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Sensitivity of Dopaminergic Neuron Differentiation from Stem Cells to Chronic Low-Dose Methylmercury Exposure

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Perinatal exposure to low doses of methylmercury (MeHg) can cause adult neurological symptoms. Rather than leading to a net cell loss, the toxicant is assumed to alter the differentiation and neuronal functions such as catecholaminergic transmission. We used neuronally differentiating murine embryonic stem cells (mESC) to explore such subtle toxicity. The mixed neuronal cultures that formed within 20 days contained a small subpopulation of tyrosine hydroxylase (TH)–positive neurons with specific dopaminergic functions such as dopamine transport (DAT) activity. The last 6 days of differentiation were associated with the functional maturation of already preformed neuronal precursors. Exposure to MeHg during this period downregulated several neuronal transcripts, without affecting housekeeping genes or causing measurable cell loss. Profiling of mRNAs relevant for neurotransmitter systems showed that dopamine receptors were coordinately downregulated, whereas known counterregulatory systems such as galanin receptor 2 were upregulated. The chronic (6 days) exposure to MeHg, but not shorter incubation periods, attenuated the expression levels of endogenous neurotrophic factors required for the maturation of TH cells. Accordingly, the size of this cell population was diminished, and DAT activity as its signature function was lost. When mixed lineage kinase activity was blocked during MeHg exposure, DAT activity was restored, and the reduction of TH levels was prevented. Thus, transcriptional profiling in differentiating mESC identified a subpopulation of neurons affected by MeHg, and a pharmacological intervention was identified that specifically protected these cells.

Key Words: **embryonic stem cells; neurotoxicology; developmental neurotoxicity; metals; toxicity; chronic; dopamine.**

Differentiating stem cells are emerging as test systems to model developmental toxicity. Some differentiation protocols are well suited to create mixed populations of different subtypes of neurons to study fate decision pathways (Gaspard and Vanderhaeghen, 2010) or can be used as assay systems to detect adverse effects of chemicals on the balance of neuron types during central nervous system development. They can reproduce aspects of the formation and patterning of tissues classically studied *in vivo*, whereas they take advantage from the ease of experimental manipulation offered by *in vitro* cultures (Kuegler *et al.*, 2010; Zimmer *et al.*, 2011).

The best-studied tool compound associated with developmental neurotoxicity (DNT) is methylmercury (MeHg) (Grandjean and Landrigan, 2006). Data derived from various animal models (Onishchenko *et al.*, 2007) and *in vitro* studies (Tamm *et al.*, 2008) demonstrate that MeHg can affect the developing brain at particularly low concentrations (Castoldi *et al.*, 2008; Clarkson, 1997). The subtle effects of low-dose mercury exposure are not associated with gross morphological changes within the developing brain (Slotkin *et al.*, 1985) but rather result in altered functions specific for certain neurotransmitters such as dopamine (Gimenez-Llort *et al.*, 2001). Pathophysiological effects may manifest years or even decades after exposure to the toxicant (Newland and Rasmussen, 2000). Altered neuronal wiring or differentiation decisions are one possible explanation for such a long-term memory of toxicant exposure.

The assumed molecular mechanisms of MeHg toxicity range from oxidative stress over interference with calcium homeostasis to binding to protein sulfhydryl groups affecting, e.g., microtubule function (for review, see Johansson *et al.*, 2007). Possibly, the relevant targets differ from cell type to cell type and may be specific for certain developmental stages and exposure conditions. Higher concentrations of MeHg affect various functions of different neural cell types (Morken *et al.*, 2005). A short pulse of low nanomolar concentrations slows the differentiation of neural stem cells (NSC) via activation of metalloproteinases involved in the notch signaling pathway (Tamm *et al.*, 2008). During later development and maturation of neurons, the role of notch signaling becomes minor and MeHg may then predominantly affect other targets, which may be related to migration (Moors *et al.*, 2009) or neurite outgrowth (Radio *et al.*, 2010). For instance, kinase signaling pathways can be modified, as exemplified by the activation of

the c-jun N-terminal kinases by MeHg (Fujimura *et al*., 2009). This pathway and especially the associated mixed lineage kinases (MLK) play an important role in the degeneration of dopaminergic neurons, asubpopulation that is also particularly sensitive to long-term low-dose exposure of MeHg (Dare *et al*., 2003).

As dopaminergic neurons are usually studied as a minor fraction of mixed primary mesencephalic cultures, specific tools have been developed to selectively affect and assess these cells. For instance, the toxicant 1-methyl-4-phenyl-pyridinium (MPP^+) is specifically taken up by dopaminergic neurons via the dopamine transporter (DAT) and may therefore be used to selectively kill these cells in a mixed culture, without adverse effects on other neurons. The structurally related compound 1-methyl-4-phenyl-tetrahydropyridine (MPTP) is not transported by DAT and therefore often used as negative control. The use of MPP^+ as radioactive tracer also allows quantification of the relative abundance of DAT-positive neurons in a mixed culture, as it is accumulated only in these cells. In such short-term uptake experiments only requiring minutes, the potential toxicity of the compound plays no role (Schildknecht *et al*., 2009).

Rodents and humans have been shown to be susceptible to MeHg during the perinatal stage, including gestation and immediate postnatal periods (Castoldi *et al*., 2008; Goulet *et al*., 2003). This time in development is associated with neuronal specification, maturation, and establishment of connectivity (Rao and Jacobson, 2005). Such a distinct phase is well defined and can be modeled with stem cell–based *in vitro* models (Zimmer *et al*., 2011). In murine embryonic stem cell (mESC), it has been demonstrated that subtle changes in the neuronal composition or maturation may be detected using messenger RNA (mRNA) expression as readout (Hogberg *et al*., 2009, 2010; Kuegler *et al*., 2011; Zimmer *et al*., 2011). This study addresses the findings from animal models that low doses of MeHg lead to adverse effects related to the later function of neurotransmitter systems. It investigates whether functional correlates may be observed in a stem cell–based model. We provide proof-of-concept for the suitability of differentiating mESC to detect chronic low-dose toxicity to maturing neurons. Moreover, we studied how transcriptional readouts correlated with functional measures used classically in neurodegeneration research to approach the mechanisms of adverse developmental effects.

MATERIALS AND METHODS

Unless otherwise mentioned, cell culture media and reagents were obtained from Invitrogen (Darmstadt, Germany) and accessory reagents from Sigma (Munich, Germany). All chemicals not specified here in detail are listed in Supplementary table 1.

Cell culture and differentiation. The mouse ES cell line CGR8 (a kind gift from K. Krause, Geneva) was cultured at 37°C in 5% CO_2 in a humidified atmosphere on plastic coated with 0.1% gelatin and routinely passaged every other day. ES cells were cultured in Glasgow's modified Eagle's medium complemented with 10% heat-inactivated fetal bovine serum (FBS) (PAA, Coelbe, Germany), Glutamax, nonessential amino acids, β-mercaptoethanol, and sodium pyruvate. Leukemia inhibitory factor (Millipore) was added at a final concentration of 1000 U/ml.

For neuronal differentiation, the protocol established by (Ying and Smith 2003) was used with slight modifications. Briefly, 1 day before initiating differentiation, cells were seeded under normal culture conditions to reach ~80% confluency after 24 h. The next day, cells were transferred to gelatin-coated plastic plates in N2/B27 medium at a density of 10^4 cells per square centimeter. Medium was changed every other day. After 7 days of differentiation (DoD), cells were transferred to poly-L-ornithine– (10 μg/ml) and laminin (10 μg/ml)-coated plates at a density of 10^4 cells per square centimeter in N2/B27 medium. After a 3-day attachment phase, the medium was changed every other day until DoD20.

Mercury quantification. For determination of the mercury content of DoD20 cultures, cells were incubated with 5nM MeHg according to the standard incubation scheme from DoD14 until DoD20. On DoD20, the medium was removed and cells were washed and detached by scraping in 0.1% Triton X-100 in PBS/5% fetal calf serum. The cell suspension was homogenized by sonication, aliquoted, and frozen until analysis by atomic absorption spectroscopy. All reagents and buffer components were carefully controlled for their mercury content (below detection limit). For calibration and positive controls, control cell lysates were spiked with different MeHg amounts. The detection limit of the method was 400pM in cell lysate matrix.

MPP^+ uptake. The assay was performed as described earlier (Schildknecht *et al*., 2009). Briefly, cells differentiated as described above were washed with Hank's balanced salt solution (HBSS), containing Ca^{2+}, pH 7.4, and then treated with the DAT blocker GBR 12909 (1μM) or a solvent control 30 min before 5μM ^{1}H-MPP^+ + 4265 becquerel per well ^{3}H-MPP^+ were added. After 60 min, the supernatants were collected, cells were washed gently five times with warm HBSS, and then lysed with PBS/0.1% Triton X-100. Radioactivity in cell lysates and the respective supernatants was measured using a Beckman LS-6500 scintillation counter (Beckman Coulter, Brea, CA).

Quantitative real-time PCR. Quantifications were performed exactly as described earlier (Zimmer *et al*., 2011). Briefly, total RNA was isolated using Trizol and reverse transcribed (SuperScript II, Invitrogen). Quantitative real-time (RT)-PCR was performed using a BioRad Light Cycler (Biorad, München, Germany). RT quantification for each gene was performed using SybrGreen. Data were normalized to *Gapdh* mRNA and expressed relative to the amount of untreated/undifferentiated samples using the $2^{-\text{Delta Delta } C(T)}$ method (for a detailed primer list, see Supplementary table 1). SABioscience quantitative PCR Arrays (Neurotransmitter Receptors and Regulators, cat. # PAMM-060, SABiosciences, Frederick, MD) also used the SybrGreen method as mentioned above and are based on validated and intron-spanning primers for target genes and several housekeeping controls. Data were normalized and analyzed using the web-based SABiosciences analyzing tool (http://www.sabiosciences.com/pcr/arrayanalysis.php). The group of downregulated genes not covered comprehensively in results comprised *Chrna2*, *Chrna6*, *Chrnb3*, *Chrng*, *Gabra1*, *Gabra4*, *Gabrq*, *Galr3*, *Glra3*, *Prokr1*, *Nmur1*, *Ntsr1*, *Ppyr1*, *Prchr*, *Sstr3*, *Tacr2* and *Tacr3*.

Immunostaining and Western blotting. For Western blot analysis, total protein was isolated using Trizol according to the manufacturer's manual. The protein concentration in the samples was determined using a BCA Assay Kit (Pierce, Rockford IL). Equal amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes (GE Healthcare, Munich, Germany), incubated with the primary antibody at 4°C overnight, followed by horseradish peroxidase–conjugated secondary antibody, and detected via enhanced chemiluminescence.

Immunocytochemistry was performed as follows: cells were fixed with ice-cold methanol or 4% paraformaldehyde in PBS and permeabilized with 0.1% Triton X-100. After blocking with 10% FBS in PBS at room temperature for 1 h, cells were incubated with primary antibodies at 4°C overnight. After incubation with the appropriate secondary antibodies, images were taken on the original cell culture dishes using an IX81 inverted microscope (Olympus, Hamburg, Germany) equipped with a ×10, NA 0.3, ×20, NA 0.45, and a ×40,

NA 0.6, long-range lens and processed using CellP imaging software (Olympus). For a detailed antibody list, see Supplementary table 1

Cytotoxicity assays. Resazurin reduction and lactate dehydrogenase (LDH) release were used to exclude general cytotoxic effects after 6 days of exposure to MeHg. During the normal neuronal differentiation (described above), indicated concentrations of MeHg were added during normal medium changes (days 14, 16, and 18). On day 20, resazurin (10 µg/ml final) was added 1 h before fluorescence measurement (530 nm_{ex}:590 nm_{em}). LDH activity was determined using the same assay plates as for resazurin reduction. LDH activity was detected separately in the supernatant and the respective cell homogenate. Cells were lysed in 0.1% Triton X-100 in PBS at 4°C overnight. Ten microliters sample was added to 200 µl of reaction buffer containing nicotinamide adenine dinucleotide (100µM) and sodium pyruvate (600µM) in sodium phosphate buffer adjusted to pH 7.4 using 40.24mM K_2HPO_4 and 9.7mM KH_2PO_4 buffer. Absorption at 340 nm was detected at 37°C in 1-min intervals over 20 min.

Quantification of tyrosine hydroxylase$^{-positive}$ areas. DoD20 cultures stained with anti-tyrosine hydroxylase (TH) antibody were analyzed by three different operators blinded to the treatment schedules. Four different wells of a 24-well plate were analyzed for each concentration in three different experiments. TH-positive areas on the entire field (1.9 cm^2) of a culture well were assigned different scores (0×, 1×, or 2×, as indicated in Fig. 5B). The untreated control was set as 100%, and all other values were calculated relative to the respective untreated control.

Statistics and data mining. All data are summarized as means ± SDs from at least three independent biological experiments, with at least three technical replicates per biological experiment, unless otherwise mentioned. Data were presented, and statistical differences were tested by ANOVA with *post hoc* tests as appropriate, using GraphPad Prism 4.0 (Graphpad Software, La Jolla, CA). Published whole-genome microarray expression data (Zimmer *et al.*, 2011) were used to extract all genes upregulated at least twofold between DoD15 and DoD20. Then, this group was analyzed for statistically overrepresented gene ontologies with the web-based analysis tool g:Profiler (Reimand *et al.*, 2007).

RESULTS

Marker Expression during Neuronal Differentiation of mESC

The transcription factor *Pou5f1* (*Oct3/4*), characteristic for pluripotent stem cells, was downregulated early during the neural induction phase. The neuroectodermal marker nestin reached a peak on DoD7 and was again downregulated during the following 13 DoD (Fig. 1A). Neuronal markers like *Tubb3*, *Mtap2*, and *Ncam*, as well as synapse-associated genes including synaptophysin (*Syp*), *Psd95*, and *Sv2a*, continuously increased during the neuronal maturation phase (Fig. 1A). Neuronal subtype diversification was indicated by upregulation of different neurotransmitter metabolism-associated genes such as *Th* and *Gad2* (Fig. 1A).

Extensive immunocytochemical characterization on DoD20 confirmed that the differentiated cells expressed neuron-specific cytoskeleton proteins like MAP2 (Fig. 1B), Tuj1 (Fig. 1B), and NCAM (data not shown), as well as proteins associated with synaptic transmission such as Syp, SV2a (Fig. 1B), SNAP25, and PSD95 (data not shown). The postmitotic neuronal protein NeuN was highly expressed in 70–80% of cells in the culture (Fig. 1B). The culture consisted of different

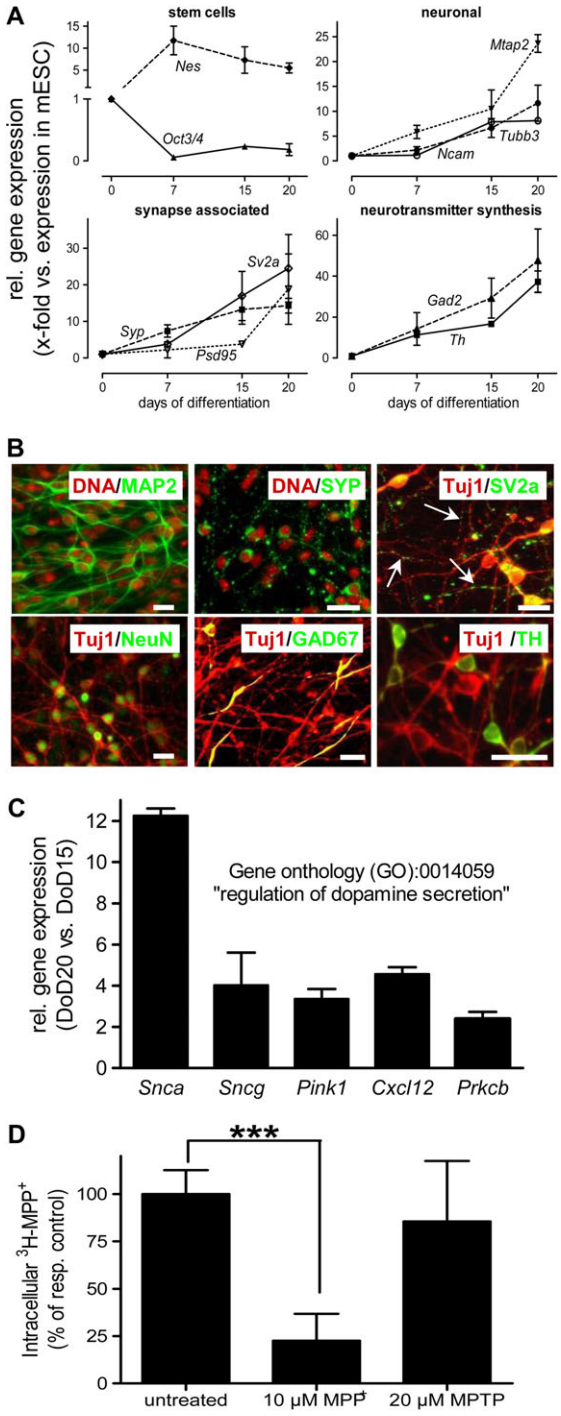

FIG. 1. Generation of a mixed neuronal population from mESC pluripotent mESC was differentiated toward the neuronal lineage and characterized at different stages. (A) Quantitative RT-PCR analysis of relative transcript levels at four time points of differentiation. The expression on DoD0 (undifferentiated mESC) was arbitrarily set to 1. Data for the markers indicated in the micrographs are averages from three independent experiments. (B) Immunocytochemical characterization of neurons on DoD20. Arrows indicate synaptic vesicles within the neurites. Scale bars: 20 µm. (C) Genome-wide analysis of genes upregulated on DoD20 versus DoD15. The GO, ''regulation of dopamine secretion'' was highly overrepresented ($p < 0.001$) and the changes of five genes of this GO, identified on the chip, are displayed. (D) DAT activity of DoD20 neurons after 72 h treatment with toxicants. ***$p \leq 0.001$.

subtypes of neurons confirmed by positive staining for γ-aminobutyric acid (GABA) (data not shown) and GAD67 (Fig. 1B) for GABAergic neurons (48 ± 5%), TH for dopaminergic neurons (< 10%), Vglut for glutamatergic neurons (data not shown), and 5-HT for serotonergic neurons (data not shown).

To obtain more information on changes taking place during the maturation phase of differentiation, a set of published (Zimmer *et al.*, 2011) whole-genome microarray expression data for cultures on DoD15 and DoD20 were queried for genes upregulated at least twofold during the last 6 days of the 20-day differentiation. This list of genes was analyzed for statistically overrepresented gene ontologies in the area of specific biological processes or pathways. The gene ontology "regulation of dopamine secretion" emerged as only highly significant hit (Fig. 1C). We next tested whether the TH-positive cells in the mixed cultures displayed dopamine-related functional properties as suggested by the expression analysis. Untreated DoD20 cells had the ability to accumulate radiolabelled MPP^+ within 60 min. This indicates the presence of functional dopamine transporters (DAT) (Schildknecht *et al.*, 2009) (Fig. 1D). Going one step further, we tested whether this neuronal subpopulation was susceptible to selective cell death triggered via their dopaminergic machinery. DoD20 cultures were treated with either 10μM of the specific toxicant MPP^+ or 20μM of the innocuous precursor MPTP for 72 h (Schildknecht *et al.*, 2009). The toxicants did not affect the viability of the overall culture (not shown) and thus did not trigger unspecific cell death. However, specific toxicity to a dopaminergic subpopulation was indicated by the decreased DAT activity triggered by MPP^+ and by the absence of such an effect after exposure to MPTP (Fig. 1D).

Specific Neurodevelopmental Disturbances Caused by MeHg during the Late Maturation Phase of Neuronal Differentiation

Our toxicity examinations focused on the period of DoD14 to DoD20 when most neuronal precursors were formed and maturation of neuronal subtypes takes place. First, the cytotoxicity of the model toxicant MeHg was assessed and found to be in the range of 60nM (half maximal effective concentration [EC_{50}]). In a next step, multiple endpoints were used to confirm the initial finding (Fig. 2A) that concentrations of $\leq$ 5nM MeHg were tolerated by the cells over a period of 6 days without measurable signs of toxicity. Neither LDH release nor mitochondrial activity (resazurin) or the amount and structure of the neuronal cytoskeleton were affected (Figs. 2B and 2C). In addition, the total amounts of various housekeeping mRNAs remained on a constant level (Fig. 2D).

In the next set of experiments, we investigated whether neuronal-specific mRNAs were affected by MeHg concentrations shown before to be noncytotoxic within the same experimental system. Cultures were exposed to the toxicant from DoD14 to DoD20. On DoD20, whole mRNA was

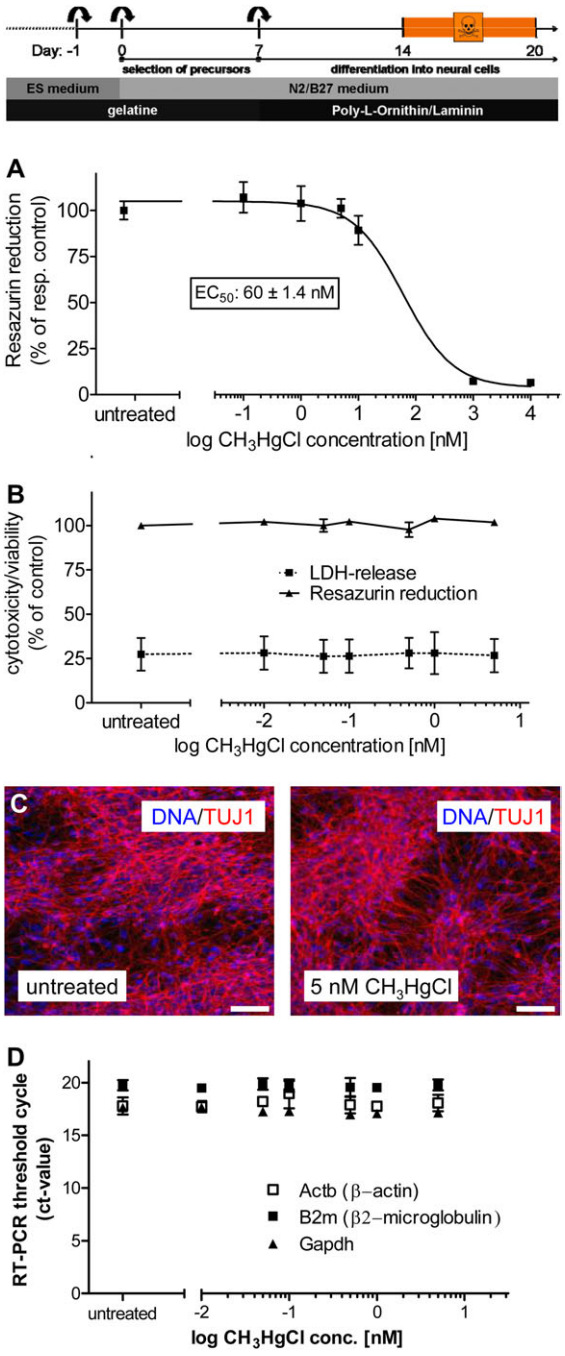

FIG. 2. Identification of CH_3HgCl concentrations that do not affect overall viability of neurons differentiating from mESC. All toxicity experiments were performed according to a standardized scheme (top). Methylmercurychloride was added in all experiments for the last 6 DoD (orange box). (A) Determination of the noncytotoxic concentration range of MeHgCl. (B) Detailed confirmation of noncytotoxic range of MeHgCl concentrations. Note the different scaling of the x-axis. (C) Immunocytochemical analysis of the neurite network density and structure after treatment with 5nM MeHgCl. Scale bar: 50 μm. (D) Quantitative RT-PCR analysis of the expression of three nonneuron-specific housekeeping genes (*Actb*, *B2m*, and *Gapdh*) after treatment with different noncytotoxic concentrations of mercury. Displayed are the threshold cycle numbers for each mRNA ($n = 2$, with three technical replicates per n) as means ± SDs.

isolated and expression levels of the synapse marker *Syp* and of neuronal cytoskeletal proteins (*Mtap2* and *Tubb3*) were analyzed. MeHg lead to a concentration-dependent decrease in expression up to about 50% (Fig. 3A). Markers related to neurotransmitter synthesis (*Th* and *Gad2*) were similarly affected (Fig. 3B). This neuron-specific adverse effect was also observed on the protein level, as we found that the neuron-specific isoform of the beta-III tubulin protein, detected by the Tuj1 antibody, also decreased with increasing concentrations of mercury (Fig. 3C). Negative control compounds, such as ascorbic acid or acetylsalicylic acid, had no effect on mRNA expression of neuron-specific genes like *Tubb3*, *Syp*, *Gad2*, or *Th* (Fig. 3D).

To obtain information on the actual cellular concentration of MeHg that triggered the specific DNT observed, cells were analyzed for their mercury content by atomic absorption spectroscopy. The content after 6 days of exposure to 5nM was 2 ± 0.15 pmol/mg cellular protein or 10 ± 0.74% of the total amount added. This suggests that MeHg had accumulated in the cells as expected from its hydrophobic properties. Based on average volume to protein ratios of brain cells, the actual cellular concentration was calculated to be in the range of 30–250 ppb, which is still in the lower end of concentrations known to cause *in vivo* effects after gestational exposure (Supplementary table 2).

Dysregulation of Genes Associated with Neurotransmitter Metabolism and Signaling

To assess potential effects of sublethal concentrations of MeHg on neurotransmitter signaling and metabolism, we examined transcripts of 84 relevant genes assembled in a PCR array. When mRNA levels were compared between control cultures and cells that had been treated with MeHg from DoD14 to DoD20, the majority of genes was observed to be unaffected (< twofold changes). This result underscores that the MeHg concentration was chosen in a way not to generally impair viability. However, the expression of two genes (cholinergic receptor, nicotinic, epsilon polypeptide [*Chrne*] and *Galr2*) was increased ≥ twofold by MeHg and the expression of 22 genes was decreased ≥ twofold (Fig. 4A). As a technical control for the validity of these data, we chose one gene from each regulation group (*Gad1* [down-regulated], melanocortin 2 receptor [no change], and *Galr2* [upregulated]), designed independent PCR primers, and reanalyzed the samples by conventional quantitative RT-PCR. This analysis fully confirmed the initial results and suggests a high validity of the overall data set (Fig. 4B). The biological heterogeneity of the downregulated genes reflects the broad *in vivo* actions of MeHg affecting different neuronal features. Interestingly, one conspicuous group of functionally related genes that were coordinately down-regulated were the dopamine receptors *Drd1a*, *Drd2*, and *Drd3*. Only *Drd4* (low overall expression) was not affected by the treatment (Fig. 4C).

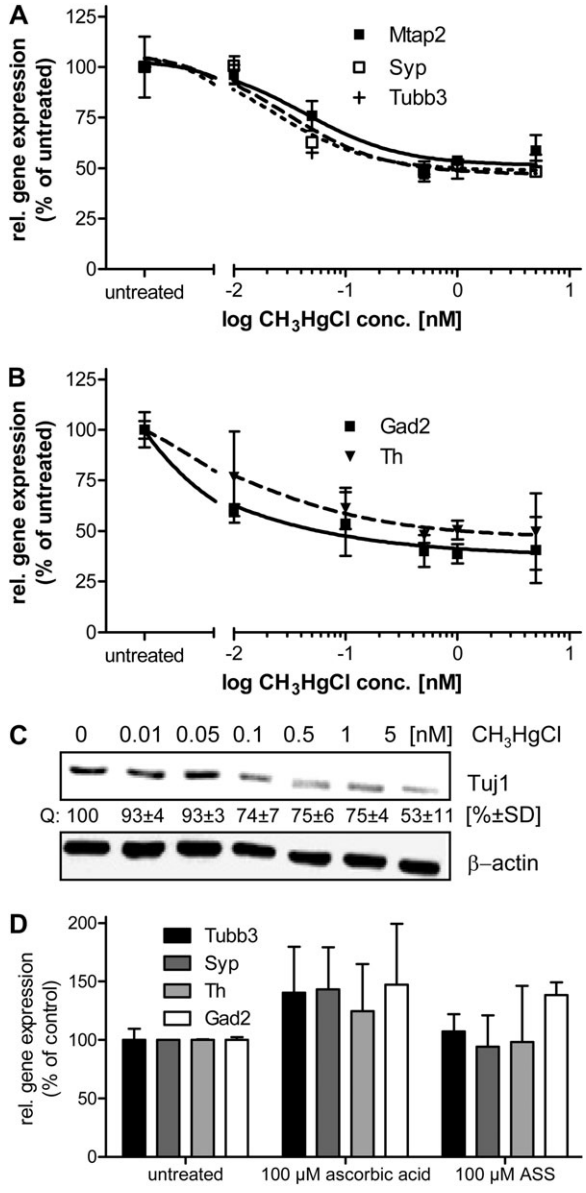

FIG. 3. Specific impairment of neuronal differentiation by noncytotoxic concentrations of CH_3HgCl. (A and B) Under the same conditions as in Figure 2, noncytotoxic concentrations of CH_3HgCl resulted in a concentration-dependent decrease of neuron-specific mRNAs. (C) Western blot analysis of the same cells indicated a decrease of beta-III tubulin protein (Tuj1 antibody). Beta-actin was used as internal loading control. A representative blot is shown. Q, quantification of the ratios of beta-III tubulin/beta-actin from two independent experiments. (D) Cells were treated instead with 100µM ascorbic acid or acetylsalicylic acid as controls, and mRNA expression of four neuron-specific mRNAs was analyzed (data are means ± SDs from two experiments [each performed in technical triplicates]).

Depletion of Dopaminergic Neurons after Treatment with Noncytotoxic Concentrations of MeHg

As different transcriptional profiling analyses pointed to the dopaminergic signaling and response system as one of the targets of MeHg in differentiating mESC, we investigated

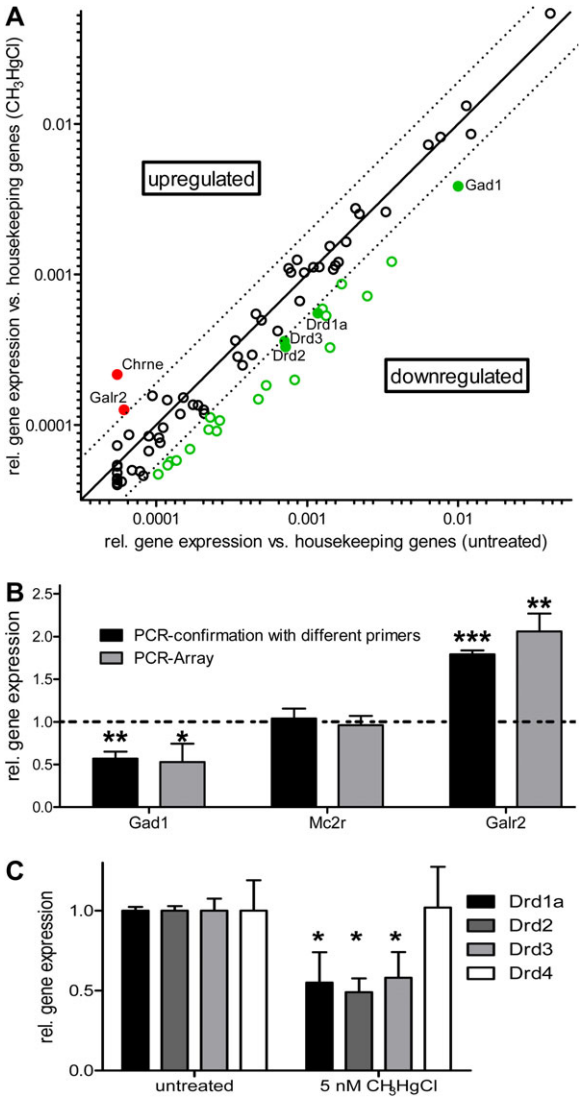

FIG. 4. Specific dysregulation of genes associated with neurotransmitter metabolism and signaling. Differentiating mESCs were exposed to 5nM CH_3HgCl for 6 days as in Figure 2. RNA from three independent differentiations was prepared from treated and control cells and each of them was analyzed twice by quantitative PCR (qPCR). (A) Scatter plot of the data obtained with a qRT-PCR array. The solid line indicates equal mRNA levels in treated and untreated cells. The area above contains genes upregulated and the area below genes downregulated by CH_3HgCl. Dashed lines indicate twofold regulation. (B) Comparison of results obtained with commercially available focused RT-PCR arrays (gray bars) and independently designed primer pairs (black bars). mRNA levels were standardized to the housekeeping gene *Gapdh* and normalized to the mean expression in the corresponding untreated sample, which was arbitrarily set to 1 (indicated by the dashed line). (C) Statistical analysis of the regulation of the expressed dopamine receptor subtypes (*Drd1–Drd4*) after CH_3HgCl treatment. $*p \leq 0.05$. All data are means of independent triplicates. *Mc2r*, melanocortin 2 receptor.

whether the development of functional dopaminergic features was directly affected. We therefore measured DAT activity of untreated cells (positive control) in comparison to cells treated from DoD14 until DoD20 with MeHg or ascorbic acid (negative control). Cultures treated with 500pM or 5nM MeHg demonstrated a significantly decreased DAT activity compared with control cells, whereas 50pM MeHg and ascorbic acid showed no effect (Fig. 5A). In addition to this biochemical endpoint, we also investigated whether the number of TH-positive neurons formed in the cultures was affected by MeHg. The distribution of these cells in the culture dish was characterized by clustering within small islands separated by areas devoid of TH staining (Fig. 5B). To obtain a quantitative measure, we manually scored the total number of islands per well by blinded operators. According to the island size, they were given the scores ×0, ×1, or ×2, with ×0 designating areas with none or only randomly distributed TH cells, ×1 referring to clusters of up to 10 cells, and ×2 designating larger clusters (Fig. 5B). This scoring resulted in robust and reproducible results across different observers and showed that MeHg (5nM) significantly decreased the amount of TH-positive areas, whereas the negative control substance ascorbic acid did not show any effect (Fig. 5C).

Effects on TH Expression and Its Regulators by Chronic MeHg Exposure

MeHg ($\leq$ 5nM) did not affect *Th*, *Tubb3*, or *Syp* expression after 48 h of acute exposure of differentiated neurons and even 72 h affected the viability only with an EC_{50} of 12 ± 0.3μM (data not shown). We therefore hypothesized that dopaminergic neurons were not affected by the toxicant directly but due to interference with their differentiation program and/or factors required for their development. As this may require chronic exposure to the toxicant, we examined how MeHg affected *Th* and its differentiation factors after incubation of DoD14 neurons for 2, 4, and 6 further days. First, the time course of the gene expression of sonic hedgehog (*Shh*), *Wnt1*, *Th*, transforming growth factor beta, fibroblast growth fator-8 (*Fgf8*), and glial-derived neurotrophic factor (*Gdnf*) was examined in untreated cells in this time period (Supplementary fig. 1). After MeHg treatment, *Shh*, *Gdnf*, and *Wnt1* were affected after 6 days ($p < 0.01$), but not after 2–4 days. The relative expression of *Fgf8* was decreased by MeHg already after a 4-day exposure (Fig. 6). This was paralleled by the kinetics of *Th* downregulation (Fig. 6). Various notch inhibitors (10–25μM of the metalloprotease inhibitor GM6001 and 1μM of the potent gamma secretase inhibitor LY450139) had no rescuing effect at all in our model system (not shown). Thus, the mechanism of this chronic developmental toxicity appeared to be different from the reported MeHg effects on early NSC (Tamm *et al.*, 2008).

Pharmacological Inhibition of Neurodevelopmental Effects of MeHg

We next examined several potential mechanisms for the effect of MeHg on TH neuron development. Although we did not find unambiguous evidence for the role of oxidative stress

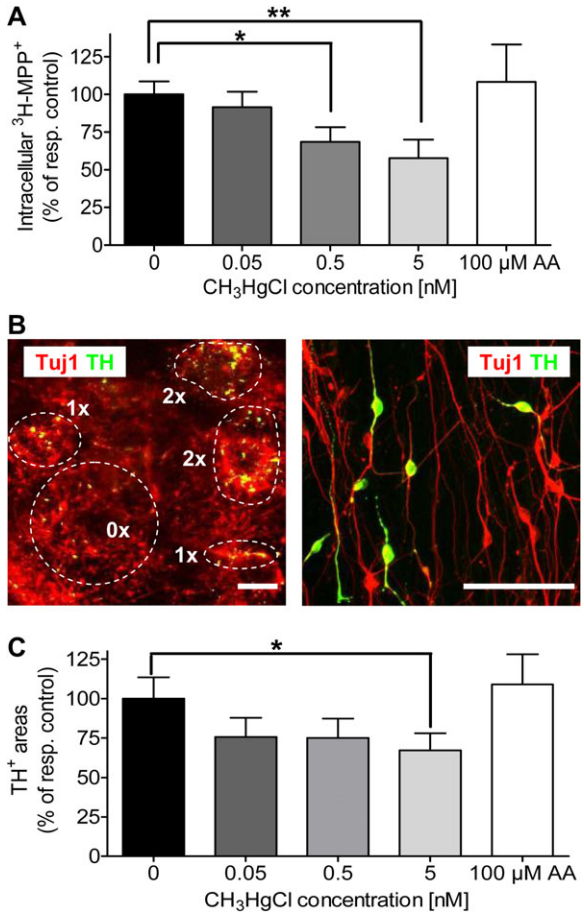

FIG. 5. Loss of dopaminergic neurons after chronic treatment with low concentrations of CH_3HgCl. (A) DAT activity (ability to take up MPP^+) was measured in DoD20 cultures after 6 days of exposure to CH_3HgCl or ascorbic acid (AA). $*p \leq 0.05$, $**p \leq 0.01$. (B) Representative images illustrating the quantification method for TH-positive areas. Left: Score assignment for the different sizes of the TH^+ areas ranging from ×0 up to ×2. Areas were scored according to their size and density of TH-positive neurons. Right: Higher magnification of a ×0 area showing individual TH-positive neurons, but no clustering. Scale bar: 100 µm. (C) Quantification of TH^+ areas obtained in a double-blinded approach as illustrated in (B). The amount of TH-positive cell areas in the respective untreated control was set to 100%. $*p \leq 0.05$.

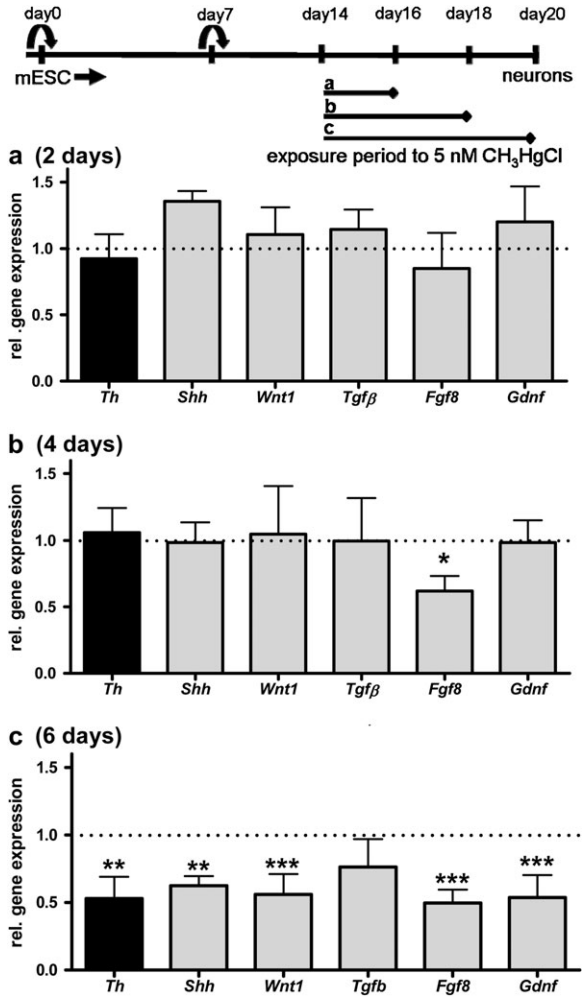

FIG. 6. Requirement of chronic exposure for developmental effects of CH_3HgCl. The treatment schedule to assess the windows of sensitivity for MeHg exposure is displayed on top. Cells were exposed to 5nM MeHg from DoD14 for either 2 (a), 4 (b), or 6 days (c). The mRNA was isolated after the exposure period (indicated by diamonds) and analyzed by RT-PCR for *Th*, sonic hedgehog (*Shh*), *Wnt1*, transforming growth factor beta (*Tgfβ*), *Fgf-8*, and *Gdnf*. The data were normalized to the levels in untreated controls (arbitrarily set to 1, indicated by the dashed line). $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$.

or the heat shock response (not shown), a clear and significant restoration of DAT activity was observed when MLKs were inhibited by CEP-1347, an inhibitor known to reduce dopaminergic degeneration under stressful culture conditions (Boll *et al.*, 2004). Inhibitor concentrations as low as 0.1–0.3µM were sufficient to prevent the effects of 6 days of exposure to 5nM MeHg (Fig. 7A), and the drug effect correlated with reduced c-jun phosphorylation, as detected by Western blot (not shown). Finally, we examined whether the observed functional rescue correlated with restoration of *Th* mRNA expression levels as a potential sign of a restored differentiation process even in the continued presence of MeHg. We analyzed *Tubb3* as general neuronal marker and *Gad1* as marker of the GABA subpopulation and *Th*. As expected from previous findings, all four genes were downregulated by a 6-day exposure to MeHg. Only the level of *Th* was significantly rescued by coincubation with CEP-1347 (Fig. 7B). Thus, although *Th* neurons are only one of the populations that may be affected by MeHg, the role of MLKs in the developmental toxicity of MeHg may be specific for this particular neuronal subpopulation.

DISCUSSION

Here, we demonstrated that neuronally differentiating ESCs can be used to obtain functional and mechanistic information on the toxicity of industrial chemicals like MeHg. We identified and characterized the particular adverse effects on

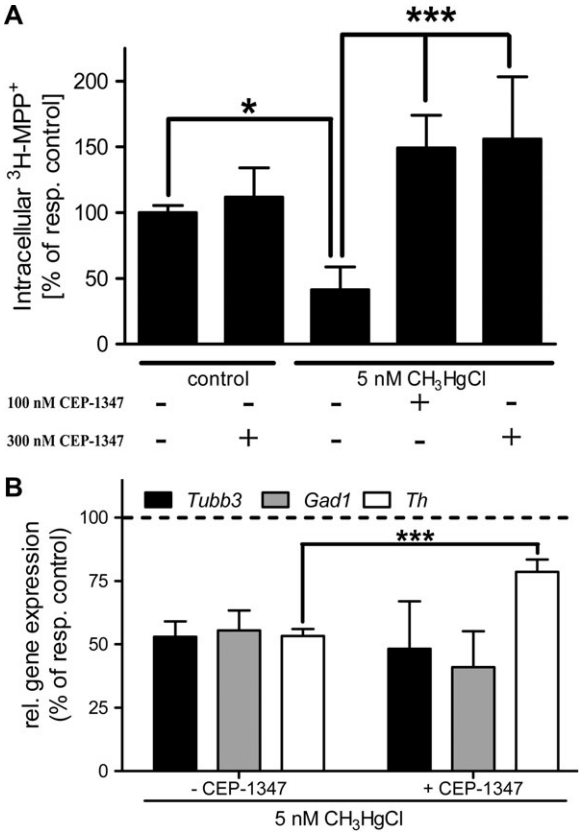

FIG. 7. Protection of dopaminergic neurons from MeHgCl-induced toxicity using CEP-1347. (A) DAT activity (evaluated by MPP^+ uptake) after exposure of DoD14 cells for 6 days to 5nM CH_3HgCl alone or in combination with CEP-1347 as indicated. DAT activity in untreated samples was set to 100%. Data are means ± SDs from four independent experiments. $*p \leq 0.05$, $***p \leq 0.001$. (B) Quantitative RT-PCR analysis of neuronal genes in cells treated for 6 days with 5nM CH_3HgCl in the presence or absence of CEP-1347. The expression in untreated cells was set to 100%, indicated by the dashed line. $n = 3$ independent experiments; $***p \leq 0.001$.

the development of dopaminergic neurons. MeHg affected not only a large variety of mRNAs but also functional properties of the neurons. Importantly, the adverse effects of the toxicant on developmental neuroplasticity were not related to growth inhibition or cytotoxicity. MeHg affected already formed neuronal precursors during their maturation phase and required chronic exposure during this culture period to trigger its effects. We also identified inhibition of MLKs as a strategy to specifically reduce the developmental toxicity of MeHg to differentiating dopaminergic neurons. Thus, this study represents a first step toward using stem cell–derived cells to address questions on mechanisms underlying toxicity.

As biological basis for our test method, we used a differentiation protocol by (Ying and Smith 2003), which yields a mixed neural culture on DoD20. The relative size of cellular populations in this system was very robust (Zimmer *et al.*, 2011) and therefore allowed the detection of toxicant effects on small subpopulations. We showed earlier that the late phase of this differentiation protocol (DoD15–DoD20) is mainly characterized by the maturation of the neuronal system (Zimmer *et al.*, 2011), and we now provide additional evidence that dopaminergic neurons differentiate during this period. The functionality of this neuronal subtype was corroborated by their selective susceptibility to the toxicant MPP^+, which is known to preferentially target dopaminergic neurons *in vivo* (Di Monte *et al.*, 1996) and *in vitro* (Schildknecht *et al.*, 2009) and by functional measurements of DAT activity.

The toxicity of MeHg is widely known from environmental disasters as in Minamata bay. However, human beings encounter the environmental toxicant usually over prolonged periods of time, e.g., during gestation, and at very low-dose exposure. The actually measured final "cellular concentration" of MeHg was 0.3 ppm (Supplementary table 2). This correlates well with the threshold of toxicant brain concentrations resulting in observable clinical effects in humans, which is in the range of 0.3 ppm (Burbacher *et al.*, 1990). In animal models, effects have been reported at brain concentration as low as 0.1 ppm (Burbacher *et al.*, 1990). Thus, the model system presented here was able to identify and characterize toxicity at cellular levels corresponding to the low end of the reported effective concentration range *in vivo*. Furthermore, our *in vitro* model reflects a low-dose, multiple exposure scenario, not unlike the gestational exposure in animal experiments. Only mercury exposure for the full period of 6 days resulted in a decrease of expression of mRNAs important for the development of dopaminergic neurons such as *Shh* or *Wnt1*. The downregulation of *Fgf8* might be one of the early biomarkers of mercury-induced developmental toxicity. In a previous study, it was found that such a disruption of patterning signals can lead to a shift in culture composition without any signs of cytotoxicity (Zimmer *et al.*, 2011).

Our study adds to earlier knowledge on potential effects by MeHg by showing that differentiating mESCs were sensitive to the toxicant during the neuronal maturation phase. The combination of expression profiling and biochemical analysis allowed the identification of a neuronal subtype and function affected. This marks a turning point in the use of ESC-derived systems in toxicology, as the study not only corroborated earlier findings but also showed a potential applicability of the model. It rather yielded new data on the pattern of transcripts affected, correlated the mRNA measurements with functional data, and identified a potential pathway of toxicity and its counterregulation.

It is still unclear, which *in vitro* endpoints correspond best to functional impairments *in vivo*. For instance, rodents exposed to MeHg may display an altered locomotor activity without histopathological changes. A possible correlate may be the decrease of general neuronal markers such as *Tubb3*, in the absence of any signs of cytotoxicity (resazurin reduction, LDH release, expression of housekeeping genes, and neurite network density). Our favored hypothesis is that the reduction of these

general neuronal markers is rather due to a lower expression of these genes in most neuronal cells of the culture and rather not due to a loss of cells. This implies that the affected cells on DoD20 would still be defined as neurons, but with a lower expression of neuronal markers. In some cells, this might lead to altered physiological performance or, in extreme cases, to a loss of function.

The decreased amount of dopaminergic neurons in our model might therefore be explained either by an impaired differentiation or by selective toxicity. The exact mechanisms may be very complex and need further investigation in the future. Our findings that the total DAT activity was decreased after a 6-day MeHg exposure is in line with reports from *in vivo* experiments, reporting a decreased [^{3}H]-dopamine uptake in mercury-treated rat synaptosomes (Rajanna and Hobson, 1985). Our observation of a decrease in TH^+ areas after mercury treatment, in combination with nondetectable cytotoxicity, might be explained by a shift in neuronal culture composition, e.g., by an impaired differentiation of dopaminergic neurons which results in cells with strongly reduced expression of some of their marker genes. Such effects have been demonstrated elsewhere. For instance, human dopaminergic neuronal precursors can differentiate to neurons with strongly decreased TH expression in suboptimal culture environments (Paul *et al*., 2007), and a surviving dopaminergic neuronal subpopulation in mice treated with MPTP can transiently lose TH expression (Sager *et al*., 2010).

A prominent group of receptors affected by MeHg treatment in our model were the dopamine receptors. This correlates well with animal studies (Dare *et al*., 2003; Gimenez-Llort *et al*., 2001; Rossi *et al*., 1997) and demonstrates that stem cell–based systems may detect changes *in vitro*, which are normally only detectable in an entire organism. A more detailed analysis of mRNAs associated with neurotransmitter synthesis and regulation revealed additional changes in the relative expression of mRNAs that have been described earlier in *in vivo* studies (Johansson *et al*., 2007). For instance, the genes for *Galr2* and *Chrne* (member of the nicotinic receptor family) were upregulated. We assume that this counterregulation may serve as an endogenous protective function, as described earlier for MeHg-induced antioxidant systems (Ni *et al*., 2010; Woods and Ellis, 1995). The protective function of galanin in experimental nerve injury is often promoted via the galanin receptor 2 (*Galr2*) (Elliott-Hunt *et al*., 2007; Lang *et al*., 2007). *Chrne* and related receptors have also been shown to play a role in neuroprotection in a large variety of circumstances, ranging from brain injury over MPTP toxicity to ethanol exposure (Bencherif, 2009).

Although MeHg is one of the best-known DNT compounds, the mechanisms underlying its DNT in the low-dose range are still unclear. In our system, notch signaling did not appear to be a relevant target, as neither the alpha secretase inhibitor GM6001 nor complete gamma secretase inhibition resulted in any protection from MeHg (data not shown). Instead, we explored the MLK signaling pathway, as it has been demonstrated earlier that CEP-1347 is able to increase the survival of immature TH-positive cells under stressful culture conditions (Boll *et al*., 2004). Our data show that overall DAT activity was brought back to control levels by the MLK inhibitor CEP-1347 and that the amount of *Th* mRNA was significantly increased. In contrast to that, the MeHg-induced decrease of *Tubb3* and *Gad1* mRNA expression levels was not altered by cotreatment with CEP-1347. Thus, the rescuing effect of the MLK inhibitor appeared specific for the dopaminergic subpopulation and suggests a specific effect of MeHg on this particular neuronal subtype.

To our knowledge, the present study is the first to investigate functional readouts to detect DNT in an ESC-based *in vitro* system and to identify an intervention that reduced the functional impairment by a toxicant. Such studies have been hampered in the past by the low robustness and high variability of stem cell systems, despite their great theoretical potential in developmental toxicology. Thanks to improved protocols developed by others (Ying and Smith, 2003), we have been able to ask this new type of questions. Our data suggest that some toxicant effects may only be noticeable late in life when the normal number of dopaminergic neurons declines. It has been hypothesized that impaired development of TH neurons early in life is linked to neurodegenerative diseases such as Parkinson's disease (Calne *et al*., 1986; Landrigan *et al*., 2005; Weiss *et al*., 2002). New sensitive assay systems, like the one described here, may contribute to clarify the role of early exposure to industrial chemicals such as MeHg in neurodegenerative diseases.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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