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Molecules and Cells

ATG5 Expression Induced by MDMA (Ecstasy), Interferes with Neuronal Differentiation of Neuroblastoma Cells

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The amphetamine derivative 3, 4-methylenedioxymethamphetamine (MDMA) has become a popular recreational drug, and has also been shown to cause serotonergic neurotoxicity. This report shows that MDMA impairs brain development in a whole mouse embryo culture. The results of quantitative real-time PCR analysis showed that autophagy-related protein 5 (Atg5) expression is elevated in mouse embryo and neuroblastoma cells after MDMA treatment. This elevated Atg5 expression interferes with the neuronal differentiation of neuroblastoma cells such as SH-SY5Y and PC12 cells. Thus, our results suggest that the use of MDMA during pregnancy may impair neuronal development via an induction of Atg5 expression.

INTRODUCTION

Autophagy is an evolutionarily conserved pathway in which the cytoplasm and organelles are engulfed within double-membraned vesicles, referred to as autophagosomes, in preparation for the turnover and recycling of these cellular constituents (Levine, 2005). The principal role of autophagy is the provision of nutrients necessary for survival, as has been demonstrated in yeast and early neonatal mice. Autophagy also plays a role in cellular remodeling during differentiation and the development of multicellular organisms, including dauer formation in *Caenorhabditis elegans* and metamorphosis in *Drosophila melanogaster* (Baehrecke, 2003; Melendez et al., 2003). To date, seven autophagy-related "Atg" proteins have been determined to directly regulate the formation of autophagic vacuoles (AV). Firstly, the Atg12 ubiquitin-like conjugation system, comprised of Atg 5, 7, 10, and 12, regulates the elongation of the AV isolation membrane, which is known as a phagophore. Secondly, the Atg8 conjugation system (Atg 3, 4, 7 and 8) regulates the attachment of phospholipids (Levine and Klionsky, 2004).

The amphetamine derivative 3,4-methylenedioxymethamphetamine (MDMA, ecstasy) has become a popular recreational drug, as humans consume it for its acute psychotropic effects, which include euphoria, a reduction in negative ideation, and increased sociability and energy (Cole and Sumnall, 2003). MDMA is known to cause serotonergic neurotoxicity in a number of species, and this effect may result in sleep disorders, depression, enhanced impulsivity, impaired decision-making, anxiety disorders, and memory impairment. It also induces long-term changes in several markers of the integrity of the serotonin neurotransmitter system (Sumnall et al., 2004). Whereas, in many studies, the neurotoxicity, pharmacokinetics, and neurobehavioral consequences of MDMA dosage have been studied extensively (Campbell et al., 2006), the detailed effects in the offspring of MDMA-using mice, as well as the relevant cellular mechanisms, have yet to be systematically addressed.

In this study, we have determined that mouse embryo development upon treatment with MDMA impairs brain development and induces the expression of the Atg5 gene. We have also shown that MDMA increases the expression of the autophagic gene, Atg5, which interferes with the neuronal differentiation of neuroblastoma cells including SH-SY5Y and PC12. These results indicate that the neuronal toxicity associated with MDMA may possibly be involved in the level of neuronal Atg5 expression.

MATERIALS AND METHODS

Whole embryo culture method

Pregnant dams of mice were sacrificed by cervical dislocation on GD 8.5, and embryos were explanted according to a modification of the method of New (New, 1978). The uterus was removed from the dam and rinsed in pH 7.6 Tyrode's salt solution (Sigma, USA) maintained at 37°C. The decidua, trophoblast, parietal endoderm, and Reichert's membrane were removed using No.4 watchmaker's forceps (Dumont and Fils, Switzerland), leaving the amnion, visceral yolk sac, and ectoplacental

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cone intact. Core is taken to avoid disrupting the visceral yolk sac, amnion and ectoplacental cone intact.

Three embryos were cultured in 3 ml of rat serum in each bottle for 48 h at 37°C using a roller system (Ikemoto Rika Kogyo, Japan) at 25 rpm. MDMA (2.5, 5, 10 μg/ml) were administered to each mouse embryo culture bottle. MDMA was dissolved in filtered saline. Gas was sterilized by filtration using a membrane with a porosity of 0.22 μm and the flow rate of the gas is continued at 150 ml/min. Gas was used three kinds of gas, Mixture of 5% O_2, 5% CO_2 and 90% N_2 for first 17 h, 20 % O_2, 5% CO_2 and 75% N_2 for next 7 h and 40% O_2, 5% N_2 for last 24 h.

Cell culture and neuronal differentiation

HEK293A, SH-SY5Y and PC12 cells were grown in DMEM medium supplemented with 10% fetal bovine serum. Transfections into HEK293A and PC12 cells were conducted using Welfect transfection reagent (Welgene, Korea), in accordance with the manufacturer's instructions. Transfections of SH-SY5Y cells were conducted using Lipofectamine transfection reagent (Invitrogen, USA).

In order to differentiate the neuroblastoma cells, retinoic acid was added to SH-SY5Y cells on the day after plating at a final concentration of 2 μM in DMEM, and PC12 cells were incubated with 50 ng/ml of Nerve Growth Factor (R&D Systems, USA) (Heese et al., 2004). Cells exhibiting one or more neuritis of a length more than twice the diameter of the cell body were considered differentiated (Kim et al., 2006). In order to quantify cell differentiation, at least 300 cells were counted, and the average percentage of differentiated cells was calculated from triplicate experiments.

Real time quantitative RT-PCR

Total RNA was isolated from the embryos or cultured cells using easy-spin kit (iNtRON, Korea) in accordance with the manufacturer's instructions. First-strand cDNA was prepared from total RNA and oligo dT using I Script cDNA synthesis kit (Bio-Rad, USA). Real-time PCR was conducted with a Mini Opticon System (Bio-Rad) and SYBR Green (Bio-Rad). The primers for mouse Atg5 were AAGTCTGTCCTTCCGCAGTC and TGAAGAAAGTTAT CTGGGTAGCTCA, and the primers for human Atg5 were TGGATTTCGTTATATCC CCTTTAG and CCTAGTGTGTGCAACTGTCCA. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was utilized to calibrate the original concentration of mRNA. The concentration of mRNA in the cell was defined as the ratio of target mRNA copies versus GAPDH mRNA copies. Data from three separate experiments were averaged.

Immunoblotting and immunofluorescence

The cells were harvested and lysed in lysis buffer (150 mM NaCl, 50 mM HEPES [pH 8.0], 0.5% NP-40) containing a protease inhibitor cocktail (Roche, Germany). For the protein immunoblots, polypeptides in the whole cell lysates were resolved via SDS-PAGE and transferred to nitrocellulose membrane filters. Immunoblot detection was conducted with a 1:2000 dilution of primary antibody with an enhanced chemiluminescence (ECL) system. Antibody for LC3 was purchased from MBL International (Woburn, USA).

For immunofluorescence microscopy, the cells were grown on sterilized glass coverslips, transfected with GFP-LC3, and fixed in 4% paraformaldehyde after 24 h of transfection. Finally, the slides were washed three times in PBS and mounted in mounting media (Vector). Images were obtained using a Leica confocal microscope (Leica, Germany).

RESULTS

MDMA induces teratogenesis in whole mouse embryo culture

In order to determine whether MDMA affects embryo development, we prepared *in vitro* whole mouse embryo cultures and treated the mouse embryos with increasing concentrations of MDMA (2.5-10 μg/ml) (Fig. 1A). Incubation of the embryos with MDMA (5 μg, 10 μg) resulted in severe teratogenesis and fetal brain damage (Fig. 1B). These results suggest that MDMA administration during pregnancy may result in damage to brain development in mouse embryos.

We subsequently performed microarray analysis in order to identify the differentially expressed genes in the MDMA-treated mouse embryos. Among the differentially expressed genes observed on microarray analysis, we found that Atg5 expression had been increased significantly (data not shown). To confirm the increased expression of the Atg5 gene in the MDMA-treated embryos, we evaluated Atg5 mRNA expression via real-time RT-PCR analysis. The mouse embryos were either mock-treated or treated with MDMA (10 μg/ml). After 48 h of incubation, mRNA was extracted from the whole embryos, and cDNAs were synthesized and subjected to real-time RT-PCR analysis. Real-time quantitative RT-PCR analysis showed that Atg5 expression had been increased by 2-fold, thereby indicating that Atg5 expression is upregulated in the mouse embryo upon treatment with MDMA.

MDMA induces Atg5 expression in SH-SY5Y neuroblastoma cells

Because MDMA impaired fetal brain development, we attempted to determine whether MDMA also influences neuronal differentiation. First, we attempted to determine whether MDMA induces Atg5 expression in the neuronal cells in a manner similar to that seen in the mouse embryo. We utilized human neuroblastoma, SH-SY5Y cells for the neuronal differentiation assay, because retinoic acid evokes the neuronal differentiation of SH-SY5Y cells. In order to evaluate Atg5 expression in the SH-SY5Y cells, we conducted real-time quantitative RT-PCR analysis using SH-SY5Y cells which had been either mock-treated or treated with 10 g/ml of MDMA. After 48 h of MDMA treatment, Atg5 mRNA expression increased by 1.8-fold in the SH-SY5Y cells (Fig. 1D). These results show that MDMA induces Atg5 expression in SH-SY5Y neuroblastoma cells.

Ectopic expression of Atg5 induces autophagy in neuroblastoma cells

As MDMA upregulates Atg5 expression in SH-SY5Y cells, we attempted to characterize the effect of Atg5 expression in the neuronal cells. To determine the function of Atg5 expression, we cloned Atg5 into the eukaryotic expression plasmid, and the plasmid encoding for Atg5 was transfected into HEK293 cells. As expected, Atg5 was readily detected at approximately 30 kDa, and we further assessed the level of endogenous LC3-II, the autophagosomal marker (Fig. 2A). The western blot with anti-LC3 antibody showed that the level of endogenous LC3-II is increased by Atg5 expression, thereby indicating that Atg5 expression induces autophagy.

Next, we attempted to determine whether Atg5 expression induces autophagy in neuronal cells by examining the cytoplasmic GFP-LC3 punctuates, which reflect the induction of autophagy and are commonly used to monitor autophagy. Either a control plasmid or the Atg5 expression plasmid with the GFP-LC3 expression plasmid were transfected into SH-SY5Y, and 36 h after transfection, the cells were fixed. While GFP-

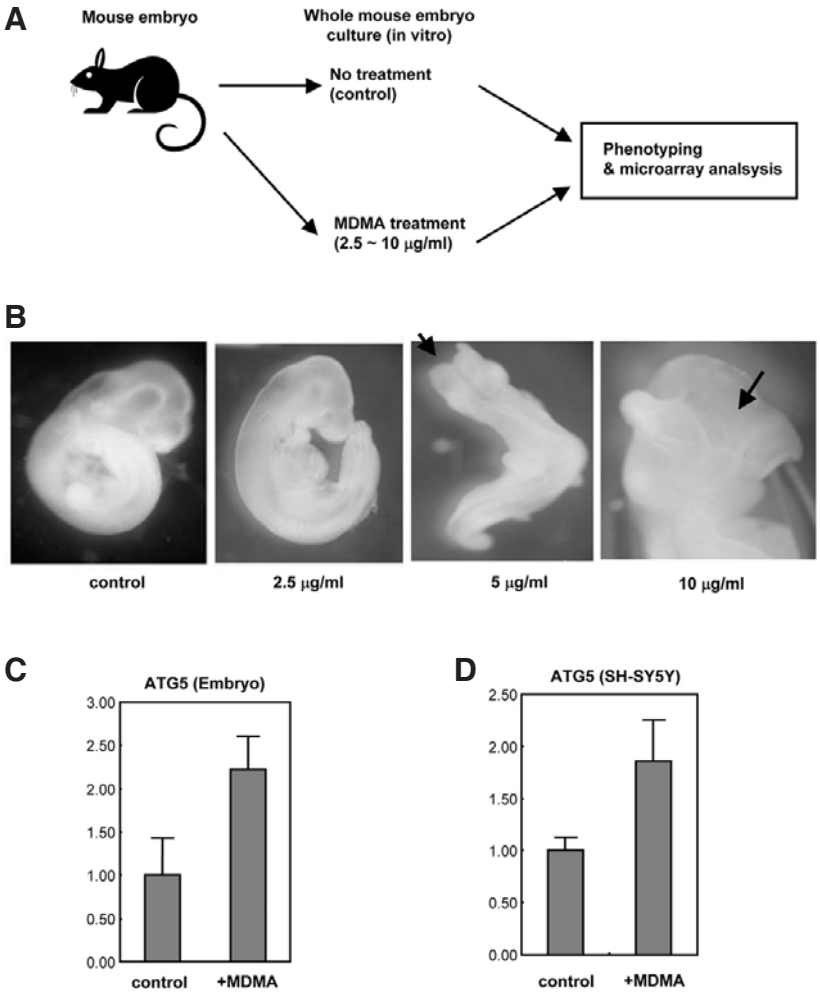

Fig. 1. MDMA induces Atg5 expression in embryo and neuroblastoma cells. (A) The animal experiment scheme. (B) The fetus brain development was imaged after MDMA (2.5, 5, 10 μg/ml) in the whole mouse embryonic culture. The brain development of embryonic mouse was impaired, and evidenced teratogenesis concordant with the MDMA concentration. (C) MDMA was administered to the embryo and then the Atg5 mRNA level was evaluated via real-time quantitative RT-PCR. (D) MDMA was administered to the SH-SY5Y cells after which the level of Atg5 mRNA was determined.

LC3 with the control plasmid was localized largely in the cytoplasm, Atg5 induced the cytoplasmic punctuates, which are reflective of the autophagosomes (Fig. 2B). These results collectively show that the increased expression of Atg5 resulted in autophagy.

Atg5 represses the neuronal differentiation of neuroblastoma cells

Recently, it has been reported that autophagy performs an important function in neuronal differentiation (Zeng and Zhou, 2008). As the level of Atg5 in the neuronal cells was increased upon MDMA treatment, we hypothesized that Atg5 may modulate neuronal differentiation. To evaluate our hypothesis, we examined the neuronal differentiation of SH-SY5Y cells via fluorescence microscopy.

SH-SY5Y cells were transfected with GFP vector in the absence or presence of the plasmid encoding for Atg5, and 2 μM retinoic acid (RA) was administered for 5 d. In the SH-SY5Y cells, 5 days of RA treatment induced morphologic differentiation with neurite extension and a profuse neuritic arborization forming extensive networks over the culture dish surface. By way of contrast, the cells expressing Atg5 protein evidenced neither neurite extension nor neuritic arborization (Fig. 3A). These results indicated that the Atg5 protein strongly inhibited the neuronal differentiation of neuroblastoma cells.

To confirm our results, we further investigated whether Atg5 modulates cell differentiation in PC12 cells via immunofluorescence microscopy. PC12 cells were transfected with GFP vector in the absence or presence of the plasmid encoding for Atg5, and 50 ng/ml of NGF was administered for 7 d (Heese et al., 2004). In the PC12 cells, 7 days of NGF treatment induced morphologic differentiation with neurite extension, whereas the PC12 cells transfected with the Atg5 protein differentiated very slowly (Fig. 3B). After 7 days of NGF treatment, the differentiated cells were counted. Cells having one or more neuritis of a length more than twice the diameter of the cell body were considered differentiated (Kim et al., 2006). PC12 cells were differentiated by over 40%, whereas less than 10% of the PC12 cells transfected with the plasmid encoding for Atg5 were differentiated after 7 days (Fig. 3C). These results show that increased Atg5 expression disrupts neuronal differentiation in neuroblastoma cells.

DISCUSSION

MDMA has been associated with serotonergic neurotoxicity in a number of species. The administration of MDMA to rats on embryonic day 14 was previously shown to result in fetal elimination of MDMA (Campbell et al., 2006). Recent investigations have demonstrated that prenatal MDMA exposure in rats induces persistent alterations in the developing brain, including changes in dopamine and serotonine metabolism (Galineau et

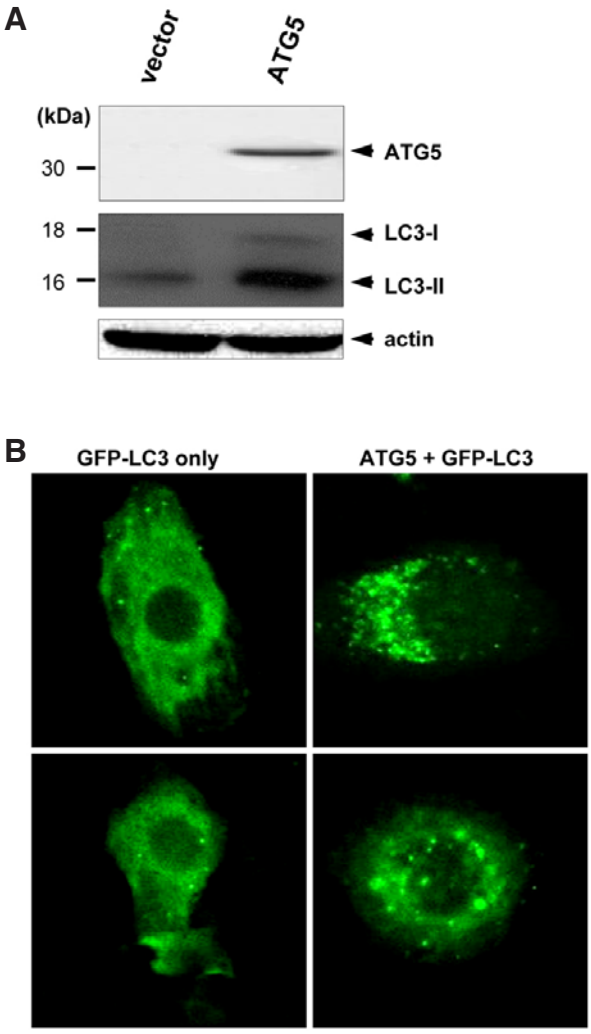

Fig. 2. Atg5 expression induces autophagy. (A) HEK293 was transfected with vector or the plasmid encoding for Atg5 and the cell lysates were subjected to Western blotting with anti-LC3 antibody. (B) SH-SY5Y cells were transfected with GFP-LC3 vector in the presence or absence of the plasmid encoding for Atg5.

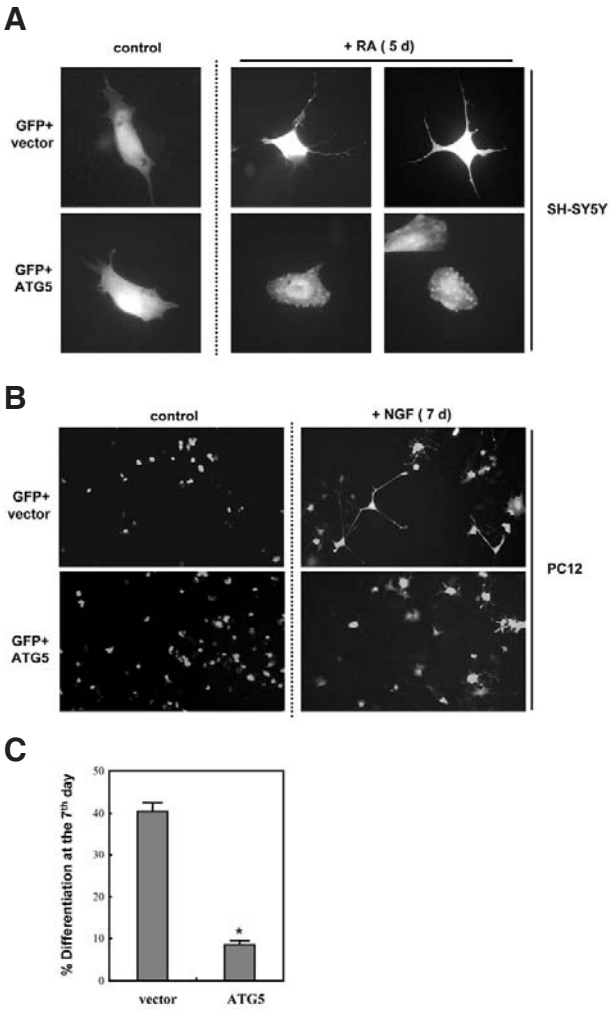

Fig. 3. Effects of Atg5 on neuronal differentiation. (A) SH-SY5Y cells were transfected with GFP vector in the absence (top panel) or presence (bottom panel) of the plasmid encoding for Atg5 and 2 μM RA was administered for the indicated days. (B) PC12 cells were transfected with GFP vector in the absence (top panel) or presence (bottom panel) of the plasmid encoding for Atg5 and NGF (50 ng/ml) was administered for the indicated days. (C) Differentiation rate of PC12 cells in the absence or presence of Atg5. Cells evidencing one or more neuritis of a length more than twice the diameter of the cell body were considered differentiated. Control vector versus Atg5 expression, *, $P < 0.001$.

al., 2005). In this report, we investigated the toxicological effect of MDMA during mouse embryogenesis by using the mouse embryo and neuroblastoma cells. We treated the mouse embryo with increasing concentrations of MDMA (2.5-10 μg/ml) and at 2.5 μg/ml MDMA, there were slight change of short tail torsion on microscopic inspection and yalk sac diameter decrease in growth and developmental stages (data not shown). Meanwhile, 5 and 10 μg treated embryos exhibited the nonclosure of neural tube, and severe decrease incidence in overall embryonic growth. Since higher concentration of MDMA (10 μg/ml) resulted in more severe teratogenesis, we assume that the teratogenic effect of MDMA is dependent on MDMA concentration.

Via real-time PCR analysis, we showed that MDMA treatment in the mouse embryo increased Atg5 gene expression. In addition, cDNA microarray results using MDMA treated neuroblastoma cell line, SH-SY5Y showed increased Atg5 gene expression in a dose-dependent manner (data not shown).

In order to identify the cellular mechanism by which MDMA affects neuroblastoma cells, we administered MDMA to the SH-SY5Y cells and to the embryos. The result, namely that MDMA treatment stimulates Atg5 mRNA expression in both the SH-SY5Y cells and the embryo, was similar to the results of microarray analysis.

Autophagy-related "Atg" proteins are known to directly induce autophagy in the central nervous system, whereas autophagy is a critical factor in the cellular clearance of toxic protein aggregates; impairments of autophagy have been implicated in the pathogenesis of neurodegenerative disorders (Degenhardt et al., 2006; Hara et al., 2006). Therefore, it is difficult to determine from those reports whether autophagy is induced in these diseases as a cytoprotective response or as a pathogenic manifestation. The level of autophagy in mice is low during the

embryogenesis, however, autophagy is immediately activated after birth to overcome the severe starvation caused by the interruption of the trans-placental nutrient supply (Kuma et al., 2004). Thus mice deficient for ATG5, die shortly after birth without forced milk feeding (Kuma et al., 2004). Our results demonstrated that the increased level of ATG5 expression in the neuroblastoma cells was clearly shown to impair the neuronal differentiation. Collectively, these results suggest that the regulation of ATG5 expression in mouse embryo is critical for mouse embryogenesis and brain development.

In an attempt to determine whether Atg5 modulates neuronal differentiation, SH-SY5Y cells and PC12 cells were transfected with the plasmid encoding for Atg5, after which differentiation was induced. The differentiated cells evidenced morphologic changes, with neurite extension and a profuse neuritic arborization forming extensive networks (Encinas et al., 2000; Kim et al., 2002), whereas the cells transfected with Atg5 either did not differentiate, or differentiated at a significantly slower rate. These results indicated that the Atg5 protein profoundly inhibited the neuronal differentiation of neuroblastoma cells.

In some brain regions, more than half of the neurons die as the result of apoptosis during brain development (Raff et al., 1993). Many apoptosis-related factors have been shown to be upregulated in the immature brain, including caspase-3, Apaf-1, Bcl-2, and Bax (Encinas et al., 2000; Kim et al., 2007; Lee et al., 2007; Yamazaki et al., 2000; Zhu et al., 2000). The activation of apoptotic mechanisms contributes to the pathogenesis of brain damage in acute neuropathological disorders in the immature brain (Lee et al., 2007; Zhu et al., 2000). Autophagy is another mechanism of cell death with distinct morphological features, whereby the lysosome-mediated engulfment of injured cells or cellular fragments can occur (Pyo et al., 2005). It appears that the specific cell types and regions involved are determined by the developmental level of the brain at the time of injury, as well as by the intensity of the injury.

Collectively, our findings illustrate the mechanism by which embryonic MDMA consumption results in an increase of Atg5 levels in the brain of the mouse embryo, and the elevated Atg5 level disrupts the neuronal differentiation of neuroblastoma cells.

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