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Effects of Pesticides on Neuronal and Glial Cell Differentiation and Maturation in Primary Cultures

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1. Introduction

Since many pesticides are developed to target the nervous system of different organisms their effects on the human brain are of great concern, in particular the effects on the immature brain. The acute neurotoxicity of pesticides is well-known from occupational exposure studies, poisoning events and suicide data (Kimbrough *et al.,* 1989). Furthermore, developmental neurotoxicity (DNT) effects, such as reduced short-term memory, hand-eye coordination, drawing ability and visuospatial deficits have been observed in several epidemiological studies (Grandjean *et al.,* 2006; Guillette *et al.,* 1998; Ruckart *et al.,* 2004).

It is widely accepted that the developing central nervous system (CNS), is much more vulnerable to injury induced by different classes of chemicals, including pesticides, than the adult CNS. This is partly due to the fact that the adult brain is well protected against chemicals by the blood brain barrier (BBB) while children's BBB is not fully differentiated until around 6 months after the birth (Adinolfi, 1985; Tilson, 2000) and at the same time they have increased absorption and a diminished ability to detoxify many exogenous compounds in comparison to that of adults (NRC, 2000).

Moreover, the development of the CNS is a very complex process involving several different important events, *e.g.* differentiation of progenitor cells, proliferation and cell migration, synaptogenesis, myelination, cell death, synthesis of neurotransmitters, formation of receptors, trimming of connections or electrical activity stimulation. These events are occurring within strictly controlled time frames and therefore each event creates different windows of vulnerability to xenobiotic exposure (Rice and Barone, 2000; Rodier, 1994; Rodier, 1995). Furthermore, the brain consists of many different cell types (*e.g.* neuronal, glial and endothelial cells) that have specific functions and different roles. Also each cell type is produced at a defined moment during the development and is therefore susceptible to environmental disturbances at different developmental time periods. Some events take place during a very short time period and interference by chemicals during these stages could have serious consequences for the individual.

Exposure to pesticides, industrial chemicals, or drugs, might contribute to the increasing incidences of neurodevelopmental disorders (Boyle et al., 1994; Grandjean and Landrigan, 2006; Lein et al., 2007; Schettler, 2001). There are some studies suggesting that one out of every six children has developmental disabilities and many of them are neurodevelopmental disorders such as learning disabilities, dyslexia, attention deficits, hyperactivity disorders and autism (Boyle et al., 1994; Grandjean and Landrigan, 2006; Lein et al., 2007; Schettler, 2001). Moreover, a report from the National Academia of Science (NAS) suggests that 28 % of all major developmental disorders in children are linked entirely or partly to environmental exposures (NRC, 2000).

A vast amount of papers in the peer-reviewed scientific literature recommends that the exposure to toxic substances during development should be considered as a non-negligible risk factor for triggering neurodevelopmental disorders in children. However, due to lack of studies only a very few substances have been identified as developmental neurotoxicants so far (Grandjean and Landrigan, 2006). Furthermore, in the case of pesticides there are no general requirements that they have to be tested for DNT effects before their registration in Europe or the US. The US Environmental Protection Agency (EPA) does only require developmental and reproductive toxicity studies for registration of pesticides used in food. Currently, DNT testing is only recommended on a case-by-case basis and at regulatory level discussions are ongoing to establish a number of criteria in the different regulatory frameworks (for chemicals, pesticides, food additives *etc.*) to be used as triggers for deciding when testing is needed (Coecke *et al.,* 2007). Moreover, the current DNT guidelines (Organisation for Economic Co-operation and Development (OECD) TG 426 and US EPA 712-C-98-239) (OECD, 2007; US EPA, 1998) are based entirely on *in vivo* studies that are time consuming, complex, costly and not suitable for the testing of large numbers of substances. In addition, the interpretation of the data from these studies can be difficult to predict human toxicity and often does not provide sufficient amount of information to facilitate regulatory decision-making. This puts pressure on all stakeholders involved (academia, industry and regulatory bodies) to look for alternative methods such as *in vitro* testing, *in silico* modeling and application of non-mammalian models that could accelerate the process of DNT testing for regulatory requirements. Both *in vitro* and non-mammalian test systems offer the possibility of providing early screening tools for a large number of substances, to identify the chemicals with DNT potential and additionally could be particularly useful in characterizing the compound-induced mechanisms of toxicity of various developmental processes.

We have evaluated an *in vitro* approach to detect DNT-induced effects after exposure to pesticides using primary cultures of rat cerebellar granule cells (CGCs). Pesticide toxicity was evaluated at the level of critical neuro-developmental processes, measured by gene expression and immunocytochemistry staining. Five pesticides with different mode of actions were selected to evaluate if our *in vitro* approach could be a useful tool for detection of DNT effects. Indeed the results allowed us to identify which cell type (neuronal or glial) and which state of development (proliferation, differentiation and maturation) was affected by pesticide exposure.

2. Material and methods

2.1 Chemicals and reagents

Reagents for cell cultures were purchased from Gibco Invitrogen (Milano, Italy); DMEM, fetal bovine serum, horse serum, L-glutamine, gentamicin, versene, HEPES and from Sigma-Aldrich (Milano, Italy); Poly-L-lysine, D (+) glucose and potassium chloride.

2.2 Primary cultures of rat cerebellar granule cells (CGCs)

The primary cultures of cerebellar granule cells (CGCs) were prepared from 7-day old Wistar rat pups as described previously (Hogberg *et al.,* 2009). The cerebella were dissociated in versene solution (1:5000) and plated at 0.25 x 10^6 cells/cm^2 in 12- or 96-well plates (Costar) coated with poly-L-lysine (0.01% diluted 1:10 (v/v) in sterile MilliQ water). Cultures were maintained in DMEM supplemented with 5% heat inactivated horse serum, 5% heat inactivated fetal bovine serum, 13 mM glucose, 0.5 mM HEPES buffer, 2 mM L-glutamine, 25 mM KCl and 10 µg/ml gentamicin. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2. The medium of CGCs was not changed throughout the whole experimental period as these cells have to be cultured in self-conditioned medium. Cell samples from control (non-treated) and treated cultures were prepared at 1, 4 and 12 Days *In Vitro* (DIV) for real time PCR analysis of mRNA expression.

2.3 Pesticide treatments of CGCs

Five pesticides (parathion, dichlorvos, parquat, pentachlorophenol and cycloheximide) and one non-neurotoxic compound (aspirin) were studied. All compounds were purchased from Sigma-Aldrich (Milano, Italy). To prepare the stock solutions toxicants were dissolved in culture medium or dimethyl sulphoxide (DMSO). The concentrations of tested pesticides were chosen based on preliminary range-finding experiments, where wide ranges of concentrations have been tested using the Alamar Blue (AB) (resazurin, Sigma, Milano, Italy) cell viability assay (data not shown). In final experiments three non-cytotoxic concentrations (shown in Table 1-7) were selected based on the AB assay results. In the case of pesticides dissolved in DMSO, a non-cytotoxic concentration (0.5% (v/v)) of DMSO was used and it was constant in all wells of the test plates, independently from the studied chemical concentrations. Twenty-four hours after isolation, the neuronal cultures were exposed to the chemicals for up to 12 DIV, to cover critical developmental processes at various stages of cell maturation. To determine whether the presence of the pesticides influenced the selected gene expression, cell samples were prepared for real time PCR analysis after 4 DIV exposures (immature culture) and 12 DIV (mature culture).

2.4 Assessment of cell viability using Alamar Blue

Cell viability was determined after exposure to the selected pesticides at 4 and 12 DIV using the AB (resazurin) assay (O'Brien *et al.,* 2000) (data not shown). The blue coloured indicator dye resazurin is reduced into fluorescent resorufin by red-ox reactions in viable cells. Resazurin (10 µl of 100 µM stock) in Hank's Buffered Salt Solution was added directly to the 96-well plates, without removing the medium (100 µl). The plates were incubated for 2 h at 37°C, 5% CO_2. After incubation the fluorescence of the resazurin metabolite, resorufin was measured at 530 nm/ 590 nm (excitation/emission) in a multiwell fluorometric reader (Fluoroskan Ascent, Labsystem, Helsinki, Finland).

2.5 Selection of genes for DNT evaluation in CGCs

Seven different genes that could identify the presence of key processes during brain development such as neuronal differentiation (neurofilament (NF) 68 and 200) and functional maturation (N-methyl D-aspartate glutamate receptor (NMDA-R) and gamma-aminobutyric acid A receptor ($GABA_A$-R), proliferation and differentiation of astrocytes (S100β) as well as the presence of neural precursor cells (nestin) were studied.

2.6 RNA purification, reverse transcription and quantitative real-time PCR

Cell samples for analysis of mRNA expression were lysed and total RNA extraction was performed according to the manufacturer's protocol of RNeasy Mini Kit (Qiagen, Milan, Italy). Any contaminating DNA was removed by digestion using an RNase-free DNase set (Qiagen). RNA concentration and protein contamination were assessed spectrophotometrically (Biophotometer; Eppendorf, Milan, Italy). Reverse transcription was performed as follows: 500 ng RNA was incubated with 2.5 mM PCR Nucleotide Mix (Promega, Milan Andorra, Italy) and 12.5 µg/ml random primers (Promega) for 5 min at 65°C using a Perkin-Elmer Geneamp PCR system 9600. Subsequently 2 units/µl RNaseOut inhibitor (Invitrogen), 10 units/µl M-MLV reverse transcriptase (Promega) were added with the respective M-MLV buffer (Promega) and the samples were incubated for 10 min at 25°C for annealing, 60 min at 37°C for cDNA synthesis and 15 min at 70°C for inactivation of enzymes. An AbiPrism 7000 sequence detector system in conjunction with TaqMan® Universal PCR Master Mix and TaqMan® Real-Time PCR Assays-on-Demand (Applera Italia, Monza, Italy) was used for investigating the gene expression and the house keeping gene according to the manufacturer's protocol. The primers used were: 18S ribosomal RNA (18S rRNA, Hs99999901_s1) (TaqMan® Gene Expression Assays ID), nestin (Nes, Rn00564394_m1), neurofilament, light polypeptide 68kDa (Nfl, Rn00582365_m1), neurofilament, heavy polypeptide 200kDa (Nefh, Rn00709325_m1), ionotropic glutamate receptor N-methyl D-aspartate 1 (GRIN1, Rn00433800_m1), gamma-aminobutyric acid A receptor delta (Gabrd, Rn01517015_g1), and S100 protein, beta polypeptide (S100β, Rn00566139_m1). Relative RNA quantification was performed using the comparative C_T method, normalizing the data to a standard calibrator (a mixture of samples from the different time points of the cell proliferation and differentiation), and to the 18S rRNA content (Livak and Schmittgen, 2001).

2.7 Immunocytochemistry

CGCs cultures for immunocytochemistry were fixed for 20 minutes with 4 % paraformaldehyde in PBS at room temperature at 1, 4 and 12 DIV. The cells were permeabilised for 15 minutes with 0.1 % TritonX100 and were followed by a blocking step (10 % goat serum) for 2h at room temperature. Primary antibodies (all from Sigma, Milano, Italy) diluted in 1 % goat serum in PBS against GFAP (mouse monoclonal 1:800), nestin (rabbit, 1:200) and NF-200 (rabbit, 1:1000) were applied to the cells over night at 4°C. Subsequently, the secondary antibodies, goat anti-mouse IgG Alexa 546 (1:1000) and IgG Alexa 488 (1:1000) (Gibco Invitrogen, Milano, Italy) were applied. Cell nuclei were stained by Hoechst 33342 (10 µg/ml) purchased from Molecular Probes Europe (Leiden, The Netherlands). Controls for specific immunostaining were performed by omitting the primary antibodies from the procedure. All stained cultures were examined by fluorescent microscopy (Olympus IX70, Hamburg, Germany).

2.8 Statistical analysis

The GraphPad Prism 5.0 (GraphPad software, San Diego, USA) program was used for statistical analyses. All data given are the means of at least three independent experiments performed in duplicates ± standard error of the mean (S.E.M.). Two-way ANOVA was performed to assess differences between treated and non-treated cultures.

All data were log-transformed to achieve Gaussian distribution. Statistical significance was indicated as follows * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ treated vs. Control.

3. Results

3.1 Characterization of primary cultures of CGCs during development

Initially, control (non-treated) cultures were characterized over time using phase-contrast microscopy and immunocytochemistry. The morphology of the cell cultures (as seen by phase-contrast microscopy) was dramatically changing over the time starting with round cell bodies at 1 DIV (Fig. 1A). At 4 DIV (Fig. 1D) the cultures started to differentiate into a characteristic neuronal morphology with neurite outgrowths which by 12 DIV formed an intense network (Fig. 1G). Immunocytochmistry staining for NF-200 (green) increased over the time, being barely expressed at 1 and 4 DIV (Fig. 1B and E) while the staining showed rich neuronal connections at 12 DIV (Fig. 1H). Similarly, the immunocytochemistry staining for the astrocytic protein GFAP (red) was increasing over the time, not expressed at 1 DIV (Fig. 1C), increased expression at 4 DIV (Fig. 1F) and highly expressed at 12 DIV (Fig. 1I). Only a few cells stained positive for the neural precursor protein nestin (green) with the same intensity at 1 DIV (Fig. 1C), 4 DIV (Fig. 1F) and 12 DIV (Fig. 1I). The observed changes in protein staining for NF-200, GFAP and nestin correlated well with the observed changes in the mRNA expression over the time (data not shown).

3.2 Exposure to the selected pesticides decreased the gene expression of the neuronal markers (NF-68 and NF-200)

To evaluate if exposure to the selected pesticides was affecting the neuronal differentiation in primary cultures of CGCs, two neuronal cytoskeleton proteins, the earlier expressed NF-68 and the later expressed NF-200 were studied. Exposure to all pesticides significantly down-regulated the mRNA level of both NF-68 (Table 1 and Fig. 2A) and NF-200 (Table 2 and Fig. 2B), however, at different concentrations and time points. The effects on the gene expression of NF-68 were as follow, with increasing toxicity starting from pentachlorophenol (50μM), dichlorvos (50μM), parathion (25μM), paraquat (2.5μM) and cycloheximide (0.05μM) (Table 1 and Fig. 2A). Moreover, the significant decrease in gene expression of NF-68 was already observed at 4 DIV for parathion (25μM), dichlorvos (50μM) and cycloheximide (0.05μM) with persisting decrease after exposure to parathion at 12 DIV. In contrast the exposure to dichlorvos and pentachlorophenol reached the control level after the prolonged exposure of 12 DIV. Only the 12 days exposure to paraquat (2.5μM) and pentachlorophenol (50μM) induced significant decrease in the gene expression of NF-68. Increasing toxicity observed at the mRNA level of NF-200 was induced by pentachlorophenol (50μM), dichlorvos (50μM), parathion (10μM), paraquat (2.5μM) and cycloheximide (0.05μM) (Table 2 and Fig. 2B). The decrease was already observed at 4 DIV for all studied pesticides. However, after 12 days exposure to dichlorvos and cycloheximide the gene expression of NF-200 went up again. The observed effects suggest that the neuronal differentiation and morphology could be affected by exposure to pesticides and was confirmed by phase-contrast microscopy after the exposure to 2.5 μM paraquat for 12 DIV (Fig. 1J). Moreover, the decreased expression of NF-200 was confirmed at the protein level by immunocytochemistry after exposure to the same concentration of paraquat (2.5 μM) for 12 DIV (Fig. 1K). Exposure to aspirin (negative control) up to 500μM did not induce any changes in the gene expression of the studied neuronal cytoskeleton proteins.

3.3 The mRNA level of the NMDA receptor and the $GABA_A$ receptor was affected differently by exposure to the various pesticides

To evaluate the neuronal maturation, subunits of the NMDA and the $GABA_A$ receptors were studied. Paraquat (2.5μM), parathion (25μM) and pentachlorophenol (50μM) exposure significantly down-regulated the mRNA expression of the NMDA receptor subunit 1 after 12 DIV (Table 3 and Fig. 2C). No significant effect at studied concentrations could be observed after the exposure to dichlorvos and cycloheximide. The mRNA level of the $GABA_A$ receptor subunit delta was affected to a higher degree than the NMDA receptor, as it was down regulated by all pesticides except cycloheximide (Table 4 and Fig. 2D). The decrease was observed at 4 DIV after exposure to (given with increasing toxicity) dichlorvos (50μM), parathion (25μM) and paraquat (2.5μM) and at 12 DIV by pentachlorophenol (50μM), parathion (25μM) and paraquat (2.5μM) exposure (Table 4 and Fig. 2D). Exposure to aspirin up to 500μM did not alter the gene expression of the $GABA_A$ receptor subunit delta or the mRNA expression of the NMDA receptor subunit 1. The obtained results indicate that the neuronal maturation could be affected by exposure to selected pesticides at given concentrations as any disturbance in the expression of NMDA (excitatory receptor) and GABA (inhibitory receptor) could lead to the changes in neuronal function.

3.4 The gene expression of the astrocytic marker S100β was decreased after exposure to paraquat, pentachlorophenol and cycloheximide

Proliferation and differentiation of astrocytes are of great importance during the brain development as they play an important role in the neuronal-glial interaction. To evaluate whether astrocytes were affected by the exposure to pesticides the zinc and calcium binding protein S100β expressed in astrocytes was studied. Exposure to paraquat (2.5μM) at 4 DIV, pentachlorophenol (50μM) at 12 DIV and cycloheximide (0.05μM) at 4 and 12 DIV significantly down-regulated the gene expression of S100β (Table 5 and Fig 2E). These results indicate that the proliferation and differentiation of astrocytes were affected by the exposure to these pesticides that consequently could lead to neuronal and DNT effects. No effects on the mRNA level of S100β were observed after exposure to parathion, dichlorvos and aspirin (negative control) at the selected concentrations (Table 5 and Fig 2E).

3.5 Exposure to parathion and paraquat induced an up-regulation in the mRNA level of the neural precursor marker nestin

To identify the presence of neural progenitor cells, nestin, a neural precursor cytoskeleton protein was studied. Exposure to parathion (50μM) at 4 and 12 DIV and paraquat (2.5μM) at 12 DIV significantly up-regulated the gene expression of nestin (Table 6 and Fig. 2F). Nestin has also been reported to be re-expressed in activated astrocytes after brain injury or neuronal damage and has been recognized as a sensitive marker for reactive astrocytes in the CNS (Chen *et al.,* 2002; Clarke *et al.,* 1994; Rutka *et al.,* 1999). The observed increase in the mRNA expression of nestin could therefore be due to proliferation of precursor cells, higher expression of nestin per cell or and most likely because of re-expression of nestin in astrocytes that became activated in response to the possible neuronal damage. In contrast exposure to pentachlorphenol (50μM) significantly decreased the gene expression of nestin. The increased expression of nestin was confirmed at the protein level by immunocytochemistry after exposure to 2.5 μM paraquat for 12 DIV (Fig. 1L). Exposure to

dichlorvos and cycloheximide at the selected concentrations as well as exposure to aspirin (500μM) did not induce any changes in the mRNA level of nestin (Table 6 and Fig. 2F).

4. Tables and figures

NF-68	DIV 4	DIV12	DIV 4	DIV12	DIV 4	DIV12
Parathion	**10μM**		**25μM**		**50μM**	
% of control	70±8	57±14	38±10***	40±13***	13±9***	2±1***
Dichlorvos	**10μM**		**50μM**		**75μM**	
% of control	111±6	84±3	55±3*	95±5	60±10*	86±3
Paraquat	**0.16μM**		**0.63 μM**		**2.5 μM**	
% of control	89±11	86±13	91±15	67±10	68±11	23±6***
Pentachlorophenol	**10μM**		**25μM**		**50μM**	
% of control	116±17	87±7	95±4	84±11	84±6	22±5***
Cycloheximide	**0.01μM**		**0.05μM**		**0.075μM**	
% of control	71±7	97±12	52±5*	83±7	53±2**	86±2
Aspirin	**100μM**		**250μM**		**500μM**	
% of control	97±4	92±6	99±6	93±7	93±9	108±4

Table 1. Changes in mRNA levels (expressed as % of control) of the neuronal marker NF-68 in primary cultures of CGCs exposed to pesticides (parathion, dichlorvos, paraquat, pentachlorophenol or cycloheximide) or to aspirin (negative control). *P<0.05, **P<0.01 and ***P<0.001 comparing to untreated culture.

NF-200	DIV 4	DIV12	DIV 4	DIV12	DIV 4	DIV12
Parathion	**10μM**		**25μM**		**50μM**	
% of control	51±8***	39±12***	25±7***	26±8***	41±9***	2±1***
Dichlorvos	**10μM**		**50μM**		**75μM**	
% of control	111±4	75±4	42±3***	76±4	33±9***	65±9**
Paraquat	**0.16μM**		**0.63 μM**		**2.5 μM**	
% of control	77±6	97±16	74±13	71±10	49±5**	15±3***
Pentachlorophenol	**10μM**		**25μM**		**50μM**	
% of control	111±27	86±8	81±9	63±8	52±7**	9±5***
Cycloheximide	**0.01μM**		**0.05μM**		**0.075μM**	
% of control	71±9	75±10	47±5***	66±6	35±4***	72±5
Aspirin	**100μM**		**250μM**		**500μM**	
% of control	99±9	99±7	95±10	88±7	95±11	95±9

Table 2. Changes in mRNA levels (expressed as % of control) of the neuronal marker NF-200 in primary cultures of CGCs exposed to pesticides (parathion, dichlorvos, paraquat, pentachlorophenol or cycloheximide) or the non-neurotoxic chemical aspirin. **P<0.01 and ***P<0.001 comparing to untreated culture.

NMDA-R	DIV 4	DIV12	DIV 4	DIV12	DIV 4	DIV12
Parathion	**10µM**		**25µM**		**50µM**	
% of control	87±3	78±15	65±12	46±15***	43±7***	2±1***
Dichlorvos	**10µM**		**50µM**		**75µM**	
% of control	121±8	86±5	97±7	94±5	108±11	91±6
Paraquat	**0.16µM**		**0.63 µM**		**2.5 µM**	
% of control	92±9	89±16	95±17	62±7	72±10	24±7***
Pentachlorophenol	**10µM**		**25µM**		**50µM**	
% of control	105±6	107±15	99±9	94±15	94±5	21±5***
Cycloheximide	**0.01µM**		**0.05µM**		**0.075µM**	
% of control	99±8	111±9	107±8	113±9	107±7	113±4
Aspirin	**100µM**		**250µM**		**500µM**	
% of control	102±9	93±7	104±9	95±5	103±9	100±8

Table. 3 Changes in mRNA levels (expressed as % of control) of subunit 1 of the NMDA receptor in primary cultures of CGCs exposed to pesticides (parathion, dichlorvos, paraquat, pentachlorophenol or cycloheximide) or the non-neurotoxic chemical aspirin. ***P<0.001 comparing to untreated culture.

$GABA_A$-R	DIV 4	DIV12	DIV 4	DIV12	DIV 4	DIV12
Parathion	**10µM**		**25µM**		**50µM**	
% of control	90±9	63±14	47±10**	41±13***	24±13***	1±0.5***
Dichlorvos	**10µM**		**50µM**		**75µM**	
% of control	113±11	83±5	51±3*	85±3	47±14**	68±12*
Paraquat	**0.16µM**		**0.63 µM**		**2.5 µM**	
% of control	73±8	76±9	71±11	50±6	56±10	28±7***
Pentachlorophenol	**10µM**		**25µM**		**50µM**	
% of control	146±40	97±11	99±4	107±15	107±8	47±13**
Cycloheximide	**0.01µM**		**0.05µM**		**0.075µM**	
% of control	90±10	97±11	93±8	103±5	89±5	109±6
Aspirin	**100µM**		**250µM**		**500µM**	
% of control	96±8	84±6	93±10	87±5	84±9	100±8

Table 4. Changes in mRNA levels (expressed as % of control) of subunit delta of the $GABA_A$ receptor in primary cultures of CGCs exposed to pesticides (parathion, dichlorvos, paraquat, pentachlorophenol or cycloheximide) or the non-neurotoxic chemical aspirin. *P<0.05, **P<0.01 and ***P<0.001 comparing to untreated culture.

S100β	DIV 4	DIV12	DIV 4	DIV12	DIV 4	DIV12
Parathion	**10μM**		**25μM**		**50μM**	
% of control	110±17	116±23	112±19	123±32	101±14	80±15
Dichlorvos	**10μM**		**50μM**		**75μM**	
% of control	132±17	104±10	104±9	89±10	117±10	76±4
Paraquat	**0.16μM**		**0.63 μM**		**2.5 μM**	
% of control	62±6	116±18	63±9	96±16	61±9*	97±15
Pentachlorophenol	**10μM**		**25μM**		**50μM**	
% of control	135±26	129±11	110±32	144±11	88±17	47±5**
Cycloheximide	**0.01μM**		**0.05μM**		**0.075μM**	
% of control	104±17	99±21	61±9*	52±17***	61±13*	25±7***
Aspirin	**100μM**		**250μM**		**500μM**	
% of control	107±14	111±11	111±12	112±8	107±12	91±3

Table 5. Changes in mRNA levels (expressed as % of control) of the astrocytic marker S100β in primary cultures of CGCs exposed to pesticides (parathion, dichlorvos, paraquat, pentachlorophenol or cycloheximide) or the non-neurotoxic chemical aspirin. *P<0.05, **P<0.01 and ***P<0.001 comparing to untreated culture.

Nestin	DIV 4	DIV12	DIV 4	DIV12	DIV 4	DIV12
Parathion	**10μM**		**25μM**		**50μM**	
% of control	89±5	127±25	94±5	142±14	263±106**	338±86***
Dichlorvos	**10μM**		**50μM**		**75μM**	
% of control	123±8	103±4	110±5	104±11	128±7	93±10
Paraquat	**0.16μM**		**0.63 μM**		**2.5 μM**	
% of control	69±4	120±17	82±10	127±21	76±6	228±31***
Pentachlorophenol	**10μM**		**25μM**		**50μM**	
% of control	130±22	117±11	93±13	104±18	71±4	34±10***
Cycloheximide	**0.01μM**		**0.05μM**		**0.075μM**	
% of control	116±12	125±15	119±11	94±11	136±17	83±10
Aspirin	**100μM**		**250μM**		**500μM**	
% of control	105±12	121±16	97±9	124±13	97±8	92±10

Table 6. Changes in mRNA levels (expressed as % of control) of the neural precursor marker nestin in primary cultures of CGCs exposed to pesticides (parathion, dichlorvos, paraquat, pentachlorophenol or cycloheximide) or the non-neurotoxic chemical aspirin. **P<0.01 and ***P<0.001 comparing to untreated culture.

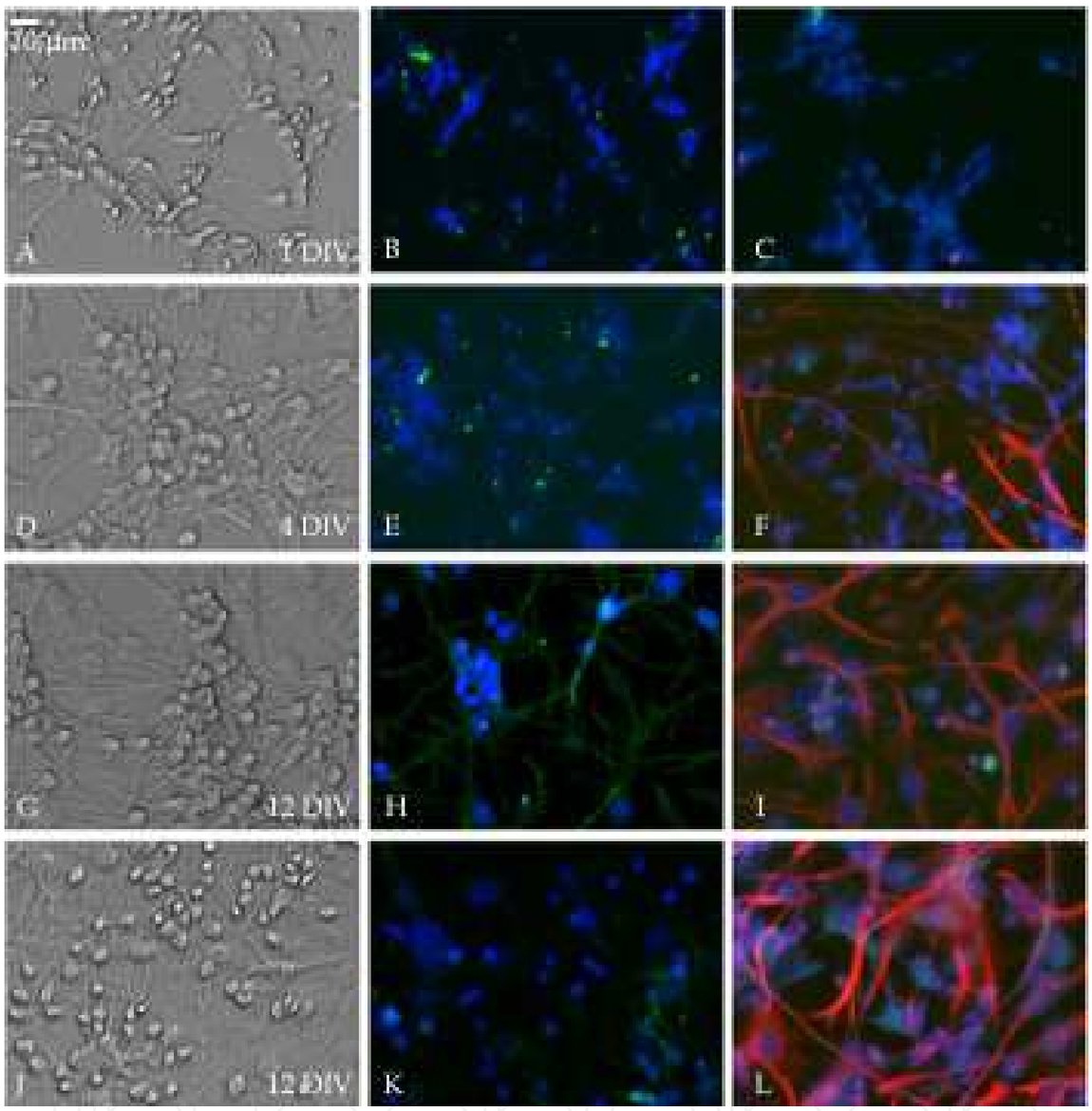

Fig. 1. Characterisation of CGCs by phase-contrast microscopy and immunocytochemistry in control (non-treated) and exposed to paraquat cultures. Neurons with round cell bodies (A) at 1 DIV progressively differentiated into neuronal phenotype showing outgrowth of neuritis at (D) 4 DIV and a dense neuronal network over the time (G) 12 DIV. In control cultures, protein expression of NF-200 (green) was very low at (B) 1 DIV and (E) 4 DIV but was higher expressed at (H) 12 DIV. The astrocytic protein GFAP (red) was not expressed at (C) 1 DIV but the expression increased over the time at (F) 4 DIV and (I) 12 DIV. The neural precursor protein nestin (green) was low expressed and at the same level over the time (C) 1 DIV, (F) 4 DIV and (I) 12 DIV. Exposure to paraquat (2.5 μM) at 12 DIV decreased the (J) neuronal network and (K) the protein expression of NF-200 (green) while the protein expression of (L) nestin (green) was increased. Nuclei were co-stained with Hoechst 33342 (blue).

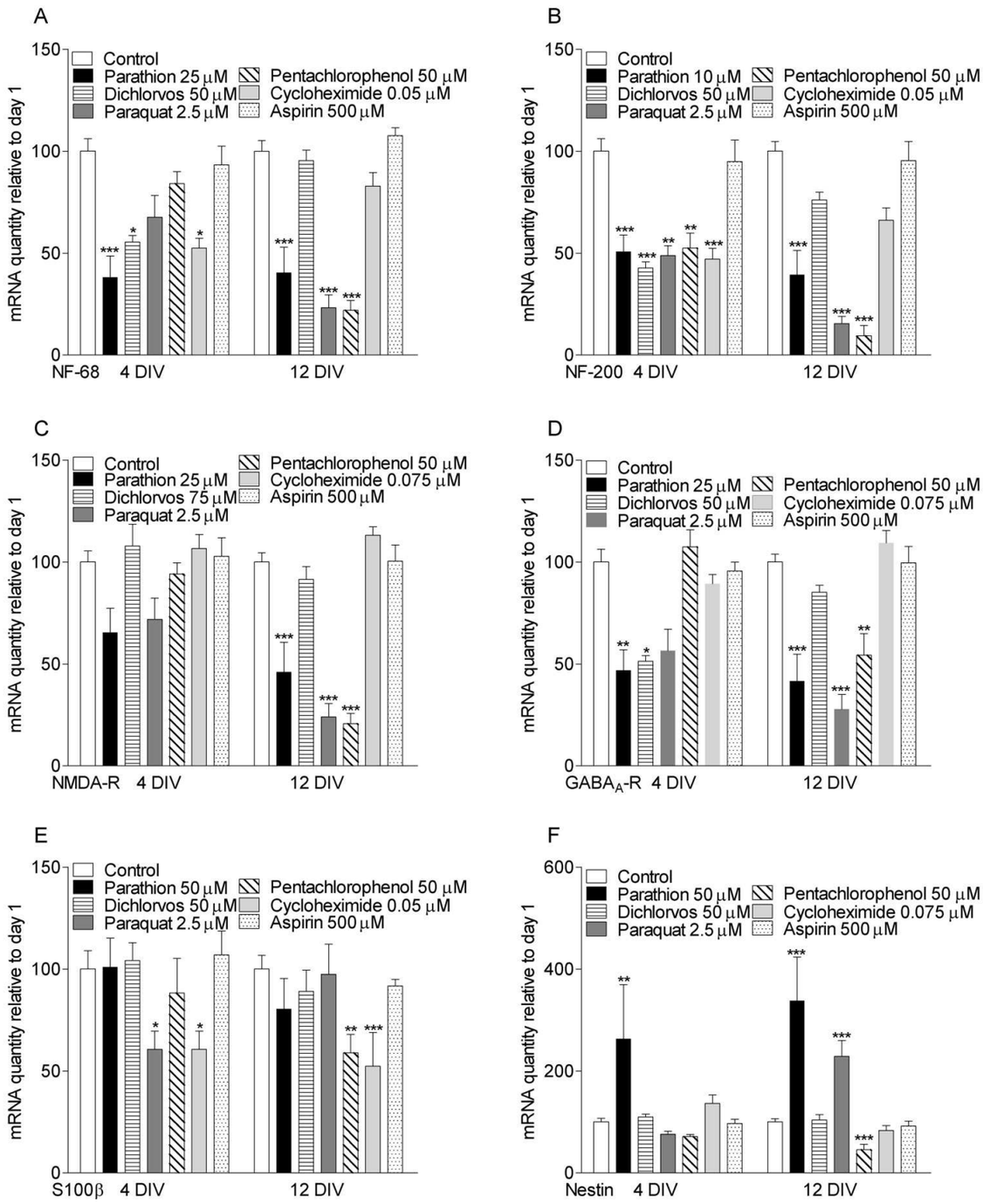

Fig. 2. The lowest concentration that induced the changes in mRNA levels (expressed as % of control) after exposure to pesticides (or aspirin). The neuronal markers: (A) NF-68 and (B) NF-200, (C) NMDA-R and (D) GABA$_A$-R. The astrocytic marker (E) S100β and the neural precursor marker (F) nestin. In the case of no observed changes the highest concentration tested is shown. *P<0.05, **P<0.01 and ***P<0.001 comparing to untreated culture.

5. Discussion

It is well known that a wide range of pesticides (~45%) can cause neurotoxicity. Taking into consideration the vulnerability of the developing brain it is likely that many of these substances could also cause developmental neurotoxicity. So to protect children's health it is important from a regulatory and public health prospective to have reliable tools in order to identify pesticides (and other chemicals) with DNT potential. In these studies we have demonstrated that gene expression is a relevant and promising tool to detect compounds with potential to induce DNT. Interestingly we could identify pesticides with different toxic mechanisms and at concentrations lower than those found in human plasma (Hogberg et al., 2009) suggesting that it is both a specific and sensitive endpoint.

To evaluate if we could identify toxic effects induced by various classes of pesticides we selected five pesticides with diverse mechanisms of toxicity. Since insecticides are especially known to induce neurotoxic effects, two insecticides (parathion and dichlorvos) from the most widely used class, organophosphates, were studied. Organophosphates were developed in the 1940's and 50's (Costa, 1988) and their main target is the acetyl cholinesterase enzyme. Inhibition of this enzyme causes accumulation of acetylcholine at the cholinergic synapses leading to overstimulation of cholinergic receptors that can give various effects in the CNS. Young animals and presumably children are more sensitive to the acute toxicity of these inhibitors than adults, possibly due to lower detoxification abilities (Costa, 2006; Mileson *et al.,* 1998; Pope, 1999; Thullbery *et al.,* 2005). In addition, the adverse effects on the developing brain could also be mediated by additional mechanisms, such as damage to DNA and RNA synthesis (Crumpton *et al.,* 2000a; Crumpton *et al.,* 2000b; Song *et al.,* 1997), deregulation of signal transduction pathways (Ehrich, 1995; Song *et al.,* 1997), oxidative stress (Crumpton *et al.,* 2000b) and astroglial cell proliferation (Garcia *et al.,* 2001; Guizzetti *et al.,* 2005). Indeed, in our study exposure to parathion and dichlorvos induced several changes in gene expression of both neuronal and glial cells indicating multiple mechanisms of toxicity (besides the inhibition of acetyl cholinesterase). Moreover, the neurotoxicity of these two organophosphates seems to differ in mechanism as they affected the gene expression differently, with parathion being the most toxic since it affected more genes at lower concentrations and at earlier time points.

Herbicides and fungicides are in general less toxic for the brain than insecticides, with the exceptions of paraquat (herbicide) which was studied here. Paraquat induces neurodegeneration of dopaminergic neurons (Castello *et al.,* 2007; Thiruchelvam *et al.,* 2002; Wu *et al.,* 2005) and can cause oxidative stress. The obtained results show that neurons are more affected by paraquat than the astrocytes as the gene expression of the neuronal markers (NF-68, NF-200, NMDA-R and $GABA_A$-R) were more affected than the astrocytic marker (S100β). This correlates well with the toxic mechanism, as neurons are known to be particularly vulnerable to oxidative stress. Interestingly, the mRNA level of nestin was up-regulated suggesting the presence of activated astrocytes possibly due to the neuronal damage. In fact, activated glial cells (astrocytes and microglia) can enhance the neurotoxcity as they release a variety of proinflammatory and neurotoxic factors such as cytokines and free radicals (Bal-Price and Brown, 2001).

In addition, two pesticides (pentachlorophenol and cycloheximide) with more general toxic mechanisms affecting any kind of cell type were studied. The mechanism of toxicity induced by pentachlorophenol is through the uncoupling of the mitochondrial phosphorylation leading to decreased levels of ATP production which is an essential source of energy for all

fundamental cell functions (Godfraind *et al.,* 1971). Indeed, in our study the expression of all genes were altered by the exposure to pentachlorophenol indicating that all cells (neurons, astrocytes and neural precursor) were affected. The fungicide cycloheximide is inhibiting the protein biosynthesis, which could lead to serious effects on the brain development (Harris *et al.,* 1968). Our results show that the mRNA expression of NF-68 and NF-200 was decreased by the cycloheximide exposure, however, only at the early time point (4 DIV). This indicates a delay in the synthesis of the neuro cytoskeleton proteins that might affect the neuronal morphological differentiation and final neuronal function. Moreover, the gene expression of S100β was down regulated at both 4 and 12 DIV suggesting that the proliferation and/or differentiation of the astrocytes were affected by cycloheximide exposure.

One non-neurotoxic chemical (aspirin) was selected as a negative control to evaluate if the gene expression could be a selective endpoint to detect specific neurotoxic effects. Indeed, aspirin exposure (up to 500μM) did not induce any changes suggesting that only neurotoxic compounds were detected by this endpoint. However, more non-neurotoxic chemicals should be tested to evaluate the robustness of this endpoint.

Since the development of the brain is based on precise events in space and time (such as proliferation, migration and differentiation) that are strictly controlled, *e.g.* by neurotransmitters, electrical activity and hormones, the pesticides-induced mechanisms of toxicity identified in the above studies are likely to have a potential to cause DNT. There are more than 600 pesticides registered on the market, including insecticides, fungicides and rodenticides, and several of these are produced in high volumes (Grandjean and Landrigan, 2006). Even though the uses of many pesticides are restricted and an increase in DNT testing is demanded, the general lack of DNT data for agricultural chemicals is of particular concern because of their widespread use and ubiquitous exposure (Whyatt *et al.,* 2004). A population based study has reported that over 90 % of the children in the US have detectable urinary remains of neurotoxic pesticides (Schettler, 2001). This exposure might be a risk to children's health, as these substances are suggested to contribute to developmental disorders such as attention deficits, hyperactivity disorders, autism and learning disabilities (Costa *et al.,* 2008; Jurewicz and Hanke, 2008; Shafer *et al.,* 2005). To protect children's health the reliable testing strategy has to be built up, as the current one that is based on *in vivo* approaches is too complex and not effective enough. Incorporation of alternative approaches such as *in vitro* studies, together with *in silico* modeling (Coecke et al., 2007) and non-mammalian animal models (zebra fish, medaka or *caenorhabditis elegance*) could significantly facilitate the decision making process for regulatory purposes.

6. Conclusion

The results from our study suggests that adverse effects induced by studied pesticides (parathion, dichlorvos, paraquat, pentachlorophenol and cycloheximide) were mediated by multiple toxicity mechanisms. Indeed, our *in vitro* approach allowed us to determine which cell type (neuronal or glial) and at which stage of development (proliferation, differentiation or maturation) was affected by the exposure to the different pesticides. Furthermore, the induced toxicity was observed at concentrations relevant to environmental exposure as compared to concentrations found in human plasma (Hogberg *et al.,* 2009). This suggests that gene expression could be used as a sensitive endpoint for testing DNT effects of pesticides. Incorporation of this endpoint together with other neuronal/glial specific assays

into an *in vitro* DNT testing strategy could be useful for an initial identification and further prioritization of compounds that have DNT potential. Such a testing strategy could speed up the process of DNT chemical assessment for regulatory purposes leading to their restricted use and a tighter control of children's exposure to potential DNT compounds.

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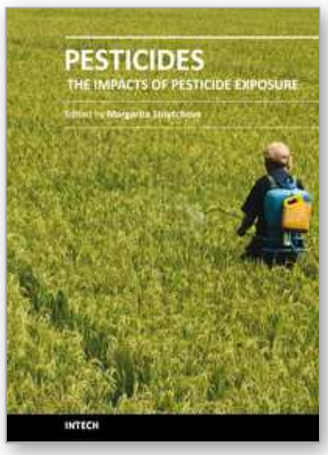

Pesticides - The Impacts of Pesticides Exposure
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Pesticides are supposed to complete their intended function without â€œany unreasonable risk to man or the environmentâ€. Pesticides approval and registration are performed â€œtaking into account the economic, social and environmental costs and benefits of the use of any pesticideâ€. The present book documents the various adverse impacts of pesticides usage: pollution, dietary intake and health effects such as birth defects, neurological disorders, cancer and hormone disruption. Risk assessment methods and the involvement of molecular modeling to the knowledge of pesticides are highlighted, too. The volume summarizes the expertise of leading specialists from all over the world.

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