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# Cadmium induces alterations in the human spinal cord morphogenesis

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**Abstract** The effects of cadmium on the central nervous system are still relatively poorly understood and its role in neurodegenerative diseases has been debated. In our research, cultured explants from 25 human foetal spinal cords (10–11 weeks gestational age) were incubated with 10 and 100 μM cadmium chloride ($CdCl_2$) for 24 h. After treatment, an immunohistochemical study [for Sglial fibrillary acidic protein (GFAP) and choline acetyltransferase (ChAT)], a Western blot analysis (for GFAP, $\beta$-Tubulin III, nerve growth factor receptor, Caspase 8 and poly (ADP-ribose) polymerase), and a terminal deoxynucleotidyl transferase biotin-dUTP nick end labelling (TUNEL) assay (for detection of apoptotic bodies) were performed. The treatment with $CdCl_2$ induced a significant and dose-dependent change in the ratio motor neurons/glial cells in the ventral horns of human foetal spinal cord. The decrease of the choline acetyltransferase-positive cells (motor neurons) and the reduction of $\beta$ Tubulin III indicate that $CdCl_2$ specifically affects motor neurons of the ventral horns. While the number of motor neurons decreased for the activation of apoptotic pathways (as shown by the increased expression of Caspase 8, nerve growth factor receptor, and poly (ADP-ribose) polymerase), glial cells, both in the subependymal zone and in the gray matter of the ventral horns, increased (as shown by the increase of GFAP expression). These results provide the evidence that during human spinal cord development, $CdCl_2$ may affect the fate of neural and glial cells thus, being potentially involved in the etiopathogenesis of neurodegenerative diseases.

**Keywords** Neurodegenerative diseases · Human spinal cord · Cadmium

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## Introduction

A growing list of potential environmental risk factors is proposed for developing of amyotrophic lateral sclerosis (ALS) and other neurodegenerative diseases (Sutedja et al. 2008). The most consistent associations are exposure to pesticides, heavy metals, intense physical activity including playing professional soccer, head injury, cigarette smoking, and electromagnetic field. Among heavy metals, cadmium (Cd) is one of the most toxic heavy metals to which man can be exposed at work or in the environment (Bernard 2008) and it has been found to produce several toxic effects in animals and humans (Godt et al. 2006). Kidney, lung, liver, and bone are the principal target organs of chronic Cd toxicity (Nogawa 1981) whereas the toxic effects of Cd on the central nervous

system (CNS) are still inadequately understood. Cd, together with copper, seems to be, more than other metals, involved in the pathogenesis of neurodegenerative diseases (Matés et al. 2010) and some papers hypothesized that several diseases including Parkinson disease (Dhillon et al. 2008), as well as malformations (Paniagua-Castro et al. 2008) such as spina bifida (Kalter 1985) and forelimb ectrodactyly (Schreiner et al. 2009) might be related to Cd exposure. Concerning the role of Cd in the etiopathogenesis of sporadic and familiar ALS, some data identify it as a candidate risk factor (Huang et al. 2006). Nevertheless, more studies are required to provide an authoritative answer related to the cause–effect relationship between Cd and ALS.

Difficulties in defining the role of Cd in CNS is principally due to the fact that the blood–brain barrier (BBB) modulates the possibility for many substances to reach the CNS. Even though the anatomical structure of the BBB is analogous in the adult and in the newborn, it has been widely demonstrated that BBB in the newborn possesses different functional characteristics. In fact, during brain development there is a decline in BBB permeability to some molecules due to a drop in the intrinsic permeability of the BBB and blood cerebrospinal fluid interfaces (Saunders et al. 2000). These considerations explain the fact that Cd, and other compounds, could pass the BBB of the foetus and the newborn, exploiting the natural transporters of the essential metals (Bressler et al. 2007).

Our current knowledge of the morphological and molecular mechanisms of CNS development stems principally from experiments in chick, rat, and mouse embryos as well as in *Xenopus*; furthermore, after Cd treatment, differences in life stage specific teratogenic response have been observed in several mouse strains (Robinson et al. 2009). Cd exposure induces a great reduction in the expression of nervous system development related genes indicating a role for Cd in neural tube defects and formation. In addition, Cd induces neurological deficits during early embryonic stages in zebrafish and these affect the regionalization of the neural tube, the pattern formation and the cell fate determination, the commitment of proneural genes, and the induction of neurogenesis (Chow et al. 2008).

Several studies indicate that children of women exposed to Cd during pregnancy show lower motor and perceptual abilities, and that a high Cd body burden in children is also related to impaired intelligence and lowered school achievement (Thatcher et al. 1982; Bonithon-Kopp et al. 1986). However, little is known about the molecular and cellular basis of Cd's developmental neurotoxicity in the early life stage of animals and humans. In this study we focus on the role of Cd in affecting the human foetal spinal cord during the early stage of its morphogenesis and in particular we study the modifications of motor neurons and glial cells in the ventral horns.

## Materials and methods

### Tissue collection

Foetal tissue collection and preparation have been previously described (Lazzeri et al. 2007; Gallina et al. 2008).

Briefly, 25 spinal cord samples were obtained from the nervous system of legally aborted human foetuses. In order to avoid significant morphological and physiological differences, only foetuses of 10–11 weeks gestational age were chosen. Estimates of foetal gestational ages were determined by multiple parameters, based on time from the last menstrual period, ultrasound estimation of foetal size (crown rump length) converted to foetal age, and morphometric measurement of foetal parts. Legal abortions were performed in authorized hospitals, and permission to collect tissue was obtained from the maternal donors at the end of the abortion procedure. The study protocols were approved by the University of Firenze Ethical Committee (Protocol no. 6783-04).

### Organ culture

Each foetal spinal cord and ganglion were dissected from the spine and, immediately after isolation, were washed with phosphate-buffered saline (PBS) pH 7.4. The whole spinal cord and spinal ganglia were collected and prepared by a stereomicroscope provided for a millimetric scale. From the overlapping of spine's length and the spinal cord at this stage of development, an identification of the lumbar tract was performed. After, the last 6 mm of the lumbar tract was collected and then cut into three fragments (cross sections). The fragments were then explanted onto a

piece of Millipore filter supported by a stainless grid platform in an organ culture dish. One piece was explanted upon each dish. Organ cultures were grown in serum-free Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) at 37°C in 5% $CO_2$ for 24 h. After removal of the medium, the samples were incubated for 24 h in a fresh medium in the presence or absence of increasing concentrations of $CdCl_2$ (10 μM, 100 μM). $CdCl_2$ solution (Sigma-Aldrich) pH 5.3 was diluted in the same serum-free culture medium used to growth the explants. The range of concentrations of $CdCl_2$ and the experimental procedures used in this study derived from previous experiments performed on the same experimental model.

After incubation, some fragments were fixed in 4% buffered formalin, embedded in paraffin, and used for morphological studies and for immunohistochemical procedures. Other fragments were frozen and then processed for Western blot analysis.

## Immunohistochemistry

Immunohistochemical studies were performed as previously described (Gulisano et al. 2009). Gliosis represents a remarkable reaction of astrocytes to all types of injuries in the adult and those in a developing CNS. One of the hallmarks of astrocyte activation and the resulting reactive gliosis is the up regulation of the intermediate filament system, mainly composed of the glial fibrillary acidic protein (GFAP) (Pekny and Nilsson 2005). The immunohistochemical analysis of this marker was used to evaluate the possible activation of gliosis induced by the spinal cord samples'exposure to $CdCl_2$.

Neuronal nuclear antigen (NeuN), a highly specific marker of human fetal neuronal nuclei, has been chosen to study neuronal distribution in spinal cord and in dorsal root ganglion after $CdCl_2$ treatment. Furthermore, the immunohistochemical analysis of choline acetyltransferase (ChAT), a marker for cholinergic neurons in both peripheral and CNS (Oda 1999), was utilized to specifically evaluate the effect of $CdCl_2$ on the distribution and number of motor neurons in the spinal cord samples.

Briefly, transversal sections of human foetal spinal cord samples were incubated overnight at 4°C with the following primary antibodies: mouse monoclonal antibodies to human (GFAP) (clone 2A5, dilution 1:100, Santa Cruz Biotechnology), rabbit polyclonal antibody to human (ChAT) (dilution 1:150, Abcam), and mouse monoclonal antibody to human NeuN (dilution 1:100, Chemicon). The sections were rinsed in PBS, incubated with biotinylated secondary antibodies and submitted to a streptavidin–biotin peroxidase complex (Ultravision large volume detection system anti-polyvalent, LabVision). The reaction product was developed with the 3′,3′-diaminobenzidine tetrahydrochloride as chromogen (Sigma-Aldrich). The slides were examined with a Nikon Microphot-FX microscope (Nikon). Computer-assisted quantification of GFAP staining has been made using Quantity One Software (Bio-Rad Laboratories).

## Western blot analysis

The foetal spinal cord samples were homogenized in an ice-cold lysis buffer [50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.25% sodium dodecyl sulfate (SDS)] supplemented with a protease inhibitor cocktail (Sigma-Aldrich), and after centrifugation for 15 min at 4°C at 10,000×$g$. The supernatant was collected and the protein concentration was measured using a Coomassie Bio-Rad protein assay kit (Bio-Rad Laboratories). Aliquots containing 20 μg of proteins were diluted in 4× reducing Laemmli's sample buffer (250 mM Tris–HCl, pH 6.8, 20% glycerol, 8% SDS, 20% 2-mercaptoethanol, 0.008% bromophenol blue) and loaded onto 10 or 12% SDS-PAGE. Then, proteins were transferred onto polyvinylidene difluoride membranes (Hibond-P, Amersham). Membranes were blocked 1 h at room temperature in a 5% BSA–TTBS buffer (0.1% Tween-20, 20 mM Tris–HCl, 150 mM NaCl, pH 7.5), washed in TTBS, and incubated at 4°C overnight with the following primary antibodies: anti-$\beta$ Actin (dilution 1:10,000), anti-GFAP (dilution 1:1,000), anti-$\beta$ Tubulin III (dilution 1:2,000), anti-Caspase 8 (dilution 1:600), anti-NGFRp75 (dilution 1:1,000), and anti-PARP (dilution 1:1,000) (Santa Cruz Biotechnology). GFAP and $\beta$ Tubulin III were used for the quantification of the glial and neuronal components, respectively, in the control spinal cord samples and after $CdCl_2$ exposure (Gulisano et al. 2009). The activation of the apoptotic pathway following $CdCl_2$ treatment was studied analysing NGFRp75, PARP and Caspase 8 expression (Pehar et al. 2007; Gulisano et al. 2009; Keane et al. 2001). The primary antibodies' incubation

was followed by peroxidase conjugated secondary IgG treatment (Santa Cruz Biotechnology). Finally, the reacted proteins were revealed by the enhanced chemiluminescence system (ECL plus; Amersham Bioscience). Image acquisition and densitometric analysis were performed with Quantity One software on a ChemiDoc XRS instrument (Bio-Rad Laboratories Inc.). The densitometric analysis of each protein was normalized to $\beta$ Actin signal. To determine whether motor neuron $CdCl_2$ induced apoptosis was mediated by the NGFRp75 receptor pathway, a blocking assay was performed by pre incubating the fragments at 37°C for 30 min with 50 μg/ml of affinity purified neutralizing anti-NGFRp75 monoclonal antibody (Santa Cruz Biotechnology) before $CdCl_2$ treatment.

### Apoptotic assays

The terminal deoxynucleotidyl transferase biotin-dUTP nick end labelling (TUNEL) assay, performed using the in situ cell death detection kit (Roche Applied Science), was utilized to detect apoptotic bodies at the single cell level of the human foetals' spinal cords and dorsal root ganglia. Briefly, tissue sections were permeabilized with Proteinase K (20 μg/ml in 10 mM Tris–HCl, pH 7.6) for 15 min and stained with the TUNEL mixture according to the manufacturer's instructions. Slides were visualized using a fluorescence microscope (Nikon Microphot-FX, Nikon). The number of TUNEL positive cells (green) was counted in 15 separate fields for each slide of three independent experiments.

### Statistical analysis

Results are expressed as mean ± SD. Comparison between groups was carried out employing the Mann–Whitney test or the Wilcoxon test, as appropriate. $P < 0.05$ or $P < 0.01$ was considered statistically significant.

## Results

Treatment of human foetal spinal cord specimens for 24 h with $CdCl_2$ 10 and 100 μM deeply affects the morphology and the cytoarchitecture of the spinal cord (Fig. 1). In fact, after haematoxylin eosin staining, the ventral horns showed relevant changes both in number and cell distribution after $CdCl_2$ treatment as compared to the untreated specimens. Increasing the $CdCl_2$ concentration (10 and 100 μM) significantly lowered the cell number in the ventral horns and deeply modified the shape characteristic of the ventral horns (Fig. 1). In the dorsal horns, after $CdCl_2$ treatment, a dorsally directed enlargement of the central canal and a progressive deformation of the dorsal median septum clearly appeared (Fig. 1). After $CdCl_2$ 100 μM treatment the dorsal median septum showed a disarrangement of the spinal cord dorsal region morphology (Fig. 1). On the contrary, the morphology and the cytoarchitecture of the dorsal root ganglion, considered as control tissue, do not appear significantly modified after $CdCl_2$ treatment (Fig. 1).

Up regulation of intermediate filament proteins, in particular the GFAP, is one of the best known hallmark of reactive astrocytes and reactive gliosis induced by neuronal degeneration (Pekny and Nilsson 2005). Therefore, the GFAP expression in the spinal cord after $CdCl_2$ treatment, was evaluated by immunohistochemistry and by Western blot analysis (Fig. 2). Immunohistochemical study showed a dose-dependent increase in the expression of GFAP positive cells after $CdCl_2$ treatment in comparison to the untreated specimens (Fig. 2a–c). These data were confirmed by Western blot analysis and relative densitometric quantification (Fig. 2d).

Since gliosis often occurs after neuronal damages, the reduction of the neuronal compartment was investigated and the expression of $\beta$ Tubulin III, a marker of foetal neurons (Gulisano et al. 2009) evaluated. The Western blot analysis demonstrated a significant decrease of Tubulin III expression after $CdCl_2$ exposure in comparison to control specimens (Fig. 3a) thus suggesting a loss of neural cells following the treatment. The reduction of the neuronal compartment was associated to the activation of several apoptotic pathways, as demonstrated by the Western blot analysis of some apoptotic markers (Fig. 3b–d). In fact, the exposure of human foetal spinal cord specimens to 10 and 100 μM $CdCl_2$ caused an increase of the expression level of NGFRp75 (Fig. 3b), specifically involved in motor neuron cell death (Pehar et al. 2004), furthermore, an up regulation of Caspase 8 (Fig. 3c), not commonly

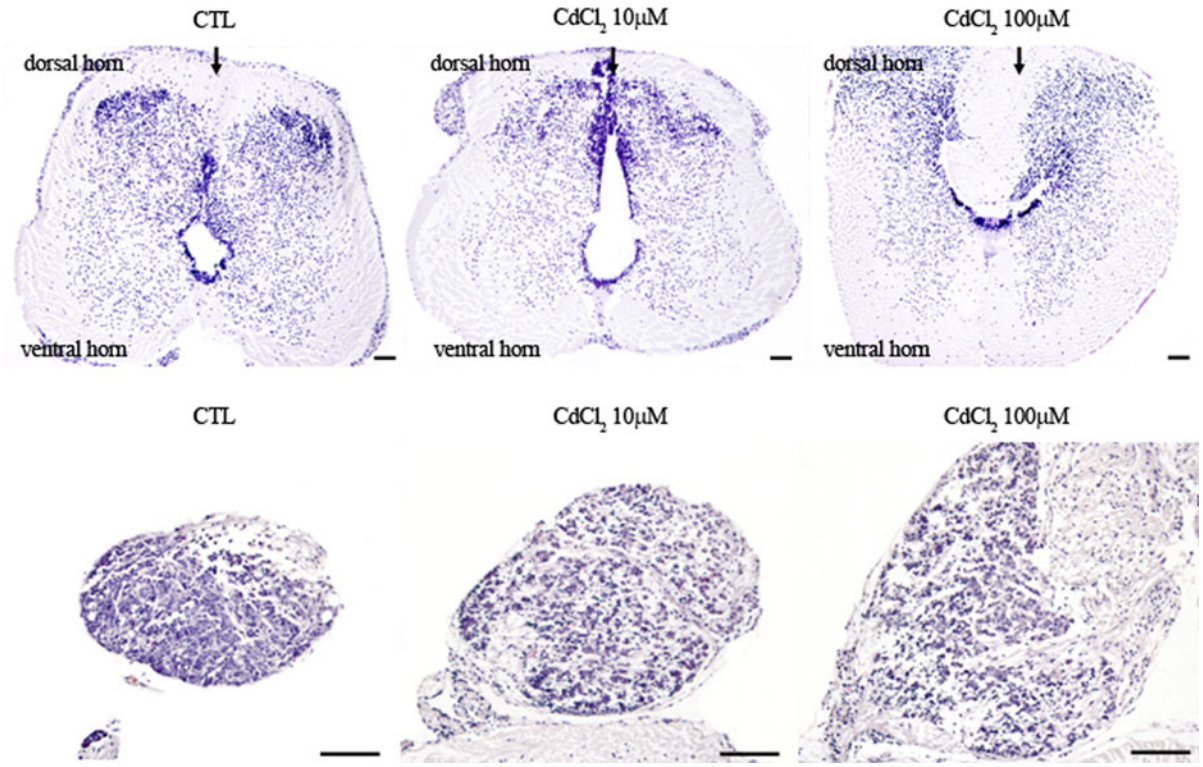



**Fig. 1** Morphology of human foetal spinal cord and dorsal root ganglion after treatment with $CdCl_2$. Sections of spinal cord and of spinal ganglion from the lumbar region were cultured on organ-culture dishes in serum-free medium for 24 h and then untreated or incubated with $CdCl_2$ (10 and 100 μM). *Upper panels* human spinal cord sections conducted at the lumbar level. In control specimens (*CTL*) the typical shape of dorsal and ventral horns was observed; dorsal median septum, ventral median fissure and ependymal canal were correctly formed. After the treatment with 10 μM $CdCl_2$ the cell density in the ventral horns was significantly reduced and the white matter appeared more evident. Dorsally, the median septum *downwards arrow* showed a degenerative aspect. When human foetal spinal cord was treated with 100 μM $CdCl_2$, the decrease of cell density in the ventral horns was more evident. In the posterior area, the dorsal median septum, after a total dehiscence, was occupied by a rarefied zone with a scant number of cells and with the aspect of white matter. *Lower panels* human dorsal root ganglion at the lumbar level. In control specimens (*CTL*) the typical shape of a dorsal root ganglion is evident; after 10 and 100 μM $CdCl_2$ treatment the structure of the spinal ganglion was not significantly modified. haematoxylin eosin. *Scale bar* 100 μm

expressed in neural precursor cells and required for initiating the apoptotic cascade (Ricci-Vitiani et al. 2004) and of both full-length and cleaved PARP (Fig. 3d) were evident after $CdCl_2$ exposure. To better understand, which of these apoptotic pathway plays a major role in inducing motor neurons death, we blocked the NGFRp75 mediated apoptotic pathway; a blocking assay of NGFRp75 was performed by pre incubating the fragments with the affinity purified neutralizing anti-NGFRp75 monoclonal antibody before 100 μM $CdCl_2$ treatment. The blocking assay, followed by the analysis of PARP expression and its densitometric evaluation, showed a significant decrease of the cleaved PARP, thus suggesting a crucial role for NGFRp75 pathway in inducing motor neurons apoptosis (Fig. 3f).

In order to determine if the spinal cord motor neurons were more susceptible to $CdCl_2$ treatment than neurons of the dorsal root ganglion, taken as control tissue, we evaluated the expression and the distribution of NeuN, a specific marker of neuronal cells, recognizing a specific nuclear neuronal protein (Sarnat et al. 1998). While increasing concentrations of $CdCl_2$ induced a significant loss of the neuronal population in the ventral horn of the spinal cord, no differences were observed in neuronal density of the spinal ganglion (Fig. 4). To further demonstrate that the $CdCl_2$ dependent neuronal decrease observed in

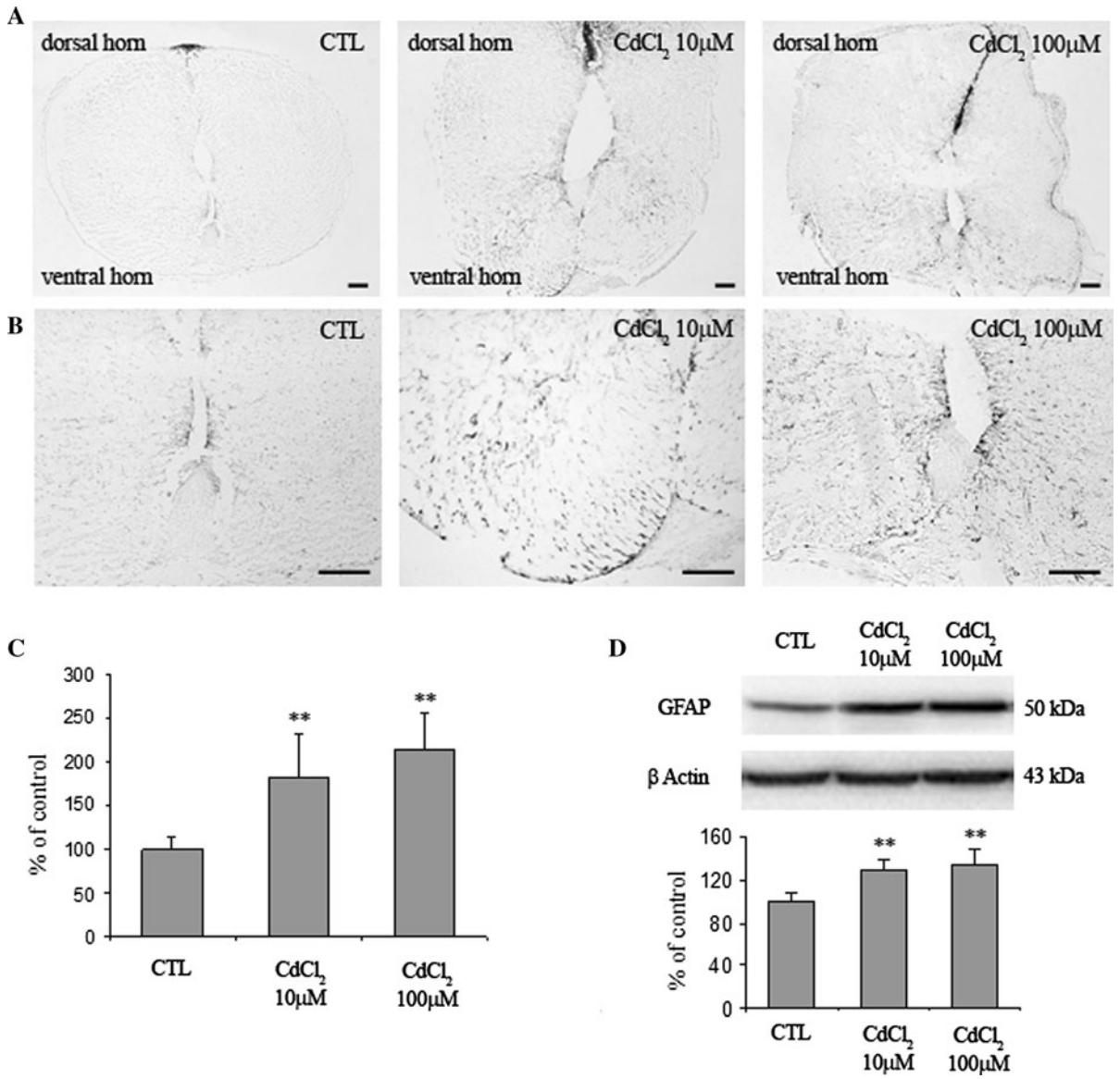



**Fig. 2** GFAP expression in human foetal spinal cord after $CdCl_2$ treatment. **a** In control specimens (*CTL*) only few GFAP positive cells were detected (in black), while the GFAP positivity after stimulation for 24 h with 10 and 100 μM $CdCl_2$ was significantly increased in a dose-dependent manner both in the ventral horns and around the central canal. Immunohistochemistry. *Scale bar* 100 μm. **b** Particular of the ventral horns. *Scale bar* 100 μm. **c** Computer-assisted quantification of GFAP staining using "Quantity One Software" in three slides for each sample and 15 fields for each slide. Data are expressed as percent increase ± SD over the control value taken as 100% (**$P < 0.01$ when comparing $CdCl_2$ 10 and 100 μM vs. CTL). **d** Western blot analysis and densitometric quantification for GFAP. Tissues were untreated (*CTL*) or treated for 24 h with $CdCl_2$. Western blot analysis with anti-GFAP antibody revealed a single band migrating approximately at the expected 50 kDa molecular mass. Note the increased expression of GFAP after the increasing $CdCl_2$ treatment. The densitometric quantification of the bands, expressed as percentage over control (taken as 100%), confirmed the increased GFAP expression following $CdCl_2$ exposure. *β* Actin signal was used for the normalization of GFAP protein expression. (**$P < 0.01$ when comparing $CdCl_2$ 10 and 100 μM vs. *CTL*)

the ventral horns of the spinal cord was due to a loss of neurons and specifically of motor neurons, we performed an immunohistochemical analysis of ChAT, a specific marker for motor neurons (Fig. 5a). The number of ChAT positive neurons was significantly decreased in a dose-dependent manner after the

treatment with different concentrations of $CdCl_2$ (Fig. 5b). According to the decrease of ChAT positive cells, the number of apoptotic cells in the ventral horns, as revealed by TUNEL assay (Fig. 6a), increased in a dose-dependent manner in the specimens treated with $CdCl_2$ in comparison to control, untreated specimens. The number of apoptotic cells in the spinal ganglion (Fig. 6b), considered a control tissue, were not significantly varied after $CdCl_2$ treatment.

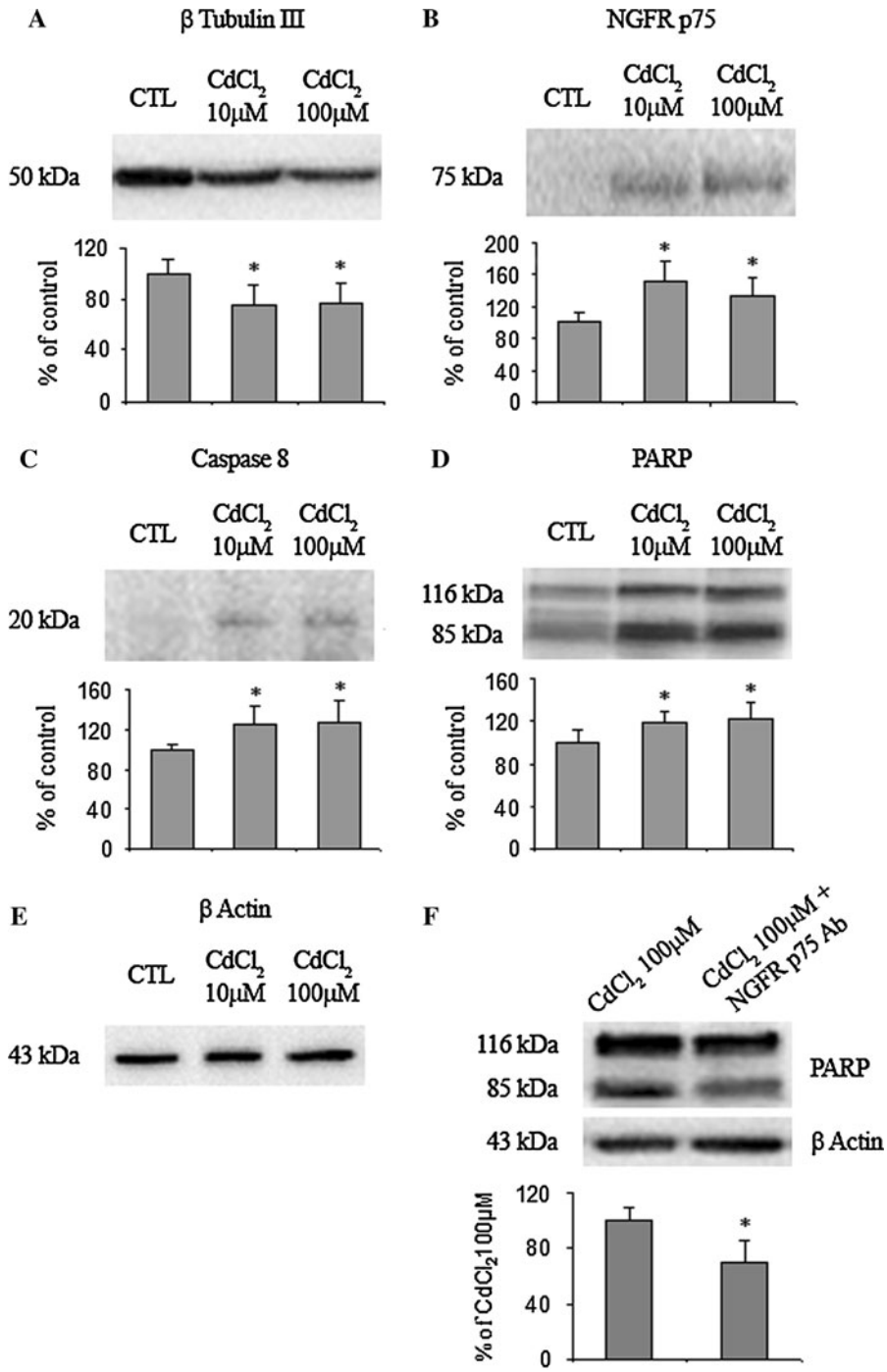
## Discussion

It is well established that multiple gene-environmental interactions which govern different biochemical pathways are linked to the etiopathogenesis of neurodegenerative diseases (Johnson and Atchison 2009). Among the environmental agents linked to neurodegenerative diseases, noxious industrial pollutants play a crucial role. Although studies exist demonstrating that industrial chemicals cause CNS damages during its development, only few known chemicals have been labelled as being toxic to human neurodevelopment (Grandjean and Landrigan 2006) and Cd is among these. The heavy metals such as Cd are certainly likely candidates for investigation because of their proven neurotoxicity, ubiquitous nature and causal epidemiological link with neuropsychological disorders and neurodegenerative diseases (Johnson and Atchison 2009).

The neurotoxic effects of Cd have been reported in neonatal mouse brain and young rat brain but only meager data are available relative to the role of Cd on the human developing CNS. In humans, occupational exposure to Cd is associated with neuropsychological disorders (Hart et al. 1989), and Cd exposure is reported to be a possible cause of ALS (Bar-Sela et al. 2001). Cd has been shown to selectively damage striatum (Fernandez-Perez et al. 2010), and Parkinsonism has been reported in a 64-year-old man exposed to a high dose of Cd (Okuda et al. 1997).

Damages Cd-induced on CNS may be explained by the Cd ability of activating apoptotic pathways. Recently, several reports have shown that Cd can induce apoptosis of many tissues and cells both in vivo and in vitro, such as the cells of the respiratory system, the testis, the kidney, the liver, and the immune system (Li et al. 2000). This evidence indicates that apoptosis probably plays a key role in acute and chronic intoxication by Cd. In human lung epithelial fibroblast cell line, Cd has been shown to cause apoptosis by the Caspase-8-dependent Bid cleavage, activation of Caspase-9 and -3, and PARP cleavage (Oh et al. 2004). In human lymphoma cells it has been demonstrated that Caspase 8 may be the most apical Caspase induced by Cd in the apoptotic Caspase-dependent pathway (Li et al. 2000), furthermore, a recent research has demonstrated that the activation of NGFRp75 induces motor neuron apoptosis in rat embryonic spinal cord (Pehar et al. 2007). Besides the activation of different apoptotic pathways, recent researches have demonstrated that astrocytes play a complex role in repair after spinal cord injury. The vicinity of a traumatic injury, environmental cues associated with cell damage and neuroinflammation induce astrocytes to proliferate, migrate, differentiate, forming a dense network bordering the lesion site. This response not only contributes to the formation of the glial scar (White and Jakeman 2008) but it also provides support and guidance for axonal growth and aid in improving functional recovery after spinal cord injury. Several studies using GFAP-deficient mice demonstrated that animals lacking of GFAP have increased hippocampal degeneration after brain injury (Otani et al. 2003), form an abnormal glial scar after brain and spinal cord lesions (Pekny et al. 1999), produce astrocytes with impaired migratory abilities in vitro (Lepekhin et al. 2001), and are more susceptible to death after cervical spinal cord injury (Nawashir et al. 1998). Reactive astrocytes can arise either from astrocytes that are already present at the time of injury, or from progenitor cells that are found either in regions surrounding the central canal (Beattie et al. 1997) or the subpial region of the spinal cord (Wu et al. 2005).

In this study we investigated the effects of Cd on human spinal cord at early stages of development, when its vulnerability to neurotoxicants is high in comparison to dorsal root ganglion, taken as control tissue. We used spinal cord slices in vitro, an experimental model in which the tissue cytoarchitecture and extracellular matrix connections are preserved, and the interactions between neuronal and glial cells analogous to those in vivo. This study provided the first evidence that Cd is able to affect two distinct cell populations in developing human spinal cord; in fact, important signs of apoptosis are




evident in motor neurons and, at the same time, a significant gliosis arises.

Our findings indicate that Cd may activate different signal cascades mainly involving NGFRp75 but also Caspase 8 and PARP with consequent specifically reduction of the motor neuronal compartment. Neuronal Cd-induced apoptosis is associated to a significant gliosis as demonstrated by a significant increase of GFAP expression. The number of GFAP positive cells increased after Cd treatment surrounding the

◀**Fig. 3** Western blot analysis of spinal cord protein extract stained with antisera to $\beta$ Tubulin III, NGFRp75, Caspase 8 or PARP. Western blot analysis of human foetal spinal cord samples showed a significant decreased expression of the neuronal marker $\beta$ Tubulin III following $CdCl_2$ exposure, indicating a neuronal loss after the treatment (**a**). These data were confirmed by the analysis of some apoptotic markers such as NGFRp75 (**b**), Caspase 8 (**c**), and PARP (**d**) which resulted upregulated after $CdCl_2$ exposure. The densitometric computer-assisted quantification of bands corresponding to the proteins examined by Western blot was expressed as percentage over control (taken as 100%). Concerning PARP, the densitometric evaluation was performed on the 85 kDa cleaved fragment. All the panels derived from one blot and $\beta$ Actin signal was used for the normalization of each protein expression (**e**). ($^*P < 0.05$ when comparing $CdCl_2$ 10 and 100 μM vs. CTL). Western blot analysis and densitometric quantification performed after the block of the NGFRp75 pathway showed a significant decrease of cleaved PARP after treatment with 100 μM $CdCl_2$ (**f**). In this panel the unblocked sample is considered as control (taken as 100%). ($^*P < 0.05$ when comparing blocked $CdCl_2$ 100 μM sample vs. the unblocked one)

area of motor neuron loss, as well as in the proximity of the ependymal canal. The significance of this increased GFAP expression is not yet clear. It is possible that Cd may activate astrocyte, thus playing a role in the organizational events of glial scar formation. This increase in GFAP expression may also be related to the attempt of glial cells to be supportive of recovery.

Motor neurons death and consequent glial activation observed in this study after Cd treatment, may be explained considering the molecular mechanism of Cd. In fact, Cd ion easily substitutes for the Calcium and Zinc ions in several biological systems because it carries the same charge and it has a similar radius. For its ability in substituting and/or displacing calcium and zinc, Cd is involved in the generation of reactive oxygen species, inhibition of DNA repair enzymes, deregulation of cell proliferation as well as DNA methylation (Beyersmann and Hartwig 2008).

Disregulation of Calcium and abnormal Calcium homeostasis are decisive in long-term neurodegeneration diseases (Hidalgo and Carrasco 2011) such as Alzheimer's disease, Parkinsons's disease, Huntington's disease and ALS (Zündorf and Reiser 2011). During CNS development, when the permeability of BBB is changing, Cd, interfering with biochemical events where calcium or zinc are involved, might deeply affect the developing of nervous structures.

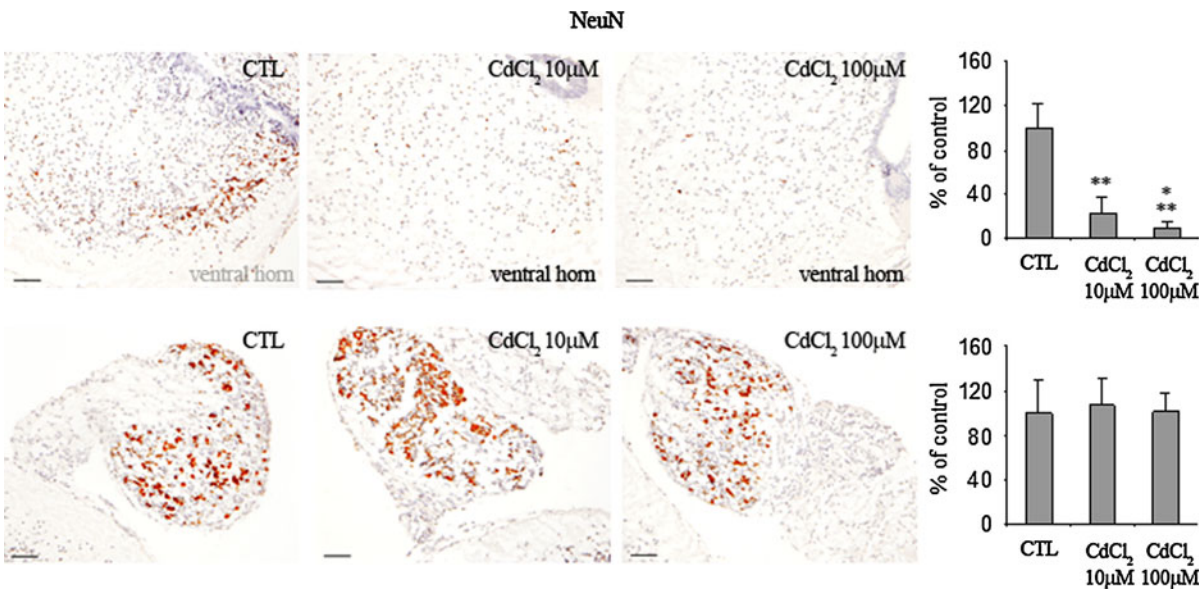



**Fig. 4** Immunohistochemical analysis of NeuN in the ventral horns of human foetal spinal cord and ganglion samples. *Upper panels* NeuN positive cells were detected before $CdCl_2$ treatment in the ventral horns of the foetal spinal cord (*CTL*); after stimulation for 24 h with 10 and 100 μM a significant dose-dependent decrease of NeuN positive cells was observed. *Lower panels* the number of the NeuN positive cells appears unaffected after $CdCl_2$ treatment. Appropriate densitometric evaluation was performed for each tissue and treatment. *Scale bar* 50 μm. ($^{**}P < 0.01$ when comparing $CdCl_2$ 10 and 100 μM vs. CTL; $^*P < 0.05$ when comparing $CdCl_2$ 100 μM vs. $CdCl_2$ 10 μM)

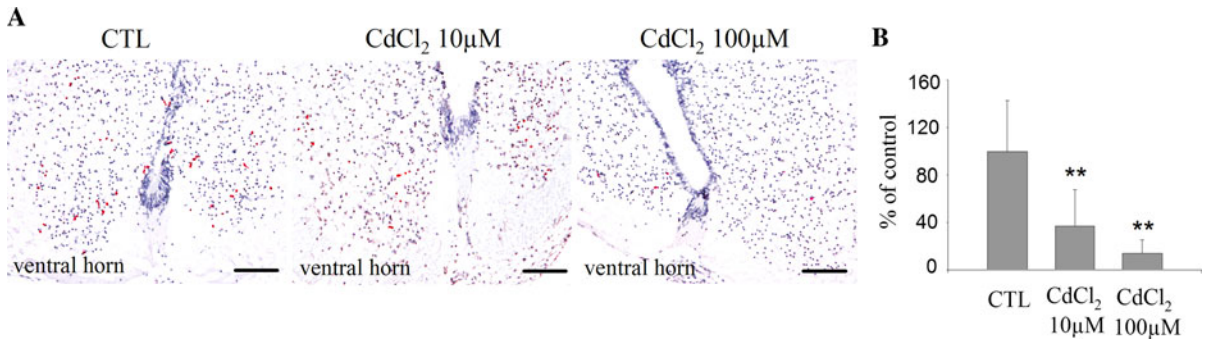



**Fig. 5** Immunohistochemical analysis of ChAT in the ventral horns of human foetal spinal cord samples. **a** Specimens of human foetal spinal cord were immunostained with anti-ChAT, a specific marker of motor neurons. Cell nuclei were counterstained with haematoxylin. *Scale bar* 100 μm. **b** The number of ChAT positive cells in control and treated specimens was counted and expressed as percentage over control (taken as 100%). ChAT positive motor neurons significantly decreased in a dose-dependent manner when specimens were treated with $CdCl_2$ 10 and 100 μM in comparison to control specimens. Data were obtained counting 15 separate fields for each slide of three independent experiments (**$P < 0.01$ when comparing $CdCl_2$ 10 and 100 μM vs. CTL)

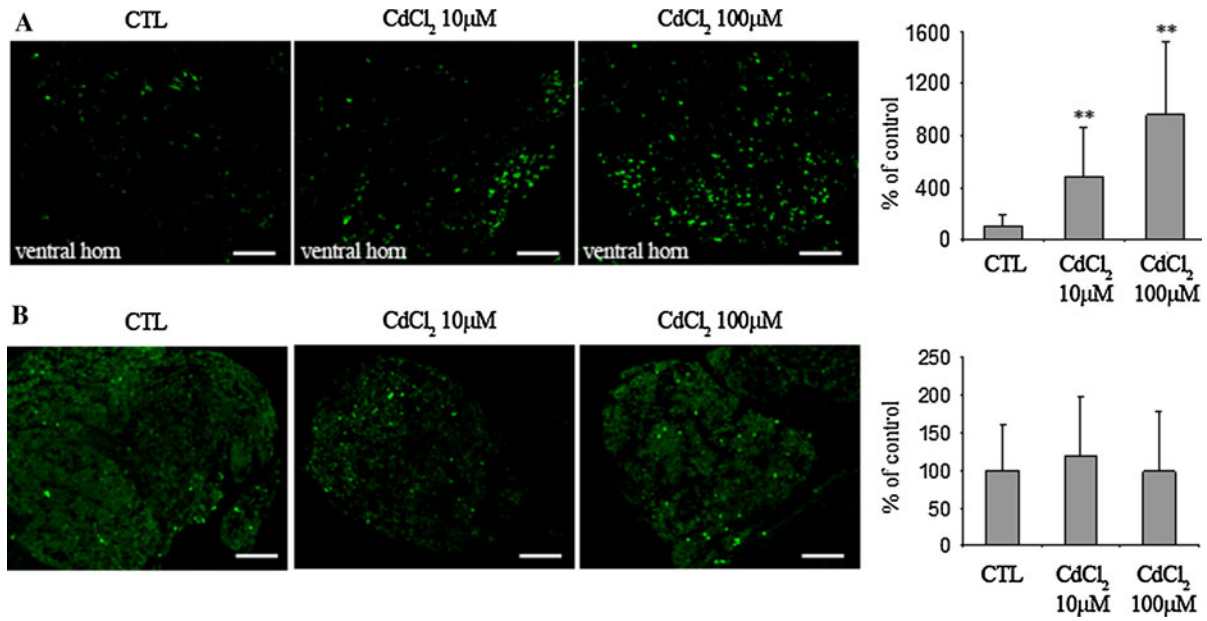



**Fig. 6** TUNEL assay in the ventral horns of human foetal spinal cord and spinal ganglion samples. **a** TUNEL assay for the staining of apoptotic cells in ventral horns of human foetal spinal cord. Nuclei of apoptotic cells were stained with fluorescent dye. The density of apoptotic cells significantly increased after $CdCl_2$ treatment. *Scale bar* 100 μm. The number of apoptotic cells in control and treated specimens was counted and expressed as percentage over control (taken as 100%). TUNEL positive cells significantly increased after $CdCl_2$ treatment in comparison to control untreated specimens in a dose-dependent manner. Data were obtained counting 15 separate fields for each slide of three independent experiments (**$P < 0.01$ when comparing $CdCl_2$ 10 and 100 μM vs. CTL). **b** TUNEL assay for the staining of apoptotic cells in dorsal ganglion. Nuclei of apoptotic cells were stained with fluorescent dye. The number of apoptotic cells did not significantly vary after $CdCl_2$ treatment. *Scale bar* 100 μm

Based on the results shown above, and consistent with previous observations, we assume that Cd, due to its chemical characteristics and its influence on cell homeostasis, might be one, even though not the only one, metal playing a key role in neuronal degeneration and concomitant gliosis, i.e. those pathologic

features typically observed in common neurodegenerative diseases.

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