Brain Research Bulletin 110 (2015) 54–67

Contents lists available at ScienceDirect

Brain Research Bulletin

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

Research report

Prenatal nicotine alters the developmental neurotoxicity of postnatal chlorpyrifos directed toward cholinergic systems: Better, worse, or just "different?"

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ARTICLE INFO

Article history:
Received 5 November 2014
Accepted 5 December 2014
Available online 12 December 2014

Keywords:
Acetylcholine
Brain development
Chlorpyrifos
Nicotine
Organophosphate pesticides

ABSTRACT

This study examines whether prenatal nicotine exposure sensitizes the developing brain to subsequent developmental neurotoxicity evoked by chlorpyrifos, a commonly-used insecticide. We gave nicotine to pregnant rats throughout gestation at a dose (3 mg/kg/day) producing plasma levels typical of smokers; offspring were then given chlorpyrifos on postnatal days 1–4, at a dose (1 mg/kg) that produces minimally-detectable inhibition of brain cholinesterase activity. We evaluated indices for acetylcholine (ACh) synaptic function throughout adolescence, young adulthood and later adulthood, in brain regions possessing the majority of ACh projections and cell bodies; we measured nicotinic ACh receptor binding, hemicholinium-3 binding to the presynaptic choline transporter and choline acetyltransferase activity, all known targets for the adverse developmental effects of nicotine and chlorpyrifos given individually. By itself nicotine elicited overall upregulation of the ACh markers, albeit with selective differences by sex, region and age. Likewise, chlorpyrifos alone had highly sex-selective effects. Importantly, all the effects showed temporal progression between adolescence and adulthood, pointing to ongoing synaptic changes rather than just persistence after an initial injury. Prenatal nicotine administration altered the responses to chlorpyrifos in a consistent pattern for all three markers, lowering values relative to those of the individual treatments or to those expected from simple additive effects of nicotine and chlorpyrifos. The combination produced global interference with emergence of the ACh phenotype, an effect not seen with nicotine or chlorpyrifos alone. Given that human exposures to nicotine and chlorpyrifos are widespread, our results point to the creation of a subpopulation with heightened vulnerability.

1. Introduction

Protection of the human population from environmental toxicants requires consideration of subpopulations that may be especially vulnerable. This is particularly important for developmental neurotoxicity, given the widespread use of thousands of neurotoxic chemicals and their likely contribution to the increased incidence of neurodevelopmental disorders (Grandjean and Landrigan, 2006). In the case of the organophosphate pesticides, which represent a large proportion of total worldwide insecticide use (Casida and Quistad, 2004), we already know that there are polymorphisms of PON1, the enzyme that breaks down these neurotoxicants, clearly defining a sensitive subpopulation (Povey, 2010). In recent studies, we have explored how non-genetic factors, particularly an individual's "chemical history," might similarly create differential susceptibility, namely, whether prenatal exposures to neuroactive drugs or chemicals sensitize an individual to neurotoxicants encountered later in life. In particular, we showed how drug therapies commonly used in preterm labor enhance the subsequent vulnerability of the developing brain to chlorpyrifos, a commonly-used organophosphate pesticide (Aldridge et al., 2005; Levin et al., 2014; Meyer et al., 2005; Rhodes et al., 2004; Slotkin et al., 2013, 2014a). However, by far the most widely-encountered prenatal drug exposure is nicotine. Maternal cigarette smoking during pregnancy is the single most identifiable and preventable cause of neonatal morbidity and mortality (DiFranza and Lew, 1995), and the nicotine contained in tobacco smoke produces abnormalities of brain development leading to neurobehavioral deficits such as attention deficit/hyperactivity disorder, conduct

Abbreviations: ACh, acetylcholine; ChAT, choline acetyltransferase; HC3, hemicholinium-3; nAChR, nicotinic acetylcholine receptor; PN, postnatal day.

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http://dx.doi.org/10.1016/j.brainresbull.2014.12.003
0361-9230/

disorder and affective disorders (Ernst et al., 2001; Herrmann et al., 2008; Slotkin, 2008). Besides active maternal smoking, significant nicotine exposures occur through second-hand smoke or through the use of nicotine replacement products in smoking cessation, or more recently, through the use of "vaping" devices for inhaling nicotine. Nicotine's direct actions on nicotinic acetylcholine receptors (nAChRs), preempt the timing and intensity of the natural trophic signals ordinarily controlled by acetylcholine (ACh), leading to abnormalities of neuronal cell replication, differentiation and synaptic connectivity (Dwyer et al., 2008; Hohmann and Berger-Sweeney, 1998; Lauder and Schambra, 1999; Slotkin, 2004, 2008). Likewise, chlorpyrifos and other organophosphates target ACh systems as one of their primary modes of action, involving not only inhibition of cholinesterase (the mechanism for systemic toxicity), but also mechanisms that compromise ACh receptors and cell signaling at exposures below the threshold for cholinesterase inhibition (Slotkin, 2004, 2005).

The convergence of nicotine and chlorpyrifos on ACh pathways makes this a likely combination for enhanced vulnerability. At the same time, however, nicotine possesses neuroprotective properties that can attenuate the actions of other neurotoxicants (Belluardo et al., 2000; Ferrea and Winterer, 2009; Kawamata and Shimohama, 2011). Using in vitro models, we found that nicotine can protect neuronotypic cells from the antimitotic actions and oxidative stress induced by chlorpyrifos, while at the same time nicotine itself had adverse effects on neural cell replication and differentiation (Abreu-Villaça et al., 2005; Qiao et al., 2003b, 2005; Slotkin et al., 2007b, 2014b); the balance between protection and damage was highly dependent on the specific developmental stage of the cells (Slotkin et al., 2007b). It is thus difficult to predict whether prior exposure to nicotine might worsen or improve the outcome of subsequent developmental exposure to chlorpyrifos.

Accordingly, in the present work, we examined the impact of fetal nicotine exposure on the subsequent effects of postnatal chlorpyrifos directed toward the development of ACh systems in the rat brain. Nicotine was given throughout gestation via implanted osmotic minipump, at a dose (3 mg/kg/day) that produces nicotine plasma levels similar to those in moderate smokers (Lichtensteiger et al., 1988; Murrin et al., 1987; Trauth et al., 2000); we specifically chose a lower dose than in our previous work simulating heavy smoking (Slotkin, 2004, 2008), so as to produce submaximal changes in order to leave room for additional effects of chlorpyrifos. Chlorpyrifos was then given daily on postnatal days (PN) 1–4 at a dose of 1 mg/kg, a regimen that produces just-detectable inhibition of brain cholinesterase and that disrupts neurobehavioral development, but that is not systemically toxic (Slotkin, 1999, 2004, 2005; Song et al., 1997). This exposure model successfully predicts both the neurobehavioral deficits and abnormalities of brain structure seen in children exposed prenatally to chlorpyrifos (Bouchard et al., 2011; Engel et al., 2011; Rauh et al., 2006, 2011, 2012). Our study thus encompassed four treatment groups: control, nicotine alone, chlorpyrifos alone, and nicotine followed by chlorpyrifos.

We assessed the impact on brain regions comprising the major ACh projections and their corresponding cell bodies, focusing on well-established markers of ACh synaptic function: the concentration of α4β2 nAChRs, binding of hemicholinium-3 (HC3) to the presynaptic high-affinity choline transporter, and activity of choline acetyltransferase (ChAT). The α4β2 nAChR is the most abundant nAChR subtype in the mammalian brain (Flores et al., 1992; Happe et al., 1994; Whiting and Lindstrom, 1987, 1988) and underlies the ability of ACh systems to release other neurotransmitters involved in reward, cognition and mood (Buisson and Bertrand, 2001, 2002; Dani and De Biasi, 2001; Fenster et al., 1999; Quick and Lester, 2002). High-affinity choline transporters and ChAT are both constitutive components of ACh nerve terminals but they differ in their regulatory mechanisms and hence in their functional significance. ChAT is the enzyme that synthesizes ACh, but is not regulated by nerve impulse activity, so that its presence provides an index of the development of ACh projections (Happe and Murrin, 1992; Kreider et al., 2005, 2006; Slotkin, 2004, 2008). In contrast, HC3 binding to the choline transporter is directly responsive to neuronal activity (Klemm and Kuhar, 1979; Simon et al., 1976), so that comparative effects on HC3 binding and ChAT enables the characterization of both the development of innervation and presynaptic impulse activity. Accordingly, in addition to assessing HC3 binding and ChAT activity, we determined the HC3/ChAT ratio as an index of presynaptic activity relative to the number of cholinergic nerve terminals (Abreu-Villaça et al., 2004; Slotkin et al., 1994, 2007a). We also contrasted the specific effects on ACh synaptic development with assessments of systemic toxicity (maternal weight gain, litter characteristics, postnatal body and brain region weights), and determinations of whether prenatal nicotine treatment enhanced the subsequent ability of chlorpyrifos to inhibit cholinesterase.

2. Materials and methods

2.1. Animal treatments

All experiments were carried out humanely and with regard for alleviation of suffering, with protocols approved by the Institutional Animal Care and Use Committee and in accordance with all federal and state guidelines. Timed-pregnant Sprague-Dawley rats were shipped on the second day of gestation by climate-controlled truck (total transit time < 1 h), housed individually and allowed free access to food and water. There were four treatment groups, each comprising 10–12 litters: controls (prenatal vehicle infusion + postnatal vehicle injections), nicotine treatment alone (nicotine infusion + vehicle injections), chlorpyrifos treatment alone (vehicle infusion + chlorpyrifos injections), and those receiving the combined treatment (nicotine infusion + chlorpyrifos injections). On gestational day 4, before implantation of the embryo in the uterine wall, each animal was quickly anesthetized with ether, a small area on the back was shaved, and an incision made to permit s.c. insertion of a Model 2ML2 Alzet minipump, after which the incision was closed with wound clips. The pumps contained nicotine bitartrate dissolved in bacteriostatic water so as to deliver 3 mg/kg/day of nicotine free base, with the dosage determined by the initial body weights of the dams (215 ± 2 g); control pumps contained bacteriostatic water and equivalent concentrations of sodium bitartrate. Because weights increased with gestation, the dose rate fell accordingly to 2 mg/kg/day, but the dose rates remained well within the range that produces nicotine plasma levels similar to those in moderate smokers (Fewell et al., 2001; Trauth et al., 2000). It should be noted that the pump, marketed as a two week infusion device, actually takes approximately 17 days to be exhausted completely (information supplied by the manufacturer) and thus the nicotine infusion terminates on the 21st day of gestation; in earlier work, we confirmed the termination of nicotine delivery coinciding with the calculated values (Trauth et al., 2000). Parturition occurred during gestational day 22, which was also taken as PN0.

After birth, pups were randomized within treatment groups and litter sizes were culled to 10 (5 males and 5 females) to ensure standard nutrition. Control and nicotine-treated litters were then assigned to either the vehicle or chlorpyrifos postnatal treatment groups. Chlorpyrifos was dissolved in dimethylsulfoxide to provide consistent absorption (Whitney et al., 1995) and pups were injected subcutaneously at a dose of 1 mg/kg in a volume of 1 ml/kg once daily on postnatal days 1–4; control pups received equivalent injections of the dimethylsulfoxide vehicle. This regimen has been shown previously to produce developmental