



Pesticide exposure during pregnancy, like nicotine, affects the brainstem $\alpha 7$ nicotinic acetylcholine receptor expression, increasing the risk of sudden unexplained perinatal death



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ABSTRACT

This study indicates the impact of nicotine and pesticides (organochlorine and organophosphate insecticides used in agriculture) on neuronal $\alpha 7$ -nicotinic acetylcholine receptor expression in brainstem regions receiving cholinergic projections in human perinatal life. An in-depth anatomopathological examination of the autonomic nervous system and immunohistochemistry to analyze the $\alpha 7$ -nicotinic acetylcholine receptor expression in the brainstem from 44 fetuses and newborns were performed. In addition, the presence of selected agricultural pesticides in cerebral cortex samples of the victims was determined by specific analytical procedures. Hypodevelopment of brainstem structures checking the vital functions, frequently associated with $\alpha 7$ -nicotinic acetylcholine receptor immunopositivity and smoke absorption in pregnancy, was observed in high percentages of victims of sudden unexpected perinatal death. In nearly 30% of cases however the mothers never smoked, but lived in rural areas. The search for pesticides highlighted in many of these cases traces of both organochlorine and organophosphate pesticides. We detain that exposition to pesticides in pregnancy produces homologous actions to those of nicotine on neuronal $\alpha 7$ -nicotinic acetylcholine receptor, allowing to developmental alterations of brainstem vital centers in victims of sudden unexplained death.

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1. Introduction

Although the number of pregnant women who smoke has declined during the last years in the western world, thanks to widespread information campaigns, a large number of fetuses are still exposed to nicotine in utero [1–4]. Among the wide variety of neurotoxic chemicals contained in tobacco smoke, nicotine is known for its adverse effects in particular on the activity of many transmitter systems, including cholinergic transmission, during the central nervous system development [5,6]. Accumulating evidence suggests that acetylcholine (ACh), the major cholinergic neurochemical transmitter with a fundamental trophic role in the brain development, acts through synaptic mechanisms mainly mediated by nicotinic ACh receptors (nAChRs) [7,8].

The molecular biology of neuronal nAChRs features a multitude of potential subtypes. To date, various homomeric or heteromeric combinations of twelve different nicotinic receptor subunits ($\alpha 2$ – $\alpha 10$

and $\beta 2$ – $\beta 4$) have been described on the basis of their molecular structure and activity [9–11]. Nicotine can mimic the effect of ACh and incorrectly promote the cholinergic activity of the nAChRs, leading to neuronal damage because of an inappropriate timing or intensity of stimulation [12]. In particular, in the developing brain, the most toxic effects of nicotine affect the $\alpha 7$ subunit, given the role played by this specific receptor in neuronal differentiation, axogenesis and synapse formation [13,14]. This raises the possibility that during key critical developmental periods $\alpha 7$ -nAChRs could be vulnerable and potential targets also for other neurotoxicants in addition to nicotine, such as environmental pollutants, that could cause significant disruptions of the ACh synaptic turnover. In support of this hypothesis, Slotkin demonstrated in experimental studies that chlorpyrifos, a commonly used organophosphate insecticide in agriculture, shows homologous actions to those of nicotine in eliciting $\alpha 7$ nAChR stimulation [15–17].

Therefore, the first goal of this study was to compare the expression of $\alpha 7$ -nAChRs in the brainstem, where the main nuclei checking the vital functions are located, from fetuses and newborns who died of known and unknown causes, with smoker and nonsmoker mothers, in order to assess a possible correlation between a hyperactivation of these receptors promoted by nicotine during pregnancy and sudden unexplained perinatal death.

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The second goal was to evaluate, in the same cases, whether the insecticides and, more generally, the pesticides commonly used in the areas of origin of the victims, produce comparable effects to those of nicotine on $\alpha 7$ nAChR expression during the brainstem development.

A final intent was to assess possible cumulative effects of a combination of nicotine and pesticide absorption in utero on $\alpha 7$ nAChR function.

2. Material and methods

In total, 45 brains were collected from 23 fetuses (26–40 gestational weeks, with a peak from 36 to 39 weeks) and 22 infants aged 1–10 months (mean age: 3 postnatal months).

This was a selected set of cases sent to our Research Center in conformity with the 2006 guidelines stipulated by Italian law n.31 “Regulations for Diagnostic Post Mortem Investigation in Victims of SIDS and Unexpected Fetal Death”. This law decrees that all infants with suspected SIDS who died suddenly within the first year of age, as well as all fetuses who died after the 25th week of gestation without any apparent cause, must undergo an in-depth anatomic-pathological examination, particularly of the autonomic nervous system paying particular attention to the brainstem, where the main neuronal structures controlling the vital functions are located.

Permission from the Ethics Committee was not required for this study as the “Lino Rossi” Research Center is the national referral center for sudden unexplained fetal and infant death, in accordance with the above-mentioned Italian law n.31. Parents provided written informed consent to the study procedures.

All the 45 cases of the study were processed for the neuropathological examination, the $\alpha 7$ nicotinic receptor immunohistochemical detection and for the agricultural pesticide chemical research.

2.1. Neuropathological examination

Following the guidelines provided by the Italian law n.31 (available at the webpage <http://users.unimi.it/centrolinorossi/en/guidelines.html>), firstly, before the anatomic-pathological procedures, fresh pieces of about 1 cm² of the cerebral cortex were collected from each case for the investigations on environmental pollutants, and frozen at -20°C until analysis (see below).

All the brainstems were processed after a rigorous time of fixation in 10% phosphate-buffered formalin for two weeks, that is the optimal time not only verified by us but also reported in literature [18] to obtain the best immunohistochemical assays.

Then, after fixation, three specimens from the brainstem were obtained and subsequently embedded in paraffin. The first specimen, ponto-mesencephalic, included the upper third of the pons and the adjacent portion of the mesencephalon. The second extended from the upper third of the medulla oblongata to the portion adjacent to the pons. The third specimen included the obex, 2–3 mm above it and below it. Transverse serial sections were obtained from the samples at intervals of 50–60 μm . At each level two of these sections were routinely stained for histological examination using hematoxylin–eosin and Klüver–Barrera techniques.

The microscopic evaluation was focused on the locus coeruleus and the Kölliker–Fuse nucleus in the rostral pons/caudal mesencephalon, on the retrotrapezoid nucleus, the superior olivary complex and the facial/parafacial complex in the caudal pons; on the hypoglossus, the dorsal motor vagus, the tractus solitarius, the ambiguus, the pre-Bötzinger, the inferior olivary, the raphe and the arcuate nuclei in the medulla oblongata. Plates in the human brainstem atlas of Olszewski and Baxter’s [19] were used as reference. As regards several nuclei not represented in this atlas, such as the Kölliker–Fuse, the pre-Bötzinger and the retrotrapezoid nuclei, we relied to our previous specific studies aimed to their identification [20–22].

The $\alpha 7$ nAChR expression in these nuclei and/or structures was determined by immunohistochemistry on additional sections.

2.2. $\alpha 7$ -nAChR immunohistochemistry

The immunohistochemical method used in order to evaluate the expression of $\alpha 7$ nicotinic receptors was applied using the specific rabbit polyclonal antibody (aa 22–71, Abcam Ltd, UK, cod. ab10096) on the selected transverse brainstem sections. After dewaxing and rehydration, sections were immersed and boiled in Tris–EDTA buffer for antigen retrieval with a microwave oven, after blocking the endogenous peroxidase by 3% hydrogen peroxide treatment. Then, sections were incubated with diluted 1:167 primary antibody overnight in a wet chamber. Samples were washed with PBS buffer and incubated with a biotinylated goat anti-rabbit IgG secondary antibody (PK-6101, Vector Laboratories, CA, USA) and then processed with the avidin–biotin–immunoperoxidase technique (VEDH-4000, Vector Laboratories, CA, USA). Finally, each section was counterstained with Mayer’s hematoxylin and coverslipped.

A set of sections from each study group was used as negative control: the tissue samples were stained using the same procedure but omitting the primary antibody in order to verify that the immunolabeling was not due to non-specific labeling by the secondary antibody. In fact, if specific staining occurs in negative control tissues, the immunohistochemical results should not be considered valid.

nAChR immunohistochemistry quantification — The degree of immunoreactivity was evaluated in each selected nucleus and/or structure in the brainstem as the number of neuronal cells showing a dark brown color, divided by the total number of neurons, and expressed as percentage (nAChR immunopositivity index: nAChR-I). nAChR-I was classified as: “Class 0” for no or light staining (negativity); “Class 1” when the index was $<10\%$ (weak positivity); “Class 2” with a percentage of immunopositive cells ranging from 10 to 40% (moderate positivity); and “Class 3” with an index of $>40\%$ of the counted cells (strong positivity).

Comparison among the observations, carried out by two independent, blinded pathologists, was performed by employing Kappa statistics (Kappa Index — KI) to evaluate inter-observer reproducibility, according to the Landis and Koch system (KI value of 0 to 0.2 = slight agreement; 0.21 to 0.40 = fair agreement; 0.41 to 0.60 = moderate agreement; 0.61 to 0.80 = strong agreement; 0.81 to 1.00 = very strong or almost perfect agreement, with a value of 1.0 corresponding to perfect agreement) [23]. The Kappa Index obtained from the application of this method in the present study was very satisfactory (KI = 0.85).

2.3. Chemical characterization

The determination of selected agricultural pesticides in brain samples was performed according to the method proposed by Cappiello et al. [24].

Each frozen brain sample, appropriately stored for this analysis, was homogenized with 2 mL of n-hexane and transferred into a solid phase extraction (SPE) cartridge (Thermo Scientific, Bellefonte, USA) containing 500 mg of C-18 sorbent to retain most of the matrix impurities and release the compounds of interest with hexane. The SPE cartridge was conditioned with 4 mL of n-hexane before the purification step, and was washed with 1 mL of n-hexane followed by 1 mL of dichloromethane after the elution step.

2.3.1. Chemicals and materials

All brain tissues were subjected to analytical procedures to determine the level of 20 organochlorine pesticides (OCPs) and other pesticides and xenobiotic compounds belonging to the class of organophosphates (OPPs), carbamates and phenols (including chlorpyrifos, chlorfenvinfos, captan, boscalid and bisphenol A) (Sigma-Aldrich, Milan, Italy). All solvents used (n-hexane and dichloromethane) were pesticide residue

analysis grade, supplied by Merk (Suprasolv, 99% purity, Merk, Germany). Stock solutions were prepared in n-hexane at a concentration of 100 µg/mL. A standard mixture containing all compounds was prepared by appropriate dilution and stored at 4 °C in the dark.

Analyses of the extracted samples were performed with an Agilent Technologies gas chromatograph 6890N equipped with a single quadrupole mass spectrometer 5975C TAD/MS working in electron ionization.

Chromatographic separation was carried out using an HP-5MS (Agilent J&W GC columns, Folsom, CA, USA), 30.0 m × 0.25 mm I.D., containing 5% phenyl-methylsiloxane, with a phase thickness of 0.25 µm. Carrier gas was helium at 1 mL/min (constant flow). The GC oven temperatures were programmed as follows: 80 °C, held for 1 min, ramped at 30 °C/min to 180 °C, ramped at 3 °C/min to 225 °C, held for 4 min, ramped at 20 °C/min to 300 °C, and held for 4.08 min, (total acquisition time: 25 min). Splitless sample injection of 1 µL at 250 °C was selected. The transfer line and ion source temperature were kept at 290 °C and 300 °C, respectively.

2.4. Clinical diagnoses

In 28 cases, after the in-depth autoptic examination no specific pathology has been identified. A diagnosis of SIUDS (sudden intrauterine unexplained death syndrome) was established for 14 fetuses, and of SIDS (sudden infant death syndrome) for 14 infants who died within the first year of life. In the remaining 17 cases, 9 stillbirths, and 8 infant deaths, a precise cause of death was formulated at autopsy. These cases were considered as controls. Specific diagnoses among the fetal deaths were: “chorioamnionitis” (detected in 3 cases) and congenital heart disease (in 6 cases). The related infant death diagnoses included 4 cases of “congenital heart disease”, 3 cases of “bronchopneumonia” and 1 case of “pericarditis”.

For every victim of the study, a complete clinical history was collected, also considering the place of origin. Thirty-three cases came from northern Italy and 12 from central and southern regions. Fourteen cases, in particular, were from polluted rural areas of Lombardy and Trentino. Additionally, mothers were asked to complete a questionnaire probing smoking habit, and if they were smokers, detailing the number of cigarettes smoked before, during and after pregnancy. Smoking habit was assigned to two categories (smokers vs. nonsmokers). Overall, 18 of the 45 mothers (40%) were smokers of more than 3 cigarettes/day already before the onset of pregnancy, and 27 were nonsmokers. Since the retrospective assessment of the smoking habit of a mother, mainly after the death of his son, is sometimes unavoidable, the negative self-reports were validated by the urinary measuring of cotinine, the main metabolite of nicotine.

Fifteen women in the smoker group were mothers of a victim of sudden unexplained death and 3 of control cases.

Table 1 presents the case profile of this study, including the Italian region of origin and maternal smoking habit.

2.5. Statistical analysis

Results were tabulated and analyzed for differences comparing pairs of groups by analysis of variance (ANOVA). Statistical calculations were carried out with SPSS statistical software (version 11.0; SPSS Inc., Chicago, IL, USA). The threshold level set for statistical significance was $p < 0.05$.

3. Results

3.1. Neuropathological examination

Several alterations were observed at routine histological examination of the brainstem in 13 SIUDS and 11 SIDS cases, including the hypodevelopment of different nuclei and/or structures checking the vital functions (hypoplasia/agenesis of one or more nuclei of the raphe system, of the parafacial/facial complex, the pre-Böttinger, the Kölliker–Fuse and the arcuate nuclei). Frequently, individual victims displayed any combination of these alterations. The hypoplasia of the parafacial/facial complex, in terms of a decreased neuronal number and area of both facial and parafacial nuclei, was in particular the more frequent finding in SIUDS cases (11/14) (Fig. 1). Notably, in 2 SIUDS all the above-mentioned alterations were together present.

There was no histological evidence of brainstem developmental abnormalities in any of the control victims, except for hypoplasia of the arcuate nucleus, detected in 35% of cases (6/17). However, we do not regard the hypodevelopment of this nucleus as a significant pathological result, given its presence with comparable incidences in SIUDS/SIDS cases and also in older people who died of different causes, such as cardiomyopathies and cancers (our observations, data not published). We can then speculate that the arcuate nucleus, though regarded as a chemoreceptorial structure, doesn't have essential functions for life given, above all, the presence of hypoplasia in subjects who died in old age. Likely, its hypofunction resulted by the hypoplasia is counterbalanced by the hyperfunction of different chemoreceptorial nuclei in the brainstem, such as the raphe nuclei and the retrotrapezoid nucleus.

3.2. Immunohistochemical expression of $\alpha 7$ receptors

Positivity for $\alpha 7$ -nAChR subunits was observed in 13 SIUDS, 10 SIDS and 3 controls. In the positive cases, immunoreactivity was present in brainstem nuclei and/or neuronal complexes with both normal and delayed maturation. In detail, a percentage of dark-stained neurons ranging from 33% to 38% (corresponding to the “Class 2” of nAChR-1) was detected in 8 SIUDS, 4 SIDS and in the 3 positive control cases. In

Table 1
Case profiles of the study.

Victims	Age range	Italian region of provenience		Death diagnosis	Maternal smoking habit	
		From rural areas			Smokers (no.18)	Nonsmokers (no.26)
Fetuses (n.23)	26–40 gw	Trentino (n.9)	(n.4)	SIUDS (n.14)	8	6
		Lombardy (n.6)	(n.5)			
		Others (n.7)	(n.1)			
Infants (n.22)	1–10 pm	Trentino (n.10)	(n.2)	Control cases (n.9): Chorioamnionitis (n.3) Congenital heart diseases (n.6)	2	7
		Lombardy (n.7)	(n.2)			
		Others (n.5)	–			
Infants (n.22)	1–10 pm	Trentino (n.10)	(n.2)	SIDS (n.14)	7	7
		Lombardy (n.7)	(n.2)			
		Others (n.5)	–			
Infants (n.22)	1–10 pm	Trentino (n.10)	(n.2)	Control cases (n.8): Bronchopneumonia (n.3) Congenital heart diseases (n.4) Pericarditis (n.1)	1	7
		Lombardy (n.7)	(n.2)			
		Others (n.5)	–			

gw = gestational weeks; pm = postnatal months.

SIUDS = sudden intrauterine unexplained death syndrome; SIDS = sudden infant death syndrome.

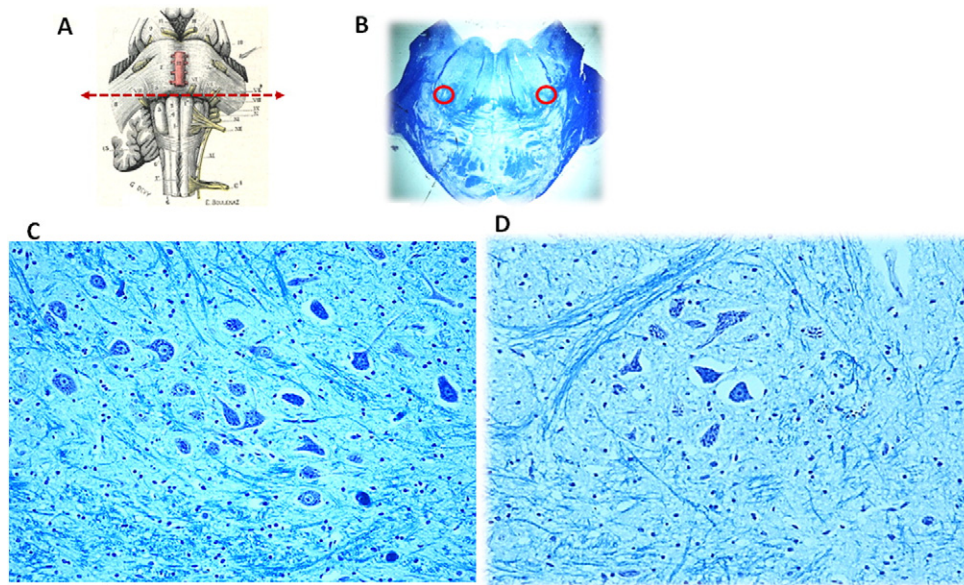


Fig. 1. A) Schematic representation of brainstem ventral surface: arrow indicates the optimal level in the caudal pons to obtain histological sections for the study of the facial/parafacial complex (F/PFc); B) example of full histological section at this level showing the F/PFc localization; C) normal cytoarchitecture of the F/PFc in a control fetus (39 gestational weeks); D) F/PFc hypoplasia in an age-matched SIUDS case. B) C) and D) Klüver–Barrera staining; magnification: B) 0.5 \times ; C) and D) 20 \times .

the remaining 5 SIUDS and 6 SIDS the percentage of immunostained neurons was very high, between 82 and 100% (values belonging to the “Class 3” nAChR-I). Fig. 2 shows examples of “Class 3” nAChR-I in the parafacial/facial complex, the neuronal center mostly involved in developmental alterations in fetal deaths.

3.3. Pesticide detection

Different compounds from organochlorine pesticides (OCPs) and organophosphate pesticides (OPPs) (heptachlor, α and γ -chlordane, chlordfeninfos, chlorpyrifos, p,p-DDT, p,p-DDE, Endrin, α and β -endosulfans) were detected at various concentrations in cerebral cortex samples of 13 cases (9 SIUDS, 3 SIDS and 1 fetus of the control group), all from the Trentino and Lombardy agricultural areas, where these pollutants are intensely used (Table 2).

3.4. Correlations among overall pathological results

The immunopositive expression pattern of $\alpha 7$ receptors was higher in fetuses and infants with smoker mothers compared with nonsmoker mothers ($p < 0.05$). In particular, a consistently enhanced $\alpha 7$ -immunoreactivity was diffusely present in brainstem sections of 15 victims of sudden death (8 SIUDS and 7 SIDS) with the hypodevelopment

of nuclei involved in the regulation of the vital functions and a smoker mother (54%). Likewise, the mother of 1 of the 3 victims of the control group with $\alpha 7$ subunit hyperexpression had the habit of smoking. Traces of organic pollutants were detected in cerebral cortex samples of 5 SIUDS, 3 SIDS and 1 fetal control with class 3 $\alpha 7$ -nAChR-I classification (i.e. strong immunopositivity) whose mothers never smoked, but who live in rural areas. The findings in 5 SIUDS cases with smoker mothers of multiple developmental alterations of the brainstem, $\alpha 7$ receptor overexpression and positivity for pesticides, were remarkable.

Table 3 summarizes the neuropathological results.

4. Discussion

To our knowledge, this is the first human study examining the impact of specific neurotoxicants (i.e. nicotine and pesticides) on $\alpha 7$ -nAChRs, a specific class of receptors that play essential roles in the cytoarchitectural development of brainstem regions receiving cholinergic projections, in sudden unexplained fetal and infant deaths (SIUDS and SIDS). Here we also present new data ever reported in the literature indicating that organochlorine and organophosphate insecticides commonly used in rural areas of origin of the victims of sudden death, exert homologous actions to those of nicotine on $\alpha 7$ -nAChRs, similarly interfering with the cholinergic system during the brain development.

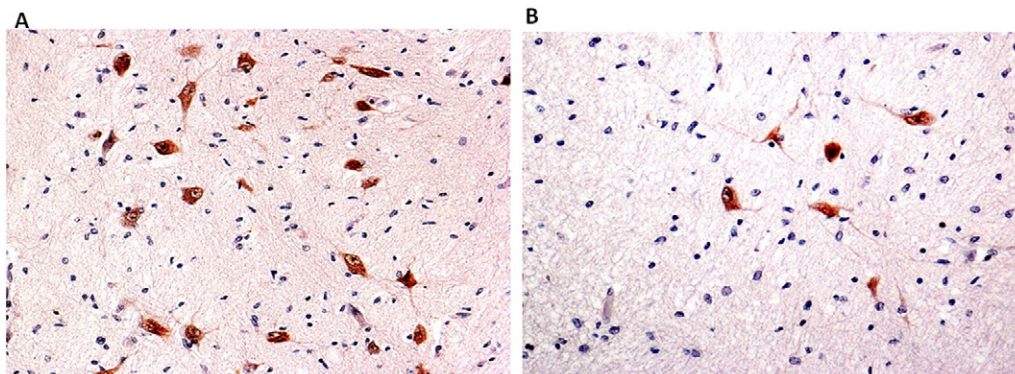


Fig. 2. Strong $\alpha 7$ -nAChR immunopositivity (“Class 3” of $\alpha 7$ -nAChR-Index) in the facial/parafacial complex (F/PF) to: A) SIDS case (3 month-old) with normal F/PF cytoarchitecture; B) SIUDS case (40 gestational weeks) with F/PF hypoplasia. $\alpha 7$ -nAChR immunostaining; magnification A) B) and C) 20 \times .

Table 2
Positivity for persistent pollutants in 13 cerebral cortex samples out of the 45 analyzed cases.

Diagnosis	Age	Compounds	Concentration (ng/g) ^a
SIUDS	33 gw	γ-Chlordane	1.5
		p,p-DDT	10.3
SIUD	33 gw	γ-Chlordane	1.5
SIUDS	34 gw	Chlorfenvinfos	31.5
SIUDS	35 gw	γ-Chlordane	25.0
SIUDS	37 gw	α-Endosulfan	667.0
		β-Endosulfan	993.0
SIUD	39 gw	α-Chlordane	<LOQ
SIUDS	40 gw	Endrin	63.5
		p,p-DDE	3.5
SIUDS	40 gw	γ-Chlordane	10.2
SIUDS	41 gw	γ-Chlordane	10.2
		α-Chlordane	11.3
Control fetus	35 gw	γ-Chlordane	25.0
		α-Chlordane	34.0
SIDS	1 pm	Heptachlor	7.4
		γ-Chlordane	6.6
		α-Chlordane	6.9
SIDS	4 pm	Chlorpyrifos	8.7
SIDS	6 pm	Chlorfenvinfos	0.8
		p,p-DDE	3.3
		Chlorpyrifos	1.6

gw = gestational weeks; ng/g = nanogram per tissue gram; LOQ = limit of quantification. pm = postnatal months.

^a Unfortunately, the “acceptable range” for humans of pesticide concentration in “ng/g” is not known for humans. Few data are available only expressed in ng/L (see Ref. [40]).

The α7 subunit of the nAChRs plays a fundamental role in regulating growth, differentiation and plasticity of the developing neurons through Ca²⁺-dependent mechanisms mainly aimed to regulate receptor gene expression [13,25,26].

The first result of the present study was the significantly increased α7-nAChR expression in the brainstem of fetuses exposed to smoking during pregnancy. This is in agreement with the current recognition that a hallmark of the developmental neurotoxicity of nicotine is the upregulation of these specific receptors, via significant strengthening of the α7-mRNA level [13]. Animal research, in particular, showed that nicotine administered prenatally increases the number of ³H-nicotinic binding sites in the brain both pre- and postnatally, leading to an increased release of Ach [27,28]. Excessive amounts of Ach may, in turn, inappropriately interfere with α7-nAChR gene expression, affecting their function [29]. It is important to emphasize that the α7 subunits are characterized, as compared with other nAChR subtypes, by faster kinetics, being more rapidly activated by nicotine. They are also more permeable to Ca²⁺ than any other ligand-gated ion channel receptor prenatally exposed to nicotine, influencing neuronal plasticity and many physiological processes in the developing nervous system [13].

Table 3
Pathological results in SIUDS and SIDS victims and controls.

Alterations [§]	Group I Fetal control n.9 [2]	Group II SIUDS n.14 [8]	Group III Infant control n.8 [1]	Group IV SIDS n.14 [7]
Brainstem alterations ^a				
Raphe nuclei hypoplasia	0/9	8/14 **	0/8	7/14 **
Parafacial/facial complex hypoplasia	0/9	10/14	0/8	1/14
Pre-Bötzing nucleus hypoplasia	0/9	4/14	0/8	6/14
Kölliker–Fuse nucleus hypoplasia	0/9	7/14	0/8	0/14
arcuate nucleus hypoplasia	3/9	7/14	3/8	5/14
α7-nAChR immunopositivity ^b	1/9 [0]	13/14 [8]**	2/8 [1]	10/14 [7]**
Presence of pesticides in cerebral cortex	1/9 [0]	9/14 [6]**	0/8	3/14 [2]*

In brackets are indicated the number of cases with smoking mother.

[§] Statistically-significant differences, SIUDS or SIDS vs. corresponding control (Group II vs. I, or Group IV vs. III): *p < 0.05 and **p < 0.01.

^a Individual victims may display any combination of the brainstem morphological alterations. A total of 24 out of the 28 SIUDS/SIDS cases had one or more developmental defects of the brainstem.

^b “Class 2” or “Class 3” of immunoreactivity grading (see “nAChR immunohistochemistry quantification” in the Material and methods section).

The overexpression of α7-nAChR, measured by the percentage of neurons with strong immunostaining, we observed in victims of SIUDS and SIDS with smoker mothers, was for the first time associated with developmental alterations of brainstem structures involved in the control of vital functions, precisely with hypoplasia/agenesis of one or more raphe nuclei, of the parafacial/facial complex, the pre-Bötzing and the Kölliker–Fuse nuclei, already by us previously reported as related to unexplained perinatal death [20,21,30,31]. In particular the high percentage of hypoplasia of the parafacial/facial complex, exclusively observed in SIUDS, confirms our opinion that this alteration is a specific marker of unexplained stillbirth and that a normal development of this structure is of essential importance for the extra-uterine life [21].

Therefore we assume that nicotine perturbs structural maturation of the brainstem nuclei by impairing the central cholinergic system through the upregulation of nicotinic receptor bindings. Accordingly, several animal studies reported that the stimulation of nAChRs by nicotine causes neuronal inhibition of DNA synthesis, mitotic abnormalities and apoptosis, so compromising the normal differentiation and development of brain structures essential for life [32,33]. Interestingly, Gospe et al. [34,35] and Slotkin et al. [36] reported in experimental studies on pregnant rats that the main effect of prenatal nicotine exposure is a deficit in cell number particularly in specific vulnerable regions of the hindbrain, the embryonic structure that gives rise to the major components of the brainstem (pons and medulla oblongata). These data support our hypothesis that the high incidence of “hypoplasia” (a term that properly refers to a below-normal number of cells) observed by us in many structures of both the medulla oblongata and pons in SIUDS/SIDS cases, could be linked to nicotine exposure in pregnancy.

Previously, a few authors have studied the effect of maternal smoking in pregnancy on nicotinic receptors in infant died of SIDS, with different results. Two studies, using quantitative receptor autoradiography, found no difference in ³H-nicotinic binding in brainstem nuclei related to cardiorespiratory control between SIDS cases with or without smoke exposure [37,38]. Machaalani et al. [39] on the contrary reported increased α7 receptor immunostaining in several brainstem nuclei of SIDS cases exposed to cigarette smoke compared to a non-SIDS cohort, however without any hint to morphological developmental alterations. It should specify furthermore that they did not study the brainstem nuclei involved in the control of vital functions such as the raphe, pre-Bötzing and Kölliker–Fuse nuclei.

In a significant proportion of cases of sudden death (25%) with hypoplasia of one or more brainstem nuclei and a high percentage of α7-nAChR immunostained neurons, the mothers were non-smokers. Toxicological examination of the cerebral cortex samples detected, in all these cases, the presence of organochlorine pesticides (OCPs) and organophosphate pesticides (OPPs) that are intensely used in the specific agricultural areas of origin of the victims. These neurotoxicants were found in variable concentrations and expressed as nanogram per

tissue grams (from 0.8 to 993 ng/g). It would be very useful in this regard to know the “acceptable range” of the pesticide concentrations for humans but these data are partially available only in terms of nanogram per liter, given the easy dissolution of these compounds in water. The European Union (EU) for the protection of human health, in particular, established the maximum acceptable concentration of 100 ng for water liter (ng/L) for individual pesticides [40] but does not provide the possibility of converting these measures in nanograms per gram of tissue. However, Slotkin in his experimental studies on rodents [15–17] demonstrated that early neonatal rat exposure to 1 mg/kg body weight of chlorpyrifos, one of the most widely used organophosphate insecticides in agriculture, elicited an upregulation of brain $\alpha 7$ -nAChR. Nevertheless, also if a similar threshold is not known for humans, we can suppose the same effects of pesticides on the maturation and function of brainstem nicotinic receptors in SIUDS/SIDS victims.

Persistent environmental OCPs, and certain compounds like nicotine, can pass through the placental barrier into the fetal blood stream, and pose health risks to fetuses and neonates, who are believed to be more vulnerable to the effects of environmental xenobiotics, as their organs and detoxification enzymatic systems are relatively immature [41–43]. Therefore, exposure to OCPs in utero has been linked to the same adverse effects on developing fetuses as those elicited by nicotine, such as intrauterine growth retardation, low birth weight, neurocognitive deficits and hormonal dysfunctions [43–45].

An important finding, in 5 SIUDS cases with smoker mothers, was the extensive combination of brainstem developmental alterations, including $\alpha 7$ -nAChR over-expression, and the presence of endosulfans and chlorpyrifos in the cerebral cortex. These results, even if very limited, strongly suggest that the effects of a combination of nicotine and pesticides are likely cumulative.

Nevertheless, our opinion is in contrast with experimental studies showing that simultaneous exposure to both neurotoxic agents produced less-than-additive effects and the combination of nicotine and chlorpyrifos had lesser effects than chlorpyrifos alone [46]. For this reason we are now planning to examine the behavior of combinations of these neurotoxicants particularly on $\alpha 7$ -nAChRs in a wider set of perinatal deaths.

5. Conclusions

Our results indicate that $\alpha 7$ -nAChRs are specific targets in the developing brain not only of nicotine but, more generally, of all cholinergic neurotoxicants, including pesticides. Thus, both cigarette smoke and persistent pollutant absorption in utero independently affect the expression of $\alpha 7$ -nAChRs in the brainstem of the fetus and newborn and can lead to developmental alterations in neuronal centers that are essential to support life. Ultimately, the $\alpha 7$ upregulation in our study provides a biological explanation of the mechanisms by which nicotine and pesticides, together or individually, perturb neuronal development increasing the risk of SIUDS and SIDS.

Then, first of all pregnant women should be severely advised that smoking places their unborn children in danger. In addition we suggest that pregnant women should avoid not only cigarette smoking but also pesticide exposure whenever possible.

Abbreviations

ACh	acetylcholine
nAChR-I	nicotinic acetylcholine receptor index
nAChRs	nicotinic acetylcholine receptors
OCPs	organochlorine pesticides
OPPs	organophosphate pesticides
SIDS	sudden infant death syndrome
SIUDS	sudden intrauterine unexplained death syndrome
SPE	solid phase extraction

Authors' contributions

AML planned the study, analyzed the data and wrote the manuscript with collaborative input and extensive discussion with LM. AC and VT designed and performed the chemical characterization of pesticides in brain samples. MFC carried out the immunohistochemical study of the nicotinic receptors and participated in the evaluation of the results. All Authors read and approved the final manuscript.

Conflict of interest

All authors declare that they have no conflicts of interest, financial or otherwise to declare.

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