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Altered Expression of Nicotinic Receptors in Perinatal Life Related to Prenatal Exposure to Toxics—An Overview of the Research Carried Out on This Topic at the "Lino Rossi" Research Center of the Milan University

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Abstract

The article aims to underline the impact of nicotine and pesticides on neuronal *α*7-nicotinic acetylcholine receptors expression in brainstem regions receiving cholinergic projections, given their fundamental role during the neuronal development. The in-depth histopathological/immunohistochemical examination of the autonomic nervous system performed at the "Lino Rossi" Research Center of the Milan University on a wide group of sudden unexpected fetal and infant deaths, highlighted the frequent hypodevelopment of brainstem structures checking the vital functions associated to altered expression of *α*7-nicotinic acetylcholine receptors and smoke absorption in pregnancy. A dysregulation of the catecholamine system was also observed in the cerebellar cortex of the same cases. However, in a not negligible percentage of sudden deaths with altered expression of *α*7-nicotinic receptors, the mothers never smoked but lived in rural areas. Specific analytical procedures showed the presence of agricultural pesticides in cerebral cortex samples of these victims. Therefore, it is possible to believe that the exposition to pesticides during pregnancy can produce the same harmful effects as nicotine on the nicotinic acetylcholine receptors. Moreover, alterations of *α*7-nicotinic acetylcholine receptors receptor expression were also detected in the lungs of many sudden perinatal death victims, allowing to consider even these findings as possible consequence of maternal exposure to toxic factors.

Keywords

Nicotine, Pesticides, Endocrine Disruptor Compounds (EDCs), *α*7-Nicotinic Acetylcholine Receptors, Neuropathology, Sudden Infant Death Syndrome

(SIDS), Sudden Intrauterine Unexplained Death (SIUDS)

1. Introduction

In this paper I would like to present and discuss the main findings obtained in many years of research carried out at the "*Lino Rossi Research Center for the study and prevention of the unexpected perinatal death and Sudden Infant Death Syndrome* (*SIDS*)" of the Milan University (Italy) on a wide group of sudden fetal and infant deaths, related to alterations of the *α*7-nicotinic acetylcholine receptors expression as consequence of prenatal exposure to toxics.

I start from a premise: the "Lino Rossi" Research Center is the national referral center for the application of the Italian Law no. 31/2006 "Regulations for diagnostic post mortem investigation in victims of Sudden Infant Death Syndrome (SIDS) and Sudden Intrauterine Unexplained Death Syndrome (SIUDS)" [1]. This law states that all infants dying suddenly of suspected SIDS within the first year of age, as well as all fetuses who died after the 25th week of gestation without any apparent cause in Italian regions, must undergo an in-depth post mortem examination, in accordance with specific guidelines, as reported below.

2. Methods

Overall, since 2006, the year of entry into force of the aforementioned law, around 200 cases of SIDS and 300 of SIUDS have been examined at the Lino Rossi Research Center. The applied pathological protocol was mainly focused on the in-depth examination of the autonomic nervous system [2] [3] [4] [5].

For every case, a complete medical history was collected, including information related to the main potential risk factors (especially maternal smoking, maternal alcohol and drug abuse before and during pregnancy and, when available, data on air pollution in the mother's area of residence). The mothers were asked in particular to complete a questionnaire on their smoking habits with details regarding the number of cigarettes smoked before, during and after pregnancy. The negative responses were validated by toxicological analyses on fetal and infant hair in order to search for traces of cotinine, which is a highly stable metabolite of nicotine.

2.1. Neuropathological Procedure for the Examination of the Brainstem and Cerebellum in SIUDS/SIDS

Figure 1 shows a scheme of the methodology for the brainstem examination. At the right, the sampling of three specimens is shown. The first specimen, ponto-mesencephalic, includes the upper third of the pons and the adjacent portion of caudal mesencephalon. The second extends from the upper portion of the medulla oblongata to the adjacent caudal portion of the pons. The third specimen includes the obex. A fourth specimen is taken from the rostral tract of

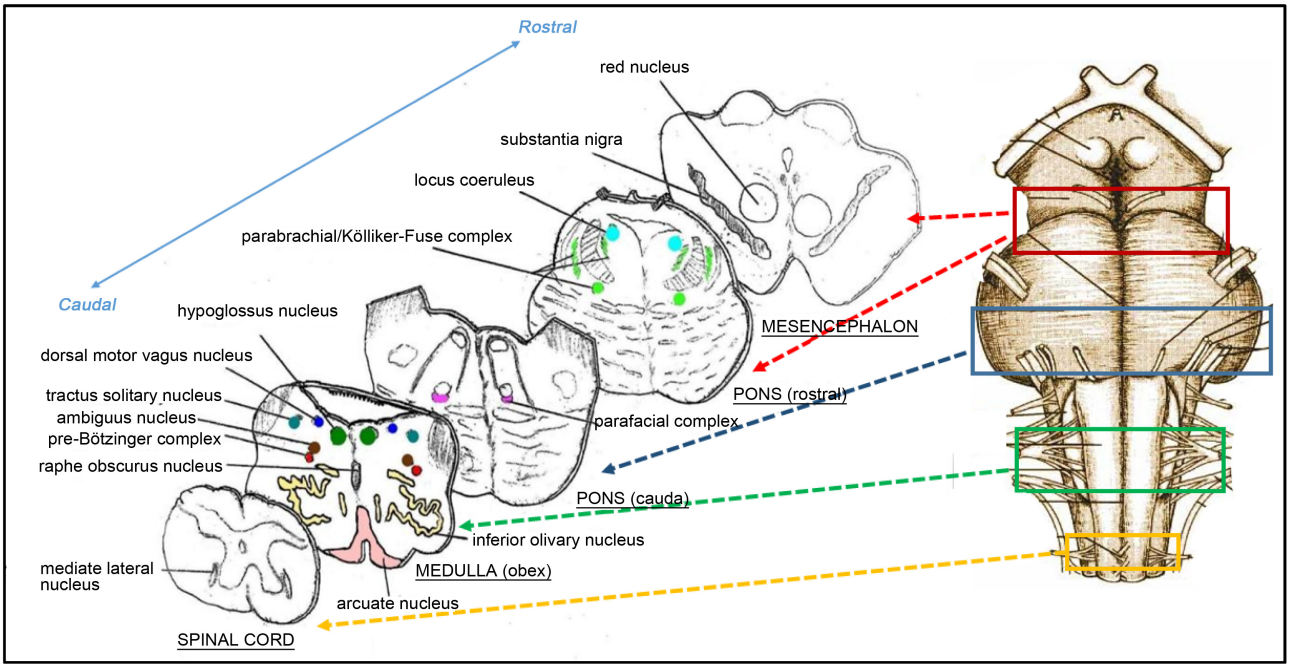

Figure 1. *At the right*, schematic representation of the sampling from the brainstem. *At the left*, the histological sections obtained from the specimens, with the indication of the main nuclei and structures to be examined.

spinal cord.

Transverse serial sections of each specimen are made at intervals of 60 μm. For each level, eight to ten 5-μm-thick sections are obtained, two of which are stained using hematoxylin-eosin and Klüver-Barrera for histological examination, by using a light microscope with a system of objective lenses to progressively magnify the images. The brainstem structures that can be recognized are: the hypoglossus, dorsal motor vagal, tractus solitarii, ambiguus, inferior olivary, pre-Bötzinger, arcuate, dorsal and ventral cochlear, medial inferior vestibular, obscurus and pallidus raphé nuclei in the medulla oblongata; the locus coeruleus, facial/parafacial complex, retrotrapezoid nucleus, superior olivary complex, superior and lateral vestibular nuclei, Kölliker-Fuse, median and magnus raphé nuclei in the pons; the inferior colliculus, substantia nigra, dorsal and caudal linear raphé nuclei in the caudal mesencephalon. In the spinal cord the intermediolateral nucleus is of great interest.

Figure 1, at the left, shows the representative histological sections obtained from the four specimens, with the indication of the most important nuclei and structures to be examined, given their frequent involvement in SIUDS/SIDS, in terms of delayed development (hypoplasia/agenesis) or malformations.

The cerebellum is excised from the brainstem by cutting the peduncles. Then, transversal samples of 60 μm of both cerebellar hemispheres, including all the lobules (anterior, posterior and flocculonodular lobule) are obtained. The microscopic analysis of the cerebellum included the examination of the cortex layers, *i.e.* the external granular layer (EGL), molecular layer (ML), Purkinje cell layer (PCL), internal granular layer (IGL) and the medullary deep nuclei (den-

tate nucleus, fastigial nucleus, globose nucleus and emboliform nucleus).

2.1.1. Immunohistochemical Protocol

The remaining sections obtained from each sample are treated, according to the needs, with specific immunohistochemical techniques in order to evaluate the expression of functional markers, whose altered expression can represent a very significant finding in SIUDS/SIDS. Among these markers are worthy of mention: the neuronal nuclear antigen, the nicotinic acetylcholine receptors, the serotonin, the somatostatin and the orexin. Here we report the methodology only related to the nicotinic receptors, and in particular to the *α7* nicotinic receptors, as this article wants to focus on the importance of these receptors in the perinatal life and on the damage that their altered expression can cause.

2.1.2. *α*7-nAChR Immunohistochemistry

The expression of *α7* nicotinic receptors is evaluated by using a specific rabbit polyclonal antibody (aa 22-71, Abcam Ltd., UK, cod. ab10096) on the selected histological transverse sections. After dewaxing and rehydration, sections are immersed and boiled in TRIS-EDTA Buffer for antigen retrieval with a microwave oven, after blocking endogenous peroxidase by 3% hydrogen peroxide treatment. Then, sections are incubated with diluted 1:500 primary antibody overnight in a wet chamber. Samples are 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 is counterstained with Mayer's Hematoxylin.

nAChR immunohistochemistry quantification-The degree of immunoreactivity is evaluated in each selected nucleus and/or structure in the brainstem and cerebellum 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). The nAChR-I is classified as: "Class 0" for no or light staining (negativity); "Class 1" when the index is <10% (weak positivity); "Class 2" with a percentage of immunopositive cells ranging between 10 and 40% (moderate positivity); "Class 3" with an index of >40% of the counted cells (strong positivity). **Figure 2** shows many *α7*-nAChR immunopositive neurons in the brainstem.

2.2. Procedure for the Examination of the Lungs in SIUDS/SIDS

Samples are obtained from each lung lobe by cutting parallel to the frontal plane and passing through the hilus. The histological examination, performed on the routinely stained sections by hematoxylin-eosin, includes the radial alveolar count (RAC), a reliable index of lung maturation closely related to the gestational age for fetuses and to postnatal age for newborns. RAC is based on the evaluation of the number of airspaces cut by a straight line drawn from the most peripheral bronchiole to the nearest connective tissue septum or pleura border. The

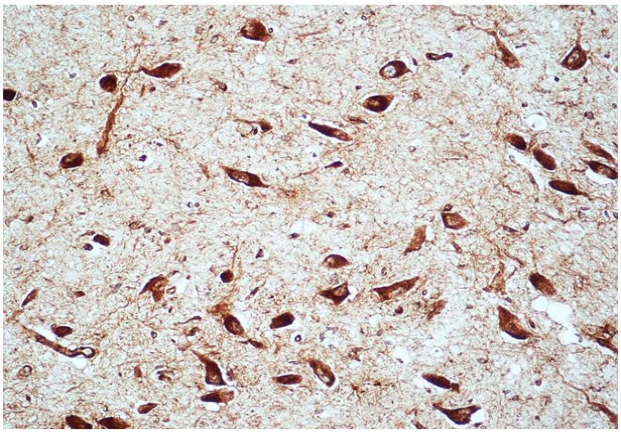

Figure 2. Strong *α7*-nAChR immunopositivity ("Class 3" of *α7*-nAChR-Index) in the brainstem of a SIDS case (3 month-old). Magnification: 40×.

same immunohistochemical procedure for the evaluation of the *α7* nicotinic receptor expression in the nervous system is applicable also for the lungs. **Figure 3** shows many *α7*-immunopositive nAChR neurons in the lung parenchyma.

2.3. Procedures for the Toxicological Examination in SIUDS/SIDS

Protocol for the Chemical Characterization

Each brain sample (approximately 0.5 g) is homogenized with 2 mL of *n*-hexane to obtain a dense, reach supernatant. The homogenized tissue is transferred into a solid phase extraction (SPE) cartridge containing 500 mg of C_{-18} sorbent, in order to retain most of the matrix impurities and to release the compounds of interest with hexane (34). The SPE cartridge is conditioned with 4 mL of *n*-hexane, before purification step, and then washed with 1 mL of *n*-hexane followed by 1 mL of dichloromethane after elution step. The extraction method is developed and validated in terms of accuracy, precision, limit of quantification (LOQ), limit of detection (LOD), and linearity. Nine isotopically labeled internal standards (ISTDs) are used for method validation.

1) Chemicals and materials: a mixture of 20 organochlorine compounds (EPA CLP mix), chlorpyrifos, chlorfenvinfos, captan, boscalid and bisphenol are purchased from Sigma-Aldrich (Milan, Italy). All solvents used (*n*-hexane and dichloromethane) are supplied from Merk (Suprasolv, 99% purity, Merk, Germany). Stock solutions are prepared in *n*-hexane at a concentration of 100 μg/mL. A standard mixture containing all compounds (25 specific EDCs and 9 ISTD) was prepared by appropriate dilution and stored at 4˚C in the dark. SPE cartridges HyperSep-C18 (500 mg/6mL) are purchased by Thermo Scientific (Bellefonte, USA).

2) Instrumentations: the analyses on the extracted samples are performed by an Agilent Technologies gas chromatograph 6890N, equipped with a single quadrupole mass spectrometer 5975C TAD/MS, working in electron ionization. All brain tissues are subjected to analytical procedure in order to determine the

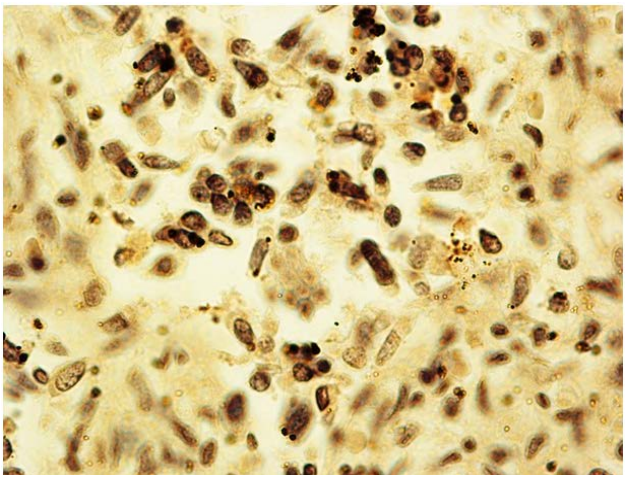

Figure 3. Positive *α7* nAChR immunoexpression of a group of cells in the lung parenchyma of a SIUDS case (38 gestational weeks). Magnification: 40×.

level of the 25 selected compounds. The chromatographic separation is carried out using an HP-5MS (Agilent J&W GC columns, Folsom, CA, USA), i.d. 30.0 m × 0.25 mm, containing 5% phenyl-methylsiloxane, with a phase thickness of 0.25 μm. As carrier gas, helium at 1 mL/min (constant flow) is adopted. The GC oven temperatures are 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). The transfer line and ion source temperature are kept at 290˚C and 300˚C, respectively.

3. Results and Discussion

Through the application of the above-reported guidelines, the harmful effects of prenatal nicotine absorption on the autonomic nervous system were highlighted in a wide case study of sudden fetal and infant deaths. More precisely, a significantly increased incidence of hypodevelopment of important brainstem and cerebellum structures was observed in fetuses and infants of smoking mothers, compared to victims with nonsmoking mothers [6]-[11].

The defects consisted mainly in hypoplasia of the neuronal centers involved in breathing control such as the Kölliker-Fuse nucleus and the facial/parafacial complex in the pons, the pre-Bötzinger nucleus in the medulla oblongata and the intermediolateral nucleus in the upper spinal cord. These nuclei, linked together in a "respiratory network" (RN), under normal conditions, function in ordered sequences: the intermediolateral nucleus coordinates during pregnancy episodic breathing movements aimed at promoting the fetal lung maturation. At the same time the Kölliker-Fuse nucleus plays an important role as it inhibits the response of central and peripheral chemoreceptors and therefore the respiratory reflexes *in utero*, whilst allowing occasional respiratory activity of the intermediolateral nucleus. After birth, the Kölliker-Fuse nucleus abruptly reduces its inhi-

bitory effects and becomes active as promoter center of breathing. In detail, the Kölliker-Fuse nucleus is able to stimulate the facial/parafacial complex and the pre-Bötzinger nucleus to trigger the first inspiratory impulse as well as those thereafter [12] [13] [14] [15]. All the RN centers, through excitatory and/or inhibitory connections, according to the need, can also coordinate the pulmonary motor responses to hematic oscillations of pO_2, pCO_2 and pH, presumably due, in many cases, to nicotine absorption [16] [17].

In cases of maternal smoking in pregnancy, carbon monoxide, a gaseous combustion product of nicotine, may readily cross the placenta and bind to fetal hemoglobin [18] [19] [20] [21]. The consequent carboxyhemoglobin is not able to release oxygen, thus hindering the physiological development of fetal organs, especially those more susceptible to hypoxic damage, including the brain. Moreover, nicotine is one of the few lipid-soluble substances that can pass through the blood-brain barrier by passive diffusion and act directly on the expression of specific receptors which are essential for the development of the nervous system, such as the nicotinic acetylcholine receptors (nAChRs). A single nAChR is an integral membrane protein composed of five subunits, with a central small ionic pore which normally opens when binding to the ACh agonist and closes in the resting state. The five subunits are combined to form two main receptor groups: the "homomeric receptors" containing only α subunits ($\alpha 7$-$\alpha 9$) and the "heteromeric receptors" that contain two or more α and β subtypes in the same complex ($\alpha 2$-$\alpha 6$ and $\beta 2$-$\beta 4$) [22] [23] [24] [25].

Nicotine behaves as an ACh antagonist. In particular it elicits its toxic effects above all on the $\alpha 7$-nAChR subunit, due to its faster kinetics compared with other nAChR subtypes and its feature of primary target for neurotoxicants [26] [27]. So, ACh cannot perform its normal function, since the corresponding receptors are made unavailable by nicotine. As results, a significant synaptic turnover disruption and severe defects of the nervous system developmental process can happen.

In one of our studies [28] we compared the expression of $\alpha 7$-nAChRs in the brainstems of 23 fetuses and 22 newborns who died of known and unknown causes, with both smoking and nonsmoking mothers, in order to assess the possible relationship between hyperactivation of these receptors caused by nicotine absorption during pregnancy and sudden unexplained perinatal death. An overexpression of $\alpha 7$-nAChRs, associated to developmental alterations of brainstem structures involved in the breathing control (mainly hypoplasia of the parafacial/facial complex and the pre-Bötzinger and the Kölliker-Fuse nuclei), was highlighted in many SIDS and SIUDS victims with smoking mothers.

To date several Authors have focused on the effects of maternal smoking in pregnancy on the nicotinic receptors of infants who died of SIDS but with conflicting results. While Duncan *et al.* [29] and Nachmanoff *et al.* [30] did not find any differences in the receptor expression in brainstem nuclei in SIDS cases both exposed and not exposed to tobacco smoke, Macahalani *et al.* [31] reported intense $\alpha 7$-nAChRs immunostaining in several brainstem nuclei in SIDS cases

with smoking mothers, according to the results of our study [28]. Then, we can sustain that nicotine is able to perturb the structural maturation of brainstem nuclei by impairing the central cholinergic system through the upregulation of nicotinic receptor bindings. This hypothesis is also supported by experimental studies showing that stimulation of nAChRs by nicotine causes neuronal inhibition of DNA synthesis, mitotic abnormalities and apoptosis of brain structures essential for life, thus compromising their normal development [32] [33].

Many SIUDS and SIDS cases included in our study [28] with hypoplasia of one or more brainstem nuclei and high percentage of *α7*-nAChR immunopositive neurons, were born from non-smoking mothers. Therefore, we speculated that other environmental risk factors besides tobacco smoke, such as persistent pollutants, could have caused these alterations. We then used appropriate chemical procedures to analyze the cerebral cortex samples of these cases in order to evaluate the possible presence of agricultural pesticides that were used in the area of the mothers' residence, especially the endocrine disrupting compounds (EDCs), which are organic chemicals characterized by high degradation resistance and ability to interfere with the functionality of the endocrine system.

The chemical analysis, through gas chromatography-mass spectrometry (GC-MS) [34] [35], showed the presence of EDCs such as organochlorine and organophosphate pesticides (more precisely *α* and *γ*-chlordane, chlorfenvinfos, chlorpyrifos, p,p-DDT, p,p-DDE, endrin, *α*- and *β*-endosulfans) in the brain samples. The exposure to persistent EDCs in pregnancy most likely produces the same effect of nicotine on *α7*-nAChR expression and functionality. Likewise to nicotine, pesticides, when absorbed by the mothers, can pass through the placental barrier into the fetal blood stream and then, through the blood-brain barrier, into the fetal brain. Here pesticides may interfere with the expression of genes controlling the nervous system development and cause specific molecular alterations in the DNA, RNA and antigenic proteins of the neurons [36] [37].

Our findings indicate that *α7*-nAChRs are specific targets in the developing brain for nicotine as well as for cholinergic neurotoxicants, including EDCs. Therefore, prenatal absorption of cigarette smoke and pesticide, individually or together, can perturb the expression of *α7*-nAChRs in the brain of fetuses and newborns so allowing developmental alterations of neuronal centers that are essential for life. These results show that the altered expression of *α7*-nAChRs due to exposure to toxins *in utero* increases the risk of SIDS/SIUDS.

Nicotinic receptors are present not only in the central nervous system but also in other human tissues and organs with the same susceptibility to toxic substances [38] [39]. We have reported the presence of high *α7*-nAChR immunopositivity in the lung parenchyma and airway vessel walls in many SIUDS/SIDS victims with smoking mother [40]. This article emphasizes the extreme vulnerability of the developing lung to maternal cigarette smoke absorption, as also reported by the literature [41] [42] [43]. So, the pulmonary hypoplasia in fetuses and many respiratory disorders in infancy may be linked to the interaction of xenobiotics, including pesticides, with the lung *α7*-nAChRs.

4. Conclusion

A multitude of research studies have highlighted the presence of developmental disorders of brain structures which play an important role in the control of vital functions, especially in fetal and infant deaths, associated to maternal exposure to environmental risk factors. This article, summarizing the research carried out at the Lino Rossi Center of the University of Milan in this field, adds new information on the potentially dangerous effects of cigarette smoke and air pollutants on the functionality of the nicotinic receptors during the development not only of the nervous system but also of the lungs. It follows the need to warn pregnant women that cigarette smoking places their unborn children in severe danger for life. In addition, they should not only avoid smoking but also EDC exposure whenever possible.

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Author Contribution

A.M.L. conducted the research summarized here, planned and wrote the present article.

Conflicts of Interest

The author declares no conflict of interest.

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