Journal of the Neurological Sciences 348 (2015) 94–100

Contents lists available at ScienceDirect

Journal of the Neurological Sciences

journal homepage: www.elsevier.com/locate/jns

Pesticide exposure during pregnancy, like nicotine, affects the brainstem α7 nicotinic acetylcholine receptor expression, increasing the risk of sudden unexplained perinatal death

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ARTICLE INFO

Article history:
Received 15 August 2014
Received in revised form 24 October 2014
Accepted 10 November 2014
Available online 18 November 2014

Keywords:
Nicotine
Pesticides
Nicotinic receptors
Neuropathology
SIDS
SIUDS

ABSTRACT

This study indicates the impact of nicotine and pesticides (organochlorine and organophosphate insecticides used in agriculture) on neuronal α7-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 α7-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 α7-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 α7-nicotinic acetylcholine receptor, allowing to developmental alterations of brainstem vital centers in victims of sudden unexplained death.

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 (α2–α10 and β2–β4) 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 α7 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 α7-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 α7 nAChR stimulation [15–17].

Therefore, the first goal of this study was to compare the expression of α7-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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http://dx.doi.org/10.1016/j.jns.2014.11.014
0022-510X/

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 α7 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 α7 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 anatomo-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 α7 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 anatomo-pathological procedures, fresh pieces of about 1 cm^2 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 α7 nAChR expression in these nuclei and/or structures was determined by immunohistochemistry on additional sections.

2.2. α7-nAChR immunohistochemistry

The immunohistochemical method used in order to evaluate the expression of α7 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