

1 Growth and neurite stimulating effects of the neonicotinoid pesticide
2 clothianidin on human neuroblastoma SH-SY5Y cells

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29 *Abbreviations:* nAChR, nicotinic acetylcholine receptor; CTD, clothianidin; ACE,
30 acetamiprid; IMI, imidacloprid; NOAEL, no observed adverse effect level; DH β E,
31 dihydro- β -erythroidine; MEC, Mecamylamine; MLA, methyllycaconitin

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25 **ABSTRACT**

26 Neonicotinoids are one of most widely used pesticides targeting nicotinic acetylcholine
27 receptors (nAChRs) of insects. Recent epidemiological evidence revealed increasing amounts
28 of neonicotinoids detected in human samples, raising the critical question of whether
29 neonicotinoids affect human health. We investigated the effects of a neonicotinoid pesticide
30 clothianidin (CTD) on human neuroblastoma SH-SY5Y cells as *in vitro* models of human
31 neuronal cells. Cellular and functional effects of micromolar doses of CTD were evaluated by
32 changes in cell growth, intracellular signaling activities and gene expression profiles. We
33 examined further the effects of CTD on neuronal differentiation by measuring neurite
34 outgrowth. Exposure to CTD (1–100 μ M) significantly increased the number of cells within
35 24 hours of culture. The nAChRs antagonists, mecamylamine and SR16584, inhibited this
36 effect, suggesting human α 3 β 4 nAChRs could be targets of neonicotinoids. We observed a
37 transient intracellular calcium influx and increased phosphorylation of extracellular
38 signal-regulated kinase 1/2 shortly after exposure to CTD. Transcriptome analysis revealed
39 that CTD down-regulated genes involved in neuronal function (e.g., formation of filopodia
40 and calcium ion influx) and morphology (e.g., axon guidance signaling and cytoskeleton
41 signaling); these changes were reflected by a finding of increased neurite length during
42 neuronal differentiation. These findings provide novel insight into the potential risks of
43 neonicotinoids to the human nervous system.

44
45 *Keywords:* Neonicotinoid; Pesticide; Clothianidin; Neuroblastoma cell; Nicotinic
46 acetylcholine receptor; Intracellular signaling.

1 47 **1. Introduction**

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3 48 Neonicotinoids are the most recent and widely used class of pesticides to control harmful
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6 49 insects in the world. With expanding use of the pesticides in farming, multiple neonicotinoids
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9 50 are present in most vegetables, fruits and crops at parts-per-billion concentrations (Chen et al.,
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11 51 2014; Ikenaka et al., 2018). In fact, neonicotinoids were detected in urine samples from most
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14 52 adults and children (Ueyama et al., 2015; Ikenaka et al., 2019) and the urinary concentration
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16 53 of total neonicotinoids in Japanese children has been reported to be in the hundreds-level
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19 54 nanomolar (Osaka et al., 2016). Neonicotinoids act as nicotinic acetylcholine receptor
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21 55 (nAChR) agonists with as much as hundreds fold greater affinity for insect nAChRs than for
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24 56 mammalian receptors (Tomizawa and Casida, 2005); however, adverse effects on
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26 57 physiological function of non-target vertebrates have been reported over the last decade
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29 58 (Hoshi et al., 2014; Gibbons et al., 2015; Wang et al., 2018). *In vitro* studies have shown that
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32 59 potency and specificity for mammalian nAChRs are critically different among types of
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34 60 neonicotinoids (Casida, 2018). In particular, Kimura-Kuroda et al. (2012) firstly showed that
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37 61 acetamiprid (ACE) and imidacloprid (IMI), earlier chloropyridylmethyl neonicotinoids,
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39 62 caused neural excitation in cerebellar cells from neonatal rats mediated by nAChRs. These
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42 63 ligand-gated ion channel receptors play a variety of roles in multiple areas of the mammalian
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44 64 brain, including not only cholinergic transmission but also neural excitability and synaptic
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47 65 plasticity (Gotti, et al., 2006; Dani and Bertrand, 2007). Thus, there is increasing concern
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49 66 about the risks of neonicotinoids on the central nervous system in mammals.

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52 67 Recent rodent studies indicated that a variety of neonicotinoids have neurobehavioral
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54 68 effects on mammals depending on the timing of exposure. We previously reported that at or
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57 69 below the no observed adverse effect level (NOAEL) of a later-developed
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60 70 chlorothiazolylmethyl neonicotinoid, clothianidin (CTD) resulted in anxiety-like behavior

1 71 and human-audible vocalization in a novel environment in mature mice (Hirano et al., 2015,
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3 72 2018). Gestational and postnatal exposure of an early-type of neonicotinoid, ACE induced
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5 73 behavioral changes including increasing of sexual and attacking behavior in mature offspring
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8 74 (Sano et al., 2016), whereas IMI decreased social aggression behavior (Burke et al., 2018). In
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11 75 addition, pre- and postnatal exposure to the latest neonicotinoid, dinotefuran increased the
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13 76 number of dopaminergic and serotonergic neurons in the midbrain of mature mice (Takada et
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16 77 al., 2018; Yoneda et al., 2018). Although no common effects associated with the
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18 78 developmental neurotoxicity were observed among neonicotinoids (Sheets et al., 2016),
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21 79 differentiative neurons in the mammalian developing brain could be a potential target of
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24 80 neonicotinoids.

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26 81 It has been noted that the amount and detection rate of neonicotinoids in human-derived
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29 82 samples are increasing gradually every year (Ueyama et al., 2015). Although human
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32 83 population studies reported that environmental exposure to neonicotinoids associated with
33
34 84 adverse neurological outcomes such as memory loss and finger tremor, experimental studies
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36 85 of risks of neonicotinoids in humans are still far from sufficient (Cimino et al., 2017). Most
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39 86 toxicological data for evaluating the effects of neonicotinoids were obtained from *in vitro* and
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42 87 *in vivo* studies using rats and mice as animal models; however, there are evolutionary
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44 88 differences in the amino acid sequence of nAChRs subunits between rodents and human
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47 89 (Tsunoyama and Gojobori, 1998; Stokes et al., 2015). Therefore, the intensity and dose
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50 90 responses of nAChRs to nicotinic agonists can be different across species (Papke and Porter
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52 91 Papke, 2002; Anderson et al., 2008), suggesting that there is some uncertainty to predict the
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55 92 risk of modulating the function of human-type nAChRs from other species.

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57 93 In the present study, we focused on CTD which has been banned in the European Union
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60 94 since 2013; however, it became a first-line pesticide for farming in many countries.

1 95 Intraperitoneally-administered CTD was immediately absorbed and reached throughout the
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3 96 body in mice including the brain at least a few hours (Ford and Casida, 2006). The aim of the
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5 97 present study is to provide novel experimental data for assessing whether CTD could affect
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8 98 human nervous system structure or function, using a human neuroblastoma cell line
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11 99 SH-SY5Y cells known to have high expression levels of neuronal types of nAChRs that
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13 100 contain $\alpha 3$, $\alpha 7$, $\beta 2$, and $\beta 4$ subunits (Lukas et al., 1993; Groot Kormelink and Luyten, 1997;
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16 101 Kovalevich and Langford, 2013) instead of animal models. We investigated the potential
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18 102 effects of CTD on cell growth and development, and examined mechanisms of action
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21 103 focusing on intracellular signaling and gene expression profiles.
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26 105 **2. Materials and methods**

28 106 *2.1. Cell culture and chemical treatments*

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31 107 Human neuroblastoma SH-SY5Y cells (ECACC, No. 94030304) were maintained in
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34 108 Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS), 100
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36 109 U/ml penicillin and 100 $\mu\text{g/ml}$ streptomycin at 37°C in humidified air with 5% CO₂.
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39 110 Clothianidin (CTD; purity > 99.8%; Sigma-Aldrich, St. Louis, MO, USA),
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41 111 dihydro- β -erythroidine hydrobromide (DH β E; Tocris Bioscience, Ellisville, MO, USA) and
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44 112 SR 16584 (Cayman Chemical Co., Ann Arbor, MI, USA) were dissolved in dimethyl
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46 113 sulfoxide (DMSO). Mecamylamine hydrochloride (MEC; Sigma-Aldrich) and
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49 114 methyllycaconitine citrate (MLA; Cayman Chemical Co.) were dissolved in sterilized water.
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52 115 In all experiments, cells treated with corresponding vehicle (0.1% v/v) were used as a control.
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57 117 *2.2. Cell growth assay*

59 118 For cell counting assays, cells were subcultured in 12-well plates at a seeding density $5.0 \times$

1 119 10^4 cells/mL with 1 mL of the culture medium for 24 h, and then exposed to 1 or 100 μ M
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3 120 CTD with reference to previous *in vitro* studies (Kimura-Kuroda et al., 2012; Christen et al.,
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5 121 2017). After washing once with phosphate-buffered saline (PBS) and subsequent
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8 122 trypsinization, the number of cells was counted using a hemacytometer on Day 1 and 2. For
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10 123 antagonist assays, cells were exposed to nAChR antagonists concomitantly with 100 μ M
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12 124 CTD and counted on Day 1. For microplate assays, cells were plated into 96-well plates at a
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14 125 seeding density 5.0×10^4 cells/mL with 100 μ L of the culture medium for 24 h, and then
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16 126 exposed to various concentrations of CTD (1 nM to 100 μ M) for 24 h. Cells were then
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18 127 incubated with
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21 128 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium
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24 129 (WST-8; Dojindo Laboratories, Kumamoto, Japan) solution for 3 h and the absorbance at 450
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26 130 nm was measured by a BioRad 680 microplate reader (BioRad, Hercules, CA, USA).
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32 33 34 132 2.3. *Fluo-4 calcium flux assay*

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36 133 Changes in intracellular calcium concentration were measured with a fluo-4 kit (Dojindo
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39 134 Laboratories) according to the manufacturer's instructions. Briefly, semi-confluent cells in a
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41 135 96-well microplate were loaded with 10 μ M Fluo-4 AM in 100 μ L of recording buffer [20
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43 136 mM HEPES buffer containing 115 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$,
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45 137 13.8 mM glucose] and 0.02% Pluronic F-127 for 60 min at 37°C. Cells were washed with
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47 138 PBS, and then 90 μ L of the recording buffer was added. After measuring baseline
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49 139 fluorescence for 1 min, test compounds dissolved in the recording buffer (10 μ L) were added
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52 140 and changes in fluorescence (excitation 488 nm; emission 530 nm) were kinetically measured
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55 141 by a microplate reader (SpectraMax i3, Molecular Devices, Sunnyvale, CA, USA) for 2 min.
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2.4. Western blotting

After 1 h of CTD treatment, semi-confluent cells were dissolved in ice-cold lysis buffer [50 mM Tris-HCl buffer (pH 8.0) containing 150 mM NaCl and 1% NP-40, supplemented with 1:1000 protease inhibitor cocktail and phosphatase inhibitor cocktail (Sigma-Aldrich)] and homogenized by an ultrasonic disruptor. Total protein concentrations of supernatant were determined using a Pierce BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA), and then equal amounts of proteins from each sample were boiled in SDS sample buffer [in final concentrations of 60 mM Tris /HCl, 10% glycerol, 2% SDS, 5% mercaptoethanol, 0.025% bromophenol blue, pH 6.8] at 94°C for 5 min. Samples containing 10 µg of protein extract were separated in SDS-polyacrylamide gels and transferred to PVDF membranes. Membranes were blocked by PVDF Blocking Reagent for Can Get Signal (Toyobo Co., Ltd., Osaka, Japan) for 2 h at room temperature, and then incubated overnight at 4°C in Can Get Signal Immunoreaction Enhancer Solution 1 (Toyobo Co., Ltd.) with the rabbit anti-pERK antibody and mouse anti-ERK antibody (1:2000, #9101 and #9107, Cell Signaling Technology, Danvers, MA, USA). After washing 3 times with Tris-buffered saline containing 0.1% Tween 20, membranes were then incubated in Can Get Signal Immunoreaction Enhancer Solution 2 (Toyobo Co., Ltd.) with IRDye 680RD donkey anti-mouse IgG and IRDye 800CW goat anti-mouse IgG (1:2000; LI-COR Biosciences, Lincoln, NE, USA) for 2 h at room temperature. The Odyssey infrared imaging system (LI-COR Biosciences) was used to scan the infrared signal on membranes, and Image Studio 5.1 software (LI-COR Biosciences) was used to quantify the band intensity.

2.5. RNA isolation and Clariom S assay

After 24 h of 1 µM CTD treatment, total RNA was extracted from semi-confluent cells

1 167 using a NucleoSpin plus RNA isolation kit (Macherey-Nagel GmbH & Co., Düren, Germany)
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3 168 following the manufacturer's instructions. The quality of the RNA was analyzed using a
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5 169 Bioanalyzer 2100 and an RNA6000 Nano LabChip kit (Agilent Technologies, Inc., Santa
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8 170 Clara, CA, USA) and samples with RIN (RNA integrity number) values above 9.8 were
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11 171 considered acceptable. All RNA samples (500 ng) were amplified and labeled using the
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13 172 GeneChip WT PLUS Reagent Kit and hybridized with the Clariom S human arrays
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16 173 containing 21,448 probe sets (Affymetrix, Inc., Santa Clara, CA, USA). All microarrays were
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18 174 washed and stained on the GeneChip Fluidics Station 450 using the GeneChip Hybridization,
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21 175 Wash, and Stain Kit, and then scanned on the GeneChip Scanner 3000 (Affymetrix, Inc.). The
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23 176 raw intensity data were normalized and analyzed using GeneSpring GX 14.9 software
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26 177 (Agilent Technologies, Inc.). To examine the molecular functions of differentially expressed
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28 178 genes, data were analyzed using Ingenuity Pathways Analysis (IPA) tools (Ingenuity Systems,
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31 179 Mountain View, CA, USA). The microarray data (.CEL files) were deposited in a public
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34 180 database (Gene Expression Omnibus, accession number: GSE126103).
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39 182 *2.6. Quantitative reverse transcription PCR*

41 183 RNA samples (1 µg) were reverse-transcribed to cDNA using a PrimeScript RT Master
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44 184 Mix (Takara Bio Inc., Shiga, Japan) following the manufacturer's instructions. Gene
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46 185 expression was quantified using SYBR Green Premix Ex Taq II (Takara Bio Inc.) with
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49 186 specific primers (Table 1) on an Mx3005P Real-Time QPCR System (Agilent Technologies,
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52 187 Inc.). Cycling conditions were as follows: An initial degeneration of 95°C for 30 sec,
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54 188 followed by 40 cycles of denaturing at 95°C for 5 sec, annealing at 55°C for 30 sec, and
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57 189 elongation at 72°C for 30 sec. Copy number of genes were calculated by standard curves and
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59 190 the relative gene expression levels are normalized by a housekeeping gene,
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1 191 glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using a MxPro software (version 4.10,
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3 192 Agilent Technologies, Inc.). All samples were measured in duplicate and the specificity of the
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6 193 PCR products was confirmed by melting curves.
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11 195 *2.7. Neurite outgrowth assay*

13 196 Cells were plated on glass coverslips precoated with poly-L-lysine in 24-well plates at a
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16 197 seeding density of 5.0×10^4 cells/mL with 500 μ L of the culture medium. For differentiation,
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18 198 cells were treated with FBS-free DMEM containing 10 μ M all-trans retinoic acid
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21 199 (Sigma-Aldrich) for 3 days, followed by FBS-free DMEM containing 50 ng/mL of
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23 200 brain-derived neurotrophic factor (Alomone lab, Jerusalem, Israel) for 3 days with or without
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26 201 1 or 100 μ M CTD. After washing with PBS, cells were immediately fixed by 4%
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29 202 paraformaldehyde for 10 min and then permeabilized in 0.1% Triton-X in PBS for 10 min.
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31 203 Cells were blocked for 1 h with Blocking One Histo (Nacalai Tesque, Inc., Kyoto, Japan) and
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34 204 then incubated overnight at 4°C in Can Get Signal Immunoreaction Enhancer Solution A
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36 205 (Toyobo Co., Ltd.) with rabbit anti-MAP2 antibody (1:200, #4542, Cell Signaling
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39 206 Technology). After washing 3 times with PBS containing 0.1% Tween 20, cells were
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42 207 incubated for 2 h in Can Get Signal Immunoreaction Enhancer Solution B (Toyobo Co., Ltd.)
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44 208 with anti-rabbit donkey IgG (H&L) antibody conjugated to DyLight 549 (1:1000,
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47 209 #611-742-127, Rockland Immunochemicals, Inc., Gilbertsville, PA, USA). Cell nucleus was
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49 210 counterstained with 4'-6-diamidino-2-phenylindole (DAPI). The coverslips were mounted on
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52 211 microscope slides in ProLong Glass antifade mountant (Thermo Fisher Scientific, Waltham,
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54 212 MA, USA) and fluorescence images were acquired with a BX61/DP70 microscope (Olympus,
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57 213 Tokyo, Japan). Neurite length was measured in at least 100 cells in three randomly chosen
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59 214 fields using ImageJ software (version 1.51) with the NeuronJ plugin.
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3 216 *2.8. Statistical analyses*

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5 217 Results from at least three independent experiments are expressed as the mean \pm standard
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8 218 deviation (SD). IBM SPSS statistics 23 software (IBM Co., Somers, NY, USA) was used to
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11 219 perform statistical analyses. The dose-response curve was fitted using the sigmoidal
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13 220 (4-parameter) equation with JMP13 Pro software (SAS Institute, Cary, NC, USA). The EC50
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16 221 value was calculated by determining the concentration at which 50% of maximum activity
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18 222 was reached using the sigmoidal fit equation. One-way analysis of variance (ANOVA)
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21 223 followed by Dunnett's post-hoc test was used to analyze the effects of CTD. Two-way
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23 224 ANOVA followed by Tukey's post hoc test was used to analyze the interaction between CTD
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26 225 and nAChR antagonists. The Welch's *t* test was used to validate the microarray results by
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29 226 quantitative reverse transcription PCR. The results were considered significant when the
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31 227 *p*-value was less than 0.05.

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36 229 **3. Result**37
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39 230 *3.1. Effects of CTD on the cell number of SH-SY5Y cells*

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41 231 To determine the potential effects of CTD on human neuroblastoma SH-SY5Y cells, the
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44 232 cell growth assays were conducted. As shown in Fig. 1A, CTD dose-dependently stimulated
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47 233 the cell growth and one-way ANOVA showed that there were significant differences at Day 1
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49 234 [F(2, 12) = 11.150, *p* < 0.01] and Day 2 [F(2, 12) = 5.764, *p* < 0.05]. The high concentration
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52 235 of CTD (100 μ M) significantly increased the relative cell number at Day 1 (*p* < 0.01) and
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54 236 Day 2 (*p* < 0.05). Low concentrations of CTD (1 μ M) also increased the relative cell number
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57 237 at Day 1 (*p* < 0.05), but not significantly at Day 2. The dose-response relationship between
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60 238 the exposure concentration and the growth stimulating effect of CTD was evaluated by the

1 239 WST-8 assay. The dose-response curves showed that CTD had growth stimulating effects on
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3 240 SH-SY5Y cells with an EC50 of 577 nM (Fig. 1B).
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7 8 242 *3.2. Antagonists to nAChR blocked the growth stimulating effect of CTD on SH-SY5Y cells*

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11 243 We therefore investigated whether the effects of CTD on the SH-SY5Y cells were
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13 244 mediated by human-type nAChRs. Although 10 μ M mecamylamine (MEC), a broad
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16 245 spectrum non-competitive nAChRs antagonist, had no effect on the SH-SY5Y cells alone, it
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18 246 inhibited the growth stimulating effect of 100 μ M CTD (Fig. 2A). Two-way ANOVA showed
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21 247 that there was no effect of MEC [$F(1, 16) = 3.295$] and significant effect of CTD [$F(1, 16) =$
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23 248 $7.853, p < 0.05$] and significant interaction [$F(1, 16) = 12.887, p < 0.01$]. According to a
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26 249 previous report showing that SH-SY5Y cells are known to express $\alpha 3, \alpha 7, \beta 2$ and $\beta 4$ subunit
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29 250 of nAChRs (Groot Kormelink and Luyten, 1997), we further examined which subunit of
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31 251 nAChRs is involved in the effects of CTD with using subunit specific competitive nAChR
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34 252 antagonists. As shown in Fig. 2B and 2C, the $\alpha 7$ and $\beta 2$ subunit specific antagonists
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36 253 methyllycaconitine (MLA, 100 nM) and dihydro- β -erythroidine (DH β E, 10 μ M) did not
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39 254 suppress the effects of CTD. There was no effect of antagonists [MLA: $F(1, 16) = 1.964$;
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41 255 DH β E: $F(1, 16) = 4.191$], and significant effects of CTD were observed [MLA: $F(1, 16) =$
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43 256 $15.817, p < 0.01$; DH β E: $F(1, 16) = 16.005, p < 0.01$]. No interaction was detected between
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45
46 257 CTD and antagonists [MLA: $F(1, 16) = 4.743$; DH β E: $F(1, 16) = 0.009$]. In contrast, an $\alpha 3\beta 4$
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49 258 specific antagonist, SR 16584 (10 μ M), blocked the effect induced by 100 μ M CTD (Fig. 2D).
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52 259 Two-way ANOVA showed that there was no effect of SR 16584 [$F(1, 16) = 2.602$] and
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54 260 significant effect of CTD [$F(1, 16) = 10.179, p < 0.05$] and significant interaction [$F(1, 16) =$
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57 261 $8.312, p < 0.05$].
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3.3. CTD evoked intracellular calcium flux in SH-SY5Y cells

As nAChRs are ligand-gated ion channels permeable to calcium ions, we next evaluate the CTD-induced changes of intracellular calcium levels in SH-SY5Y cells loaded with calcium-sensitive dye Fluo-4 AM. The temporal changes of intracellular calcium levels were monitored by fluorescence intensity of fluo-4 (F) normalized to the average of baseline intensity (F₀). As shown in Fig. 3A, transient increases of fluorescence intensity were observed immediately after exposure to CTD. Quantitative analyses showed that both the maximum amplitude (F_{max}-F₀) [F(3, 11) = 5.674, $p < 0.05$] and the area under the curve [F(3, 11) = 8.415, $p < 0.01$] were significantly increased by at least 10 μ M of CTD (Fig. 3B and 3C).

3.4. Effects of CTD on phosphorylation levels of ERK in SH-SY5Y cells

To examine the modulation of the intracellular signaling subsequent to intracellular calcium influx induced by CTD, we analyzed phosphorylation levels of extracellular signal-regulated kinase 1/2 (ERK), which is known to be important in proliferation of neuroblastoma cells (Stafman and Beierle, 2016). SH-SY5Y cells were exposed to CTD for 1 h, and total and phosphorylated ERK were detected on the same membrane at once by western blotting. As shown in Fig. 4A, although the band intensity of total ERK did not change, CTD increased that of phosphorylated ERK (p-ERK) compared to the control samples. Quantitative analyses showed that CTD dose-dependently and significantly increased the p-ERK/ERK ratio [F(3, 11) = 4.053, $p < 0.05$] (Fig. 4B).

3.5. Effects of CTD on global gene expression of SH-SY5Y cells

To understand changes of gene expression involved in the mechanisms of effects of CTD,

1 287 the transcriptome analysis was performed in cells exposed to 1 μ M CTD for 24 h using
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3 288 GeneChip system and the clariom S human array with 21,448 probe sets. After normalization
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5 289 of obtained intensities of probes using the RMA algorithm using the GeneSpring software,
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8 290 low intensity probes (<10% expression level) were cutoff as noise. We then identified
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11 291 differentially expressed genes (174 up-regulated and 151 down-regulated) with at least
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13 292 1.5-fold change compared with the control group (0.1% DMSO), and then conducted
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16 293 bioinformatical analyses to reveal biological functions, canonical pathways, and networks of
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18 294 differentially expressed genes based on Ingenuity Pathway Analysis (IPA) knowledge base
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21 295 software. We obtained little findings related to the phenotypic changes observed in this study
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23 296 from up-regulated genes (Supplemental Materials). The top 25 biological functions with
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26 297 positive z-scores related to the down-regulated genes are summarized in Table 2. Notably,
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28 298 CTD significantly decreased the expression of genes associated with neural functions with
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31 299 annotations of “Formation of filopodia,” “Brain lesion,” “Quantity of neurons,” “Behavior,”
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34 300 and “Differentiation of nervous system.” Additionally, multiple annotations associated with
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36 301 calcium signaling, “Flux of Ca^{2+} ,” “Influx of Ca^{2+} ,” and “Ion homeostasis of cells,” were also
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39 302 listed. The differentially activated or suppressed canonical pathways in down-regulated genes
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41 303 are shown in Fig. 5A. Canonical pathways including “Axonal Guidance Signaling” and
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44 304 “Synaptic Long Term Depression” relating to neural function were enriched in
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46 305 down-regulated genes. Cytoskeletal pathways, “G α 12/13 Signaling,” “PAK Signaling,”
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49 306 “Actin Cytoskeleton Signaling,” and “Signaling by Rho Family GTPases,” were significantly
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52 307 suppressed. Network analyses revealed gene networks with 40 molecules involved in
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54 308 “Cardiovascular System Development and Function,” “Organismal Development” and
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57 309 “Cell-To-Cell Signaling and Interaction” (Fig. 5B) and several down-regulated genes
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59 310 indirectly affected signal transduction molecules such as ERK, ras-related protein 1 (Rap1)

1 311 and cAMP response element binding protein (Creb).
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5 313 *3.6. Validation of microarray results by quantitative reverse transcription PCR* 6 7

8 314 To confirm the microarray results, the expression levels of down-regulated genes were
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10 315 measured by quantitative reverse transcription PCR. We chose three genes significant in the
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12 316 bioinformatical analyses by the IPA software including neuronal differentiation 4
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14 317 (NEUROD4), adrenoceptor beta 2 (ADRB2), and neurotensin (NTS). As shown in Fig. 6,
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16 317 (NEUROD4), adrenoceptor beta 2 (ADRB2), and neurotensin (NTS). As shown in Fig. 6,
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18 318 quantitative results showed that 1 μM of CTD significantly suppressed the expression of
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21 319 these genes ($p < 0.05$), which were the same pattern in microarray results.
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26 321 *3.7. Effects of CTD on neurite outgrowth of SH-SY5Y cells* 27

28 322 In order to examine the neurodifferentiative effects of micromolar concentrations of CTD,
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30 323 we treated differentiating cells with CTD and morphologically evaluated the neurite
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32 324 outgrowth. As shown in Fig. 7A, neurites in differentiated cells were visualized by
33
34 324 outgrowth. As shown in Fig. 7A, neurites in differentiated cells were visualized by
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36 325 immunofluorescent staining with the neurite marker MAP2 (microtubule-associated protein
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38
39 326 2). Quantitative analyses revealed that at least 1 μM of CTD significantly increased neurite
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41 327 length compared to the control group (Fig. 7B) [$F(3, 13) = 12.711, p < 0.01$]. The number of
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43 328 neurites per cell was not significantly changed by CTD (Fig. 7C) [$F(3, 13) = 3.345$].
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49 330 **4. Discussion** 50

51 331 In the present study, we investigated whether CTD affected human neuroblastoma
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53 332 SH-SY5Y cells to obtain information about the risks of neonicotinoids in the human nervous
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56 333 system. Unexpectedly, CTD dose-dependently increased the number of cells and nAChR
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59 334 antagonists inhibited the growth stimulating effect of CTD. We clarified some of the
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1 335 underlying mechanisms of the effects of CTD mediating intracellular calcium flux,
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3 336 phosphorylation of signal transduction molecules and alteration of global gene expression.
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5 337 Although cytotoxic effects of millimolar concentrations of neonicotinoids on SH-SY5Y cells
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8 338 were previously reported (Skandrani et al., 2006; Şenyildiz et al., 2018), these results firstly
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11 339 demonstrated that micromolar concentrations have functional effects on human-derived
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13 340 neuronal cells, in part by changing the intracellular signaling. Taken together, our data would
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16 341 provide a new perspective into understanding the effects of non-lethal doses of
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18 342 neonicotinoids on human nervous systems.

21 343 One of the interesting findings of this study is the growth stimulating effects of CTD,
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23 344 consistent with previous work evaluating the effects of nicotine in the same cell line (Serres
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25
26 345 and Carney, 2006). At the cellular level, cholinergic signaling by exogenous stimulation
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28
29 346 regulates cellular activities such as apoptosis, cell survival, proliferation and differentiation
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31 347 (Resende and Adhikari, 2009). Excessive amounts of nicotinic agonists are lethal, but
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34 348 sublethal doses consistently had proliferative effects in nAChR-expressing cell lines such as
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36 349 HT29 colon cancer cells and A549 lung cancer cells (Wong et al., 2007; Mucchietto et al.,
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39 350 2018). Additionally, activation of nAChRs has neuroprotective effects in SH-SY5Y cells; for
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41 351 example, nAChRs activation alleviated neurotoxicity of okadaic acid and amyloid- β (Del
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44 352 Barrio et al., 2011; Xue et al., 2015). Although the proliferation of neural cells was observed
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47 353 in limited areas in the mature brain, an *in vivo* study showed that an $\alpha 7$ nicotinic agonist
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49 354 reactivated adult neurogenesis in cortex and hippocampus in mice (Narla et al., 2013). Taken
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52 355 together, further studies should be carried out focusing on the effects of neonicotinoids on
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54 356 proliferative and differentiative neural stem cells in developing brains.

57 357 Our results showed that concomitant exposure to nAChR antagonists inhibited the growth
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59 358 stimulating effects of CTD. Given that these antagonists are active against human nAChRs,
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1 359 our findings raise the likelihood that human-type nAChRs could be affected by
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3 360 neonicotinoids. Neuronal nAChRs are composed of pentamer structure and largely divided
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6 361 into four groups by subunit composition: (i) $\alpha 7$ homomers, (ii) $\alpha 4$ and $\beta 2$ heteromers, (iii) $\alpha 3$,
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8 362 $\beta 4$ and $\beta 2$ heteromers and (iv) $\alpha 2$, $\alpha 4$ and $\beta 4$ heteromers (Albuquerque et al., 2009). To date,
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11 363 previous studies have reported that CTD modulates electrophysiological responses to
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13 364 acetylcholine in human embryonic kidney (HEK293) cells expressing human $\alpha 4\beta 2$ nAChRs,
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16 365 and in *Xenopus* oocytes expressing rat $\alpha 7$ nAChRs (Li et al., 2011; Cartereau et al., 2018). In
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18 366 this study, we used human neuroblastoma SH-SY5Y cells expressing $\alpha 3$, $\alpha 7$, $\beta 2$, and $\beta 4$
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21 367 subunits (Groot Kormelink and Luyten, 1997). Our results showed that mecamylamine and
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23 368 SR 16584 inhibited the increasing of the cell number, indicating that $\alpha 3\beta 4$ nAChRs are
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26 369 largely responsible for the effects of CTD. As described above, $\alpha 3\beta 4$ nAChRs are frequently
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29 370 called “ganglion type” nAChRs, but they also play important roles in broad regions of
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31 371 mammal brain including hippocampus, medial habenula, pineal gland, cerebellum, locus
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34 372 coeruleus, substantia nigra and ventral tegmental area (Gotti, et al., 2006). Compared to other
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36 373 types of nAChRs, the current responses of $\alpha 3\beta 4$ nAChRs to nicotinic agonists are slow but
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39 374 strong and durable (Chavez-Noriega et al., 1997), which may lead to the functional effects
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41
42 375 and phenotypic changes observed in this study. A recent study also revealed that the
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44 376 neonicotinoid IMI facilitates the expression of tyrosine hydroxylase, a marker of
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47 377 differentiation in PC12D cells mediated by rat $\alpha 3\beta 4$ and $\alpha 7$ nAChRs (Kawahata and
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49 378 Yamakuni, 2018); thus, human $\alpha 3\beta 4$ nAChRs could be significant to understanding the
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51
52 379 unexpected effects of neonicotinoids.

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54 380 Neuronal nAChRs function as non-selective cation channels permeable to calcium ions.
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57 381 Cation influx by nAChRs subsequently raises the intracellular calcium concentration by
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59 382 activating voltage-dependent calcium channels (VDCCs) with membrane depolarization and

1 383 calcium release by ryanodine receptors from endoplasmic reticulum (Shen and Yakel, 2009).
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3 384 In this study, micromolar concentrations of CTD dose-dependently evoked the transient
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5 385 increase of intracellular calcium level for a few tens of seconds. These temporal patterns are
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8 386 very similar to intracellular calcium responses to micromolar concentrations of nicotine; such
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11 387 responses are partly mediated by $\alpha 7$ nAChRs and depend to a large part on VDCCs
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13 388 (Dajas-Bailador et al., 2002a; Gilbert et al., 2009). Transcriptome analysis by Kimura-Kuroda
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16 389 et al., (2016) showed that 1 μ M nicotine and two neonicotinoids (ACE and IMI) commonly
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18 390 altered the gene expression of VDCC subunits in rat cerebellar cells. Another *in vivo* study
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21 391 consistently demonstrated that CTD-induced dopamine release in rat striatum is related to
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23 392 neuronal membrane depolarization (Faro et al., 2012). Our result also showed that gene sets
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26 393 related “Flux of Ca^{2+} ,” “Influx of Ca^{2+} ,” and “Ion homeostasis of cells” were significantly
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29 394 enriched in the CTD-down-regulated genes, which may be a result of negative feedback by
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31 395 sustained higher intracellular calcium with membrane depolarization.

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34 396 Mitogen-activated protein kinases play crucial roles in neural cells for transmitting
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36 397 exogenous stimulation to intracellular signaling. In particular, ERK regulates cell
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39 398 proliferation, differentiation, survival, and migration. In this study, we found that CTD
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42 399 dose-dependently increased the phosphorylation level of ERK, consistent with other studies
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44 400 in mouse neuroblastoma N1E-115 cells (Tomizawa and Casida, 2002) and SH-SY5Y cells
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47 401 (Dajas-Bailador et al., 2002b). These studies consistently demonstrated that phosphorylation
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49 402 states of ERK were altered by nAChR-mediated calcium signaling and membrane
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52 403 depolarization. In rat PC12h cells, ERK phosphorylation by nicotinic ligands is inhibited by
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54 404 $\alpha 3\beta 4$ nAChRs (Nakayama et al., 2006) and over-expression of $\alpha 7$ nAChRs promotes the
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57 405 basal level of p-ERK (Utsugisawa et al., 2002). Our network analyses showed that most of
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60 406 the down-regulated genes indirectly act upstream of ERK, which may support the modulation

1 407 of ERK activation induced by CTD. It also should be noted that neonicotinoids, including
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3 408 CTD are immediately metabolized inside the body of mammals, and certain amounts of
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5 409 metabolites of neonicotinoids are detected in the brain (Fold and Casida, 2006). Other studies
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8 410 demonstrated that metabolites of IMI had higher affinity for human nAChRs and induced
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11 411 strong activation of ERK than that of the original compound (Tomizawa and Casida 2002,
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13 412 2005), indicating that additional studies should be conducted focusing on the potential effects
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16 413 of metabolites of neonicotinoids. Considering that ERK signaling controls neuroplasticity and
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18 414 synaptic transmission in the brain (Thomas and Huganir, 2004), we should consider the risks
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21 415 of neonicotinoids on higher brain function such as learning and memory.

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23 416 Microarray analyses revealed different aspects of the effects of CTD, indicating that 24 h
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26 417 exposure of 1 μ M CTD could disrupt several sets of gene expression involved in cytoskeleton
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29 418 regulatory signaling. A series of studies recently showed that α 7 nAChRs coupled to
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31 419 G-proteins and regulated cytoskeletal dynamics and axon growth (King et al., 2015; Kabbani
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34 420 and Nichols, 2018), suggesting intracellular calcium flux mediated by nAChRs also
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36 421 associated with morphological changes of neuronal cells. In this study, our data indicated that
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39 422 down-regulated genes contained significantly enriched biological functions of “Formation of
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41 423 filopodia,” essential for advancing growth cones to extend neurites at the tip of the axon. It
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44 424 was also noted that “Axon guidance signaling” was the most significantly enriched pathway
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47 425 with no activity pattern available and “Differentiation of nervous system” was the
48
49 426 significantly enriched biological function in the down-regulated genes by CTD. Therefore,
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51
52 427 we investigated whether CTD had an impact on neurite outgrowth in differentiating
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54 428 SH-SY5Y cells. We observed increased length and number of neurite in differentiated
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57 429 SH-SY5Y cells by CTD exposure, both of which disagree with previous studies reporting that
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59 430 ACE and IMI suppressed neurite outgrowth in rat Purkinje cells (Kimura-Kuroda et al., 2016).

1 431 Furthermore, another study reported that four neonicotinoids had no effect on neurite length
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3 432 of PC12 cells (Christen et al., 2017). Nordman and Kabbani (2012) showed that nicotinic
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5 433 agonists inhibited neurite surface area in PC12 cells, whereas a nicotinic antagonist for $\alpha 7$
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8 434 nAChRs, α -bungarotoxin promoted it. These inconsistencies among studies may reflect
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11 435 differences in species, cell type, and receptor properties.

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13 436 In conclusion, the present study demonstrated for the first time that CTD had functional
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16 437 effects on human neuroblastoma SH-SY5Y cells that resulted in increased cell growth
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18 438 mediated by human nAChRs. Moreover, our data showed that micromolar concentrations of
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21 439 CTD acutely disrupted intracellular signaling that led to an influx of intracellular calcium,
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24 440 phosphorylation of ERK and alteration of global gene expression. Transcriptional and
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26 441 neuromorphological changes strongly suggest that CTD has impacts on neuronal
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29 442 differentiation and neurite outgrowth. Taken together, our data provide novel toxicological
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32 443 information to assess the risk of neonicotinoids on human health by using a human-derived
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34 444 cell line with neuronal properties. Additional research is needed to understand the risk of
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36 445 environmentally-relevant doses of neonicotinoids, with a focus on developmental events in
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38
39 446 the brain such as neural differentiation, migration, and neural circuit development.

40 41 447 42 43 44 448 **Conflict of interest**

45
46 449 The authors declare that they have no conflict of interest.
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1 639 **Figure legends**

2
3 640 **Fig. 1.** Effects of clothianidin (CTD) on the cell growth of SH-SY5Y cells. (A) Cell growth
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5
6 641 curve for SH-SY5Y cells exposed to 1 or 100 μM CTD. Relative cell number is determined
7
8 642 as fold change in cell number relative to the number of cells initially plated. (B)
9
10
11 643 Dose-response curve for the increases of cell number at 24 h of CTD exposure evaluated by
12
13 644 WST-8 assay. Responses are normalized to the maximum response induced by 10 μM CTD
14
15
16 645 and fitted to the 4-parameter sigmoidal equation. All data points are represented by at least
17
18 646 five independent experiments and values are shown by the mean \pm SD, $^\dagger p < 0.05$ CTD 1 μM
19
20
21 647 vs. control group, and $*p < 0.05$, $**p < 0.01$ CTD 100 μM vs. control group (one-way
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23
24 648 ANOVA followed by Dunnett's post-hoc test).

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26 649
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29 650 **Fig. 2.** Effects of nicotinic acetylcholine receptors antagonists, (A) 10 μM mecamylamine
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31 651 (MEC; broad spectrum antagonist), (B) 100 nM methyllycaconitine (MLA; $\alpha 7$ selective
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34 652 antagonist), (C) 10 μM dihydro- β -erythroidine (DH β E; $\beta 2$ selective antagonist) and (D) 10
35
36 653 μM SR 16584 ($\alpha 3\beta 4$ selective antagonist) on the growth stimulating effect of 100 μM CTD.
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38
39 654 All data are represented by at least five independent experiments and values are shown by the
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41 655 mean \pm SD, $*p < 0.05$, $**p < 0.01$ vs. other groups (two-way ANOVA followed by Tukey's
42
43
44 656 post-hoc test).

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46 657
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49 658 **Fig. 3.** Effects of CTD on intracellular calcium level of SH-SY5Y cells evaluated by fluo-4
50
51 659 assay. (A) Time course of changes in the fluorescence intensity of fluo-4 evoked by CTD.
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53
54 660 Fluorescence intensity at each time point was normalized to the average level of the baseline
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56
57 661 intensity. The summarized data, (B) Fmax-F0 and (C) area under curve were calculated to
58
59 662 quantitate the effect of CTD. F: fluorescence intensity; F0: average level of baseline

1 663 fluorescence intensity; Fmax: maximal value of fluorescence intensity after treatment. All
 2
 3 664 data are represented by at least three independent experiments and values are shown by the
 4
 5 665 mean \pm SD, * p < 0.05, ** p < 0.01 vs. control group (one-way ANOVA followed by Dunnett's
 6
 7
 8 666 post-hoc test).

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13 668 **Fig. 4.** Effects of CTD on phosphorylation levels of extracellular signal-regulated kinase 1/2
 14
 15
 16 669 (ERK) on SH-SY5Y cells analyzed by western blotting. (A) Representative images of bands
 17
 18 670 of phosphorylated ERK (p-ERK) and total ERK on the same membrane. (B) Phosphorylation
 19
 20
 21 671 levels of ERK after 1 h exposure of CTD were calculated by band intensities of p-ERK
 22
 23
 24 672 normalized by those of total ERK. All data are represented by at least three independent
 25
 26 673 experiments and values are shown by the mean \pm SD, * p < 0.05 vs. control group (one-way
 27
 28
 29 674 ANOVA followed by Dunnett's post-hoc test).

31 675

34 676 **Fig. 5.** Canonical pathways and a gene network of 151 down-regulated genes (>1.5-fold)
 35
 36 677 altered by 24 h exposure of 1 μ M CTD identified by ingenuity pathway analysis (IPA)
 37
 38
 39 678 software. (A) Signaling pathways significantly enriched by Fisher's exact test were
 40
 41
 42 679 summarized. Solid and dotted squares showed pathways related to neural and cytoskeletal
 43
 44 680 function. (B) A gene network map illustrated interactions of down-regulated genes (green
 45
 46
 47 681 colored) and other molecules.

49 682

52 683 **Fig. 6.** Validation of microarray results using quantitative reverse transcription PCR. Gene
 53
 54 684 expression levels of neuronal differentiation 4 (NEUROD4), adrenoceptor beta 2 (ADRB2),
 55
 56
 57 685 neurotensin (NTS) in the control (white bar) and of CTD-exposed group (1 μ M, blue bar)
 58
 59
 60 686 were calculated relative to the housekeeping gene, glyceraldehyde-3-phosphate

1 687 dehydrogenase (GAPDH) by comparison to the control group. All data are represented by at
2
3 688 least three independent experiments and values are shown by the mean \pm SD, $*p < 0.05$ vs.
4
5
6 689 control group (Welch's *t* test).

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8 690

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11 691 **Fig. 7.** Effects of CTD on neurite outgrowth in differentiating SH-SY5Y cells. Cells were
12
13 692 induced to differentiate for 6 days with or without CTD exposure. (A) Representative images
14
15
16 693 of immunofluorescent staining of a neurite maker, MAP2 (microtubule-associated protein 2:
17
18 694 red) with DAPI cell nuclear staining (blue) in the control, CTD 1 μ M, CTD 10 μ M and
19
20
21 695 CTD 100 μ M group. The average of (B) neurite length and (C) number of neurites per cell
22
23
24 696 were measured by the image analysis. Bar = 100 μ m. All data are represented by at least three
25
26 697 independent experiments and values are shown by the mean \pm SD, $*p < 0.05$, $**p < 0.01$ vs.
27
28
29 698 control group (one-way ANOVA followed by Dunnett's post-hoc test).

Table1. Primer sequences used in quantitative reverse transcription PCR

Gene Symbol	Gene ID	Direction	Primer sequence (5'-3')
NEUROD4	NM_021191.3	foward	gggagagctagtagcaacacac
(neuronal differentiation 4)		reverse	ttgggaccccttctcttagg
ADRB2	NM_000024.5	foward	ggattgtgtcaggccttacc
(adrenoceptor beta 2)		reverse	gatcaccaggggaacgtaga
NTS	NM_006183.5	foward	ccttcagtgtgcttctgac
(neurotensin)		reverse	gcagagtcatttccaagag
GAPDH	NM_001256799.2	foward	ccaccaactgcttagcac
(glyceraldehyde-3-phosphate dehydrogenase)		reverse	catcacgccacagtttcc

Table 2. Top 25 of biological functions of 151 down-regulated genes

Functions Annotation	<i>p</i> -Value	Predicted Activation State	Activation z-score	Number of Molecules
Size of body	9.46E-05	Decreased	-4.088	18
Formation of filopodia	4.74E-03	Decreased	-2.219	5
Flux of Ca ²⁺	5.64E-03	Decreased	-2.192	7
Binding of leukocyte cell lines	4.57E-04	Decreased	-2.000	4
Hydrolysis of nucleotide	6.18E-03		-1.949	4
Influx of Ca ²⁺	1.17E-02		-1.932	5
Ion homeostasis of cells	5.18E-03		-1.890	11
Adhesion of blood cells	7.08E-03		-1.857	8
Adhesion of tumor cell lines	7.04E-03		-1.680	7
Adhesion of immune cells	1.51E-02		-1.598	7
Leukocyte migration	4.62E-03		-1.341	15
Permeability of vascular system	1.52E-04		-1.164	7
Angiogenesis of tumor	8.02E-03		-1.131	4
Binding of hematopoietic cell lines	5.26E-05		-1.109	5
Brain lesion	4.55E-03		-0.927	15
Atherosclerosis	4.39E-03		-0.878	10
Growth of vessel	7.45E-04		-0.796	6
Advanced malignant tumor	8.25E-03		-0.594	15
Quantity of neurons	1.16E-02		-0.527	8
Cancer of cells	1.29E-02		-0.384	46
Neoplasia of cells	4.51E-03		-0.350	49
Behavior	7.85E-03		-0.046	16
Lymphatic system tumor	8.34E-03		0.926	24
Differentiation of nervous system	1.42E-02		1.342	9
Organismal death	9.36E-03	Increased	4.755	30

activation z-score; >2.0 or < -2.0 is significantly predictive

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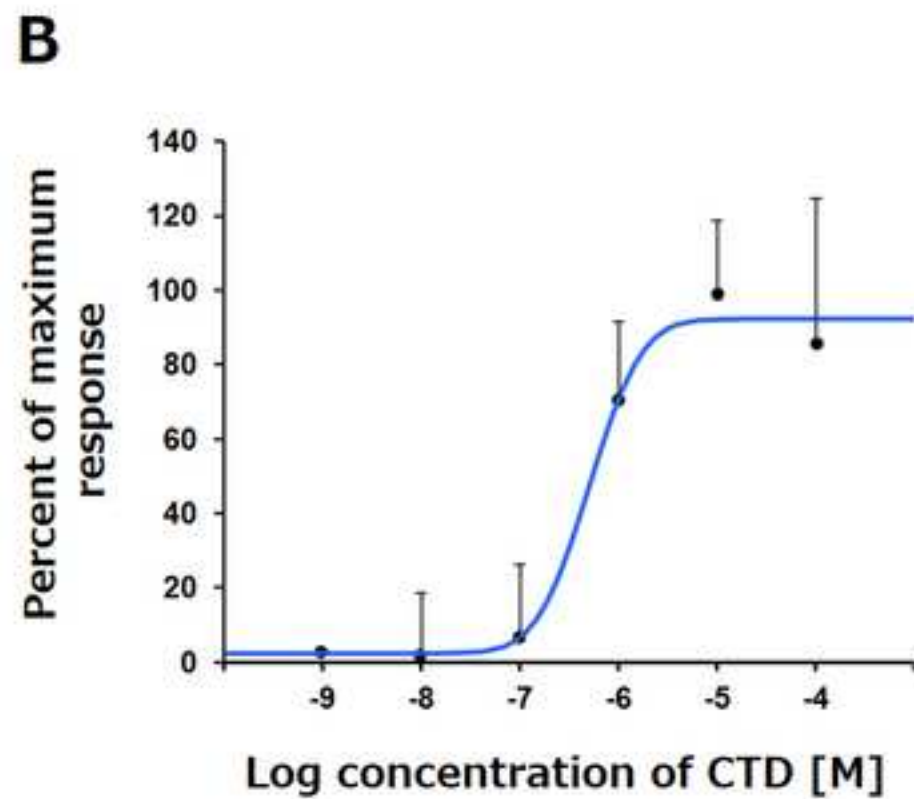
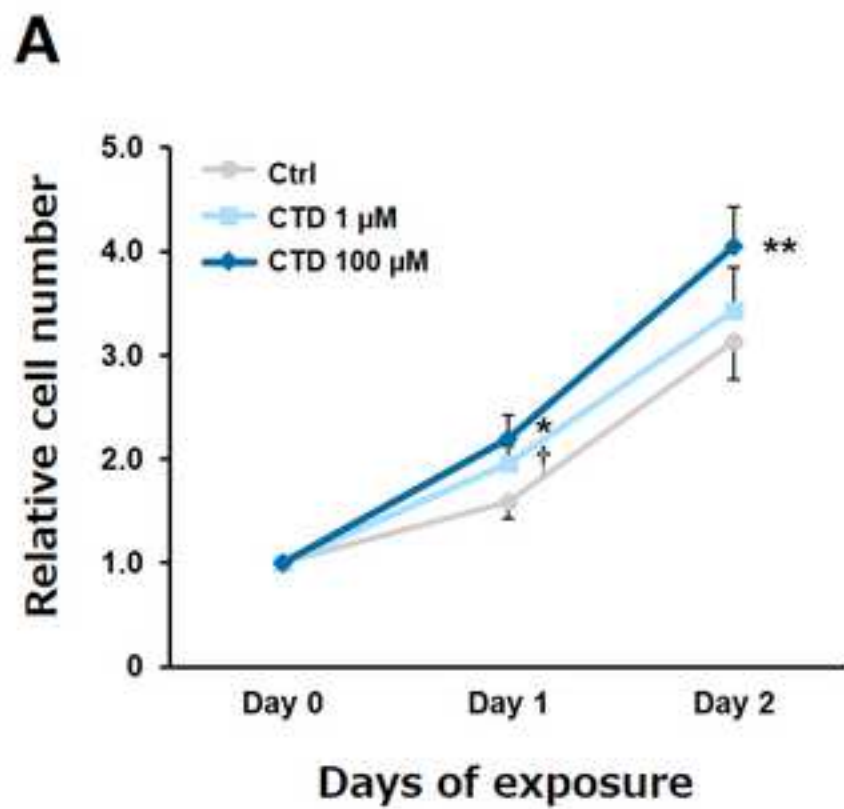
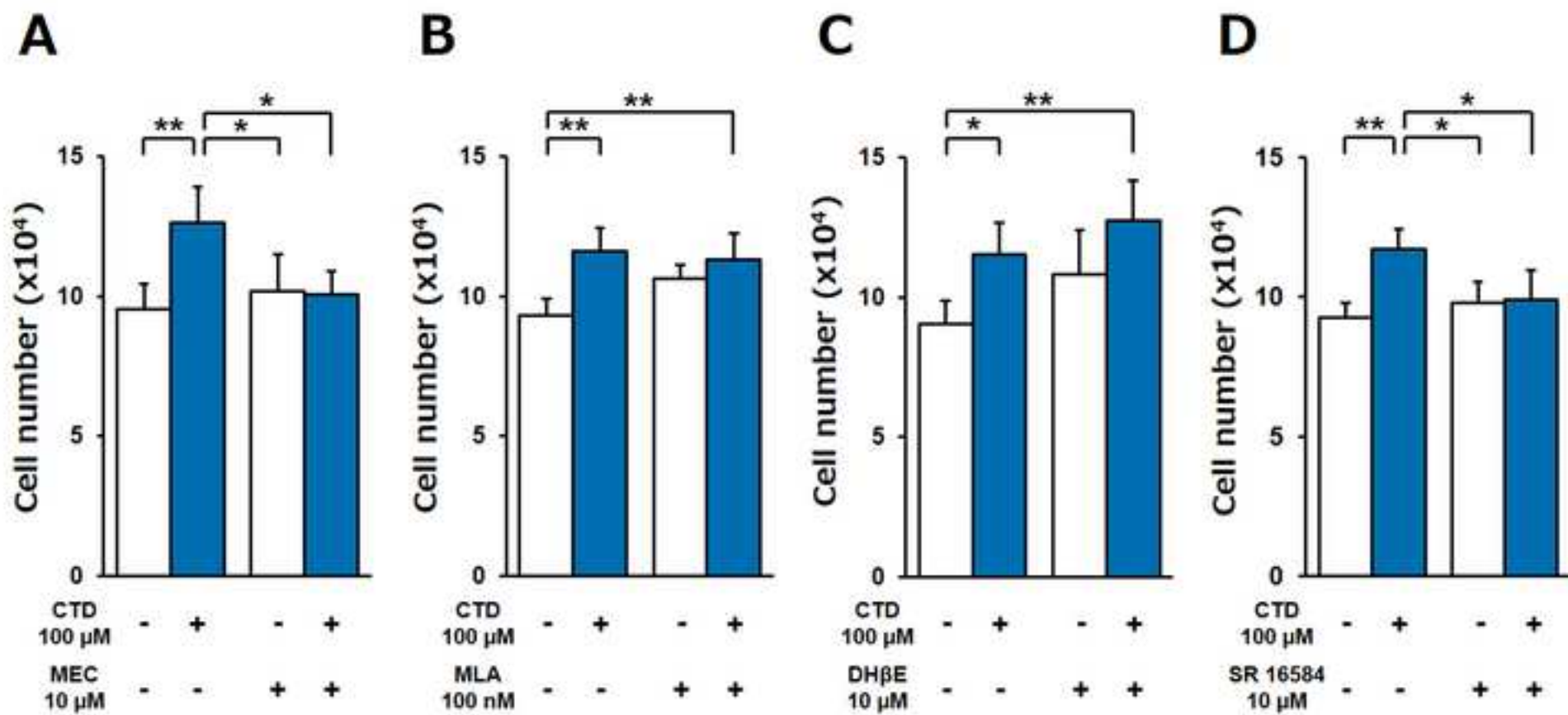
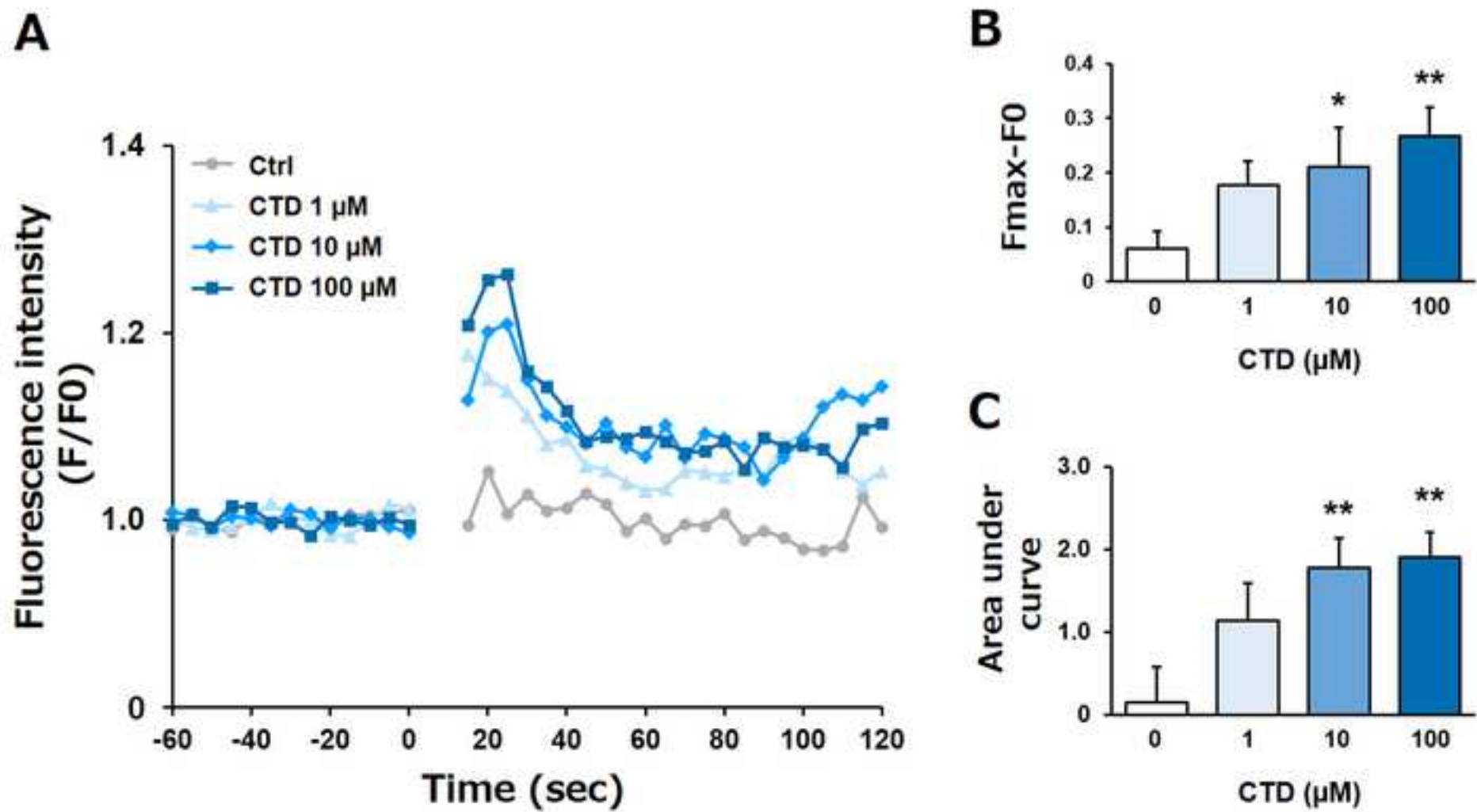
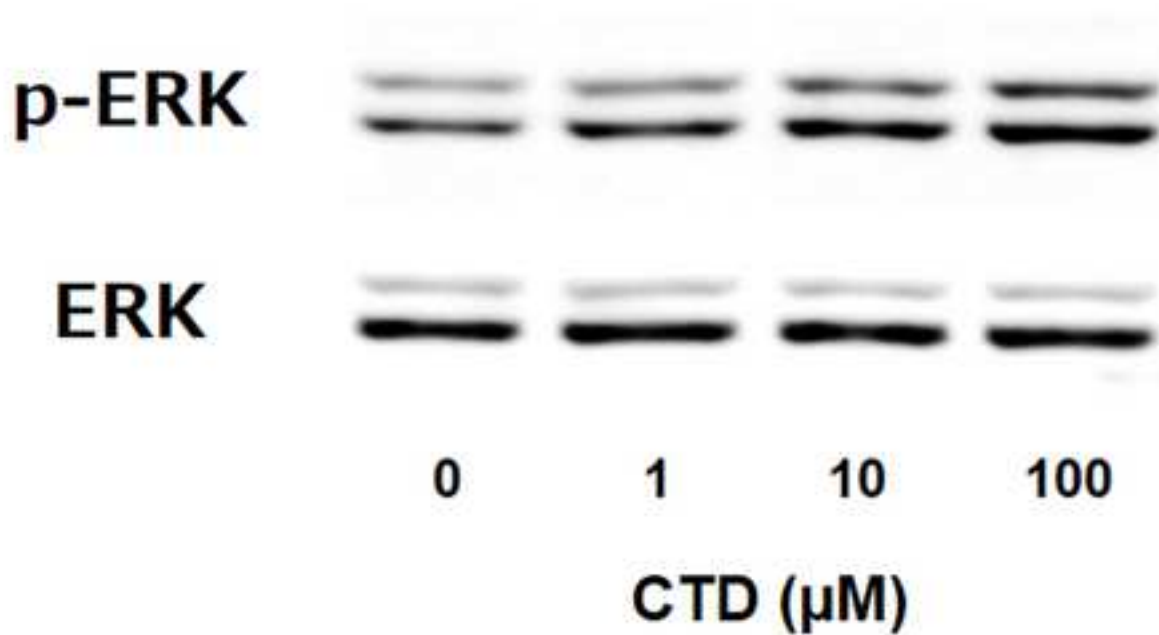


Figure
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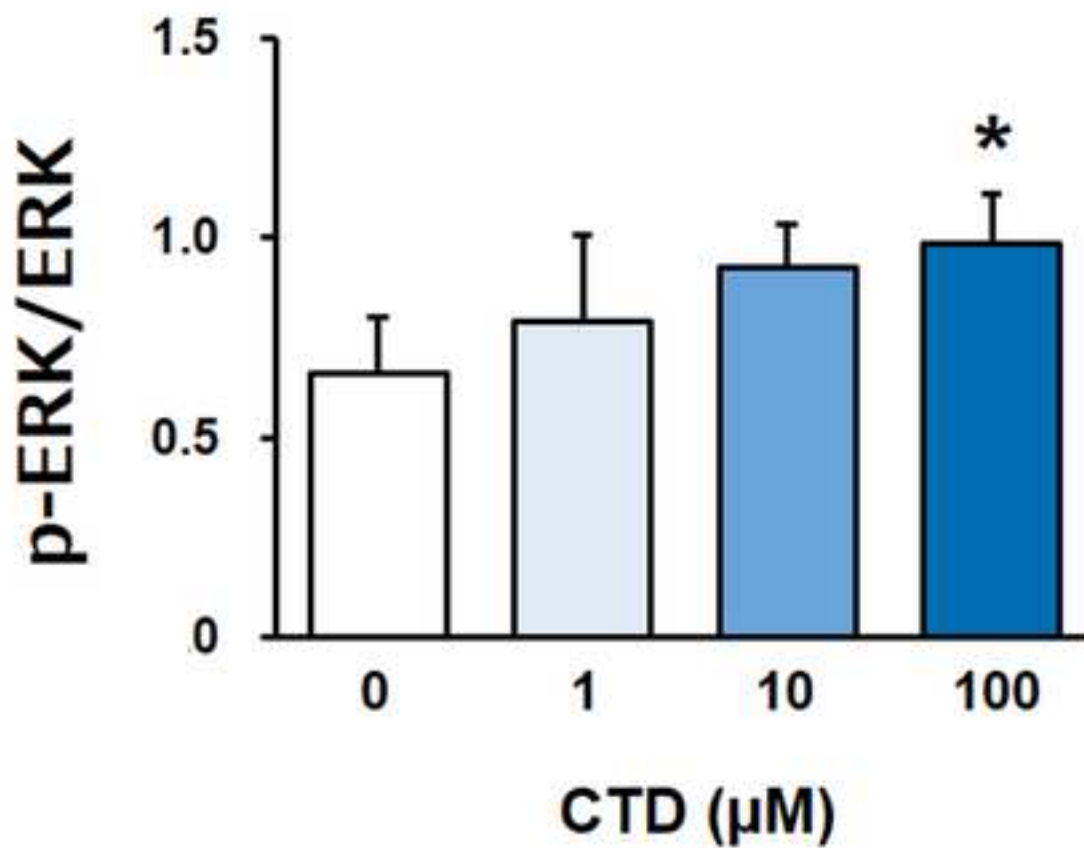




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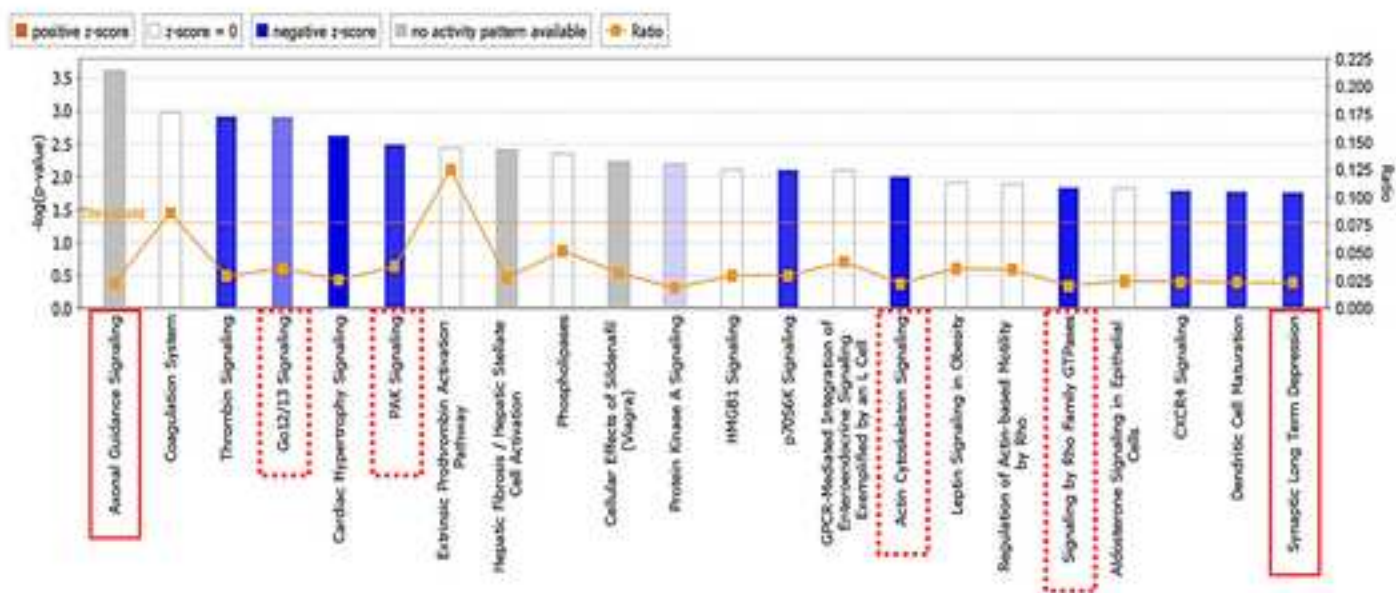
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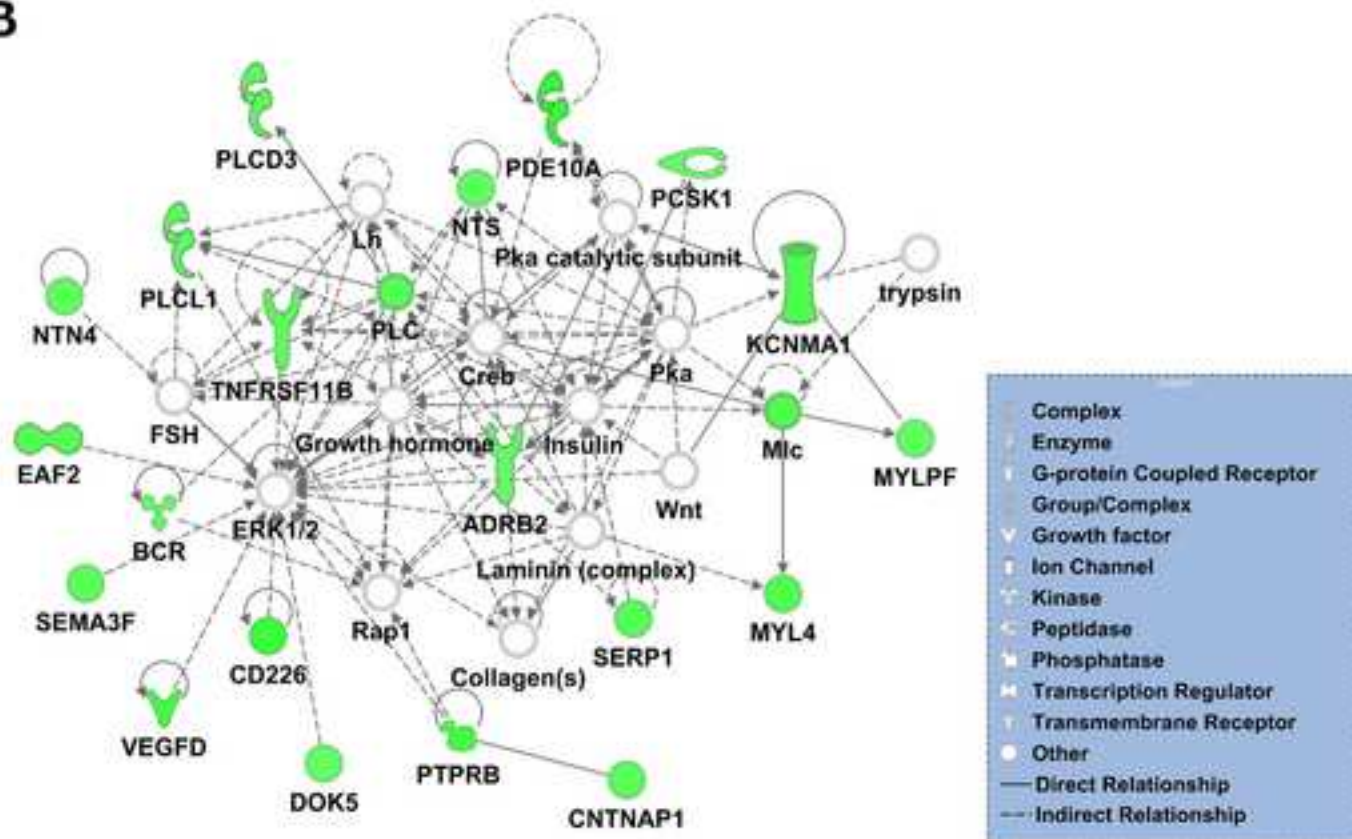
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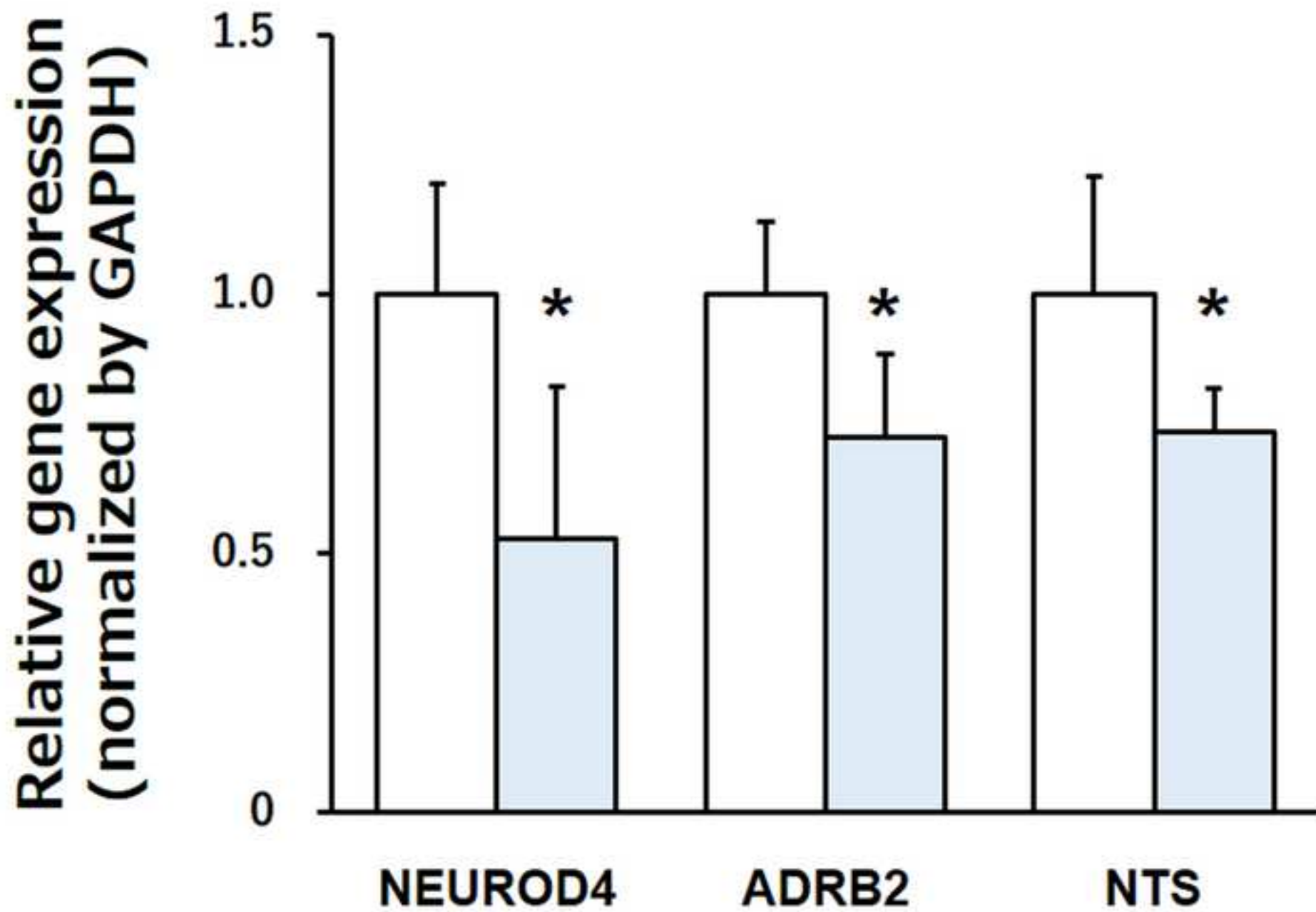
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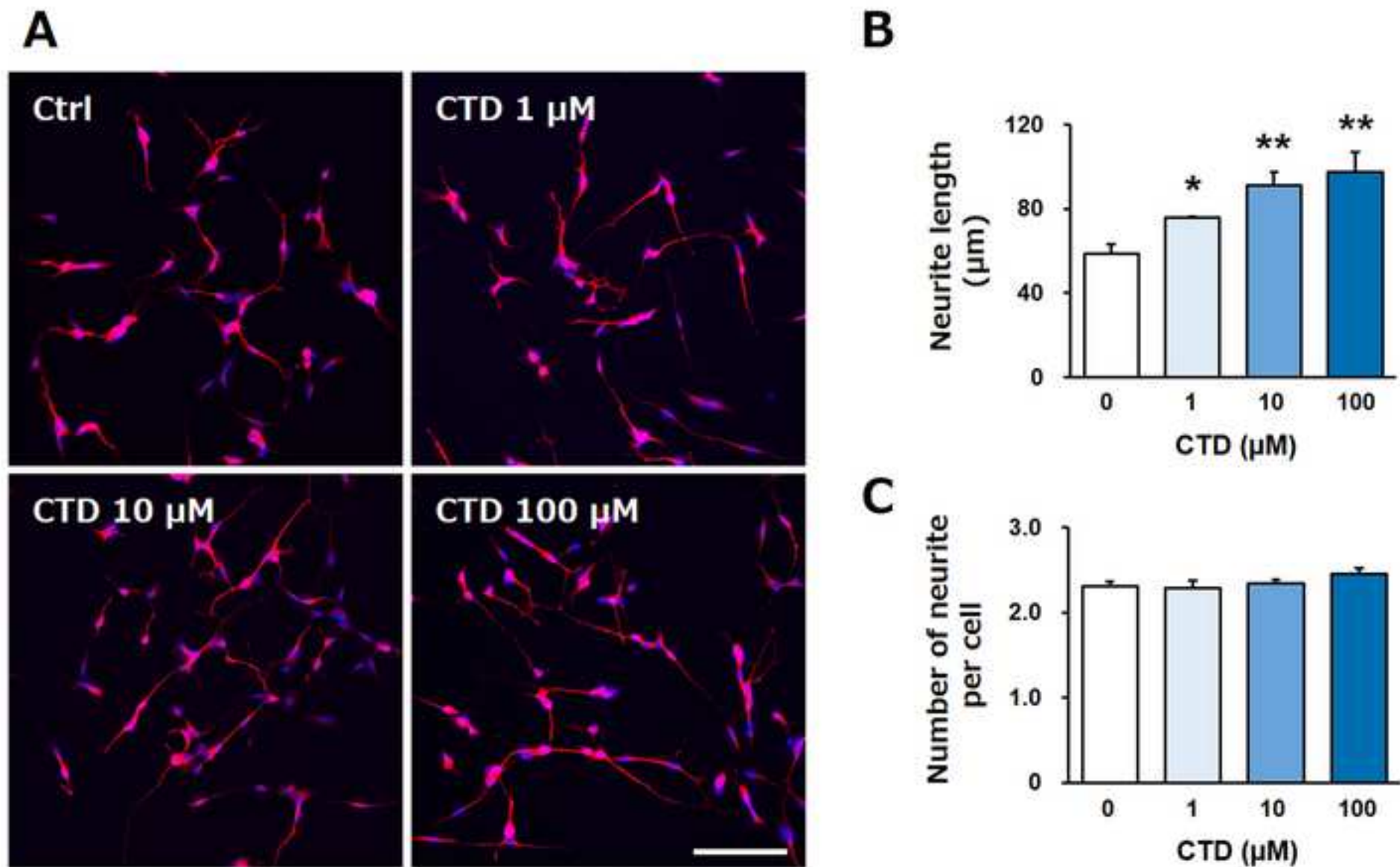
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