POLYCHLORINATED BIPHENYLS PCB 153 AND PCB 126 IMPAIR THE GLUTAMATE-NITRIC OXIDE-cGMP PATHWAY IN CEREBELLAR NEURONS IN CULTURE BY DIFFERENT MECHANISMS.

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Abstract
Polychlorinated biphenyls (PCBs) are persistent organic pollutants present in human blood and milk. Exposure to PCBs during pregnancy and lactation leads to cognitive impairment in children. Perinatal exposure to PCB 153 or PCB 126 impairs the glu-NO-cGMP pathway in cerebellum *in vivo* and learning ability in adult rats. The aims of this work were: 1) to assess whether long-term exposure of primary cultures of cerebellar neurons to PCB153 or 126 reproduces the impairment in the function of the glu-NO-cGMP pathway found in rat cerebellum *in vivo*; 2) to provide some insight on the steps of the pathway affected by these PCBs; 3) to assess whether the mechanisms of interference of the pathway are different for PCB126 and PCB153. Both PCB153 and PCB126 increase basal levels of cGMP by different mechanisms. PCB126 increases the amount of soluble guanylate cyclase while PCB153 does not. PCB153 reduces the amount of calmodulin while PCB126 does not. Also both PCBs impair the function of the glu-NO-cGMP pathway by different mechanisms; PCB153 impairs NO-induced activation of sGC and increase in cGMP while PCB126 does not. PCB126 reduces NMDA-induced increase in calcium while PCB153 does not.

Introduction
Polychlorinated biphenyls (PCBs) are persistent organic pollutants that accumulate in the food chain. PCBs are present in blood and milk of mothers and children may be exposed to PCBs during pregnancy and lactation. Children born from mothers exposed to PCBs show memory deficits and cognitive dysfunctions as well as sensory and motor disorders[1, 2]. PCBs have been classified in coplanar and non-coplanar, and also into 'dioxin-like' and 'non-dioxin-like'. Both types of PCBs have been associated to neurodevelopmental deficits[2] but it is not clear whether the mechanisms of neurotoxicity of 'dioxin-like' and non 'dioxin-like' congeners are similar or different. It has been reported that the ability to learn a Y maze conditional discrimination task is modulated by the glutamate (glu)-nitric oxide (NO)-cGMP pathway, associated to NMDA receptors. The function of this glu-NO-cGMP pathway and learning ability decrease in parallel in adult compared to young rats[3]. The function of the glu-NO-cGMP pathway is impaired in cerebellum in animal models of chronic hyperammonemia or liver failure[4]. The ability to learn the Y maze task is also reduced in these animal models[5, 6, 7, 8]. Perinatal exposure of rats to PCB 153 or PCB 126 impairs the function of the glu-NO-cGMP pathway in cerebellum *in vivo* and the ability to learn a conditional discrimination task when the rats are 2-3 months-old[9]. The authors propose that the impairment in learning ability is a consequence of the reduced function of the glu-NO-cGMP pathway[9]. The molecular mechanisms by which exposure to PCBs impairs the function of the glu-NO-cGMP pathway cannot be studied in detail in brain *in vivo* but could be analyzed in primary cultures of neurons exposed to the PCBs, if this model reproduces the effects of PCBs found in brain *in vivo*.
The aims of the present work were: (1) to assess whether *in vitro* exposure of primary cultures of cerebellar neurons to PCB153 or 126 reproduce the impairment in the function of the glu-NO-cGMP pathway found *in vivo*; (2) to provide some insight on the steps of the pathway affected by these PCBs and (3) to assess whether the mechanisms of interference of the pathway are different for the dioxin-like PCB126 and for the non dioxin-like PCB153.

Material and methods.
Primary cultures of neurons. Primary cultures of cerebellar neurons were prepared as previously described[10]. Cerebella from 7-day-old Wistar rats were rapidly dissected and incubated with 3 mg/ml dispase for 30 min in a 5% CO_2 incubator at 37ºC. The supernatant was removed, and cells were incubated with basal Eagle medium

containing 40µg/ml DNase I. The cellular suspension was filtered through a mesh with a pore size of 90µm and centrifuged at 400g for 5 min, and the cell bottom was rinsed twice with basal Eagle medium. The cells were resuspended in complete basal Eagle medium. Cells were plated onto polylysine-coated plates after 20 min at 37ºC, medium containing unattached cells was removed, and fresh medium was added. To prevent proliferation of non-neuronal cells, 10µM cytosine arabinoside was added 24h after plating. Glucose (5.6mM) was added to the culture medium twice a week.

Exposure to neurotoxicants. PCB 153 or PCB 126 were added at the indicated concentrations to the culture medium 24 hours after seeding and were present for 10-13 days. PCBs are dissolved in DMSO and the same concentration of DMSO was added to culture medium of control plates.

Determination of neuronal viability. Neuronal viability was determined as previously described[11] by double fluorescence staining with propidium iodide (PI, labels dead neurons in red, 0.46µg/ml) and fluorescein diacetate (FDA, labels living neurons in green, 15µg/ml. Neuronal viability was assessed after exposure to different concentrations of PCB153 or PCB126 to choose the doses to be used in the subsequent experiments, avoiding use of doses leading to massive death.

Determination of basal cGMP in cultured neurons. Primary cultured neurons were used 11-14 days after seeding. Monolayers in tissue culture dishes were washed three times with pre-warmed Locke's solution without magnesium. After treatments, Locke's solution was removed, the neurons were resuspended in 200µl of the kit assay buffer containing 4mM EDTA and disrupted by sonication. Samples were centrifuged (13,000g, 5 min) and cGMP was measured in the supernatant. cGMP was determined using the BIOTRAK cGMP enzyme immunoassay kit from Amersham.

Activation of the glu-NO-cGMP pathway or of soluble guanylate cyclase (sGC) in cerebellar neurons. To activate the glu-NO-cGMP pathway 0.3mM NMDA was added for 5 min at 37ºC. To activate sGC, the NO-generating agent SNAP (0.1mM) was added for 5 min. Then neurons were resuspended in acetate buffer containing 4mM EDTA and disrupted by sonication. Samples were centrifuged (14,000g, 5 min) and cGMP was measured as above.

Determination of NMDA-induced increase in free intracellular calcium concentration. Monolayers in tissue culture dishes were washed three times with pre-warmed Locke's solution without magnesium. Neurons were loaded with the fluorescent probe Fura2-AM (4µM) for 45 minutes and then washed 4-5 folds to eliminate excess of probe and incubated for 15 minutes to complete cutting of probe by intracellular estherases. To measure the increase of intracellular calcium induced by NMDA we perfused 0.3mM NMDA in Locke's solution followed by Locke's solution alone. The ratio 340/380 was analyzed with Aquacosmos software from Hamamatsu. To convert the ratio (F340/F380) to free calcium concentration (nM), we used the following formula [Ca](nM) = Kd*Fmax/Fmin (380) *(R/Rmin)/(R/Rmax), with these constants: 230*1,75*(R-0,0385)/(0,101-R) Kd is the theoretical value at temperature and pH conditions that we have in our experiments for Fura2, Rmax , Rmin and Fmax/Fmin(380) were calculated in these cultures with a solution of Locke without calcium + 10µM ionomicin + 5mM EGTA for Rmin and a solution of Locke + 10µM ionomicin + 20mM calcium for Rmax.

Immunoblotting. Neurons were homogenized in lysis buffer. Samples were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting as previously described[12] using monoclonal antibodies against NR1 subunit of NMDA receptor and calmodulin, and polyclonal antibodies against nNOS, GC alpha 1 subunit, and GC beta 1 subunit. All primary antibodies were used at 1:1000 dilution. Secondary antibodies conjugated with alkaline phosphatase were from Sigma. After development using alkaline phosphatase images were captured with a Hewlett Packard scan Scanjet 5300C. The intensities of the bands were measured using the program AlphaImager 2200

Statistical analysis. The data shown are the mean $\pm$ S.E.M. of four to six experiments. Statistical significance was estimated with one-way ANOVA and Newman-Keuls multiple comparison test and Student's t-test.

Results and Discussion

Effects of chronic exposure to PCB 153 or PCB 126 on neuronal viability.

Long-term exposure to PCB 153 induced some neuronal death at 50µM (71 ± 7% of survival Vs 86 ± 4% in control neurons). The EC_{50} for PCB153 was 120µM. Exposure to low concentrations of PCB 126 induces death of a small percentage of neurons, beginning at 1nM (75 ± 2% survival Vs 82 ± 4 in control neurons). Higher

concentrations of PCB126 increased neuronal death in a dose-dependent manner. The EC_{50} for PCB126 was 9µM (Fig. 1).

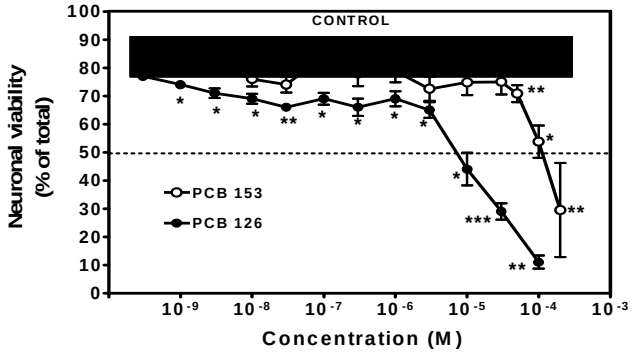

Figure 1. Effects of chronic exposure to PCB 153 or PCB 126 on neuronal viability. Values are the mean ± SEM of triplicate samples from five experiments. Values that are significantly different from control values are indicated by asterisks (*p < 0.05, **p< 0.01).

Long-term exposure to PCB 153 or PCB 126 increases basal levels of cGMP in cerebellar neurons in culture.
Long-term exposure to PCB 153 at 1µM or higher concentrations increased the basal levels of cGMP, which increased to 136% of control for 1µM PCB 153 and to 150% for 3 and 10µM PCB 153. Long-term exposure to PCB 126 also increased the basal levels of cGMP but at lower concentrations, reaching 123, 174 and 183% of control neurons at 0.1, 0.3 and 1µM PCB 126, respectively (Fig. 2).

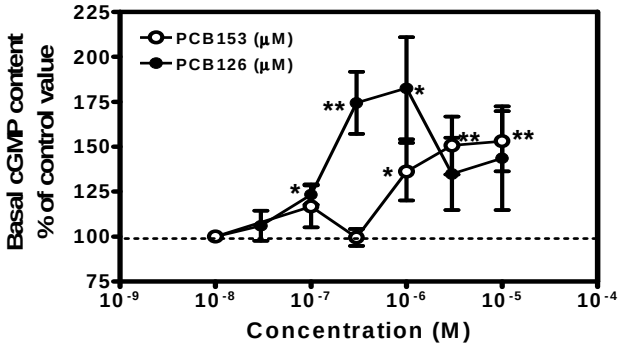

Figure 2. Long-term exposure of cerebellar neurons in culture to PCB 126 or PCB 153 increases basal levels of cGMP. Values are the mean ± SEM of triplicates samples from seven experiments. Values that are significantly different from control values are indicated by asterisks (*p < 0.05, **p< 0.01).

Long-term exposure to PCB 153 or PCB 126 impairs the function of the glu-NO-cGMP pathway.
We added 0.3mM NMDA to activate the glu-NO-cGMP pathway. NMDA induced an increase of intracellular cGMP in control neurons (470 ± 55% of basal value). In neurons treated with PCB 153 or PCB 126 NMDA also induced increase of cGMP, but the increase was significantly lower (Fig. 3). Long-term exposure to PCB 153 reduced the NMDA-induced increase of cGMP at 1µM or higher concentrations. NMDA-induced increase in cGMP in neurons exposed to 1, 3 and 10µM PCB153 was 81 ± 7, 82 ± 7 and 61 ± 8% of the increase in control neurons (Fig. 3). Long-term exposure to PCB 126 also reduced NMDA-induced increase of cGMP, NMDA-induced increase in cGMP in neurons exposed to 0.3µM PCB126 was 65 ± 11% of the increase in control neurons, the reduction was around 50% for neurons exposed to 1,3 or 10µM PCB 126 (Fig.3).

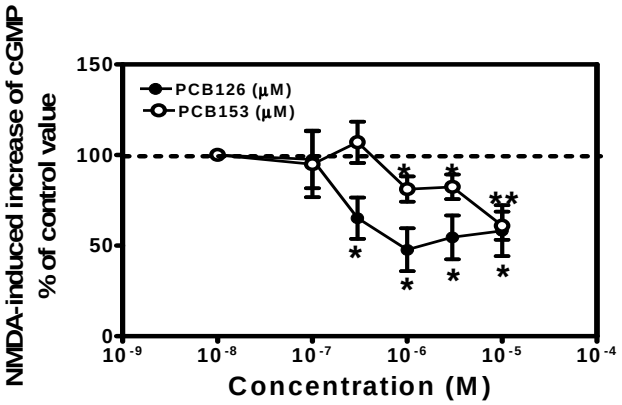

Figure 3. Long-term exposure of cerebellar neurons in culture to PCB 126 or PCB153 impairs the function of the glu-NO-cGMP pathway. Values are the mean ± SEM of triplicate samples from five experiments. Values that are significantly different from control values are indicated by asterisks (*p < 0.05, **p< 0.01).

It is shown that both PCB153 and 126 increase basal levels of cGMP in cerebellar neurons in culture and reduce the function of the glu-NO-cGMP pathway at concentrations that did not induce neuronal death. PCB126 is about 10-fold more potent than PCB153 in increasing basal cGMP levels, 3-10-fold more potent in inhibiting the glu-NO-cGMP pathway and 13-fold more potent in inducing neuronal death. Also *in vivo* PCB126 is more potent in inhibiting the glu-NO-cGMP pathway in cerebellum and 10,000-fold larger doses of PCB153 are required to induce the same effect than PCB126[9].

To assess which step of the glu-NO-cGMP pathway is affected by chronic exposure to PCB 153 or PCB 126 we analyzed the effects of exposure to PCBs on activation of sGC by a NO-generating compound, SNAP.

Long-term exposure to PCB 153 but not to PCB 126 impairs activation of sGC by NO.

Addition of 0.1mM SNAP to control neurons induces increase of intracellular cGMP (498 ±102% of basal). Long-term exposure to PCB 153 reduced the SNAP-induced increase of cGMP at 1μM or higher concentrations. Addition of SNAP to neurons exposed to 0.1, 1, 3 and 10μM PCB153 increased cGMP levels to 446± 34%; 303 ± 54%; 368 ± 49% and 399 ± 36% of basal, respectively. This represents a reduction of the increase of cGMP induced by SNAP in control neurons to 61 ± 11, 74 ± 10 and 80 ± 6 % for 1, 3 and 10μM PCB 153, while 0.1μM PCB153 did not affect significantly SNAP-induced increase in cGMP (Fig. 4). In contrast, exposure to PCB 126 did not alter SNAP-induced increase of cGMP at any of the concentrations tested (Fig. 4).

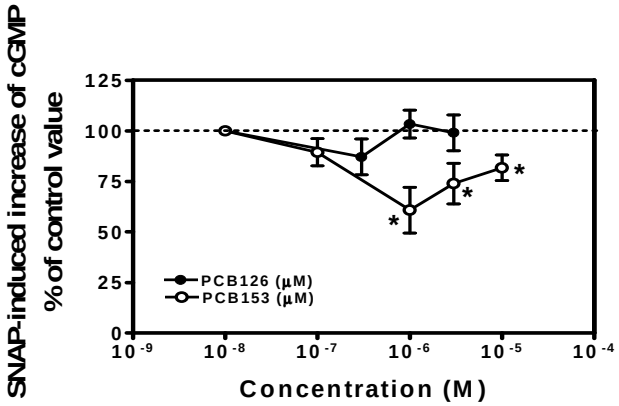

Figure 4. Long-term exposure of cerebellar neurons in culture to PCB153 but not to PCB126 reduces activation of sGC by NO. Values are the mean ± SEM of triplicate samples from five experiments. Values that are significantly different from control values are indicated by asterisks (*p < 0.05).

PCB153 reduces the direct activation of sGC by SNAP at 1, 3 and 10µM concentrations, but not at 0.1µM. As SNAP produces directly NO, which activates sGC directly without affecting NMDA receptors or intracellular calcium, this supports that the reduced formation of cGMP by sGC due to lower activation of this enzyme by NO is responsible for the reduced NMDA-induced increase in cGMP and for impairment of the function of the whole pathway by PCB153. In the case of PCB126 activation of sGC by NO is not affected.
We also assessed whether NMDA-induced increase in intracellular calcium is affected by chronic exposure to PCB 153 or PCB 126.

Long-term exposure to PCB 126 or high concentrations of PCB 153 reduces NMDA-induced increase in intracellular calcium.
Addition of 0.3mM NMDA to control neurons increased intracellular calcium (1036 ± 180% of basal). Long-term exposure to PCB 153 did not affect NMDA-induced increase in calcium at any of the concentrations tested except at 3µM, which reduces it to 53 ± 16% of control neurons. Exposure to PCB 126 at 0.3 or 3µM significantly ($p < 0.05$) reduced NMDA-induced increase in calcium to 55 ± 15 and 51 ± 3% of the increase in control neurons, respectively (Fig. 5).

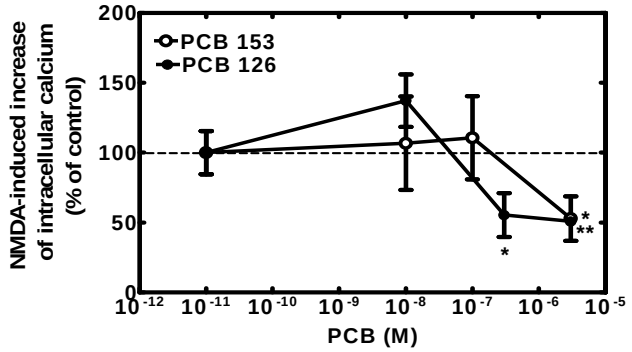

Figure 5. Long-term exposure of cerebellar neurons in culture to PCB 126 but not to PCB153 impairs NMDA-induced increase in intracellular calcium. Values are the mean ± SEM of triplicate samples from four experiments. Values that are significantly different from control values are indicated by asterisks (*$p < 0.05$, **$p< 0.01$).

A main mechanism by which PCB 126 reduces the function of the glu-NO-cGMP pathway is by reducing NMDA-induced increase in calcium, which would lead to reduced activation of NOS and formation of NO, this reduction would result in reduced activation of sGC compared to control neurons non exposed to PCB. The mechanism by which PCB126 reduces NMDA-induced increase in Ca^{2+} is not known for the moment.
To assess whether altered content of the proteins involved in the glu-NO-cGMP pathway could contribute to the impairment of the function of the pathway by PCBs, we analyzed the content of NMDA receptor subunit NR1, calmodulin, neuronal NO synthetase (nNOS) and alpha and beta subunits of guanylate cyclase in neurons exposed to PCB 153 or PCB 126.

Effect of chronic exposure to PCB 153 or PCB 126 on the content of proteins involved in the glu-NO-cGMP pathway
Exposure to PCB 153 significantly reduced ($p < 0.05$) the content of calmodulin, which was around 80% of control in neurons exposed to 1-10µM PCB 153. PCB 153 did not affect the content of NR1 subunit of NMDA receptors, nNOS or alpha subunit of sGC (Table 1). The content of the beta subunit of sGC was not affected by 1 or 3µM PCB 153 but was reduced ($p < 0.05$) to 82% of control by 10µM PCB 153. In contrast, exposure of cerebellar neurons in culture to PCB 126 did not alter the content of calmodulin (Table 1), indicating again that the mechanism by which PCBs 153 and 126 affect the glu-NO-cGMP pathway are different. PCB126 did not affect the content of the NR1 subunit of NMDA receptors or the beta subunit of sGC (Table 1). Long-term exposure to PCB126 significantly ($p < 0.05$) increased the content of the alpha subunit of sGC to 150, 153 and 134% of control at 0.3, 1 and 3µM PCB126, respectively. The content of nNOS was not affected by 0.3 or 1µM PCB 126 but was increased ($p < 0.05$) to 126% of control by 10µM PCB 126.

PCB	Concentration	Amount of protein (% of control neurons)				
		NR1	Calmodulin	nNOS	sGC-alpha1	sGC-beta1
153	1 µM	102 ± 9	**83 ± 6***	114 ± 13	86 ± 20	90 ± 7
153	3 µM	92 ± 11	**83 ± 7***	110 ± 12	95 ± 18	96 ± 7
153	10 µM	98 ± 8	**82 ± 8***	117 ± 17	117 ± 15	**82 ± 5***
126	0.3 µM	86 ± 11	105 ± 3	99 ± 13	**150 ± 19***	106 ± 11
126	1 µM	90 ± 16	103 ± 6	105 ± 8	**153 ± 25***	105 ± 13
126	3 µM	104 ± 14	98 ± 8	**126 ± 7***	**134 ± 13***	108 ± 11

Table 1. Effects of long-term exposure to PCBs 153 or 126 on the content of proteins involved in the glu-NO-cGMP pathway. NR1 subunit of NMDA receptors (NR1), calmodulin, neuronal NO synthase (nNOS) and of the alpha1 and beta1 subunits of sGC (sGC) was analyzed by immunoblotting as described in methods. Values are the mean ± SEM of at least 4 experiments. Values that are significantly different from control ones are indicated by asterisks (*$p < 0.05$).

The results reported show that chronic exposure of cerebellar neurons in culture to PCB153 or 126 impair the function of the glu-NO-cGMP pathway, thus reproducing the impairment reported in *in vivo* studies[9]. This support the idea that primary cultures of cerebellar neurons are a good model to study the mechanisms by which these PCBs impair the function of the pathway *in vivo*. We have used this model to analyze the mechanisms responsible for this impairment. It is shown that PCBs 153 and 126 also increase basal concentration of cGMP in the neurons. PCB126 is more potent than PCB153 in inducing these effects, as also occurs *in vivo*. The mechanisms by which PCB 153 and PCB 126 impair the glu-NO-cGMP pathway are different. PCB153 would affect this pathway by three different mechanisms: reducing the degradation of cGMP by phosphodiesterases; reducing the activation of sGC by NO and therefore the formation of cGMP and; at higher concentrations, reducing the increase in calcium induced by activation of NMDA receptors. PCB126 impairs the function of the glu-NO-cGMP pathway mainly by reducing NMDA-induced increase in calcium. The identification of the differential mechanisms involved in the effects of PCBs 153 and 126 would allow designing specific treatments to restore the function of the glu-NO-cGMP pathway by different pharmacological approaches. This could help to improve cognitive function in children exposed to PCBs during pregnancy or lactation.

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