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Original Article

Glial differentiation of mouse embryonic-stem-cell-derived neuronal precursors is a sensitive marker for assessing developmental neurotoxicity of insecticides

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ABSTRACT — Many epidemiological and animal exposure studies have suggested that exposure to environmental substances is a major risk factor for developmental neurotoxicity (DNT), such as in autism, and is related to the increasing relevance of neurodevelopmental disorders. Recent efforts have led to the development of various *in vitro* approaches that use cell lines and pluripotent stem cells to assess numerous environmental substances. In this study, we developed a method for assessing DNT using a mouse embryonic stem (mES) cell model that focuses on differentiation into neuronal cells (neural cells and astrocytes). Using this model system, we found that six insecticides inhibited the differentiation of neural precursor cells into astrocytes. Our data indicated that the effects of insecticides on glial differentiation were more sensitive than those of several DNT markers reported in previous studies. This mES cell model can make a quick assessment of DNT potential and may be a useful tool for screening substances with potential to induce DNT.

Key words: Developmental neurotoxicity, Insecticides, Pluripotent stem cells, *In vitro* assay, Astrocytes

INTRODUCTION

The prevalence of neurodevelopmental disorders, such as autism, attention-deficit/hyperactivity disorder, and learning disabilities, has been gradually increasing worldwide (Herbert, 2010; Baio *et al*., 2018; Maenner *et al*., 2020), and it has been suggested that exposure to environmental substances such as insecticides, flame retardants, and heavy metals, is one of the major factors responsible for the onset of neurodevelopmental disorders (Herbert, 2010; Dickerson *et al*., 2023; Ruiz-Sobremazas *et al*., 2023). Humans have been repeatedly threatened by the health impacts of intentionally and unintentionally generated hazardous substances. In particular, during the industrial revolution, increasing environmental pollution has caused various issues related to serious adverse health effects, which have motivated considerable efforts to regulate hazardous substances in the environment to protect human health. Currently, many kinds of substances are regulated by legislation (e.g., the Toxic Substances Control Act in the USA, REACH [Registration, Evaluation, Authorization and Restric-

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tion of Chemicals] in the EU, and Kashinhou [Law Concerning the Examination and Regulation of Manufacture, etc., of Chemical Substances] in Japan). Owing to these efforts, the levels of substances with high hazardous potential are gradually decreasing in the environment. However, humans are encountering emerging health issues. Over the past several decades, numerous new substances and alternatives have been developed and used worldwide instead of highly hazardous substances. While these substances provide humans with many benefits and are indispensable in daily life, the chance of constant exposure to various substances has been increasing. However, the potential toxicity and risks to public health of these substances, such as the risk of developmental neurotoxicity (DNT), remain unclear.

The assessment of the potential for DNT still relies on rodent-based *in vivo* experimental studies. However, such *in vivo* studies have a high cost and a long duration, and involve low-throughput assays. However, the number of chemical substances requiring evaluation is increasing. In response to these situations, alternative *in vitro* approaches using various cell cultures have been developed for DNT testing over the last 10 years, to prioritize substances for further *in vivo* testing. Many *in vitro* tests using various cell types (cell lines, primary cultured cells, and pluripotent stem cells) and endpoints (cell viability, neurite growth, and differentiation) have been developed to screen for DNT potential. In addition, a test battery that includes multiple *in vitro* tests is required for DNT because various cellular and molecular processes are involved in normal neurodevelopment, and distinct process targets are assumed for each substance (Behl *et al*., 2019; Sachana *et al*., 2019). For instance, neurite outgrowth tests using a cell line and derivatives from human induced pluripotent stem (iPS) cells were able to detect the effects of rotenone, carbaryl, brominated flame retardants, and metals, but not the effects of other insecticides and flame retardants that have been reported to cause DNT *in vivo* (Ryan *et al*., 2016; Delp *et al*., 2018). However, some flame retardants inhibit oligodendrocyte differentiation (Klose *et al*., 2022).

Insecticides are well-known environmental pollutants, and various types of insecticides such as organophosphorus, organochlorine, and neonicotinoids have been used for several decades. Insecticides are based on their preferential action on the nervous system of insects, and it is believed that humans and wild animals are less sensitive than insects. However, many reports, including epidemiological and animal exposure studies, indicate that most insecticides have the potential for neurotoxicity and DNT in mammals (Burns *et al*., 2013; Andersen *et al*., 2022). Additionally, several insecticides persist in the environment and easily accumulate over a long period of time in the body because they are chemically stable and lipophilic. Although these insecticides have also been examined using *in vitro* systems, the data suggest that current *in vitro* tests are not sufficient to assess their potential because there is no test that can detect all substances with the potential for DNT, as described above. Therefore, further studies are needed to develop a test that can detect DNT potential.

Here, we developed a novel DNT assay using mouse embryonic stem (mES) cells. To this end, we used six insecticides (chlorpyrifos, 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane (DDT), rotenone, carbaryl, deltamethrin, and imidacloprid) as chemicals with DNT potential (Burns *et al*., 2013; Ishido *et al*., 2017; Lan *et al*., 2017; Lee *et al*., 2015; Pitzer *et al*., 2021; Sano *et al*., 2016; Sheets *et al*., 2016), and measured their effects on differentiation into neuronal cells. Using this system, we found that all these insecticides inhibited the differentiation of neural progenitors into astrocytes, and our data were more sensitive than those from previous reports. Therefore, this system may be useful as an alternative test for detecting DNT potential.

MATERIALS AND METHODS

Chemicals

Chlorpyrifos, carbaryl, and imidacloprid were purchased from Wako Pure Chemical Industries (Osaka, Japan). DDT was obtained from AccuStandard (New Haven, CT, USA). Rotenone and deltamethrin were purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). The insecticides used in this study are listed in Table 1. The insecticides were dissolved in dimethyl sulfoxide (DMSO) and further diluted in culture medium. The final DMSO concentration in the culture medium was 0.1% (v/v).

Culture and differentiation of mES cells

The mES cell lines, B6G-2 and B6-6, were obtained from RIKEN BioResource Research Center (Ibaraki, Japan), and maintained in Glasgow's minimum essential medium supplemented with 14% knockout serum replacement (KSR), 1% fetal bovine serum (Invitrogen, Waltham, MA, USA), 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 0.5 mM monothioglycerol (MTG), 1,000 U/mL LIF, 3 μM CHIR99021, and 1 μM PD0325901 on gelatin-coated dishes. Both mES cell lines were derived from C57BL/6 mice, whereas B6G-2 cells constitutively express green fluorescent protein. There-

Table 1. List of insecticides used in this study.

Name	Abbreviation	Cas No.	Group	Mode of Action
Chlorpyrifos	CPS	2921-88-2	Organophosphate	Inhibition of acetylcholine esterase
DDT	DDT	50-29-3	Organochlorine	Activation of Na channel
Rotenone	ROT	83-79-4	Phenylpropanoid	Oxidative stress
Carbaryl	CAR	63-25-2	Carbamate	Inhibition of acetylcholine esterase
Deltamethrin	DMT	52918-63-5	Pyrethroid ester	Activation of Na channel
Imidacloprid	IMI	138261-41-3	Neonicotinoid	Activation of nicotinic acetylcholine receptors

fore, we used B6G-2 cells for real-time PCR. In contrast, we obtained immunostaining images of B6-6 cells.

For the induction toward neural commitment, cells were cultured in low-binding round-bottom 96-well plates (Sumitomo Bakelite Co., Ltd., Tokyo, Japan) in Dulbecco's modified Eagle medium containing 15% KSR, 0.5 mM MTG and 0.1 μM LDN193189. After 6 days of culture, single cells were prepared from the embryoid body (EB) using TrypLE (Invitrogen), plated at 2×10^4 cells/cm^2 in a polyornithine/laminin (P/L)-coated dish, and cultured in RHB-A medium (TaKaRa Bio, Shiga, Japan) supplemented with bFGF (20 ng/mL) and EGF (10 ng/mL). Four days later (day 10), the cells were detached by treatment with Accutase and further cultured under the same conditions until day 14.

To induce differentiation from each progenitor cell (on days 6, 10, and 14) into neural cells (neurons and glial cells), cells were plated at 5×10^4 cells/cm^2 (on day 6) or 3×10^4 cells/cm^2 (on days 10 and 14) on a P/L-coated plate and cultured in RHB-A with two growth factors (bFGF and EGF). One day after seeding, the same amount of differentiation medium was added directly without changing the medium, and the cells were exposed to each insecticide simultaneously. The differentiation medium consisted of BrainPhys (STEMCELL Technologies, Vancouver, Canada) supplemented with 2% B-27 (Invitrogen). Four days after differentiation, the cells were analyzed using real-time PCR and immunostaining.

Real-time PCR

Total RNA was isolated using the NucleoSpin RNA kit (Macherey-Nagel GmbH, Co. KG, Düren, Germany), according to the manufacturer's instructions, and cDNA was synthesized using PrimeScript RT Master Mix (Perfect Real Time) (TaKaRa Bio). Real-time PCR was performed using GeneAce SYBR qPCR Mix α (NipponGene Co., Ltd., Tokyo, Japan) and a LightCycler 96 instrument (Roche, Basel, Switzerland). Relative gene expression was calculated using the ΔCt method, and the levels of gene expression were normalized to the expression levels of a housekeeping gene (*Actb*). The primers used in this study were designed using Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/), and their sequences were as follows: *Actb* forward: cctctatgccaacacagt, *Actb* reverse: agccaccgatccacacag, *Pax6* forward: tgcccttccatctttgcttg, *Pax6* reverse: tctgttgcttttcgctagcc, Nestin forward: aaagaggtgtccgatcatctgg, Nestin reverse: tctcttcctcatcagcaaaccc, *Map2* forward: cggaaaaccacagcagcaag, *Map2* reverse: gggaggatggaggaaggtct, *Gfap* forward: tcaacgttaagctagccctgg, *Gfap* reverse: ccttctttggtgcttttgccc, *Nfia* forward: ccgaccaaggcagtttaggt, *Nfia* reverse: tggggctgtctgtctctaca, *Nr2f1* forward: ccaatactgccgcctcaaga, *Nr2f1* reverse: cattcttcctcgctgaaccg.

Cytotoxicity assay

Each precursor cell line was differentiated and exposed to each insecticide in 96-well plates as described above. Four days after differentiation and exposure, cytotoxicity was measured using Cell Counting Kit-8 (DOJINDO, Kumamoto, Japan).

Immunostaining

The cells were fixed with 4% paraformaldehyde and stained with anti-Pax6 (#PRB-278P; BioLegend, San Diego, CA, USA), anti-Map2 (M9942; Sigma-Aldrich, St. Louis, MO, USA), or anti-Gfap (MAB360, Chemicon International, Temecula, CA, USA) antibodies, followed by visualization with an Alexa-Fluo-594-labeled secondary antibody (Invitrogen) and 4′,6-diamidino-2-phenylindole (DAPI). Fluorescence images were acquired using a confocal microscope (FV-1200; Olympus, Tokyo, Japan).

Quantification of potential for specific neurotoxicity

The data obtained from each endpoint and cytotoxicity were fitted to a dose-response curve and 10% effective concentration (EC10) was calculated as previously described (Krebs *et al*. 2019), using an online tool (http://invitrotox.uni-konstanz.de/BMC/).

Genome-wide analysis of gene expression

For a comprehensive analysis of changes in gene expression, secondary neural precursor cells (NPCs; day 14) were differentiated and exposed to each insecticide in 12-well plates. Doses of each chemical were determined based on the dose-response curves of Gfap expression changes and cytotoxicity (chlorpyrifos: 10 μM, DDT: 1 μM, rotenone: 3 nM, carbaryl: 3 μM, deltamethrin: 10 μM, imidacloprid: 30 μM). One day after exposure, the cells were pooled from three wells of the 12-well plates, and total RNA was isolated using NucleoSpin RNA. RNA sequencing (RNA-seq) was performed (GeneWiz, Saitama, Japan), and transcriptome data were deposited in the Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo; series of data, GSE263683). The biological interpretation of the transcriptome data was performed using the knowledge-based functional analysis software, Ingenuity Pathways Analysis (IPA; Ingenuity Systems, Rwdwood City, CA, USA), with the cutoff set at a fold change < 1.2.

Statistical analysis

Differences were assessed using Student's t-test (StatView ver. 4.57; SAS Institute, Cary, NC, USA).

RESULTS AND DISCUSSION

Efficient induction of neural progenitor cells from mES cells

Fig. 1A shows the experimental procedure for inducing mES cells to differentiate into the neuronal lineage. RHB-A is a medium optimized for neural stem cell culture and differentiation into the neural lineage, and has been reported to effectively induce the direct differentiation of mES cells into neural cells under two-dimensional culture conditions (Ying *et al*., 2003; Abranches *et al*., 2009). Although we attempted the same experiment, we could not effectively obtain such neural cells, because most of the cells detached and died. Therefore, mES cells were first differentiated through EB formation (day 0–6), followed by culturing in RHB-A medium containing bFGF and EGF on a P/L-coated dish (day 6–14). Under these conditions, we succeeded in inducing and maintaining mES-cell-derived NPCs expressing the neural precursor markers Pax6 and Nestin (Fig. 1B and 1C). In particular, the expression levels of both genes were gradually upregulated on days 10 and 14, suggesting that culture on a P/L-coated dish induces maturation of NPCs derived from mES cells.

Previous studies have shown that repeated passages of mES-cell-derived neurospheres induce the transition from neurogenesis to gliogenesis (Okada *et al*., 2008), with changes in *Nfia* and *Nr2f1* gene expression levels (Naka *et al*., 2008). We found similar results, showing a gradual continuous increase in *Nfia* gene expression levels and transient induction of *Nr2f1* gene expression in our model using mES cells (Fig. 1D). Furthermore, $Map2^+$ neural cells were induced from each NPC, whereas $Gfap^+$ glial cells (mainly astrocytes) were induced starting from day 14 (Fig. 1E and 1F). Together, our culture system provides a model for mimicking neurodevelopmental stages, with day 10 and 14 NPCs mimicking primary and secondary NPCs, respectively (Okada *et al*., 2008).

Effects of insecticides on neural differentiation

Using this mES model, we examined whether various insecticides affected the differentiation of mES-cell-derived NPCs into neural and glial cells. In this model, *Map2* was expressed at a high level, even though primary and secondary NPCs were maintained in a medium containing bFGF and EGF. In addition, the culture of both NPC types in differentiation medium did not further increase the expression level of *Map2* (Fig. 1E). Therefore, it appears that these culture conditions spontaneously induced *Map2* expression. In contrast, day 6 EB had a relatively low expression level of *Map2*, and the addition of differentiation medium increased its expression levels (Fig. 1E). Therefore, we used day 6 EB to assess the effects on differentiation into $Map2^+$ neural cells.

To examine whether various insecticides affect differentiation into $Map2^+$ neural cells, cells were exposed to each insecticide when the differentiation of day 6 EB began, and both neural outgrowth and viability were then measured after 4 days (Fig. 2A). Of the six insecticides tested, rotenone showed the strongest cytotoxicity, starting at 10 nM, at which the viability decreased to approximately 50% (Fig. 2C). Our results also indicated that insecticides other than imidacloprid cause cytotoxicity. In terms of neural differentiation, only rotenone clearly decreased the expression level of *Map2*; however, the trend in its expression level was almost identical to the cell viability trend, suggesting that rotenone alters neural differentiation through its cytotoxicity. The expression level of *Map2* was not changed by exposure to other insecticides, such as chlorpyrifos, DDT, and carbaryl,

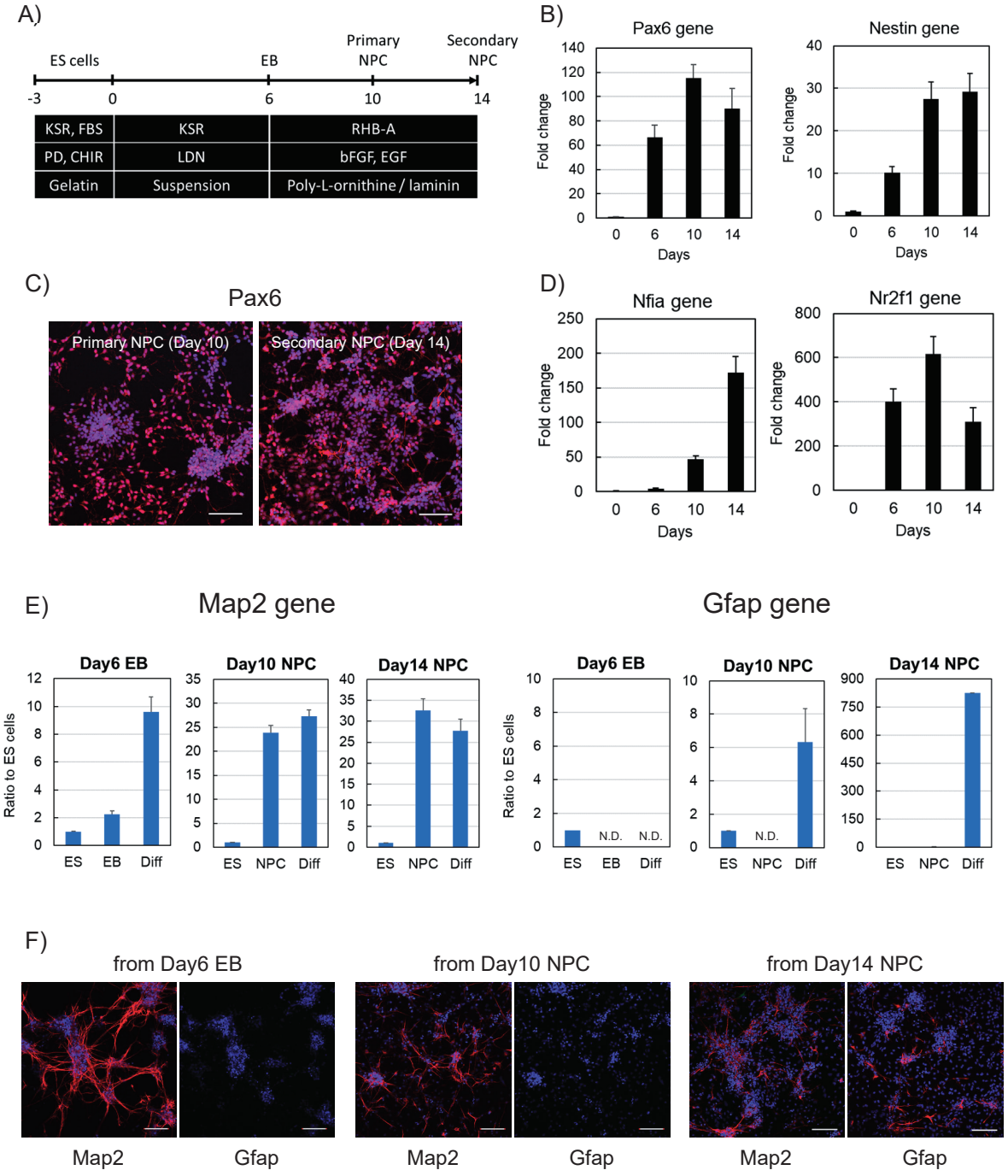

Fig. 1. Induction of neural precursors from mouse embryonic stem cells. (A) Cell culture scheme used in this study (B) Expression levels of *Pax6* and Nestin genes were measured at each time point. Data indicate the percentage of the levels on Day 0 (embryonic stem [ES] cells) ± standard deviation (SD). (C) Images of Pax6 immunostaining were taken on Day 10 and 14 (blue: 4′,6-diamidino-2-phenylindole [DAPI], red: Pax6). Bar indicates 10 µm. (D) Expression levels of *Nfia* and *Nr2f1* genes were measured at each time point. Data indicate the percentage of the levels ono Day 0 (ES cells) ± SD. (E) Expression levels of *Map2* and *Gfap* genes were measured in ES cells (ES), each neural precursor on Day 6, 10, and 14 (EB or NPC), and cells differentiated from each precursor (Diff). Data indicate the ratio to the level in ES cells ± SD. N.D.: not detected. (F) Single cells from an embryoid body (EB), Day 10 and 14 neural precursor cell (NPCs) were differentiated for 4 days, and then Map2 and Gfap were immunostained (blue: DAPI, red: Map2 or Gfap).

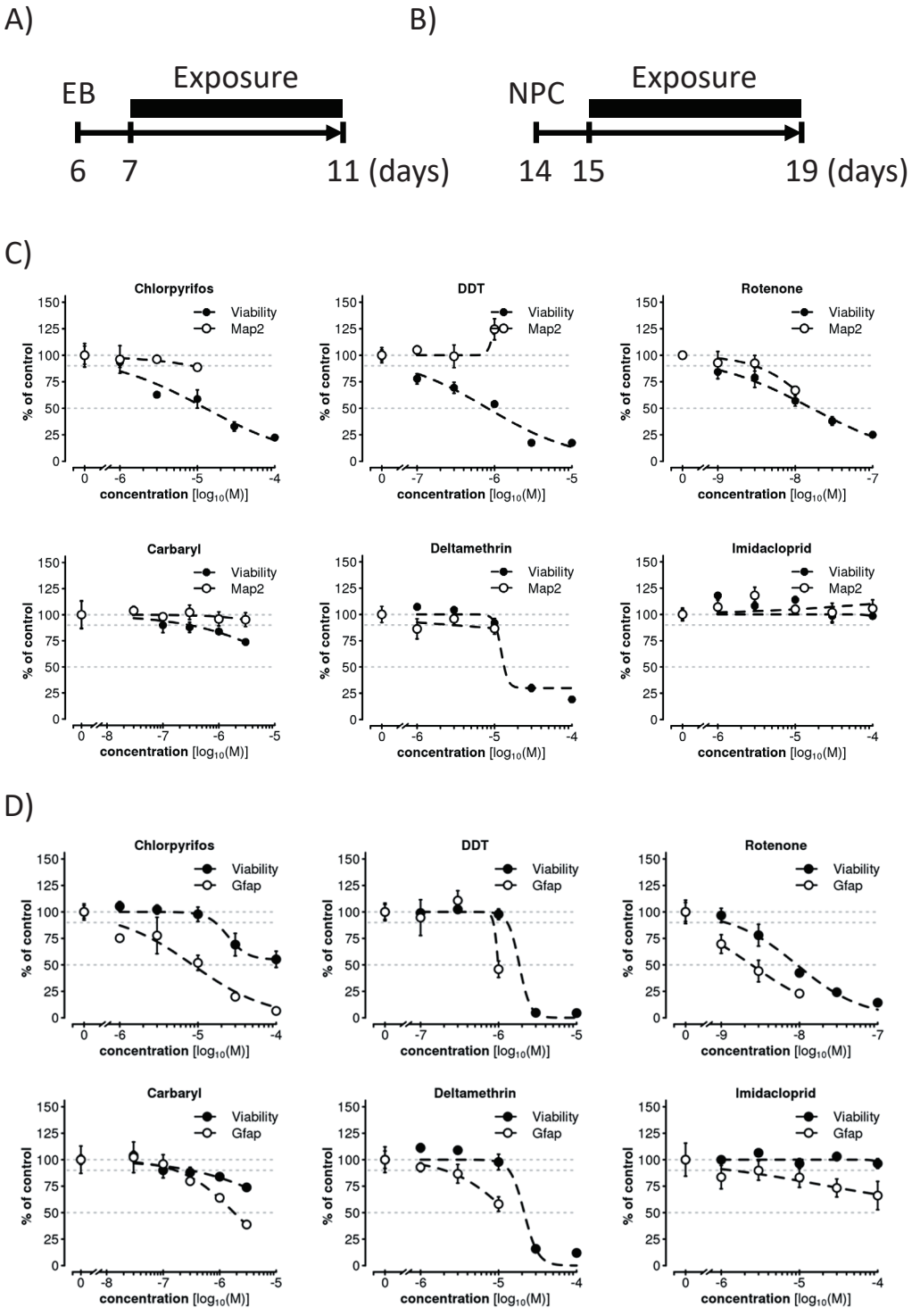

Fig. 2. Effects of insecticides on the differentiation of neural precursors. Single cells from Day 6 EB (A) and Day 14 secondary NPC (B) were differentiated into neural cells, followed by exposure to each insecticide for 4 days. Expression levels of *Map2* (C) and *Gfap* (D) genes were measured in RNA samples from day 6 EBs and day 14 secondary NPCs, respectively (open circle). Cell viability was measured after exposure (closed circles). Data indicate the percentage of control ± SD.

Table 2. Selective scores of various developmental neurotoxicity tests.

Substance	mES cell model (Mouse)		iPS cell (Human)	Primary cell (Human)	Cell line (Human)	Primary cell (Rat)
	Map2	Gfap	Neurite outgrowth			Fire rate
CPS	**0.94**	**1.35**	0.17	-	-	**0.58**
	(16.6 / 1.91)	(16.6 / 0.74)	(63.8 / 43.0)	(- / -)	(- / -)	(14.8 / 3.91)
DDT	0.13	0.17	0.06	-	-	-0.20
	(1.29 / 0.96)	(1.29 / 0.87)	(15.6 / 13.7)	(- / -)	(- / -)	(3.89 / 6.13)
ROT	-0.42	**0.74**	**1.37**	**> 4.67**	**> 2.50**	-0.42
	(11.5e-3 / 3.02e-3)	(11.5e-3 / 2.09e-4)	(38.8 / 1.65)	(- / 4.24e-4)	(- / 0.06)	(7.67e-3 / 0.02)
CAR	-0.040	0.27	**> 0.56**	**> 0.13**	-	0.45
	(0.30 / 0.33)	(0.30 / 0.16)	(- / 27.8)	(- / 14.8)	(- / -)	(8.26 / 2.93)
DMT	**0.69**	**0.82**	0.14	-	-	**> 1.42**
	(13.5 / 2.75)	(13.5 / 2.04)	(36.4 / 26.3)	(- / -)	(- / -)	(- / 1.15)
IMI	-0.47	**1.92**	NR	NR	NR	NR
	(112.2 / 331.1)	(112.2 / 1.35)				

Bold values indicate Selective scores more than 0.5.
Values in parenthesis indicate ($EC10_{viability}/EC10_{endopoint\ markers}$) in this study or ($BMC_{viability}/BMC_{endopoint\ markers}$) in the database.
Hyphen indicates the case in which benchmark concentration (BMC) is not determined.
NR means "not reported" in the database.

even though cell viability decreased in a dose-dependent manner, suggesting that these insecticides do not have effects on neural differentiation in this culture system.

Effects of insecticides on glial differentiation

Next, we examined the effects of the insecticides on glial differentiation. In this experiment, the cells were exposed to each insecticide during the differentiation of secondary NPCs into $Gfap^+$ glial cells (Fig. 2B). Rotenone had the highest cytotoxic effect on cell viability (Fig. 2D) and its pattern was almost identical to its effect on EBs (Fig. 2C). The cytotoxic effect of DDT on secondary NPCs differed slightly from that of DDT on EBs. DDT markedly decreased the viability of secondary NPCs at 3 μM (Fig. 2D) whereas it shows gradual decrease of viability of EB (Fig. 2C). In contrast, chlorpyrifos showed the reverse pattern, in which cytotoxicity during neural differentiation of EB was more sensitive. The other insecticides (carbaryl, deltamethrin, and imidacloprid) showed patterns similar to those observed during neural differentiation of EB (Fig. 2D).

Although our data indicate that these insecticides have similar cytotoxicity to both early (day 6 EB) and mature (day 14 NPC) neural precursors, we found that they have different effects on the differentiation potential of neural precursors depending on the exposure timing. In contrast to the effects on neural differentiation, the induction of *Gfap* was inhibited by all insecticides used in this study in a dose-dependent manner (Fig. 2D). These results suggested that glial differentiation was more sensitive to insecticides than neural differentiation in our model. Based on the obtained data, we estimated each EC10 value from the concentration-dependent curves of viability and differentiation markers and then calculated the selective score (SS), defined as $\log10(EC10_{viability}/EC10_{differenriation\ markers})$, as described previously (Ryan *et al*., 2016) (Table 2). If the value of SS is greater than 0.5 (3.2-fold difference between EC10 values of viability and differentiation markers), a compound is considered to have specific neurotoxicity potential independently of cytotoxicity. As shown in Table 2, the SS values of chlorpyrifos, rotenone, deltamethrin, and imidacloprid were greater than 0.5 in the case of *Gfap* gene expression (1.35, 0.74, 0.82, and 1.92, respectively), whereas only the SS values of chlorpyrifos and deltamethrin were greater than 0.5 in the case of *Map2* gene expression, indicating that *Gfap* gene induction is a sensitive marker of DNT in our experimental system. Furthermore, we compared our data with previous data published in the Developmental NeuroToxicity Data Integration and Visualization Enabling Resource (DNT-DIVER) of the National Toxicology Program database (https://sandbox.ntp.niehs.nih.gov/neurotox/). We selected data for neural outgrowth and fire rates from this database (Table 2). These two endpoints of DNT showed substance-dependent patterns. Rotenone and carbaryl clearly inhibited the neural outgrowth of human iPS-cell-derived cell lines and primary neural cells, but did not affect the fire rate. Instead, chlorpyrifos and deltamethrin affected the fire rate of neural cells but not neural outgrowth. These results indicated that each chemical affects different endpoints, which is the reason for the necessity of an *in vitro*

Table 3. Numbers of differentially expressed genes after exposure to each insecticide.

Substance	Dose	DEG	Up	Down
CPS	10 μM	883	526	357
DDT	1 μM	1163	700	463
ROT	3 nM	1142	755	387
CAR	3 μM	865	490	375
DMT	10 μM	891	517	374
IMI	100 μM	899	501	398

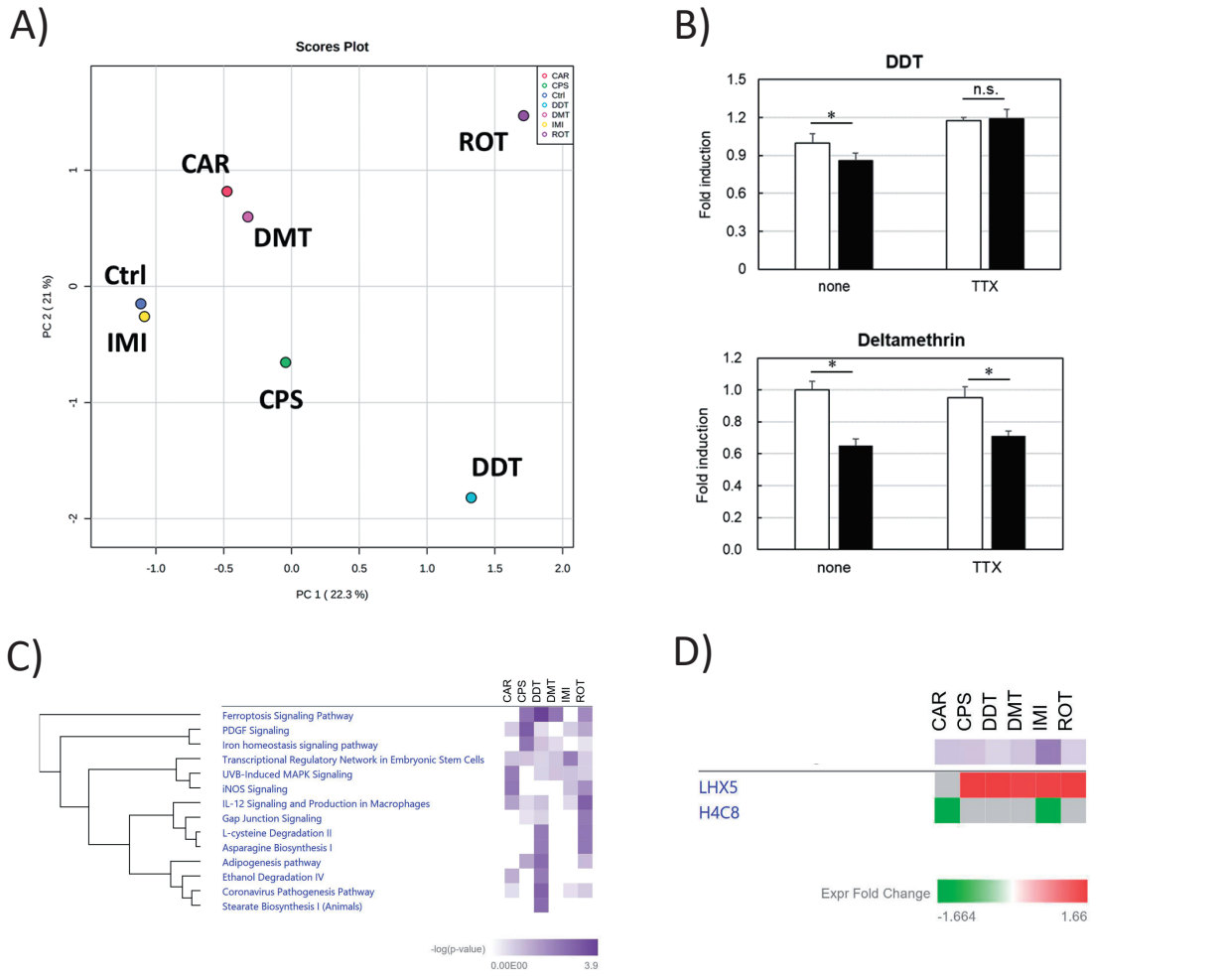

Fig. 3. Analysis of change in global gene expression pattern after exposure to insecticides. (A) Day 14 secondary NPCs were exposed to each insecticide for 1 day when cells had differentiated into neural cells. RNA sequencing was performed, and principal component analysis (PCA) results are shown. (B) Day 14 secondary NPCs were incubated with or without tetrodotoxin (TTX; 1 μM) (open and closed columns, respectively), and then exposed to DDT (1 μM) or deltamethrin (10 μM). After exposure, the expression level of Gfap was measured. Data indicate the percentage of control ± SD. Statistical significance: $*p < 0.05$, n.s.: not significance. (C) Analysis of canonical pathways associated with exposure to each insecticide was performed using Ingenuity Pathway Analysis (IPA). (D) Heatmaps indicate changes in the gene expression levels of *Lhx5* and *H4C8* included in the pathway of transcriptional regulatory network in embryonic stem cells after exposure to each insecticide.

Table 4. The biological functions of the top diseases and functions associated with exposure to each insecticide.

Substance	Score	Top Diseases and Functions
CPS	54	Auditory Disease, Carbohydrate Metabolism, Connective Tissue Disorders
	42	Lipid Metabolism, Nucleic Acid Metabolism, Small Molecule Biochemistry
	39	Cancer, Cardiac Dilation, Cardiac Enlargement
	37	Cancer, Organismal Injury and Abnormalities, DNA Replication, Recombination, and Repair
	35	Cellular Movement, Nervous System Development and Function, Embryonic Development
	33	Embryonic Development, Nervous System Development and Function, Organ Development
	31	Cell Death and Survival, Hematological System Development and Function, Hematopoiesis
	31	Developmental Disorder, Hereditary Disorder, Nephrosis
DDT	49	Amino Acid Metabolism, Small Molecule Biochemistry, Neurological Disease
	49	Cell Cycle, DNA Replication, Recombination, and Repair, Cellular Development
	41	Connective Tissue Disorders, Dermatological Diseases and Conditions, Developmental Disorder
	41	Developmental Disorder, Hereditary Disorder, Neurological Disease
	41	Lipid Metabolism, Small Molecule Biochemistry, Vitamin and Mineral Metabolism
	39	Cell-To-Cell Signaling and Interaction, Dermatological Diseases and Conditions, Developmental Disorder
	39	Gastrointestinal Disease, Inflammatory Disease, Inflammatory Response
	39	Post-Translational Modification, Cellular Function and Maintenance, Cell Death and Survival
	37	Cell Signaling, Nucleic Acid Metabolism, Molecular Transport
	35	Developmental Disorder, Hereditary Disorder, Metabolic Disease
	31	Developmental Disorder, Digestive System Development and Function, Hereditary Disorder
ROT	55	Carbohydrate Metabolism, Small Molecule Biochemistry, Connective Tissue Disorders
	50	Small Molecule Biochemistry, Cell-To-Cell Signaling and Interaction, Cellular Assembly and Organization
	45	Cell Morphology, Cellular Function and Maintenance, Molecular Transport
	42	Cardiovascular Disease, Inflammatory Disease, Organismal Injury and Abnormalities
	40	Cellular Compromise, Inflammatory Response, Developmental Disorder
	38	Developmental Disorder, Hereditary Disorder, Neurological Disease
	34	Amino Acid Metabolism, Small Molecule Biochemistry, Vitamin and Mineral Metabolism
	32	Carbohydrate Metabolism, Small Molecule Biochemistry, Organ Morphology
	32	Cell Morphology, Nervous System Development and Function, Neurological Disease
	32	Cell Signaling, Vitamin and Mineral Metabolism, Cell Cycle
	30	Cardiovascular System Development and Function, Organismal Development, Tissue Morphology
	30	DNA Replication, Recombination, and Repair, Cardiovascular Disease, Connective Tissue Disorders
CAR	50	Connective Tissue Disorders, Developmental Disorder, Gastrointestinal Disease
	40	Cardiovascular Disease, Hematological Disease, Hereditary Disorder
	38	Cancer, Developmental Disorder, Endocrine System Disorders
	36	Auditory Disease, Cellular Compromise, Cellular Development
	34	Protein Synthesis, Cancer, Cardiovascular Disease
	32	Cellular Compromise, Organismal Injury and Abnormalities, Renal Dysfunction
	30	Organ Development, Renal and Urological System Development and Function, Endocrine System Disorders
DMT	42	Cardiovascular Disease, Cardiovascular System Development and Function, Developmental Disorder
	42	Lipid Metabolism, Small Molecule Biochemistry, Vitamin and Mineral Metabolism
	42	Protein Synthesis, RNA Damage and Repair, Metabolic Disease
	33	Cellular Movement, Hepatic System Development and Function, Cancer
	33	Connective Tissue Development and Function, Connective Tissue Disorders, Organ Morphology
	33	Hematological Disease, Metabolic Disease, Organismal Injury and Abnormalities
	33	Neurological Disease, Organismal Injury and Abnormalities, Cell Death and Survival
	31	Carbohydrate Metabolism, Cell Death and Survival, Cell Morphology
	31	Organ Morphology, Developmental Disorder, Hereditary Disorder
IMI	49	Embryonic Development, Nervous System Development and Function, Organ Development
	42	Developmental Disorder, Hereditary Disorder, Neurological Disease
	42	RNA Damage and Repair, Gene Expression, Developmental Disorder
	38	Developmental Disorder, Hereditary Disorder, Metabolic Disease
	38	Lipid Metabolism, Nucleic Acid Metabolism, Small Molecule Biochemistry
	35	Lipid Metabolism, Small Molecule Biochemistry, Connective Tissue Disorders
	33	Cell Cycle, Cell Morphology, Cellular Assembly and Organization

assay battery to assess DNT, as described above (Behl *et al*., 2019). In contrast, our assay system using mES cells could detect the effects of these four insecticides, as well as imidacloprid, using a simple assay. Therefore, it is possible that this system could be useful for detecting various chemicals with DNT potential more broadly than existing tests.

Genome-wide analysis of gene expression changes after exposure to insecticides

Finally, we performed RNA-seq to investigate the mechanisms underlying the effects of insecticides on glial cell differentiation. In this series of experiments, we analyzed global gene expression changes during differentiation into glial cells. Each insecticide was also exposed at the maximum doses at which cytotoxicity was not observed (chlorpyrifos: 10 μM, DDT: 1 μM, carbaryl: 3 μM, rotenone: 3 nM, deltamethrin: 10 μM, imidacloprid: 100 μM), and differentially expressed genes (DEGs) were identified 1 day after exposure, which is an earlier time point than that used for the study of glial differentiation and cytotoxicity assays. Each insecticide induced a comparable number of DEGs (Table 3). As shown in Fig. 3A, we found similar gene expression patterns between carbaryl and deltamethrin in principal component analysis (PCA), even though these two substances have different modes of action ("Inhibition of acetylcholine esterase" and "Activation of Na channel," respectively), as described in Table 1. In contrast, DDT and deltamethrin induced different patterns of gene expression changes, even though these two substances have the same mode of action ("Activation of Na channel"). Our additional experiment using the sodium channel inhibitor tetrodotoxin (TTX) suggested that activation of the sodium channel was involved in the reduction of glial differentiation by DDT, but not by deltamethrin (Fig. 3B). These results suggest that insecticides induce different changes at the molecular level, even though they show similar effects on the differentiation of neural precursors, and do not necessarily affect glial differentiation through classical pathways. Our findings support those of a previous *in vivo* study (Lee *at al*., 2015), in which chlorpyrifos and carbaryl caused developmental neurotoxicity in mice, but these developmental neurotoxic effects were not related to the inhibition of acetylcholine esterase.

We further examined the effects of insecticides based on the changes in gene expression using IPA. Table 4 shows the "top diseases and functions" associated with DEGs following exposure to insecticides. The IPA data suggested that all the insecticides induced biological effects associated with developmental disorders and neurological diseases. Exposure to insecticides also induced gene expression changes related to other biological processes and diseases reported previously, such as auditory diseases (Dundar *et al*., 2016; Huang *et al*., 2023), lipid metabolism (Ishikawa *et al*., 2015; Olsvik *et al*., 2019; Yan *et al*., 2020; Jia *et al*., 2023), cell cycle (Yu *et al*., 2019), connective tissue disorders (Lee *et al*., 2007), carbohydrate metabolism (Karlsson *et al*., 2016; Li *et al*., 2019), and inflammation (Gao *et al*., 2013). In addition, the analysis of canonical pathways using IPA showed that insecticides commonly influenced the "transcriptional regulatory network in embryonic stem cells" pathway (Fig. 3C). Among the genes associated with this pathway, the expression of Lhx5, a transcription factor of the LIM homeobox family related to neural development, was upregulated by exposure to all insecticides tested, except carbaryl (Fig. 3D). Lhx5 is involved in the control of differentiation and development of the central nervous system (Paylar *et al*., 2001), and our data suggest that this gene is a candidate for playing an important role in the effects of glial cell differentiation. This is supported by a previous study in which another LIM homeobox family protein, Lhx2, suppressed astrogliogenesis (Subramanian *et al*., 2011). Furthermore, data from "upstream regulators" suggest that insecticides commonly activate MAP kinase (MAPK) (Table S1). The MAPK pathway is key pathway mediating the impact of environmental pollutants on the central nervous system, and the activation of MAPK pathways by exposure to various pollutants leads to various neuronal disorders through inflammation, oxidative stress, and apoptotic cell death (Ijomone *et al*., 2021). Therefore, our data suggest the involvement of MAPK in mediating the effects of insecticides on glial cell differentiation. It is well known that the perturbation of glial cell differentiation and function leads to neuronal disorders (Gzielo and Nikiforuk, 2021). However, it remains unclear whether insecticide exposure affects glial cell differentiation *in vivo*. Future *in vivo* studies will follow the effects of insecticides tested in this *in vitro* study and strengthen the validity of our model for assessing the DNT potential of various environmental pollutants.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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