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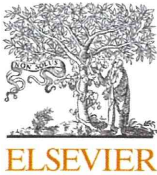

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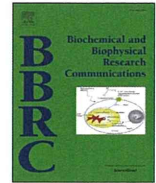

Nicotine induces mitochondrial fission through mitofusin degradation in human multipotent embryonic carcinoma cells

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ABSTRACT

Nicotine is considered to contribute to the health risks associated with cigarette smoking. Nicotine exerts its cellular functions by acting on nicotinic acetylcholine receptors (nAChRs), and adversely affects normal embryonic development. However, nicotine toxicity has not been elucidated in human embryonic stage. In the present study, we examined the cytotoxic effects of nicotine in human multipotent embryonal carcinoma cell line NT2/D1. We found that exposure to 10 μM nicotine decreased intracellular ATP levels and inhibited proliferation of NT2/D1 cells. Because nicotine suppressed energy production, which is a critical mitochondrial function, we further assessed the effects of nicotine on mitochondrial dynamics. Staining with MitoTracker revealed that 10 μM nicotine induced mitochondrial fragmentation. The levels of the mitochondrial fusion proteins, mitofusins 1 and 2, were also reduced in cells exposed to nicotine. These nicotine effects were blocked by treatment with mecamylamine, a nonselective nAChR antagonist. These data suggest that nicotine degrades mitofusin in NT2/D1 cells and thus induces mitochondrial dysfunction and cell growth inhibition in a nAChR-dependent manner. Thus, mitochondrial function in embryonic cells could be used to assess the developmental toxicity of chemicals.
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1. Introduction

Growing evidence suggest that maternal smoking during pregnancy is related to adverse neurodevelopmental outcomes in the offspring, including lower intelligence quotients and deficits in learning and memory [1,2]. Nicotine is a naturally occurring alkaloid that is present in tobacco leaves and is considered to contribute to the negative effects of cigarette smoking on health [2,3]. Nicotine exerts its cellular functions by activating nicotinic acetylcholine receptors (nAChRs), which are heterodimers composed of combinations of different types of α subunit (α1–α10) and β subunit (β1–β4) [4]. α8-nAChR has not been identified in human. Recent studies have shown that nAChRs are present in a variety of cells, such as cancer cells, vascular smooth muscle, and neural cells [3–6]. Activation of nAChRs by nicotine promotes the release of various neurotransmitters (including dopamine, norepinephrine, acetylcholine, glutamate) [7]. Altered regulation of neurotransmitter levels can adversely affect key events in normal brain development, such as the formation of neural circuits and neurotransmitter systems [7,8]. Therefore, it is necessary to elucidate the cytotoxic effects of nicotine on embryonic development.

Nicotine toxicity has been reported to affect mitochondrial function both *in vitro* and *in vivo*. For example, nicotine exposure alters mitochondrial membrane potential (MMP), increases an oxidative stress, and induces apoptosis in colon adenocarcinoma HCT-116 cell [9]. Another study has shown that nicotine exposure reduced the activity of an enzyme in the pancreatic mitochondrial respiratory chain, and impaired glucose-stimulated insulin secretion in neonatal rats [10]. However, the precise mechanisms underlying the effects of nicotine on mitochondrial function remain largely unknown.

Growing evidence suggest that mitochondria undergo continuous morphological dynamics involving fusion and fission cycles. These dynamics play a key role in maintenance of normal mitochondrial functions, such as ATP production [11]. Mitochondrial fusion and fission are regulated by several GTPases. Mitofusin 1 and 2 (Mfn1, 2) and optic atrophy 1 (Opa1) induce fusion of the outer and inner mitochondrial membranes, respectively [12,13]. In contrast, dynamin-related protein 1 (Drp1) is a cytoplasmic protein that assembles into rings surrounding the outer mitochondrial

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membrane, where it interacts with fission protein 1 (Fis1) to promote fission [14,15]. For example, pigment epithelium-derived factor is reported to improve mitochondrial function by stabilizing mitochondrial fusion in retinal pigment epithelial cells [16]. In contrast, the anti-tumor agent, doxorubicin, facilitates mitochondrial fragmentation and apoptosis by promoting Mfn2 degradation in sarcoma U2OS cells [17].

In the present study, we hypothesized a possible link between nicotine toxicity and mitochondrial function in human mulitpotent NT2/D1 cells, which have neural differentiation capability. Our results showed that exposure to 10 μM nicotine decreased intracellular ATP levels and inhibited cell growth. Moreover, nicotine exposure induced Mfn degradation and mitochondrial fragmentation via nicotinic acetylcholine receptors (nAChRs). Thus, nicotine induces toxicity through impairment of mitochondrial quality control in human NT2/D1 cells.

2. Materials and methods

2.1. Cell culture

The human multipotent embryonal carcinoma NT2/D1 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). SH-SY5Y cells were obtained from European Collection of Animal Cell Culture (Salisbury, Wiltshire, UK). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Biological Industries, Ashrat, Israel) and 0.05 mg/ml penicillin-streptomycin mixture (Life Technologies, Carlsbad, CA, USA) at 37 °C in the presence of 5% CO_2.

2.2. Cell proliferation assay

Cell viability was measured using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA), as previously described [18]. Briefly, NT2/D1 cells were seeded into 96-well plate and exposed to different concentrations of nicotine. After exposure to nicotine, One Solution Reagent was added to each well, and the plate was incubated at 37 °C for another 2 h. Absorbance was measured at 490 nm by iMark microplate reader (Bio-Rad, Hercules, CA, USA).

2.3. Measurement of intracellular ATP levels

The intracellular ATP content was measured using the ATP Determination Kit (Life Technologies), as previously described [19]. Briefly, the cells were washed and lysed with phosphate-buffered saline containing 0.1% Triton X-100. The resulting cell lysates were added to a reaction mixture containing 0.5 mM D-luciferin, 1 mM dithiothreitol, and 1.25 μg/ml luciferase and incubated for 30 min at room temperature. Luminescence was measured using a Wallac1420ARVO fluoroscan (Perkin–Elmer, Waltham, MA, USA). The luminescence intensities were normalized to the total protein content.

2.4. Assessment of mitochondrial fusion

After treatment with nicotine (10 μM, 24 h), cells were fixed with 4% paraformaldehyde and stained with 50 nM MitoTracker Red CMXRos (Cell Signaling Technology, Danvers, MA, USA) and 0.1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI; Dojin, Kumamoto, Japan). Changes in mitochondrial morphology were observed using a confocal laser microscope (Nikon A1). Images (n = 3–7) of random fields were taken, and the number of cells displaying mitochondrial fusion (<10% punctiform) was counted in each image, as previously described [20]. The number of cells showing mitochondrial fission was calculated by subtracting the number of cells with mitochondrial fusion from the total cell number.

2.5. Real-time PCR

Total RNA was isolated from NT2/D1 cells using TRIzol reagent (Life Technologies), and quantitative real-time reverse transcription (RT)-PCR with QuantiTect SYBR Green RT-PCR Kit (QIAGEN, Valencia, CA, USA) was performed using an ABI PRISM 7900HT sequence detection system (Applied Biosystems, Foster City, CA, USA) as previously described [21]. The relative change in the amount of transcript was normalized to the mRNA levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The following primer sequences were used for real-time PCR analysis: *nAChRα1*, forward, 5′-CTGGACCTACGACGGCTCT-3′ and reverse, 5′-CGCTGCATGACGAAGTGGT-3′; *nAChRα2*, forward, 5′-ACACTTCA-GACGTGGTGATTG-3′ and reverse, 5′-CCACTCCTGTTTTAGCCAGAC-3′; *nAChRα3*, forward, 5′-ACCTGTGGCTCAAGCAAATCT-3′ and reverse, 5′-GCAGGGACACGCATGAACT-3′; *nAChRα4*, forward, 5′-GGAGGGCGTCCAGTACATTG-3′ and reverse, 5′-GAA-GATGCGGTCGATGACCA-3′; *nAChRα5*, forward, 5′-AGATG-GAACCCTGATGACTATGGT-3′ and reverse, 5′-AAACGTCCATCTGCATTATCAAAC-3′; *nAChRα6*, forward, 5′-GGCAGGGATTCCTTCATGGG-3′ and reverse, 5′-GCCTCTCCTCAGTTGCACAG-3′; *nAChRα7*, forward, 5′-CATGGCCTTCTCGGTCTTCA-3′ and reverse, 5′-CACGGCCTCCAC-GAAGTT-3′; *nAChRα10*, forward, 5′-CAGATGCCTACCTACGATGGG-3′ and reverse, 5′-GGGAAGGCTGCTACATCCA-3′; *nAChRβ1*, forward, 5′-TGAGACCTCACTATCAGTACCCA-3′ and reverse, 5′-AGAACCACGA-CACTAAGGATGA-3′; *nAChRβ2*, forward, 5′-GGTGACAGTA-CAGCTTATGGTG-3′ and reverse, 5′-AGGCGATAATCTTCCCACTCC-3′; *nAChRβ3*, forward, 5′-TGCTGGTTCTCATCGTCCTTG-3′ and reverse, 5′-GCATCTTCATTTTCGGCGATTGA-3′; *nAChRβ4*, forward, 5′-CAGCTTATCAGCGTGAATGAGC-3′ and reverse, 5′-GTCAGGCGG-TAATCAGTCCAT-3′; *Drp1*, forward, 5′-TGGGCGCCGACATCA-3′ and reverse, 5′-GCTCTGCGTTCCCACTACGA-3′; *Fis1*, forward, 5′-TACGTCCGCGGGTTGCT-3′ and reverse, 5′-CCAGTTCCTTGGCCTGGTT-3′; *Mfn1*, forward, 5′-GGCATCTGTGGCC-GAGTT-3′ and reverse, 5′-ATTATGCTAAGTCTCCGCTCCAA-3′; *Mfn2*, forward, 5′-GCTCGGAGGCACATGAAAGT-3′ and reverse, 5′-ATCACGGTGCTCTTCCCATT-3′; *Opa1*, forward, 5′-GTGCTGCCCGCCTAGAAA-3′ and reverse, 5′-TGA-CAGGCACCCGTACTCAGT-3′; *GAPDH*, forward, 5′-GTCTCCTCTGACTTCAACAGCG-3′ and reverse, 5′-ACCACCCTGTTGCTGTAGCCAA-3′.

2.6. Western blot analysis

Western blot analysis was performed as previously reported [22]. Briefly, the cells were lysed with Cell Lysis Buffer (Cell Signaling Technology). The proteins were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to Immobilon-P (Millipore, Billerica, MA, USA). The membranes were probed with anti-Drp1 monoclonal antibodies (1:1000; Cell Signaling Technology), anti-Fis1 polyclonal antibodies (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Mfn1 polyclonal antibodies (1:1000; Cell Signaling Technology), anti-Mfn2 monoclonal antibodies (1:1000; Cell Signaling Technology), anti-Opa1 monoclonal antibodies (1:1000; BD Biosciences), and anti-β-actin monoclonal antibodies (1:5000; Sigma–Aldrich). The membranes were then incubated with secondary antibodies against rabbit or mouse IgG conjugated to horseradish peroxidase (Cell Signaling Technology). The bands were visualized using the ECL Western Blotting Analysis System (GE

Healthcare, Buckinghamshire, UK), and images were acquired using a LAS-3000 Imager (FUJIFILM UK Ltd., Systems, Bedford, UK).

2.7. Chemicals and reagents

Nicotine was obtained from Wako Pure Chemicals (Osaka, Japan). Mecamylamine hydrochloride (MCA) and m-chlorophenylhydrazone (CCCP) were obtained from Sigma–Aldrich.

2.8. Statistical analysis

All data were presented as means ± S.D. ANOVA followed by post hoc Fisher test was used to analyze data in Fig. 1A and B and Figs. 2–4C. Student's t-test was used to analyze data in Fig. 4A. P-values less than 0.05 were considered to be statistically significant.

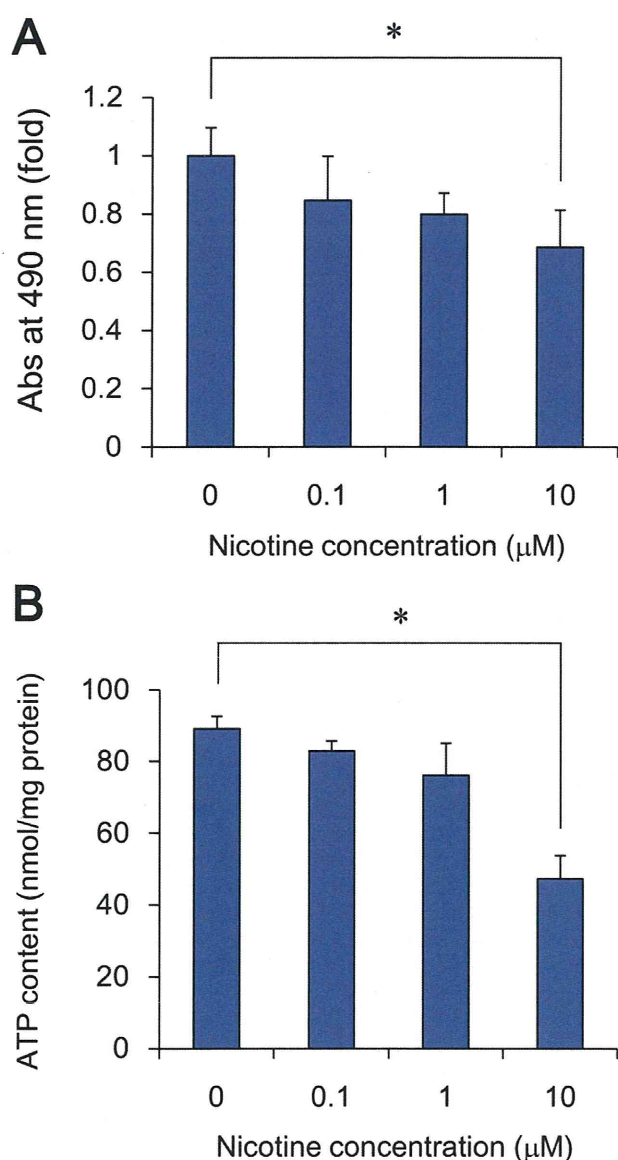

Fig. 1. Nicotine inhibits cell proliferation via intracellular ATP decrease in NT2/D1 cells. A. Cells were exposed to different concentrations of nicotine for 72 h. Cell viability was examined using the CellTiter 96 AQueous One Solution Cell Proliferation Assay. B. After treatment with different concentrations of nicotine for 24 h, intracellular ATP content was determined in cell lysates. Data represent the mean ± SD (n = 3). *P < 0.05.

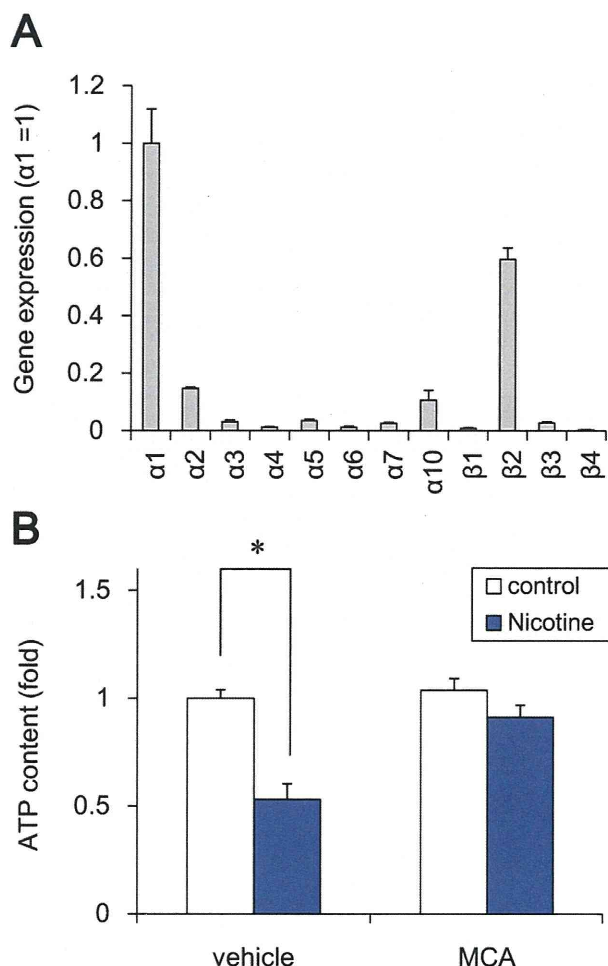

Fig. 2. Nicotine reduces intracellular ATP levels via nAChRs in NT2/D1 cells. A. Expression of AChR subtypes was analyzed by real-time PCR in NT2/D1 cells. The relative changes were determined by normalizing with GAPDH. B. After treatment with 10 μM nicotine and/or 30 μM MCA for 24 h, intracellular ATP content was determined in cell lysates. Data represent the mean ± SD (n = 3). *P < 0.05.

3. Results

3.1. Cytotoxic effects of nicotine in NT2/D1 cells

To examine the effects of nicotine on human multipotent embryonic cells, we exposed the cells to different concentrations of nicotine for 72 h and measured cell viability by MTT assay using human multipotent embryonic carcinoma NT2/D1 cells, which have an ability to differentiate into neuronal cells. We found that treatment with 10 μM nicotine significantly inhibited cell proliferation (Fig. 1A). Similarly, exposure to 10 μM nicotine significantly reduced the ATP content of the cells (Fig. 1B). To further investigate whether the nicotine effects are selective for undifferentiated cells, we used human SH-SY5Y neuroblastoma cells. We found that exposure to 10 μM nicotine had little effect on proliferation and ATP content of SH-SY5Y cells (Fig. S1).

We next examined the nAChR mRNA levels by real-time PCR and confirmed that nAChR subtypes except α9-nAChR were expressed in NT2/D1 cells (Fig. 2A). To examine whether the inhibition of ATP

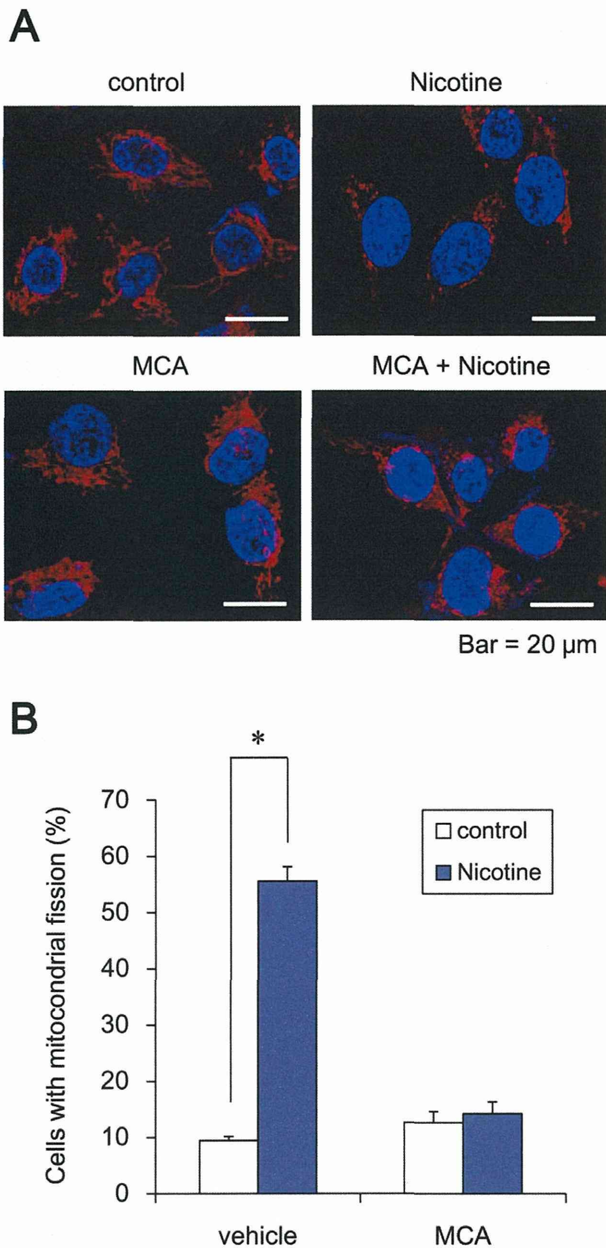

Fig. 3. Nicotine induces mitochondrial fission via nAChRs in NT2/D1 cells. A. Cells were exposed to 10 μM nicotine, in the presence or absence of 30 μM MCA, for 24 h. The cells were stained with MitoTracker Red CMXRos and DAPI and mitochondrial morphology was observed by confocal laser microscopy. Bar = 20 μm. B. The number of cells showing mitochondrial fusion (<10% punctiform) was counted in three independent captured images. The number of cells showing mitochondrial fission was calculated by subtracting the number of cells with mitochondrial fusion from the total cell number. *P < 0.05.

production is mediated via the nAChRs, we tested the effect of nAChR antagonist on the ATP content. As shown in Fig. 2B, a non-selective nAChR antagonist mecamylamine (MCA) abolished the nicotine-induced reduction of ATP content. MCA alone did not affect the ATP level. These data suggest that nicotine decreases the ATP content via its nAChR and inhibits cell proliferation in NT2/D1 cells.

3.2. *Effects of nicotine on mitochondrial morphology in NT2/D1 cells*

Mitochondrial function, including ATP production, are maintained by mitochondrial fusion and fission [11]. Since nicotine reduced intracellular ATP levels, we next focused on the mitochondrial dynamics in NT2/D1 cells. Nicotine exposure (10 μM, 24 h) significantly increased the number of fragmented mitochondria with punctate morphology, as compared to the level observed in untreated control cells (Fig. 3). Moreover, MCA abolished this nicotine-induced mitochondrial fragmentation (Fig. 3). MCA alone did not affect mitochondrial dynamics. In contrast to NT2/D1 cells, nicotine did not significantly affect the mitochondrial dynamics in SH-SY5Y neuroblastoma cells (Fig. S1). These results suggest that nicotine induces mitochondrial fission via nAChRs in NT2/D1 cells.

3.3. *Nicotine reduces Mfn1 and Mfn2 protein levels in NT2/D1 cells*

To examine the molecular mechanism by which nicotine induces mitochondrial fragmentation, we assessed its effects on mitochondrial fission (Fis1, Drp1) and fusion genes (Mfn1, Mfn2, Opa1). Real-time PCR analysis showed that each gene expression was not significantly altered by nicotine exposure (Fig. 4A). Interestingly, western blot analysis revealed that nicotine did significantly decrease the levels of Mfn1 and Mfn2 proteins (Fig. 4B and C). In contrast, the levels of other proteins, including Fis1, Drp1, and Opa1, were not affected by nicotine. These data suggest that nicotine-induced mitochondrial fragmentation is caused by the degradation of Mfn1 and Mfn2 proteins.

4. Discussion

In the present study, we demonstrated that exposure to micromolar levels of nicotine impairs mitochondrial quality control in human multipotent embryonic carcinoma cells. Exposure to nicotine induces nAChR-dependent degradation of Mfn1 and Mfn2, thereby promoting mitochondrial fragmentation. These negative nAChR-mediated effects of nicotine on mitochondrial quality control could inhibit ATP production and cell viability.

Undifferentiated embryonic cells may tend to be sensitive to the growth inhibitory effects of nicotine, whereas proliferative and protective effects of nicotine have been described in more developed somatic cells [23–27]. Our studies showed that treatment with 10 μM nicotine reduces cell growth in human embryonic cells (Fig. 1), whereas the growth of human neuroblastoma SH-SY5Y cells is not affected (Fig. S1). Previous study has also shown that exposure to more than 1.8 μM nicotine inhibits cell adhesion and induces apoptosis in human embryonic stem cells [28]. The concentrations of nicotine tested in our study were relevant to the circulating levels of nicotine in cigarette smokers, which have been reported to range from 10 nM to 10 μM [29]; these have the potential to inhibit the growth of embryonic cells. In contrast to these growth inhibitory effects, nicotine is known to stimulate the proliferation of hematopoietic and neuronal progenitors [23–25]. In addition, nicotine is reported to protect rat basal forebrain neurons or rat hippocampal neurons from the cytotoxicity of β-amyloid protein [26,27]. Taken together, nicotine effects in undifferentiated embryonic cells contains different mechanisms from developed somatic cells. Therefore, further studies are required to elucidate the mechanism of cell stage-specific effects using embryonic and differentiated cells.

Our data suggest that nicotine induces mitochondrial fission through the degradation of Mfn1 and Mfn2 (Figs. 3 and 4). Consistent with this finding, chemical stressors have been reported

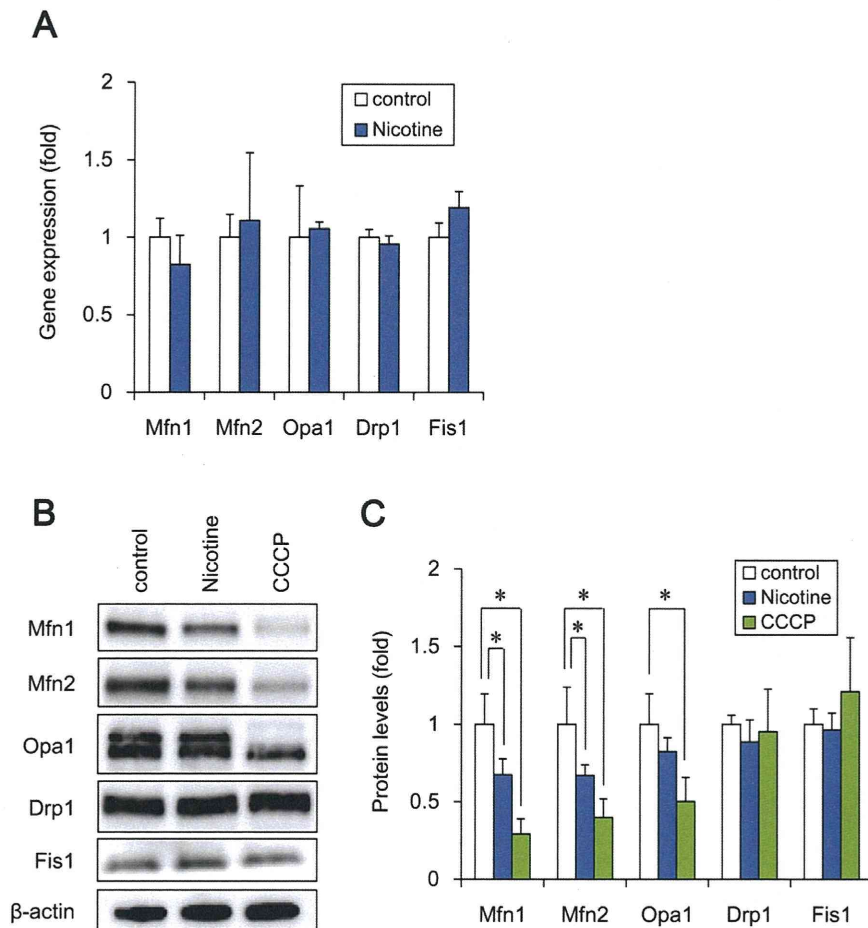

Fig. 4. Nicotine reduces Mfn1 and Mfn2 protein levels in NT2/D1 cells. A. After exposure to 10 μM nicotine for 24 h, the expression of the indicated mitochondrial genes was analyzed by real-time PCR. The relative changes were determined by normalizing with GAPDH. B. After exposure to 10 μM nicotine or 10 μM CCCP for 24 h, the expression of mitochondrial proteins was analyzed by western blot using anti-Drp1, anti-Fis1, anti-Mfn1, anti-Mfn2, anti-Opa1, or anti-β-actin antibodies. C. The band densities were analyzed by ImageJ software. Relative changes in expression were determined by normalization to β-actin. Data represent the mean ± SD (n = 3). *P < 0.05.

to cause mitochondrial fission via Mfn degradation. For example, organotin compounds such as tributyltin induce proteasomal degradation of Mfn1 and Mfn2, which facilitates mitochondrial fragmentation and growth arrest in NT2/D1 cells [30,31]. Since nicotine showed similar effects in NT2/D1 cells, nicotine exposure may also degrade Mfn1 and Mfn2 via proteasome. Moreover, an inhibitor of mitochondrial calcium efflux, CGP37157, is reported to degrade Mfn1 via E3 ubiquitin ligase and induce mitochondrial fission in prostate cancer LNCaP cells [32]. Further studies will be necessary to determine whether ubiquitin ligases are involved in nicotine-induced Mfn1 and Mfn2 degradation in embryonic cells.

Our data suggest that nicotine toxicity is mediated by dysfunctional mitochondrial quality control, which occurs via a nAChR-dependent mechanism (Figs. 2 and 3). Nicotine has been reported to evoke extracellular calcium influx through plasma membrane nAChRs [4]. Moreover, a transient increase in intracellular calcium levels is known to cause mitochondrial calcium overload, which is followed by the depolarization of the mitochondrial membrane, resulting in a loss of MMP [33,34]. In other cell lines, MMP reduction is reported to induce the mitochondrial translocation of the E3 ubiquitin ligase, Parkin, which targets the Mfn protein for proteasomal degradation [35]. Therefore, nicotine may increase intracellular calcium entry via nAChRs, thus reducing the MMP and inducing mitochondrial translocation of E3 ubiquitin ligases; this increases the proteasomal degradation of Mfn1 and Mfn2. Several reports indicate that knockdown of Mfn1 and Mfn2 in the cells induces mitochondrial fragmentation and shows severe cellular defects, including decreased ATP content and poor cell growth [36,37]. Especially, Mfn2 has been reported to be necessary for striatal axonal projections of midbrain dopamine neurons by the studies using dopamine neuron-specific Mfn2 knockout mice [38]. Taken together, Mfn1 and Mfn2 might be involved in several nAChR-mediated effects of nicotine, such as the reduction of ATP content, growth inhibition, and modulation of synaptic transmission. In future studies, it will be necessary to investigate the precise mechanism involved in nicotine-induced Mfn degradation, which results in mitochondrial fission and impaired function.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Transparency document

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2016.01.063.

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Title

Effects of 13 developmentally toxic chemicals on the migration of rat cephalic neural crest cells in vitro

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ABSTRACT

The inhibition of neural crest cell (NCC) migration has been considered as a possible pathogenic mechanism underlying chemical developmental toxicity. In this study, we examined the effects of 13 developmentally toxic chemicals on the migration of rat cephalic NCCs (cNCCs) by using a simple in vitro assay. cNCCs were cultured for 48 h as emigrants from rhombencephalic neural tubes explanted from rat embryos at day 10.5 of gestation. The chemicals were added to the culture medium at 24h of culture. Migration of cNCCs was measured as the change in the radius (radius ratio) calculated from the circular spread of cNCCs between 24 and 48 h of culture. Of the chemicals examined, 13-*cis*-retinoic acid, ethanol, ibuprofen, lead acetate, salicylic acid, and selenate inhibited the migration of cNCCs at their embryotoxic concentrations; no effects were observed for acetaminophen, caffeine, indium, phenytoin, selenite, tributyltin, and valproic acid. In a cNCC proliferation assay, ethanol, ibuprofen, salicylic acid, selenate, and tributyltin inhibited cell proliferation, suggesting the contribution of the reduced cell number to the inhibited migration of cNCCs. It was determined that several developmentally toxic chemicals inhibited the migration of cNCCs, the effects of which were manifested as various craniofacial abnormalities.

Key words

Developmental toxicity; Embryo; Migration assay; Neural crest cell; Rat

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INTRODUCTION

In vertebrate embryos, neural crest cells (NCCs) migrate to various tissues throughout the body and contribute to tissue organization; malfunction NCCs of can lead to dysmorphologies, tumors and syndromes called neurocristopathies (Hall 2009; Le Douarin & Kalcheim 1999). The inhibition of NCC migration has, therefore, been considered as a possible pathogenic mechanism underlying chemical developmental toxicity. It has been shown, for example, that all-*trans*-retinoic acid, a well-known teratogen, inhibits the migration of cephalic NCCs (cNCCs), causing branchial abnormalities in cultured mouse and rat embryos (Menegola et al. 2004).

The effects of chemicals on the migration of NCCs in mammals, however, have not been fully investigated, probably because no convenient experimental methods are available. The migration of NCCs has been examined by time-lapse video image analysis of fluorescence-labeled cells (Fuller et al. 2002; Kawakami et al. 2011), or by human neural crest stem cells with scratch assay (Zimmer et al. 2012). These methods are complicated and therefore not ideal for testing of chemicals in a common toxicity laboratory.

Recently, we established a simple in vitro assay that enabled examination of the effects of chemicals on the migration of cNCCs and trunk NCCs (tNCCs) (Usami et al. 2014b). In this method, NCCs are cultured as emigrants from isolated neural tubes of day 10.5 rat embryos. The cultured NCCs are exposed to test chemicals and their migration is determined as the radius ratio calculated from circular spread of the NCCs during the exposure period. Using this method we examined the effects of 13 developmentally toxic chemicals on the migration of cNCCs. We also examined the effects of chemicals on the proliferation of cNCCs, because this migration assay depends on the spread of cells and

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can therefore be influenced by the cell number.

We selected developmentally toxic chemicals on the basis of our interest in our related study such as proteomics of embryos (Usami et al. 2014a; Usami et al. 2009; Usami et al. 2008) and metabolomics of hepatocytes (Kim et al. 2014) since there was little information about the effects of chemicals on the migration of cNCCs. However, we considered that the chemicals include both ones might affect cNCC migration, e.g., ethanol, and selenate, and ones might not, e.g., indium, and tributyltin, which was speculated from their potential to cause craniofacial abnormality.

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MATERIALS AND METHODS

Animals

Wistar rats (Crj: WI, Charles River Japan Inc., Kanagawa, Japan) were used. Pregnant rats were obtained by mating female and male rats overnight, and the plug day was designated as day 0.5 of gestation. All the animal experiments were performed according to the guidelines for animal experiments of the National Institute of Health Sciences.

Chemicals

Acetaminophen (CAS 103-90-2), 13-*cis*-retinoic acid (CAS 4759-48-2), ibuprofen (CAS 31121-93-4), salicylic acid (CAS 54-21-7), selenate (CAS 13410-01-0), and selenite (CAS 10102-18-8) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Caffeine (CAS 58-08-2), ethanol (CAS 64-17-5), indium (CAS 22519-64-8), lead acetate (CAS 6080-56-4), phenytoin (CAS 57-41-0), and tributyltin (CAS 1461-22-9) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Valproic acid (CAS 1069-66-5) was purchased from Merck Co. (Darmstadt, Germany).

Culture of NCCs

Rat NCCs were cultured as emigrated cells from neural tubes of rat embryos at day 10.5 of gestation as previously described (Usami et al. 2014b), according to the culture schedule shown in Fig. 1. Neural tubes were excised from the rhombencephalic (for cNCCs) or trunk (for tNCCs) region of the embryos in Hanks' balanced salt solution with sharpened tungsten needles. The excised neural tubes were cultured in 35-mm culture dishes (BD Primaria; Becton, Dickinson and Company, Franklin Lakes, NJ) containing 2 ml of Dulbecco's Modified Eagle Medium with high glucose (DMEM; GIBCO, Life

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Technologies Corp., Carlsbad, CA) and 10% (v/v) fetal bovine serum (GIBCO) at 37°C with 5% CO_2 for 48 h.

Phase-contrast images of cultured NCCs were recorded digitally at a magnification of ×10 with a microscope at 24 and 48 h of culture (BZ-9000; Keyence, Osaka, Japan). In the proliferation assay, the neural tube was removed at 18 h of culture, and the cell nuclei were stained with 4',6- diaminodino-2-phenylindole (DAPI, Invitrogen) and fluorescent images were photographed with the microscope at 48 h of culture. Representative photographs of the cNCCs are shown in Fig. 2.

Addition of chemicals

The chemicals were added at 24 h of culture by replacing the culture medium. For addition to the culture medium, caffeine, ethanol, salicylic acid, selenate, selenite, and valproic acid were directly dissolved in or diluted with the culture medium. Acetaminophen, 13-*cis*-retinoic acid, phenytoin, and tributyltin were dissolved in or diluted with dimethyl sulfoxide and 5 μl each of the solutions was added to 5 ml of the culture medium. Ibuprofen, indium, and lead acetate were dissolved in pure water and 100 μl each of the solutions was added to 4.9 ml of the culture medium.

The concentrations of the following chemicals in the culture medium were their embryotoxic concentrations obtained from the literature: acetaminophen (Weeks et al. 1990), caffeine (Robinson et al. 2010; Shreiner et al. 1986), 13-*cis*-retinoic acid (Lee et al. 1991), ethanol (Usami et al. 2014a), ibuprofen (Guest et al. 1994), indium (Usami et al. 2009), lead acetate (Zhao et al. 1997), phenytoin (Winn 2002), salicylic acid (Greenaway et al. 1985), selenate (Usami et al. 2008), selenite (Usami et al. 2008), tributyltin (Cooke et al. 2008; Adeeko et al. 2003), and valproic acid (Guest et al. 1994)

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Migration assay of NCCs

The migration distance of NCCs was calculated as the increased radius of the circular spread of NCCs that emigrated from the neural tubes between 24 and 48 h of culture (Usami et al. 2014b). The outermost NCCs in each of the cultured neural tubes were connected with the polygon tool as if a rubber band were put around the cells, and its inner area was measured as a pixel count. Considering the polygon as a circle, its radius ratio was calculated: radius ratio = (radius at 48 h - radius at 24 h)/radius at 24 h. This ratio was then normalized as a percent of the simultaneous control to express the NCC migration for comparisons among experiments.

Proliferation assay for NCCs

NCC proliferation was evaluated as a ratio of the cell count at 48 h to that at 24 h of culture, and the effects of chemicals were examined. The cells were counted manually at 24 h on the phase-contrast image with the Cell Counter plugin of the ImageJ software (http://rsb.info.nih.gov/ij/, 1997–2009; Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, MD, USA). The cell count at 48 h was estimated as the count of stained cell nuclei from the fluorescence image with the Hybrid Cell Count function of the BZ-X Analyzer software (Keyence). The average cell counts in an intact control group were 170.6 at 24 h and 272.1 at 48 h (n = 10). Two proliferation indices, the cell count ratio and the cell proliferation ratio, were calculated as follows: cell count ratio = cell count at 48 h/cell count at 24 h, and cell proliferation ratio = (cell count at 48 h - cell count at 24 h)/cell count at 24 h.

Although these indices are basically the same, the latter is more suitable for representing

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the proliferation rate and the former is more useful for comparison with the migration index. These indices were normalized to the control to allow for comparisons among experiments.

Statistical analysis

Statistical significance of the difference between the experimental groups was examined by the Student *t* test at a probability level of 5%.

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RESULTS

Effects of chemicals on the migration of cNCCs

Of the 13 chemicals we tested, six chemicals, that is, 13-*cis*-retinoic acid, ethanol, ibuprofen, lead acetate, salicylic acid, and selenate, significantly inhibited the migration of cNCCs at their embryotoxic concentrations. 13-*cis*-Retinoic acid reduced the migration of cNCCs by approximately 13% at concentrations of 3 and 10 μM (Fig. 3A). Ethanol, ibuprofen, salicylic acid and selenate reduced the migration of cNCCs by 10.5% at 195 mM, 15.9% at 2 mM, 8.5% at 3 mM and 16.2% at 150 μM, respectively (Figs. 3B - E).

Lead acetate reduced the migration of cNCCs by 11.6% at 3 μM and by 30.0% at 10 μM in an initial experiment (Fig. 3F). Because evaluation of the toxic effects of lead at low exposure levels is important for human health, two lower concentrations were added stepwise so that the no-observed-effect level could be estimated. Lead acetate reduced the migration of cNCCs significantly by 8.7% at 1 μM; however, the decrease (6.4%) was not significant at 0.1 μM (Fig. 3G).

The remaining seven chemicals, that is, acetaminophen, caffeine, indium, phenytoin, selenite, tributyltin, and valproic acid, had no significant effects on the migration of cNCCs even at high concentrations (Figs. 4A-G). Indium did not affect the migration of cNCCs and tNCCs in the experiments (Fig. 4C). These experiments for indium were performed at a single concentration for cNCCs and tNCCs because indium showed no effects on the migration of cNCCs in a pilot study and because indium has been reported to cause malformation in the caudal part of rat embryos (Nakajima et al. 2008).

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Effects of chemicals on the proliferation of cNCCs

Effects on the proliferation of cNCCs were examined in the case of six chemicals (i.e., 13-*cis*-retinoic acid, ethanol, ibuprofen, lead acetate, salicylic acid, and selenate) that showed inhibitory effects on the migration of cNCCs. The effects of tributyltin on cNCC proliferation were also examined because of our interest in another research project. To reduce the number of animals to be used, two chemicals with the same vehicle were examined concomitantly when possible. In the control groups, the actual cell count increased by approximately 50% during the 24-h exposure period.

13-*cis*-Retinoic acid did not significantly reduce the proliferation of cNCCs at concentrations of 3 and 10 μM, the same concentrations at which it inhibited the migration of cNCCs, although the cell count ratio and the cell proliferation ratio were lowered by 2.5% and 9.7%, respectively, at 3 μM compared to the control group (Fig. 5A).

Ethanol, ibuprofen, salicylic acid, and selenate significantly reduced the cell count ratio by 16.3%, 14.1%, 12.3%, and 20.6%, and the cell proliferation ratio by 59.0%, 43.0%, 33.1%, and 55.4%, respectively, at the same concentrations (195 mM, 2 mM, 3 mM, and 150 μM, respectively) at which they inhibited the migration of cNCCs (Fig. 5B - D).

Lead acetate increased the cell count ratio by 2.2% and the cell proliferation ratio by 6.6% at 1 μM concentration, the lowest effective concentration for inhibiting the migration of cNCCs, although these differences were not statistically significant (Fig. 5C).

Tributyltin reduced the cell count ratio by 9.2% and the cell proliferation ratio by 27.0% at 100 nM concentration, although no reduction in the migration of cNCCs was observed (Fig. 5E).

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There was no significant correlation between proliferation inhibition and migration inhibition when the reduced migration was plotted against the reduced cell count ratio, suggesting a varied contribution of the latter to the former (Fig. 6).

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DISCUSSION

Here, we observed inhibition of the migration of rat cNCCs by six developmentally toxic chemicals including those not previously reported to have the inhibitory effects: ibuprofen, salicylic acid, and selenate. It is speculated that inhibition of the migration of cNCCs results in reduction of the number of cNCCs at their destination tissues. The inhibited migration of cNCCs by itself, however, seems insufficient as a pathogenic mechanism underlying teratogenicity because these chemicals do not necessarily cause similar malformations. It is probable that the inhibited migration of cNCCs that is not accompanied by an excessive cell shortage is compensated by accelerated cell proliferation at their destination tissues. Alternatively, these inhibitory effects may occur differently in the body of embryos.

From the results of the proliferation assay, it is considered that the reduced cell number may contribute to the inhibited migration of cNCCs to varying extents depending on the test chemicals. It is suggested that the migration-inhibitory effects of ethanol, ibuprofen, and selenate are due in part to the reduced number of cNCCs. In contrast, in the case of tributyltin, the reduced cell number did not affect the migration of cNCCs. Chemicals that did not inhibit cell proliferation, for example, 13-*cis*-retinoic acid, and lead acetate, appeared to inhibit the migration of cNCCs independent of the cell number.

13-*cis*-Retinoic acid appeared to more potently inhibit the migration of cNCCs than all-*trans*-retinoic acid, because the inhibitory concentration of the former (3 μM) was found to be lower than that of the latter (10 μM) in our previous study (Usami et al. 2014b). This is inconsistent with the teratogenic potential of the retinoic acids in rats, where 13-*cis*-retinoic acid is less teratogenic because of its faster elimination from the body (Collins et al. 1994). Isolated cNCCs themselves may be more susceptible to

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13-*cis*-retinoic acid than all-*trans*-retinoic acid, as suggested by the lower affinity of 13-*cis*-retinoic acid for cytoplasmic retinoid binding proteins, which may enable easy access to the cell nucleus (Rühl et al. 2001).

Ethanol is a well-known teratogen causing craniofacial malformations (Schardein & Macina 2006) and its toxic effects on NCCs have often been investigated. It was previously shown that ethanol caused apoptotic cell death (Yan et al. 2010) and inhibited migration (Shi et al. 2014) of NCCs. In the present study, both reduced cell number and inhibited migration of cNCCs were observed as the effects of ethanol, although the effective concentration of ethanol was relatively higher than those reported in previous studies, probably because of species and strain differences in the susceptibility to ethanol (Wentzel & Eriksson 2008).

Inhibitory effects of ibuprofen and salicylic acid, which are non-steroidal anti-inflammatory drugs (NSAIDs), on the migration of NCCs have not been reported to date. Although these NSAIDs are considered non-teratogenic in humans, their embryotoxic effects, including craniofacial malformations, observed in animal experiments (Joschko et al. 1993; Kosar 1993) may be related to their migration-inhibitory effects on cNCCs.

The migration-inhibitory effects of lead acetate in the present study are consistent with previously reported results for human NCCs derived from embryonic stem cells (Zimmer et al. 2012). In both studies, lead acetate at 1 μM (20 μg/dl) or higher concentrations inhibited the migration of NCCs without reduced cell proliferation. It is noted that this inhibitory concentration is comparable to blood lead levels (40.0 ± 16.5 μg/dl, mean ± SD) in a certain proportion of pregnant women (Ugwuja et al. 2012). Although lead caused craniofacial malformations only in cultured rat embryos (Zhao et al. 1997)

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and does not cause major malformations in humans, its migration-inhibitory effects on cNCCs, as a neuronal progenitor, may be related to functional deficiencies such as neurological alterations (Flora et al. 2011).

The effects of the two selenium compounds on the migration of cNCCs were different in the present study; i.e., selenate inhibited the migration of cNCCs while selenite did not. This difference may be related to the difference in malformed optic vesicles and the protein expression changes caused by the selenium compounds in cultured rat embryos; selenate caused enlargement of the optic vesicle (Usami et al. 2008), a destination of migrating cNCCs (Le Douarin & Kalcheim 1999), and increased the phosphorylated form (inactive form) of cofilin 1 (Usami et al. 2008), an actin-binding protein essential for the migration of NCCs (Gurniak et al. 2005), while selenite did not cause either (Usami et al. 2008). It is thus speculated that selenate inhibits the migration of cNCCs through inactivation of cofilin 1, which results in malformation of the optic vesicle.

In this context, it is intriguing that ethanol and indium also increased phosphorylated cofiline 1 in cultured rat embryos (Usami et al. 2014a; Usami et al. 2009). However, indium did not have inhibitory effects on the migration of cNCCs or tNCCs in the present study. This may indicate that the increase in phosphorylated cofilin 1 alone is not a sufficient condition for inhibition of the migration of NCCs, or that it could occur in different embryonic cells.

The proliferation-inhibitory effects of tributyltin on cNCCs without reduced migration may be related to its developmental toxicity; treatment of pregnant rats with tributyltin that caused blood concentrations comparable to those in the present study, reduced the body weights of pups without causing external malformations (Adeeko et al. 2003; Cooke et al. 2008).

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