Paraoxon and Pyridostigmine Interfere with Neural Stem Cell Differentiation

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Abstract

Acetylcholinesterase (AChE) inhibition has been described as the main mechanism of organophosphate (OP)-evoked toxicity. OPs represent a human health threat, because chronic exposure to low doses can damage the developing brain, and acute exposure can produce long-lasting damage to adult brains, despite post-exposure medical countermeasures. Although the main mechanism of OP toxicity is AChE inhibition, several lines of evidence suggest that OPs also act by other mechanisms. We hypothesized that rat neural progenitor cells extracted on embryonic day 14.5 would be affected by constant inhibition of AChE from chronic exposure to OP or pyridostigmine (a reversible AChE blocker) during differentiation. In this work, the OP paraoxon decreased cell viability in concentrations $>50 \mu\text{M}$, as measured with the MTT assay; however, this effect was not dose-dependent. Reduced viability could not be attributed to blockade of AChE activity, since treatment with $200 \mu\text{M}$ pyridostigmine did not affect cell viability, even after 6 days. Although changes in protein expression patterns were noted in both treatments, the distribution of differentiated phenotypes, such as the percentages of neurons and glial cells, was not altered, as determined by flow cytometry. Since paraoxon and pyridostigmine each decreased neurite outgrowth (but did not prevent differentiation), we infer that developmental patterns may have been affected.

Keywords

Organophosphates; Proteomics; Neural progenitor cells; Differentiation; Paraoxon

Introduction

Present in half of the pesticides used worldwide, organophosphate (OP) compounds have been widely used since the 1930s and were manufactured during World War II as highly toxic chemical warfare nerve agents [1]. OPs are potent, lipophilic neurotoxicants that irreversibly inhibit acetylcholinesterase (AChE) and cause acetylcholine (ACh) accumulation in the synaptic cleft [2]. The accumulation of ACh resulting from treatment with physostigmine [3], pyridostigmine (PY) [4], or other AChE inhibitors also has a therapeutic rationale. The purpose of this work was to analyze the cellular differentiation of neural progenitors when AChE is constantly inhibited by either an irreversible or reversible AChE inhibitor.

First, paraoxon (O, O-diethyl p-nitrophenylphosphate, POX), the bioactive metabolite of parathion, is generated in the liver by a cytochrome P450-mediated biotransformation [5]. POX inhibits AChE by a mechanism in which the OP-AChE intermediate is either

hydrolyzed to produce an OP leaving group and a functional enzyme or dealkylated to produce a phospho-AChE covalent bond in the enzyme active site. This permanent bond irreversibly inactivates the enzyme and triggers the subsequent accumulation of ACh that leads to neural impairment [6].

There is evidence that POX affects neurons in multiple ways. For example, it induces apoptosis in rat neonate hippocampal cells [7] and a significant decrease in ATP production by interfering with mitochondrial metabolism in dorsal root ganglion cells [8].

On the other hand, because of its charged quaternary amine group, PY is not expected to cross the blood–brain barrier. However, Abou-Donia et al. [9] detected changes in sensorimotor performance and a significant decrease in AChE activity in the rat midbrain after long-term exposure to PY.

The research employing constant inhibition of AChE in models that contain the three most important cell types of the brain is sparse. Therefore, we studied the effects of POX and PY on primary cultures of differentiating neural stem and progenitor cells (dNPCs). These cells can be isolated from the CNS of embryonic, neonatal, and adult rodents [10–12] and are of particular interest, because they retain their multi-lineage differentiation capacity. When they are expanded and maintained in the form of neuro-spheres [13], they can be differentiated into astrocytes, oligodendrocytes, and several types of neuron if the correct stimulus is added.

Until now, studies of the inhibition of AChE by POX and PY through irreversible or reversible mechanisms, respectively, have not been done in a cellular model that is able to differentiate into the major neural phenotypes present in the brain, which would closely reflect events during embryonic cortex development. Instead, most studies have been performed with undifferentiated neuroblastoma or neuron-like cells or using low POX concentrations. Accordingly, this study addresses the effects of toxic concentrations of POX and the clinically used PY on cell differentiation, neurite outgrowth, and protein expression patterns in a more suitable model for evaluating the consequences of continuous inhibition of AChE on differentiation.

Materials and Methods

Embryonic day-14 fetuses were removed from pregnant 14–16-week-old Sprague–Dawley rats weighing 250–300 g. Animals were kept in a temperature-controlled facility on a 12/12 h light–dark cycle. All procedures involving live animals were reviewed and approved by the IACUC and were carried out in agreement with current US National Institutes of Health guidelines.

Neurosphere Cell Culture

Primary cultures of dNPCs were aseptically obtained by dissection of the telencephalons of rat fetuses at 14.5 days post conception, as determined by vaginal smear [14]. Briefly, brain stem and meninges were removed from the telencephalon, and the resulting tissue was mechanically dissociated using a Stomacher 80 Biomaster blender (Se-ward Laboratory

Systems, Port St. Lucie, FL, USA). Following centrifugation at 200×g, cells were filtered using a 40-µm cell strainer (BD), resuspended, and allowed to grow in suspension at a density of 2×10^5 cells/ml in medium composed of 70 % Dulbecco's Modified Eagle's Medium (DMEM, high glucose, Life Technologies, Carlsbad, CA, USA) and 30 % F-12 nutrient mixture (Life Technologies) in the presence of 100 IU/ml of penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 5 µg/ml heparin, 20 ng/ml fibroblast growth factor (FGF)-2, 20 ng/ml epidermal growth factor (EGF), and 2 % B-27 supplements (v:v; Life Technologies) at 37 °C, 95 % humidity, and 5 % CO₂. Cells were grown for 10 days in culture plates, and half of the medium was replenished at 3-day intervals, followed by the removal of growth factors and subsequent culture on plates with poly-D-lysine (50 µg/ml) and laminin (1 µg/ml) for 6 days to allow differentiation. Three experimental conditions were tested using three independent replicates: 200 µM paraoxon (POX) dissolved in propylene glycol (PG, Sigma), 200 µM pyridostigmine (PY) dissolved in PG, or PG vehicle alone. For simplicity, the presence of PG is unstated in the experimental treatments labeled "POX" or "PY". The cell viabilities obtained were compared with those obtained in cells treated with a concentration range of 0–300 µM of the OP PY, a therapeutically used acetylcholine esterase inhibitor [15]. The cells were exposed to POX, PY, and PG over 6 days for all experiments, except in Fig. 1c. POX (200 µM), PY (200 µM), or the same volume of PG were added on day 0. A half volume of medium was changed on day 4 using 2 % B27 and DMEM/F12 plus 200 µM POX, 200 µM PY, or the same volume of PG.

Cell Viability Determination by the Methylthiazol Tetrazolium (MTT) Assay

Cells (6×10^4) were seeded in 96-well plates (Corning) and treated with several concentrations (50–300 µM) of POX or PY (Sigma) or propylene glycol (PG), and cells without PG were used as control. A total of 25 µl of MTT solution (5 mg/ml, Sigma) was added to each well containing 200 µl, and the cells were then incubated at 37 and 5 % CO₂ for 4 h. After incubation, the MTT was discharged, and 200 µl of dimethyl sulfoxide (DMSO) was added to each well. Subsequently, cell viability was assessed by measuring the OD₅₅₀ on a SpectraMax spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

Immunocytochemistry

Differentiating NPC cell viability was determined following incubation for 10 min with 5 µM propidium iodide (Calbiochem). For immunofluorescence staining, cells were first incubated with 4 % paraformaldehyde and then incubated for 30 min using a blocking solution (1 % BSA, 0.5 % Triton-100, 10 % fetal bovine serum [FBS], and 10 % methanol in PBS) at 25 °C and washed twice for 5 min with PBS. The cells were then incubated overnight in the presence of an anti-β3 tubulin rabbit monoclonal antibody (Abcam, 1:750 dilution) in a solution of 1X PBS, 0.5 % Triton-100, and 1 % FBS. The cells were washed three times with PBS, followed by incubation with a secondary antibody conjugated with the green fluorescence-emitting probe Alexa Fluor® 488 (1:1000; Molecular Probes) in a solution of 1X PBS and 1 % FBS for 1 h at room temperature, followed by washing with PBS. Images were taken on an Olympus IX70 microscope equipped with a QColor 5 camera (Olympus, Center Valley, PA, USA).

AChE Assay

AChE activity was measured using the Ellman assay [16]. The cells were homogenized in Triton buffer (sodium phosphate buffer, 0.1 M, pH 8.0, 1 % Triton X-100) at a concentration of 100 mg cells per ml buffer. The homogenates were centrifuged at 12,000g for 1 min. Supernatants were collected, and tetraisopropyl pyrophosphoramidate was added to inhibit butyrylcholinesterase. The samples were homogenized once again in Triton buffer and incubated in the dark for 5 min at 25 °C. Acetylthiocholine (AChI) substrate (Sigma) was added to each well, and the enzyme kinetics at OD₄₀₅ were recorded. AChE activity was measured in duplicate in at least three experiments, and enzymatic activity was normalized to the protein concentration, as determined using Bradford reagent (Bio-Rad).

Flow Cytometry

Flow cytometry procedures were in accordance with a previously published protocol, with a few modifications [10]. dNPCs were collected by centrifugation at 300×g for 5 min, dissociated to a single-cell suspension using 5 ml of trypsin (0.05 %), and treated with 2 % FBS (GIBCO) to inhibit trypsin activity. The cells were then centrifuged at 300×g for 5 min and fixed in PBS/1 % formaldehyde for 20 min on ice, followed by a triple wash in PBS/2 % FBS. Differentiation of neuronal and glial cells was probed with antibodies directed against neuronal β 3-tubulin (1:100, dilution, Abcam) or glial fibrillary acidic protein (GFAP, 1:500 dilution, Sigma). Cells were incubated for 1 h at 25 °C in PBS supplemented with 2 % FBS and 0.05 % Triton X-100. A secondary antibody conjugated with Alexa-Fluor® 488 (1:1000 dilution, Molecular Probes) was used for detection of β 3-tubulin. After 2 h, the cells were washed and analyzed by using a flow cytometer (FACSCALIBUR, BD, San Jose, CA, USA). An argon laser line was used for fluorescence excitation (FL1, 488 nm and FL2, 635 nm, with band pass filter). Background fluorescence was measured using unlabeled cells and cells labeled with secondary antibody alone, which were used to set gating parameters between positive and negative cell populations. Forward and side light-scatter gates were used to exclude cell aggregates and small debris. In a total of ten thousand events, normalized with negative controls and incubated without the primary antibody, were acquired per sample. Data were analyzed and plotted in one-parameter histogram format.

Sholl Analysis

Images obtained using antibodies against β -3 tubulin and the respective secondary antibody conjugated with Alexa Fluor® 488 were analyzed with the Image J program for changes in neurite outgrowth [17]. The plug-in Fiji creates concentric circles around the cell body to trace the primary neurites. The radius of the circles were 16, 24, 33, 41 and 66 μ m. The number of intersections between primary neurites and each of the circles of increasing radius was quantified. Neurites that crossed several circles were counted every time they intersected a circle and neurites smaller than 16 μ m were not counted at all. For each treatment, the intersections between circles and the neurites from 60 neurons were measured, and the calculated numbers were expressed as the average of neurites per neuron that crossed a circle of a given radius.

Proteomics

High-Resolution 2D Gel Electrophoresis—To obtain highly resolved 2D patterns, different optimization steps were necessary to improve focusing and to reduce gel streaking. Differentiated neurospheres treated with PG, POX, or PY, containing 150 µg of total protein, and rehydration buffer (8 M urea, 2 % CHAPS, 0.2 % carrier ampholytes, and 10 mM DTT for immobilized pH gradient [IPG] strips, pH 3–6, pH 5–8, and pH 7–10, Bio-Rad, Hercules, CA, USA), and rehydrated overnight at room temperature (RT). The samples were mixed gently for 1 h at room temperature before centrifugation at 18,000×g for 30 min to remove particulates. For the first-dimension separation, dNPC extracts were loaded onto an IPG strip by overnight passive in-gel rehydration at 20 °C for at least 12 h, after which isoelectric focusing (IEF) was run for a total of 20,000 Vh. All IEF took place on a Bio-Rad protein IEF cell with a surface temperature of 20 °C and a maximum current of 50 µA/strip. Following IEF, the strips were reduced for 15 min in equilibration buffer (6 M urea, 0.05 M Tris–HCl, pH 8.8, 2 % SDS, and 20 % glycerol) containing 2 % DTT and then alkylated for 10 min in equilibration buffer containing 2.5 % iodoacetamide. The second-dimension separation was performed on 4–20 %-gradient SDS–polyacrylamide gels. After 6 days of treatment or its absence, dNPC extracts were run on 2D-PAGE gels in triplicate. Electrophoresis proceeded at 15 mA/gel for the first 15 min and then at 30 mA/gel. For analysis, the 2D gels were routinely stained with Coomassie blue G-250.

Image Analysis of 2D Gels Using PDQuest Software—Coomassie blue-stained gels were scanned using the Ver-saDoc model 1000 image system running Quantity One version 4.3.0 software (Bio-Rad, Hercules, CA, USA). Gel image analysis was performed with PDQuest software version 7.4.0 (Bio-Rad). Differentially expressed proteins of interest were marked for excision. An experimental set was created for pI 3–10 gels and another for pI 3–6, 5–8, 7–10 gels, with each set containing images of nine gels (three images per treatment). After automatic detection of spots using PDQuest software, the files were manually edited to correct for artifacts. The software calculated individual spot “volumes” by density/area integration. To eliminate gel-to-gel variation, individual spot volumes for each gel were normalized relative to the total spot volume of the gel. Normalized spot volume data from each experimental set were exported to Microsoft Excel. Differences in expression of 2D spots between the control cells and cells differentiated in the presence of PY or POX were analyzed by Student’s *t* test using $p < 0.05$ as the criterion for statistical significance. Spots showing a >onefold increase or decrease in protein expression were selected for identification by peptide mass fingerprint. From each of the analyzed samples, three biological replicates with two independent protein extractions were performed, and three replicate two-dimensional gels were prepared from each protein extract.

Statistical Analysis

Data were analyzed for statistical significance using one-way ANOVA or Student’s *t*-test, as implemented with SigmaPlot version 11.00 software (Systat Software, San Jose, CA, USA). Statistical relevance was set at $p < 0.05$.

Results

Impact of Paraoxon and Pyridostigmine on Viability of Differentiating Neural Progenitor Cells

Cytotoxic effects of a 6-day treatment with POX and PY (50–300 μ M) on dNPC viability were evaluated by using the MTT assay in comparison with untreated cells (control) or with the PG vehicle (Fig. 1a, b, $p < 0.05$). POX also decreased the cell viability on days 2 and 4 when 200 μ M of POX or PY were added, as observed in Fig. 1c ($p < 0.001$, $n = 3$ for each treatment). The percentage of membrane damage (the intensity ratio propidium iodide/DAPI) shows a statistically significant difference between the groups ($p < 0.001$), confirming a decrease in cell viability in the presence of POX. On the other hand, PY did not affect cell viability at the concentrations used. (Fig 1b, c).

Differentiating NPCs Shows Active AChE that is Inhibited by Paraoxon and Pyridostigmine

Differentiating NPCs had detectable AChE activity that was partially inhibited by POX and PY, as measured by the Ellman assay. At the concentration of 200 μ M, POX decreased the AChE activity of dNPCs to 0.130 μ mol/min/ mg of protein $\pm$ 0.011 in comparison with 0.278 μ mol/ min/mg of protein $\pm$ 0.0298 (mean $\pm$ S.E.M., $p < 0.05$) for PG, and PY decreased the activity to 0.116 $\pm$ 0.0145 (mean $\pm$ S.E.M., $n = 3$, Fig. 2).

Impaired Neurite Outgrowth in Neurospheres Differentiated in the Presence of Paraoxon and Pyridostigmine

Apart from developmental processes where migration and neurite outgrowth are essential for completing neural connections, reestablishment of lost neuronal connections is crucial for repair of brain damage and maintaining synaptic plasticity [18]. Therefore, any inhibitory effects on neurite outgrowth can be judged as developmental toxicity [19] as well as a neurotoxic effect on physiological brain function [20]. After 6 days of treatment, dNPCs showed a reduction in the number of neurite in cells treated with 200 μ M POX or 200 μ M PY in comparison with PG controls, cultured the absence of inhibitors. The number of neurites was estimated by the number of intersections with circles of 16, 24, 33, 41 μ m radiuses. Within each of these radiuses, there was the same significant approximately 20 % decrease in intersections in comparison with the PG controls. Between 41 and 66 μ m there was a decrease in the number of intersections in the three groups, however, the difference between PG controls and cells exposed to AChE inhibitors was the same (Fig. 3). Therefore, the exposure to POX or PY decreased the number but not the length of neurites.

The Cellular Fate of Neural Progenitor Cells is Not Affected by POX or PY

Following induction of the embryonic telencephalon, NPCs differentiate into the three principal types of brain cells, with neurons and astrocytes constituting the majority, and oligodendrocytes representing <10 % of the population [10]. A flow cytometry assay was performed to assess how the differentiation of NPCs into astrocytes and neurons is affected by the continuous inhibition of AChE by 200 μ M POX or PY over 6 days. No significant difference in the relative distribution of neurons and astrocytes, as determined by the

expression of β 3-tubulin and GFAP, respectively, was observed for NPCs treated with PG, POX, or PY (Fig. 4).

Changes in Protein Expression Profiles of Differentiating NPCs in the Presence of Paraoxon and Pyridostigmine

Proteomics analysis using two-dimensional gel electrophoresis was performed for detection of altered protein expression in dNPCs in the presence of POX (Fig 5).

A Venn diagram (Fig. 6) was created to organize the number of unique and shared proteins between the different groups (control, POX, and PY). In order to merge the protein expression level of the three different 2D gels of neural progenitor cells in the presence of PG, POX, or PY, a multichannel viewer from PD quest software overlapping the three 2DS images was used (Fig. 7). Analysis of all three treatments revealed 116 proteins in common (Fig. 6), with controls and PY-treated neurospheres sharing 47 proteins, PG- and POX-treated neurospheres sharing 42 proteins, and POX- and PY-treated neurospheres having 47 proteins in common. On the other hand, 35 proteins were exclusively expressed in PG-treated cells, 30 in POX-treated cells, and 37 in PY-treated cells (Fig. 6). Fifty-nine differentially expressed proteins were further analyzed for their expression pattern and compared with the PG vehicle. In Fig. 5, 34 of these proteins are shown. A significant reduction in protein expression of POX-treated cells was observed for cofilin; heat shock protein 1; Rho GDP dissociation inhibitor (GDI) alpha; serine (or cysteine) proteinase inhibitor, clade H; germinal histone H4 gene; D-3-phosphoglycerate dehydrogenase; B-36 VDAC, a 36-kDa voltage-dependent anion channel; and glyceraldehyde-3-phosphate dehydrogenase. On the other hand, PY-treated cells showed a significant reduction in tumor-rejection antigen gp96 precursor, protein disulfide-isomerase A3, eukaryotic translation initiation factor 4H, serum albumin precursor, and actin, cytoplasmic 2. Other protein expression patterns were shared that were not diminished to the same extent; for example, ATP synthase subunit alpha, mitochondrial precursor was decreased 12.5 fold in POX and 3.57 in PY (Table 1). In sum, proteomics analysis revealed differential expression of structural, nuclear and metabolic proteins, indicating that dNPCs undergo structural and cell signaling changes in the presence of POX or PY.

Discussion

The main purpose of this study was to determine the effect of constant inhibition of AChE by high concentrations of POX or PY, which are irreversible and reversible inhibitors of AChE, respectively, in driving cell differentiation of NPCs to a specific cell type and also to evaluate whether AChE inhibition is responsible for decreased cell viability. A dose–response curve was obtained to establish the cytotoxic threshold concentrations for PY and POX by using the MTT assay (Fig. 1a–c). Exposure to POX decreased cell viability at concentrations in the range 50–300 μ M in a concentration-independent fashion. In addition, we observed that only a small population (25 %) is susceptible to concentrations >50 μ M POX, which is not in agreement with Buzanska et al. [21] and Visan et al. [22]. Buzanska used the OP chlorpyrifos in neuronal-committed human umbilical cord blood-derived neural stem cells (HUCB-NSCs) and observed decreasing cell viability in which 60 μ M decreased

viability to 16 %. Visan found that concentrations $<10\ \mu\text{M}$ of chlorpyrifos reduced cell viability of D3 cells to $<20\%$. The discrepancy between these findings and those presented in this paper, could be the result of differences between OPs. For instance, even though chlorpyrifos and POX possess similar chemical structures, there are differences in inhibitory potency against AChE at high concentrations [23]. Alternatively, there could be differences between cell types, such as cells derived from human umbilical cord versus rat neural progenitors. On the other hand, Bahrami et al. [7] showed that POX induces apoptosis in a dose-dependent manner when using isolated neonatal hippocampal cells. These discordant results could also be attributed to several other factors, including the use of neonatal versus embryonic tissue or to previous observations that hippocampal neurons are especially sensitive to OP.

Because AChE inhibition is the predominant action of OP, the next step was to verify whether POX-mediated apoptosis is triggered by AChE inhibition in dNPCs. To this end, we used $200\ \mu\text{M}$ PY, which did not change cell viability on days 2, 4, and 6. Since PY and POX decreased AChE activity of dNPCs on day 6 when compared with control dNPCs in the absence of AChE inhibitors, these results suggest that dNPC viability is independent of AChE inhibition. Having evaluated cell viability, the next step was to verify whether inhibition of AChE decreases neurite growth, as suggested by Yang for dorsal root ganglion neurons [24]. Here we evaluated whether $200\ \mu\text{M}$ POX or PY decreases neurite outgrowth during NPC differentiation. As expected, a significant decrease in neurite outgrowth was detected by continuous AChE inhibition using Sholl analysis. It is important to highlight that this analysis included the length of neurite up to a maximum of $66\ \mu\text{m}$ and did not include neurite beyond this value. Although, the decrease of the number of neurite crossing the Sholl circles can be attributed to several factors we can rule out the effect of decreased viability, since PY did not decrease the viability and still decreased the number of neurites intersecting the circles. In view of this result, the inhibition of AChE must cause alterations in the differentiation and cellular phenotype specification [25]. Therefore, the progress of neural differentiation was verified by quantification of $\beta 3$ -tubulin-positive cells (neurons) and glial fibrillary acidic protein (astrocytes) by flow cytometry, which is the method previously used for this purpose [10, 26]. No significant difference in the percentages of $\beta 3$ -tubulin-positive cells (neurons) or GFAP-positive cells (astrocytes) was observed along NPC differentiation. It can be concluded that inhibition of AChE by $200\ \mu\text{M}$ POX was not enough to affect the fate of NPC differentiation. On the other hand, POX treatment resulted in a decrease in cell viability and neurite outgrowth measured as crossings of Sholl circles, thus affecting the progress of NPC differentiation. As the involvement of non-cholinergic mechanisms in neural differentiation is far from being understood, a proteomics approach was undertaken to study differential protein expression patterns in the absence and presence of AChE inhibitors. Comparing the three treatments by analysis of two-dimensional gels, we found 35, 30, and 37 proteins expressed uniquely in the presence of PG alone, POX, or PY, respectively (Fig. 5). The results obtained suggest mechanisms of action not shared by the two AChE inhibitors, POX, and PY. A possible explanation for such differences could be that POX is an irreversible while PY is a reversible, inhibitor of acetylcholinesterase. However, in both cases acetylcholinesterase remained inhibited for 48 h after the last dose, proving this possibility to be incorrect. In fact, these results suggest that POX causes other

cellular effects that are independent of AChE inactivation. In order to identify the differentially expressed proteins for each treatment (PG, POX, and PY), a mass spectrometry analysis was performed for 59 differentially expressed protein spots, of which we show 34. Proteins related to cytoskeleton, metabolism, and glycolysis, as well as transcription factors, are differentially expressed by POX- and PY-treated cells, with PG-treated cells used as controls. Expression of cytoskeletal proteins, such as actin gamma 1 propeptide, beta tubulin, and profilin-1 (not shown), are decreased in POX- and PY-treated cells, in agreement with the observed decrease in neurite outgrowth. The ATP synthase alpha subunit is present in the mitochondrial membrane and produces ATP from ADP in the presence of a proton gradient across the membrane, which is generated by electron transport complexes of the respiratory chain. Expression of this protein is down-regulated 12.5 fold in comparison to PG exposure while following PY exposure, its expression is diminished 3.5 fold. Expression of the glycolytic protein glyceraldehyde 3-phosphate dehydrogenase is reduced, while PY exposure led to increased expression of this protein by a factor of 1.46 fold. The observed decreased expression of these proteins is expected to result in low energy production, which would explain the decreased viability of cells treated with POX. In summary, these results suggest that dNPCs treated with POX or PY undergo a series of structural and molecular changes, which may be responsible for impaired neurite outgrowth. Increased cell death was observed only in POX-treated cells, providing novel evidence for OP effects and AChE inhibition of NPC differentiation.

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Fig. 1.

Interference of paraoxon (POX) and pyridostigmine (PY) with cell viability. MTT assay of differentiating neural progenitor cells treated with POX (**a**) or PY (**b**) for 6 days (treated cells vs. PG treatment * $p < 0.05$). **c** MTT assay of differentiating neural progenitor cells treated with POX or PY and analyzed on days 2 and 4 (** $p < 0.001$). **d** Representative immunocytochemistry using antibodies against β 3-tubulin and a secondary antibody conjugated with Alexa Fluor® 488 (green), 5 μ M propidium iodide (red), and 1 μ g/ml of 4', 6-diamidino-2-phenylindole (DAPI, blue). *Left panel* quantification of the number of propidium iodide-positive cells ($p < 0.001$). Percentage of cell death determined by the intensity ratio propidium iodide/DAPI. Values are the mean number of cells in three independent experiments. Statistical analysis was performed using one-way ANOVA followed by the Tukey test (**a**, **b**, **c**) or by Student's *t*-test (**d**)

Fig. 2.

AChE activity in differentiating neural progenitor cells in the presence of propylene glycol (PG), paraoxon (POX), or pyridostig-mine (PY). The cells were harvested on day 6 after treatment. AChE activity was measured using the Ellman assay, and the results were as follows: PG, 0.28 ± 0.03 (mean $\pm$ S.E.M.), $n = 6$; POX, 0.13 ± 0.01 , $n = 6$; PY, 0.12 ± 0.015 , $n = 9$. $*p < 0.05$. Statistical analysis was performed using one-way ANOVA followed by the Student–Newman–Keuls test

Fig. 3.

Paraoxon decreases the number of neurite per neuron after 6 days of treatment with either POX or PY. Morphological (Sholl) analysis was performed on differentiating neural progenitor cells stained with an antibody against β 3-tubulin. ** $p < 0.01$. Statistical analysis was performed using one-way ANOVA followed by the Tukey test, $n = 60$, for each treatment. *Black bar* control, *Gray* POX, *White* PY

Fig. 4.

Maintenance of cellular fate decision of differentiating neural progenitor cells in the presence of paraoxon (POX), pyridostigmine (PY), or the vehicle control propylene glycol (PG). Flow cytometry analysis using antibodies against GFAP and β 3-tubulin after 6 days of differentiation with different treatments. The graphs shown are one-parameter histograms with cell counts on the y-axis and the measurement parameter on the x-axis. *Blank cells*, without primary antibodies and with control-only secondary antibody. There were no significant differences between the control-, PY-, and POX-treated groups

Fig. 5.

Overview of two-dimensional gel electrophoresis (2D-GE) showing protein profiles in differentiating neural progenitor cells in the presence of propylene glycol (PG) vehicle, paraoxon (POX), or pyridostigmine (PY). The *blue triangles* represent differentially expressed proteins in comparison with POX and PY; the *red triangles* represent the differentially expressed proteins following POX treatment; and the *green triangles* represent the differentially expressed proteins following PY treatment in comparison with PG. The spots on the 2D gels that are common to all the three conditions are indicated with *capital letters*. All experiments were performed in triplicate

Fig. 6. Venn diagram of common and uniquely expressed proteins in differentiating neural progenitor cells cultured in the presence of propylene glycol (PG), paraoxon (POX), or pyridostigmine (PY)

Fig. 7. Multichannel viewer highlighting the overlay of three 2D gels of neural progenitor cell proteins in the presence of propylene glycol (PG), paraoxon (POX), or pyridostigmine (PY). The images depict a computer-generated, color-coded comparison between the three samples and exhibit intensity variations in the x- and y-directions

Table 1

Proteins differentially expressed in differentiating neural progenitor cells treated with paraoxon (POX), pyridostigmine (PY), or propylene glycol (PG) and identified by peptide mass fingerprinting

Spots	GI#	Protein name	MW (kDa)	PI	Peptides	Protein scores	Gel (spot location)	Fold change POX compared to PG	Fold change PY compared to PG
1	8393101	Cofilin-1	18.75	8.22	11	376	PY	-1.2	1.97
2	34740335	Tubulin, alpha 1B	50.80	4.94	16	1160	PY	1.315	11.32
3	183396771	Heat shock protein 1 (chaperonin)	61.09	5.91	22	1130	PY	-1.75	1.29
4	4501887	Actin, gamma 1 propeptide	42.11	5.31	10	521	PG	Unique in PG	-2.27
5	149064296	Isoform CRA_c_rCG46767	66.87	5.11	23	441	PG	Unique in PG	-1.29
6	31982030	Rho GDP dissociation inhibitor (GDI) alpha	23.45	5.12	9	522	PY	Unique in PG	2.86
7	40538742	ATP synthase subunit alpha, mitochondrial precursor	59.83	9.22	19	677	PG	-12.5	-3.57
8	117935064	Triosephosphate isomerase	27.34	6.89	13	449	PG	Unique in PG	-7.73
9	157823517	Ladybird homeobox 2	21.27	6.63	7	53	PY	28	49
10	157818781	Protein kinase C substrate 80 K-H	60.15	4.41	15	564	PG	Unique in PG	-1.29
11	12025526	Histone H2B type 1	13.98	10.36	6	360	PG	Unique in PG	-1.31
12	149068858	Serine (or cysteine) proteinase inhibitor, clade H	45.10	9.01	17	308	PY	Unique in PG	9.2
13	157059865	Heterogeneous nuclear ribonucleoprotein 80b	34.00	9.04	14	543	POX	2.33	1.02
14	109504921	Germinal histone H4 gene	11.42	11.36	5	311	PY	Unique in PG	3.67
15	21746161	Tubulin, beta	50.38	4.78	12	317	PG	-1.38	-2.23
16	6756041	Tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation actein, zeta polypeptide	27.92	4.73	12	277	PG	-1.09	-1.51
17	9507245	14-3-3 protein gamma	28.46	4.8	6	182	POX	1.78	Unique in PG
18	5453555	Ras-related nuclear protein	24.58	7.01	8	318	PG	-2.54	-3.08
19	8394009	Peptidyl-prolyl cis-trans isomerase A	18.09	8.34	6	93	PG	-1.6	-4.74
20	25453420	Glutathione S-transferase P	23.65	6.89	8	450	PG	-1.46	-1.3
21	51859516	Heat shock protein 90 kDa alpha (cytosolic), class B	83.63	4.97	25	870	PG	-2.56	-1.28
22	210032365	Tumor rejection antigen gp96 precursor	93.00	4.72	26	523	POX	4.14	Unique in PG
23	158186649	Alpha-Enolase isoform 1	47.44	6.16	14	575	POX	2.19	2.89
24	13928850	D-3-phosphoglycerate dehydrogenase	57.26	6.28	19	726	PG	-1.64	1.46
25	1352384	Protein disulfide-isomerase A3	57.04	5.88	21	555	PY	1.55	-1.84

Spots	GI#	Protein name	MW (kDa)	PI	Peptides	Protein scores	Gel (spot location)	Fold change POX compared to PG	Fold change PY compared to PG
26	55741S53	Lukaryotic translation initiation factor 4H	27.36	6.67	1	100	POX	1.11	Unique in PG
27	299036	B-36 VDAC = 36kda voltage dependent anion channel	32.33	7.44	10	336	POX	-2.68	1.05
28	158138568	Serum albumin precursor	70.71	6.09	4	60	PG	1.25	Unique in PG
29	149021114	Vimentin, isoform CRA_b	53.73	5.06	30	804	POX	1.46	0
30	5803225	Tyrosine 3/tryptophan 5 - monoxygenase activation protein	29.33	4.63	17	714	POX	-1.57	-3.36
31	109484558	Glyceraldehyde-3-phosphate dehydrogenase	36.05	8.44	7	439	PG	-1.09	1.46
32	109492380	Actin, cytoplasmic 2	59.16	5.67	15	518	PY	8.43	Unique in PG
Spots	GI#	Protein name	MW (kDa)	PI	Peptides	Protein scores	Gel (spot location)	Fold change POX compared to PG	Fold change PY compared to PG
33	109486214	Phosphatidylinositol-3-phosphate/ phosphatidylinositol	245.02	6.35	24	64	POX	-3.86	-1.08
34	62664759	Prohibitin	27.76	5.44	12	670	PG	1.65	0