influence of fluoride on dopaminergic neuronal differentiation in an *in vitro* model. Neuroblastoma SH-SY5Y cells were established as the differentiation model by the addition of retinoic acid (RA), with or without the presence of fluoride.

2. MATERIALS AND METHODS

2.1 Reagents

Sodium fluoride (NaF) and sodium chloride (NaCl) were purchased from Merck (Darmstadt, Germany). RA was obtained from Sigma-Aldrich (St. Louis, MO, USA). All antibodies used in immunoblot analysis were acquired from Cell Signaling Technology (Beverly, MA, USA). All cell culture chemicals used for cell cultures were purchased from Gibco (Carlsbad, CA, USA). All other chemicals were obtained from Merck Millipore unless otherwise stated.

2.2 Cell cultures and treatments

The neuroblastoma cell line SH-SY5Y was obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured according to the manufacturer instructions. Cells were incubated with NaF or NaCl for 24 h before RA-induced differentiation. Afterwards, the cells were incubated with 10 μM of RA or 0.1% dimethyl sulfoxide (DMSO) (v/v) with or without NaF. The medium was changed daily.

2.3 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Cell viability was determined by MTT assay. SH-SY5Y cells (10^4 cells/well) were subcultured into 96-well plates (Costar, Corning, NY, USA). Upon completion of the given conditions, 0.5 mg/mL MTT solution was substituted. Cells were further incubated for 4 h under dark conditions. The purple formazans representing viable cells were solubilized in DMSO and measured by a microplate reader machine at 570 nm (BioTek Synergy H4, BioTek).

2.4 Hoechst 33342 staining

Chromatin condensation is one characteristic of apoptotic cells. The condensed nuclei were visualized by the nuclear staining dye Hoechst under the fluorescence microscope. After RA incubation, the cell culture medium was replaced with fixative (methanol/glacial acetic acid in a 3:1 ratio) for 25 min in a 4°C chamber, washed twice with phosphate buffered saline (PBS), and then stained for 15 min with Hoechst 33342 (1 μg/mL) at 25°C. Images of the nuclei were observed using a fluorescence microscope (IX83ZDC, Olympus Corp., Tokyo, Japan). Any cell with a bright blue nucleus was identified as a chromatin condensation positive cell. Images were randomly taken from three fields of each group of experiments for approximately 500 cells. The data were expressed as a percentage of condensed nuclei cells out of the total nuclei.

2.5 Immunoblot analysis

The neuronal markers of differentiated cells were detected using immunoblot analysis. After treatment, cells were lysed by radioimmunoprecipitation assay buffer supplemented with a protease inhibitor cocktail and quantified by BCA protein quantification assay (Thermo Scientific, MA, USA). The twenty-microgram protein of each experimental condition was separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto methanol-activated polyvinylidene fluoride membranes. To prevent non-specific antibody binding, membranes were pre-probed with 5% bovine serum albumin in tris-buffered saline with 0.1% Tween® 20 detergent and incubated overnight with primary antibodies (1:1000) against microtubule-associated protein-2 (MAP2) (M9942; Sigma-Aldrich, Darmstadt, Germany), tyrosine hydroxylase (TH) (#2792) and β-actin (A2228; Sigma, Darmstadt, Germany) at 4°C with gentle shaking overnight. Afterwards, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies; anti-rabbit (#7074) or anti-mouse (#7076) at ambient temperature for 2 h. The bands were visualized by a chemiluminescence kit (Bio-Rad, CA, USA). Images were acquired by the enhanced chemiluminescence system (UVITEC, Cambridge, UK) and quantified by densitometry analysis (Image J software).

2.6 Statistical analysis

All data were presented as mean±SEM from three independent experiments. Significant differences among the groups were statistically calculated using one-way ANOVA analysis followed by Tukey-Kramer test for multiple comparison results. All statistical analyses were calculated by using GraphPad Prism software 8.0. A *p*-value of less than 0.05 indicated statistical significance.

3. RESULTS AND DISCUSSION

3.1 Toxic effect of sodium fluoride on undifferentiated SH-SY5Y cell viability

To study the influence of sodium fluoride on SH-SY5Y cell viability, cells were pre-treated with various doses of NaF for durations of 48, 72, and 96 h. NaF was observed to significantly decrease cell viability in both time- and concentration-dependent manners. At 48 h and 40 μg/mL, NaF decreased the number of SH-SY5Y cells by approximately 20%, compared to the NaCl control group (Figure 1B). At 72 h and 96 h, incubation with NaF

significantly decreased cell viability at 40 µg/mL and 20 µg/mL (Figure 1C). Incubation with 10, 5 and 2.5 µg/mL of NaF did not statistically reduce cell viability under any experimental condition (Figure 1B-D).

Fluoride exposure above a certain concentration and duration of time could induce apoptosis in certain types of cells, such as macrophages (Hirano and Ando, 1996), hepatocytes (Gutowska et al., 2016), and neuroblastoma cells (Inkielewicz-Stepniak et al., 2012; Wei et al., 2014). In this study, cell viability was reduced by NaF in both time- and dose-dependent manners. These results were comparable to those reported by Xu et al. (2011), which showed that higher concentrations of NaF lead to increasing toxicity. Our study further demonstrated that not only a higher concentration, but also a longer period of incubation can increase NaF toxicity, as exemplified by the group treated with 20 µg/mL of NaF exhibiting an increase in toxicity over time. Our findings suggested that special caution should be taken to avoid conditions that may contribute to chronic exposure to fluoride, which may raise the risk of fluoride toxicity.

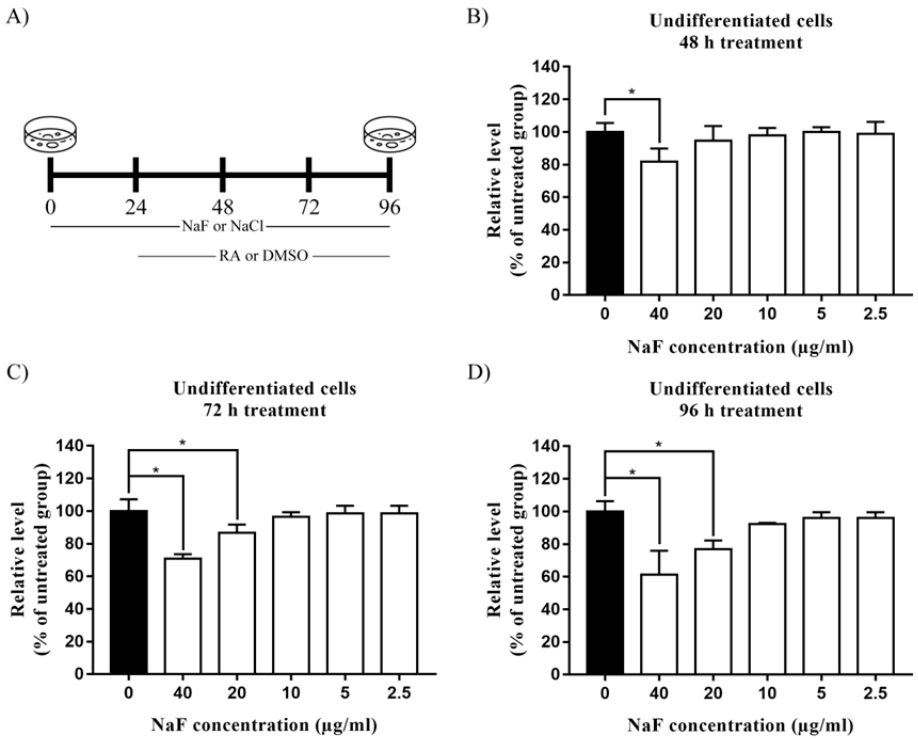

Figure 1. (A) The experimental model, NaF induced reduction of cell viability in undifferentiated SH-SY5Y cells at (B) 48 h, (C) 72 h, and (D) 96 h of incubation

Note: Each bar graph represents the mean±SEM calculated from three independent experiments. *p<0.05, compared to the NaF-untreated group.

3.2 NaF decreased cell viability and increased apoptotic nuclei in differentiating cells

To examine the effect of NaF on cell viability during neuronal differentiation, the cells were pre-treated with NaF for 24 h, followed by the addition of 10 µM of RA for 72 h. Cell viability was determined by the MTT assay. There was no significant difference observed in 2.5, 5, and 10 µg/mL NaF-treated groups, when compared to the NaCl control group (Figure 2G). However, there was a statistically significant reduction of cell viability in the group treated with 40 and 20 µg/mL of NaF.

Hoechst nuclear staining assay (Figure 2H) revealed that NaF significantly increased the number of cells with condensed chromatin at concentrations of 40 and 20 µg/mL, compared to the untreated group. In addition, 10, 5, and 2.5 µg/mL of NaF did not significantly raise the number of cells with condensed nuclei.

Sodium fluoride has previously been reported to cause apoptosis in both *in vitro* and *in vivo* models (Ribeiro et al., 2017). Characteristics of apoptosis include membrane blebbing, apoptosome formation, DNA fragmentation, and chromatin condensation (Reed, 2000). In the present study, cells were identified using Hoechst nuclear staining to detect chromatin condensation. The results indicated that, even in the presence of RA, NaF could increase chromatin condensation positive cells in differentiating neuroblastoma SH-SY5Y cells. It was recently discovered that RA is capable of exerting a protective effect against toxin-induced apoptosis and UV exposure by upregulating pro-survival pathways, such as Akt/mTOR signaling (Cao et al., 2008; Cheung et al., 2009). However, RA did not protect or diminish the deleterious effects of NaF in our experimental setup, suggesting that pre-treatment with NaF possibly prevented RA from exerting its protective effect, or RA did not confer a protective effect against NaF toxicity. However, further experimentation is needed to confirm this hypothesis.

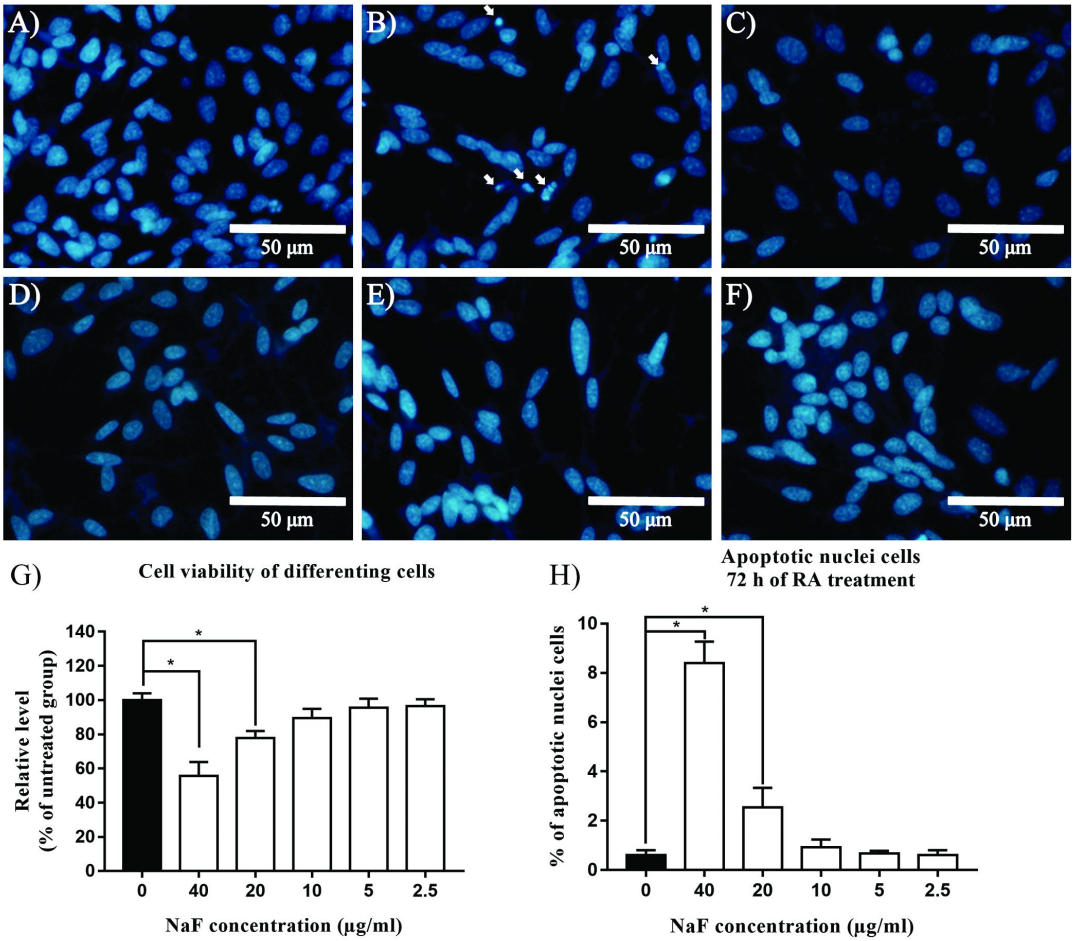

Figure 2. The number of apoptotic nuclei in cells treated with various concentrations of NaF: (A) 0 µg/mL, (B) 40 µg/mL, (C) 20 µg/mL, (D) 10 µg/mL, (E) 5 µg/mL, and (F) 2.5 µg/mL, followed by the addition of 10 µM RA for 72 h, (G) MTT assay and (H) the number of apoptotic nuclei.
Note: Each bar represents the mean±SEM calculated from three independent experiments. $^{*}p<0.05$, compared to the 0 µg/mL treated group.

3.3 NaF-attenuated neuronal markers in differentiating SH-SY5Y cells

To assess the expression of neuronal differentiation markers, cells were subjected to immunoblot analysis probing for MAP2 and TH proteins. Differentiation of SH-SY5Y cells induced by the addition of 10 µM RA significantly increased the protein expression of MAP2 and TH, compared to undifferentiated cells (Figure 3). The presence of 20 µg/mL and 10 µg/mL of NaF was observed to significantly reduce expression of MAP2 and TH. However, in the group treated with 5 µg/mL NaF, there was no statistically significant change in the expression of either MAP2 or TH.

SH-SY5Y cells could be differentiated into dopaminergic neurons by RA. The undifferentiated cells were characterized morphologically by non-polarized cell bodies with truncated processes (Figure 3C). On the other hand, differentiated SH-SY5Y cells transformed were more morphologically similar to primary neurons, with elongated processes and expression of neuronal markers (Figure 3D). Neuronal markers are endogenous tags expressed in the cell lineage during neurogenesis and differentiation. The expression pattern of markers correlates with the function of the cell state. Some neuronal markers are present in several states of neurogenesis, while others are present only in differentiated neurons. For example, expression of MAP2 is detectable in both early and late neurogenesis (Menezes and Luskin, 1994). The MAP2 protein has several isoforms (Chung et al., 1996). The MAP2b (280 kDa) isoform is expressed throughout neurogenesis, while the MAP2a (280 kDa) isoform is increasingly expressed in later states of neurogenesis. The upregulation of the juvenile isoform MAP2c (75 kDa) during neurite outgrowth and microtubule stabilization has suggested that this isoform serves a significant role in these processes (Lieven et al., 2007). Previous studies have reported that high concentrations of NaF suppressed neurite outgrowth in undifferentiated human neuroblastoma (Nakagawa-Yagi et al., 1993) and mouse hippocampal neurons (Bhatnagar et al., 2002) and were also associated with the downregulation of MAP2. In this study, RA induced the upregulation of MAP2c, which was conversely subdued with the addition of NaF. Interestingly, 10 µg/mL of NaF was shown to decrease MAP2 expression even though it did not significantly affect cell viability. These results suggested that nontoxic concentrations of NaF could adversely affect neurodifferentiation.

Dopamine is an essential neurotransmitter that plays an important role in behavior modulation, mood regulation, and motor movement (Crocker, 1997). Dopaminergic neurons produce dopamine via several enzymes, including the rate-limiting TH (Daubner et al., 2011). Fluoride has been reported to disturb the action of several neurotransmitters, including glutamate and acetylcholine (Ekambaram and Paul, 2001; Liu et al., 2013). Fluoride overexposure can also alter dopamine levels and number of dopamine receptors in the striatum (Kupnicka et al., 2020). However, the effects of fluoride on dopamine production remain unclear. The present study investigated the influence of fluoride on TH expression in dopaminergic neuronal differentiation, and the results indicated that fluoride could suppress TH expression during differentiation. TH is a rate-limiting enzyme in neurotransmitter production and is commonly used as a marker for dopaminergic neuron cell characterization (Gale and Li, 2008; Van Heesbeen et al., 2013). Since dopamine levels are correlated with cognitive function, decreased TH may possibly contribute to IQ reduction in children prenatally exposed to fluoride (González-Burgos and Feria-Velasco, 2008). Dopaminergic neurogenesis has also been shown to occur in adult mammals (Morrison, 2016). As such, chronic exposure to fluoride may have deleterious effects on dopaminergic-related neurodegenerative conditions, such as Parkinson's disease. Caution is therefore recommended when considering fluoride application for caries prevention when treating this group of special needs patients.

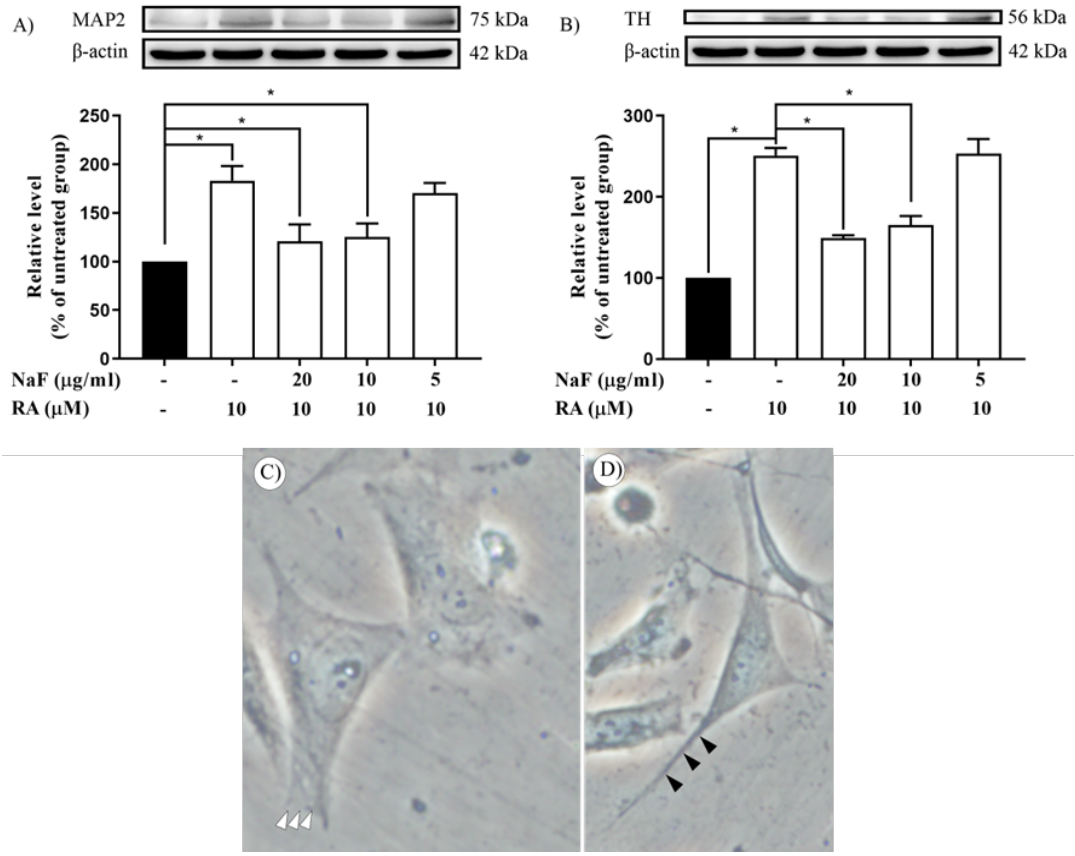

Figure 3. Immunoblot analysis showing (A) the expression of microtubule-associated protein 2 (MAP2), (B) the expression of tyrosine hydroxylase (TH). The morphology of (C) undifferentiated SH-SY5Y cells, and (D) differentiated SH-SY5Y cells.
Note: Each bar represents the mean±SEM calculated from three independent experiments. $^{*}p<0.05$. Whitehead arrows indicate a truncated process. Blackhead arrows indicate an elongated process.

4. CONCLUSION

Higher concentrations of fluoride reduced both neuroblastoma SH-SY5Y cell viability, and number of neuronal markers. At the lower concentration of 5 μg/mL, NaF did not lead to reduction of cell viability but rather to a significant decrease in MAP2 and TH expression. The results from this study indicated that a nonlethal dose of NaF could suppress neuronal differentiation, and NaF disturbed the dopamine synthesis pathway via reduction of TH expression.

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