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Subject: Any form of Nicotine during pregnancy may lead to SIDS - The UK should take note - https://www.nhs.uk/conditions/pregnancy-and-baby/smoking-pregnant/
(See attached file: Any-form-of-Nicotine-during-pregnancy-SIDS.pdf)

What the NHS UK states versus what this report offers on any form of nicotine use during pregnancy

https://www.nhs.uk/conditions/pregnancy-and-baby/smoking-pregnant/

Nicotine replacement therapy (NRT)

You can use NRT during pregnancy if it will help you stop smoking, and you're unable to stop without it. It's not recommended that you take stop smoking tablets such as Champix or Zyban during pregnancy. NRT contains only nicotine and none of the damaging chemicals found in cigarettes, so it is a much better option than continuing to smoke. It helps you by giving you the nicotine you would have had from a cigarette. You can be prescribed NRT during pregnancy by your GP or an NHS stop smoking adviser. You can also buy it over the counter without a prescription from a pharmacy. NRT is available as: patches gum inhalator nasal spray mouth spray oral strips lozenges microtabs If you have pregnancy-related nausea and vomiting, patches may be a better solution. NRT patches should be used for no more than 16 hours in any 24-hour period. The best way to stick to this is to remove the patch at bedtime. Before using any of these products, speak to your midwife, GP, a pharmacist or a specialist stop smoking adviser. By getting this specialist advice you can be sure that you are doing the best for your baby and best for you. For more information, call the NHS Smokefree advice line on 0300 123 1044. Remember, you are twice as likely to be successful at quitting if you get some support from a trained adviser.

Liquorice-flavoured nicotine products

Pregnant women are advised to avoid liquorice-flavoured nicotine products. Although there is no known risk with small amounts of liquorice flavouring, the manufacturers advise caution. This caution is based on information on the adverse effects associated with excessive amounts of liquorice root. As other flavours are available, pregnant women are advised to select an alternative, such as fruit or mint. Read more about stop smoking treatments.

E-cigarettes in pregnancy

E-cigarettes allow you to inhale nicotine through a vapour rather than smoke. Cigarettes deliver nicotine along with thousands of harmful chemicals. By itself, nicotine is relatively harmless. E-cigarettes do not produce tar and carbon monoxide, two of the main toxins in cigarette smoke. Carbon monoxide is particularly harmful to developing babies. The vapour from an e-cigarette does contain some of the potentially harmful chemicals found in cigarette smoke, but at much lower levels. E-cigarettes are fairly new and there are still some things we don't know. However, current evidence on e-cigarettes indicates they are much less risky than smoking. If using an e-cigarette helps you to stop smoking, it is much safer for you and your baby than continuing to smoke. Unlike NRT, such as patches and gum, e-cigarettes are not available on NHS prescription. If you want to use an e-cigarette, you can still get free expert help from a stop smoking adviser. Call NHS Smokefree on 0300 123 1044 for more information, or ask your midwife to refer you. Find out more about using e-cigarettes to stop smoking.

The lack of success of e-cigarettes in UK, USA and 28 European countries to date, for smoking cessation

Latest longitudinal 10 year study shows ineffectiveness of e-cigs

https://bmjopen.bmj.com/content/8/6/e016046

Latest study from USA on e-cigarettes

http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0198047

UK study shows e-cigarettes are not effective for smoking cessation at one year follow-up

https://www.ncbi.nlm.nih.gov/pubmed/25900312

E-cigarettes Associated With Depressed Smoking Cessation: A Cross-sectional Study of 28 European Union Countries

https://tobacco.ucsf.edu/e-cigarette-users-europe-including-england-are-less-likely-quit-smoking-conventional-cigarettes-results-challenge-phe-recommendation-e-cigarettes-be-used-hospitals

https://www.ajpmonline.org/article/S0749-3797(17)30749-3/fulltext

Aldehydes and acrolein DNA damage

http://www.pnas.org/content/early/2018/01/25/1718185115

http://library.med.nyu.edu/api/publications/?person=moonst01&sort=display_rank&in-biosketch=yes

https://truthinitiative.org/news/e-cigarettes-facts-stats-and-regulations?utm_source=Truth+Initiative+Mailing+List&utm_campaign=19e7444702-Newsletter_109_2018_07_19&utm_medium=email&utm_term=0_c91fd8a5c5-19e7444702-86458139

Attachments:

Any-form-of-Nicotine-during-pregnancy-SIDS.pdf

E-Cigarettes, Nicotine Patch During Pregnancy May Hike SIDS Risk

consumer.healthday.com/kids-health-information-23/sudden-infant-death-syndrome-sids-news-643/e-cigarettes-nicotine-patch-during-pregnancy-may-hike-sids-risk-735860.html

Robert Preidt

July 19, 2018

THURSDAY, July 19, 2018 (HealthDay News) -- Using any form of nicotine during pregnancy or while nursing may raise a baby's risk for sudden infant death syndrome (SIDS), new animal research suggests.

The findings indicate that nicotine patches or electronic cigarettes may not be a safe alternative to cigarettes during pregnancy, the study authors said.

The findings appear in the July 18 *Journal of Physiology*.

"Sudden infant death syndrome is such a distressing tragedy for families. We still don't fully understand the causes, but this research is important because it helps mothers reduce the risk," corresponding author Stella Lee said in a journal news release. Lee is a researcher at the Dartmouth Geisel School of Medicine in Hanover, N.H.

Smoking is known to increase the risk of SIDS. Some women who want to quit smoking during pregnancy switch to nicotine patches or electronic cigarettes, but the impact on a baby's risk of SIDS has been unclear.

In experiments with rats, the researchers found that exposing mothers to nicotine during pregnancy and nursing impaired an automatic response in their offspring called autoresuscitation. That's the ability to recover normal heart rate and breathing after gasping caused by lack of oxygen.

In human infants, problems such as getting tangled in bedding, minor illnesses or a breathing obstruction can trigger autoresuscitation. Failure of this survival mechanism has been noted in SIDS cases, according to the researchers.

Results of animal studies frequently aren't replicated in humans. Still, "we will continue to identify the possible predictors of risk and consider how we can treat infants who have a compromised autoresuscitation mechanism," said study co-senior author Dr. Aihua Li. She's an associate professor of molecular and systems biology at Dartmouth.

More information

The American Academy of Pediatrics explains how to reduce the risk of SIDS.

Use of Nicotine during pregnancy may increase risk of sudden infant death syndrome

19 July 2018. Nicotine exposure during pregnancy, whether from smoking cigarettes, or nicotine patches and e-cigarettes, increases risk of sudden infant death syndrome – sometimes known as "cot death" – according to new research published in *The Journal of Physiology.*

Sudden infant death syndrome (SIDS) is the sudden and unexpected death of an infant under 12 months of age that occurs typically while sleeping. Failure of autoresuscitation, the ability to recover normal heart rate and breathing following gasping caused by lack of oxygen in the brain, has been recorded in human SIDS cases.

Smoking increases risk for SIDS. Over the last decade, use of cigarettes has declined significantly, however, over 10% of pregnant women still smoke during pregnancy. Over recent years nicotine replacement therapies, such as nicotine patches or e-cigarettes, have been prescribed to women who wish to quit smoking during their pregnancy. However these nicotine replacement therapies may not protect infants from SIDS. With increasing numbers of nicotine patch and electronic cigarette users during pregnancy, there is an increasing urgency to better understand the impact of nicotine exposure on the development of babies during pregnancy.

The researchers showed that exposure of the mother to nicotine during pregnancy can affect the baby's central nervous system and impair the baby's cardiorespiratory responses to stressful environments, e.g. asphyxia, especially in babies who have both serotonin and serotonin receptors deficiency in the brain. This can damage a key biological mechanism called autoresuscitation that protects the infant from a severe lack of oxygen. Such failure of autoresuscitation increases the likelihood of SIDS because the infant is unable to recover from environmental stresses that cause lack of oxygen, such as getting tangled in bedding, a minor illness or a breathing obstruction.

This research suggests that the use of nicotine, e.g. nicotine patches or electronic cigarettes, are not a safe alternative to cigarettes during pregnancy, because exposure to nicotine by any route may be harmful to a baby's cardiorespiratory function and increase the risk of SIDS.

The research conducted by the Geisel school of Medicine at Dartmouth, Lebanon, New Hampshire, tested whether use of nicotine during pregnancy and nursing is more likely to elicit autoresuscitation defects in developing animals. They exposed rats to nicotine through maternal blood or milk and then looked at their response to repeated periods of severe low oxygen.

Stella Lee, the corresponding author of the study, commented on future research "Sudden infant death syndrome is such a distressing tragedy for families. We still don't fully understand the causes, but this research is important because it helps mothers reduce the

risk.”

Aihua Li, a senior author on the project added “We will continue to identify the possible predictors of risk and consider how we can treat infants who have a compromised autoresuscitation mechanism.”

END

Notes for Editors

1. Pre- and early postnatal nicotine exposure exacerbates autoresuscitation failure in serotonin deficient rat neonates: https://physoc.onlinelibrary.wiley.com/doi/10.1113/JP275885

2. The *Journal of Physiology* publishes advances in physiology which increase our understanding of how our bodies function in health and disease. http://jp.physoc.org

3. The Physiological Society brings together over 3,500 scientists from over 60 countries. The Society promotes physiology with the public and parliament alike. It supports physiologists by organising world-class conferences and offering grants for research and also publishes the latest developments in the field in its three leading scientific journals, *The Journal of Physiology*, *Experimental Physiology* and *Physiological Reports*. www.physoc.org

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The Journal of Physiology

physoc.onlinelibrary.wiley.com/doi/10.1113/JP275885

Sections

Abstract

Key points

- Sudden infant death syndrome (SIDS) is one of the leading causes of death during the first year of life and abnormalities linked to serotonin (5-HT) have been identified in many SIDS cases.
- Cigarette smoking and associated exogenous stressors, e.g. developmental nicotine exposure, may compound these serotonergic defects and any associated defects in cardiorespiratory function.
- Using neonatal rodent pups subjected to medullary 5-HT deficiency and perinatal nicotine exposure, we examined the impact of this interplay of factors on the neonates' ability to autoresuscitate at specific ages.
- In perinatal nicotine-exposed 5-HT deficient pups, impaired autoresuscitation along with significantly delayed post-anoxic recovery of normal breathing and heart rate was observed at postnatal day 10 (P10).
- We found that the interaction between 5-HT deficiency and perinatal nicotine exposure can significantly increase pups' vulnerability to environmental stressors and exacerbate defects in cardiorespiratory protective reflexes to repetitive anoxia during the development period.

Abstract

Cigarette smoking during pregnancy increases the risk of sudden infant death syndrome (SIDS), and nicotine replacements, a key ingredient of cigarettes, have been recently prescribed to women who wish to quit smoking during their pregnancy. Serotonin (5-HT) abnormalities have been consistently identified in many SIDS cases. Here we investigated the effects of perinatal nicotine exposure in mild 5-HT deficiency rat neonates on autoresuscitation, a protective cardiorespiratory reflex. The mild 5-HT deficiency was induced by a maternal tryptophan-deficient diet, and nicotine was delivered from embryonic day (E) 4 to postnatal day (P) 10 at 6 mg kg^{-1} day^{-1} through an osmotic pump. In P10 rats, nicotine exposure exacerbates autoresuscitation failure (mortality) in mildly 5-HT-deficient rats to a greater extent than in controls (P = 0.029). The recovery of eupnoea and heart rate to baseline values following repetitive anoxic events (which elicit an apnoea accompanied by a bradycardia) is significantly delayed in 5-HT-deficient rats treated with nicotine, making them more susceptible to failure of autoresuscitation (eupnoea recovery: P = 0.0053; heart rate recovery: P = < 0.0001). Neither 5-HT deficiency nor nicotine exposure alone appears to affect the ability to autoresuscitate significantly when compared

among the four treatments. The increased vulnerability to environmental stressors, e.g. severe hypoxia, asphyxia, or anoxia, in these nicotine-exposed 5-HT-deficient neonates during postnatal developmental period is evident.

Introduction

Sudden infant death syndrome (SIDS) is the sudden and unexpected death of an infant under 12 months of age that occurs typically during the sleep period, the cause of which remains unexplained following an autopsy and a death scene investigation (Krous *et al*. 2004). An increasing number of studies suggest that SIDS infants have deficits in respiratory and autonomic function that increase their vulnerability to severely hypoxic conditions (Franco *et al*. 1999; Poets *et al*. 1999; Harper, 2001; Sridhar *et al*. 2003; Poets, 2004; Kinney & Thach, 2009). Failure to autoresuscitate from events of asphyxia may result from the lack of protective responses against potentially fatal circumstances that may occur during re-breathing while in the prone position, or in other airway-obstructing conditions during sleep periods (Kemp *et al*. 1993; Mitchell *et al*. 1999). These deficits in respiratory and autonomic control have previously been associated with abnormalities in the medullary serotonin (5-hydroxytryptamine or 5-HT) system that have been documented in a majority of SIDS cases studied, including abnormal 5-HT receptor and transporter binding (Panigrahy *et al*. 2000; Paterson *et al*. 2006), reduced tissue 5-HT and tryptophan hydroxylase 2 (TPH2) levels (Duncan *et al*. 2010), and increased cell counts of immature and possibly dysfunctional 5-HT neurons (Panigrahy *et al*. 2000; Paterson *et al*. 2006; Machaalani *et al*. 2009). It is apparent these infants are more vulnerable to stressors that may trigger a life threatening apnoeic event leading to the failure of arousal and autoresuscitation. Neonatal mice and rats expressing a range of medullary 5-HT deficiencies (Hodges & Richerson, 2008; Hodges *et al*. 2008; Cummings *et al*. 2009, 2010, 2011; Penatti *et al*. 2011) demonstrate homeostatic deficits and exaggerated apnoeas and bradycardias, again implicating the medullary 5-HT system in maintaining cardiorespiratory homeostasis. Medullary 5-HT deficiencies in rodent models range from a milder 40–50% 5-HT deficit in tryptophan-deficient rat neonates (Penatti *et al*. 2011) to more severe ∼90% 5-HT deficit in transgenic *Pet-1*$^{-/-}$ mice lacking 60–70% of brainstem 5-HT cells from early embryogenesis (Cummings *et al*. 2010, 2011). Pharmacological lesion of 5-HT neurons via 5,7-DHT injections during early postnatal period (P2–3) can also induce a severe ∼80% 5-HT deficit in the brainstem of neonatal rats (Cummings *et al*. 2009).

Cigarette smoking during pregnancy increases the risk of pre-term birth and SIDS (MacDorman *et al*. 1997; Nachmanoff *et al*. 1998; Waters *et al*. 1999; Matturri *et al*. 2006; Duncan *et al*. 2008; Hakeem *et al*. 2015). In the last decade, the overall use of cigarettes has declined significantly; however, over 10% of pregnant women still smoke during pregnancy, and there is an increased trend towards using non-combusted tobacco products (snuff, dissolvable, and electronic nicotine delivery systems) that can deliver nicotine during pregnancy (England *et al*. 2016, 2017). Nicotine replacement therapies, e.g. nicotine patches or electronic cigarettes, have been prescribed to women who wish to quit smoking during their pregnancy in recent years (Coleman *et al*. 2012; Oncken, 2012), even though nicotine replacement therapies may not protect neonates from SIDS and other adverse effects of smoking tobacco (England *et al*. 2016, 2017). Perinatal nicotine exposure can directly or indirectly affect many neurotransmitter systems in the central

nervous system, e.g. the serotonergic (Xu *et al*. 2001; Duncan *et al*. 2009; Kamendi *et al*. 2009; Blood-Siegfried & Rende, 2010; Slotkin *et al*. 2015) and the cholinergic systems (Duncan *et al*. 2008), both of which are critically involved in cardiorespiratory function and autoresuscitation. Cigarette smoke can increase the likelihood of inadequate placental perfusion, chronic intra-uterine hypoxia and intra-uterine growth restriction (Lambers & Clark, 1996; Andres & Day, 2000) and nicotine itself, via interactions with the brain's endogenous nicotinic receptors, can adversely impair neural development and function (Slikker *et al*. 2005). Fetal nicotine exposure can impair cardiac and respiratory responses to hypoxia (Slotkin *et al*. 1995; St-John & Leiter, 1999; Fewell *et al*. 2001; Neff *et al*. 2004; Feng *et al*. 2010), disrupt autonomic regulation (Zeskind & Gingras, 2006) and increase the incidence of obstructive sleep apnoea (Kahn *et al*. 1994) in neonates. Any alterations in the course of development of nAChRs and the neurons expressing them, particularly medullary 5-HT neurons, will inevitably affect how they in turn function and govern cardiorespiratory homeostasis.

Medullary 5-HT neurons play a critical role in maintaining cardiorespiratory homeostasis and therefore, a dysfunctional 5-HT network will disrupt this line of defense against perturbations, such as severe hypoxia or asphyxia. During sleep, loss of appropriate responses to hypoxia exposure, e.g. arousal from sleep or gasping, becomes detrimental and makes an infant increasingly vulnerable to the risk of SIDS (Kinney *et al*. 2009).

The goal of this study was to investigate whether a mild brainstem 5-HT deficiency induced by maternal tryptophan-deficient diet would interact with perinatal nicotine exposure to increase neonates' vulnerability to environmental stressors, e.g. severe hypoxia, anoxia, or asphyxia, during postnatal developmental period. The pre–perinatal nicotine-exposed mild 5-HT-deficient neonates were challenged with repeated bouts of anoxia similar to those experienced during episodes of sleep apnoea in infants at different postnatal ages. We hypothesize that interaction between the mild medullary 5-HT and developmental nicotine exposure will exacerbate autoresuscitation failure in rat neonates when challenged with environmental anoxia at a specific age.

Methods

Ethical approval

All experimental protocols were approved by the Institutional Animal Care and Use Committee of Dartmouth College and followed the guidelines of the National Institute of Health of animal use and care. The authors have read and complied with guidelines for research in rodents outlined in the *Principles and standards for reporting animal experiments in The Journal of Physiology and Experimental Physiology* (Grundy, 2015).

Tryptophan-deficient diet treatment

Adult female Sprague-Dawley rats (Charles River Laboratories, Inc.) were housed with a 12 h light-dark cycle at an ambient temperature of 21–23°C. They were fed *ad libitum* isocaloric, amino acid-based diets containing either the required level of tryptophan (TD.99366; amino acid control diet) or those with levels reduced to approximately 55% of

required (TD.140218; tryptophan-deficient diet diet; Envigo, formerly known as Harlan Teklad). They also had unlimited access to water. The amino acid control (Ctr) diet was composed of the following (in g kg^{-1}): 3.5 L-alanine, 12.1 L-arginine HCl, 6.0 L-asparagine, 3.5 L-aspartic acid, 3.5 L-cystine, 40.0 L-glutamic acid, 23.3 glycine, 4.5 L-histidine HCl, monohydrate, 8.2 L-isoleucine, 11.1 L-leucine, 18.0 L-lysine HCl, 8.2 L-methionine, 7.5 L-phenylalanine, 3.5 L-proline, 3.5 L-serine, 8.2 L-threonine, 1.8 L-tryptophan, 5.0 L-tyrosine, 8.2 L-valine, 351.68 sucrose, 150.0 corn starch, 150.0 maltodextrin, 80.0 soybean oil, 30.0 cellulose, 35.0 mineral mix, 8.2 calcium phosphate, monobasic, monohydrate, 13.0 vitamin mix, 2.5 choline bitartrate, and 0.02 tertiary butylhydroquinone, an antioxidant. The tryptophan-deficient (TD) diet was identical except that the level of tryptophan was reduced to 1 g kg^{-1} (approximately 55% of control diet), while L-glutamic acid was increased to 41.18 g kg^{-1} in order to maintain the tryptophan-deficient (TD) diet isonitrogenous and isocaloric to the control diet (Ctr diet). Adult female Sprague-Dawley rats received the new diets for at least a week prior to mating and continued to receive the diets throughout the gestation period and post birth up to postnatal day 25.

Nicotine treatment and surgery

Pregnant dams on either Ctr or TD diet were surgically implanted subcutaneously with osmotic pumps (model 2ML4; 2.5 µl h^{-1}, 2 ml total; ALZET Osmotic Pumps, DURECT Corporation) filled with a nicotine tartrate solution (6 mg kg^{-1} day^{-1}) or vehicle (saline) on gestational day 4. The nicotine-receiving dams were control matched with dams receiving a saline vehicle infusion via the osmotic pumps. Dams were anaesthetized with 4% isoflurane for initial induction; 1–2% isoflurane was used to maintain a surgical level of anaesthesia. Each dam received a subcutaneous injection of ketoprofen (5 mg kg^{-1}) as an analgesic at this stage. While in the prone position, an area approximately 3 cm by 3 cm (fur removed) was cleaned and sterilized with 70% ethanol and topical iodine (Medline Prep Solution; topical antiseptic microbicide) in preparation for the procedure. An incision slightly wider than the width of the pump was made in the back of the rat and a pocket to house the pump was created subcutaneously by breaking apart the connective tissue below the surface of the skin using blunt dissection. Once the pump was in place, the skin was sutured and isoflurane was removed to allow the dam to recover from anaesthesia. Each dam post surgery continued to receive a subcutaneous injection of ketoprofen (5 mg kg^{-1}) as an analgesic for 3 days following pump implantation; dams were also weighed daily during this period to ensure there was no weight loss. Pump contents were released at the rate of 60 µl day^{-1} and absorbed continuously during the remainder of the gestational and early postnatal period.

HPLC analysis of monoamine medullary content

A separate group of Ctr and TD diet litters that received either vehicle (saline) or nicotine treatments were used for the HPLC analysis of monoamine medullary content. The measured monoamines are as follows: 5-HT and its metabolite 5-HIAA (5-hydroxy-indole-acetic acid), noradrenaline (NAd), adrenaline (Adr), dopamine (DA) and their respective major metabolites, HVA (homovanillic acid) and DOP (dihydroxyphenylacetic acid or DOPAC) (Fig. 1*D*). Since nicotine alone did not affect any of the monoamine levels measured, nicotine- and vehicle-treated pups were consolidated into their respective diet

groups. Ctr diet: P10 (n = 20, 6 litters), P15 (n = 20, 5 litters), and P25 (n = 20, 6 litters); TD diet: P10 (n = 20, 9 litters), P15 (n = 20, 10 litters), and P25 (n = 20, 9 litters). Pups were killed by pentobarbital overdose (>75 mg kg^{-1}, Euthasol, Virbac Inc. Fort Worth, TX, USA) and their brainstems were collected immediately after killing. The method of extracting medullary tissue was similar to those previously reported (Penatti *et al*. 2011); in short, a tissue wedge was extracted from the ventral midsection of the medulla to include the regions heavily populated by 5-HT neurons (raphe obscurus, magnus and pallidus), and was stored at −80°C. For the HPLC analysis, the tissues were isolated and homogenized, and the supernatants were harvest. HPLC was performed utilizing an Antec Decade II electrochemical detector. Twenty microlitres of supernatant were injected onto a 100 × 4.60 mm HPLC column (Phenomenex, Torrance, CA, USA). Biogenic amines are eluted with a mobile phase. HPLC data acquisition and analysis are managed by Empower software (Waters Co.) and performed by Neurochemistry Core at Vanderbilt University School of Medicine (Nashville, TN, USA).

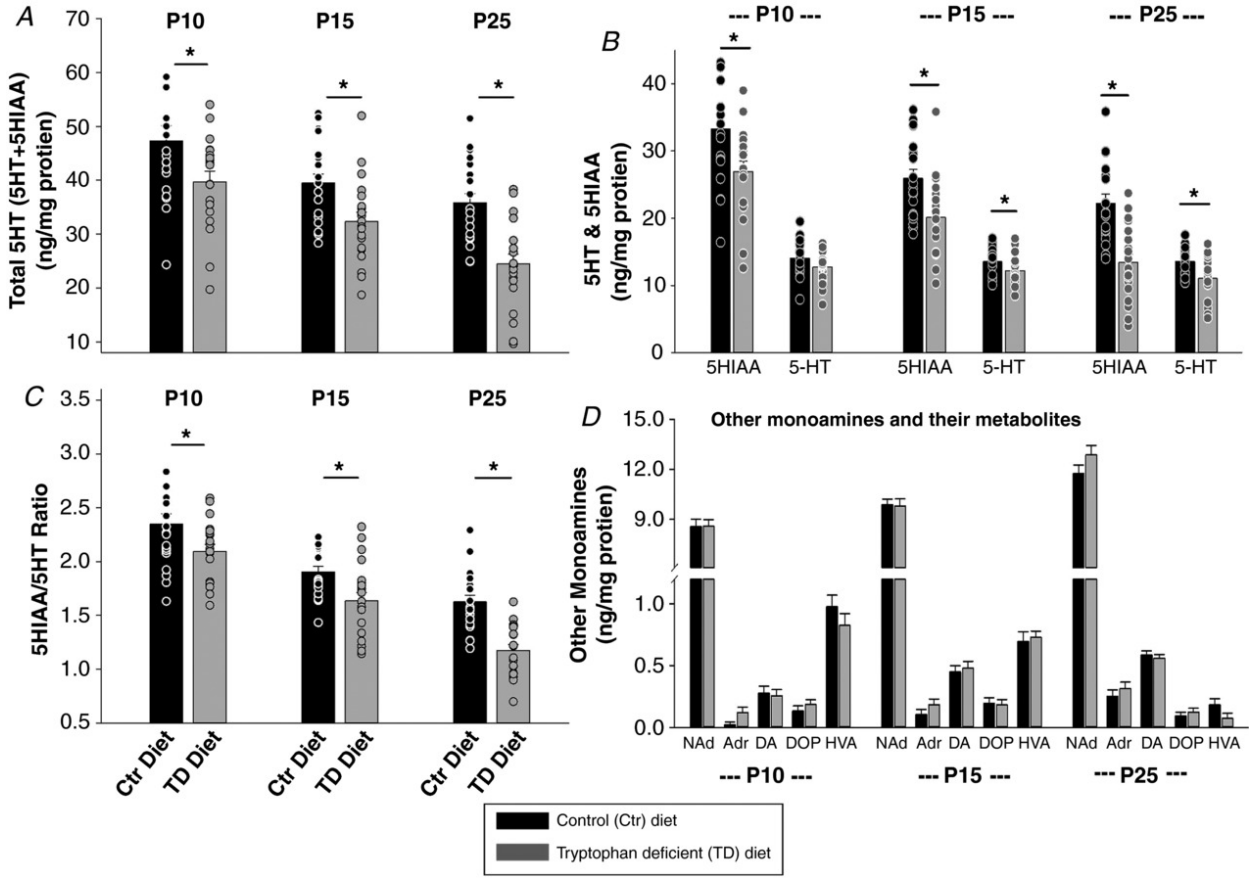

Figure 1. The effect of tryptophan-deficient (TD) on medullary monoamine levels
A–*C*, the total 5-HT (5-HT + 5-HIAA) (*A*), ratio of 5-HIAA to 5-HT (5-HIAA/5-HT) (*C*), and 5-HT and 5-HT metabolite (5-HIAA) levels (*B*) in the medulla were significantly lower in TD diet pups than control (Ctr) diet pups at P10, P15 and P25 ($^{*}P < 0.05$). Although 5-HT levels were lower in TD diet pups than Ctr diet pups at all three ages, they were only significant at P15 and P25 ($^{*}P < 0.05$). *D*, there were no significant differences with other monoamines and their metabolite levels between Ctr and TD diet groups at all three ages ($P > 0.05$). Data are presented as means ± SEM, and each circle represents an individual animal. Two-way ANOVA followed by the Holm-Sidak *post hoc* test were used for all comparisons. Black bar: Ctr diet; grey bar: TD diet. NAd, noradrenaline; Adr adrenaline; DA, dopamine; DOP, DOPAC or dihydroxyphenylacetic acid; HVA, homovanillic acid.

The values obtained are reported in nanograms per gram of tissue protein. The 5-HT + 5-HIAA data were used as a measure of total 5-HT content and the 5-HIAA/5-HT ratio was used as an index of serotonergic system activity.

ELISA analysis of serum cotinine level

To confirm nicotine exposure in pups, cotinine (a metabolite of nicotine) levels were quantified using an ELISA kit (Abnova) per the manufacturer's user manual using serum samples from pups treated with nicotine and vehicle on either Ctr or TD diet at P5, P8, P10, and P12. For serum preparation, whole blood samples were collected from the pups following the autoresuscitation experiment. Once pups were killed post experiment, whole blood samples were immediately collected from the heart by making an incision through the cardiac muscle. Blood extraction via cardiac puncture caused haemolysis and was deemed unsuitable for this analysis. Samples collected in a safe-lock tube were left to clot undisturbed at room temperature for approximately 20 min. The clot was separated from the supernatant (serum) by centrifuging at 2000 × *g* for 10 min in a refrigerated centrifuge. Serum was transferred immediately into a clean tube, being maintained at 4°C during handling. Samples with visible signs of haemolysis were discarded and those not analysed immediately for cotinine levels were stored at −80°C.

Respiratory and heart rate measurements

The experimental set-up was similar to those described in a previous study (Cummings *et al*. 2009, 2011); pups were placed in a water-jacketed glass chamber with body temperature held at 36 ±0.5°C throughout the experiment by controlling the temperature of the water perfused through the chamber. Water temperature was adjusted frequently in order to maintain a stable body temperature.

Respiratory parameters (tidal volume (V_T), breathing frequency (f_R), ventilation ($\dot{V}_E$) and oxygen consumption ($\dot{V}_{O_2}$)) were measured using head-out plethysmography; the mask separating the head from the rest of the animal was made by placing a piece of nitrile over a syringe tube (volume approximately 3 ml) and holding it in place with a fitted rubber ring (Terumo Medical Corporation, Japan). A small hole placed in the nitrile acted as an opening for the snout into the mask; the mask was sealed onto the face using Impregum F Impression Material (3M ESPE). A pneumotachograph was mounted onto the other end of the mask, through which the pups breathed in the surrounding air. Inspiratory and expiratory airflows were detected by a differential pressure transducer (Validyne Engineering Corp., Northridge, CA, USA) connected to the side-arms of the pneumotachograph. Air was drawn through the pneumotachograph and into the mask by a downstream pump to maintain a constant supply of fresh air inside the mask. The expired air was drawn through a desiccant column (Drierite Co. Ltd, Xenia, OH, USA) and then through an O_2 analyser (AEI Technologies, Pittsburgh, PA, USA). Body temperature and heart rate were measured using a rectal temperature probe and surface ECG electrodes, respectively. All data were recorded continuously using LabChart (ADinstruments, Colorado Springs, USA).

Experimental design and protocol

For the physiology experiment, animals of either sex were equally divided into four treatment groups based on the diet (Ctr or TD diet) and treatment (nicotine or vehicle). Ctr diet/vehicle group dams were treated with control diet and saline vehicle; TD diet/vehicle group dams were treated with the tryptophan-deficient diet and saline vehicle; Ctr diet/nicotine group dams were treated with control diet and nicotine; TD diet/nicotine group dams were treated with tryptophan-deficient diet and nicotine. Within each treatment group, animals were further divided into four age groups (P5, P8, P10 and P12) and each animal was tested only once at one age for the experiment. Table 1 lists the experimental groups at the four age groups including the sample size and the total number of litters used. Large litters were culled to 10 pups at P2 and any litters with fewer than eight pups were excluded from the study in order to eliminate any confounding factors that may arise from differences in litter size.

Table 1. Number of animals and litters used at each age in each treatment group

	Control vehicle		TD vehicle		Control nicotine		TD nicotine	
Age	***n***	**No. of litters**	***n***	**No. of litters**	***n***	**No. of litters**	***n***	**No. of litters**
P5	10	6	10	5	10	5	10	5
P8	13	6	15	7	13	7	13	7
P10	16	3	16	4	15	4	15	3
P12	14	8	15	9	13	6	13	6

On the day of the experiment, the animal was instrumented with a temperature probe and ECG electrodes first prior to sealing the mask onto the face. The mask was fitted onto the pre-heated water-jacketed body chamber (completing the head-out plethysmography set-up) using the same rubber ring used to fix the nitrile onto the syringe tube portion of the mask. The animal could adjust to the experimental setup for 30 min to allow the body temperature to warm to 36°C in addition to stabilizing $\dot{V}_{O_2}$. Following 10 min of baseline respiration recording, the animal underwent the autoresuscitation protocol, which consists of repeated bouts of anoxia (up to 15 bouts) separated by 5-min recovery periods of normoxia when respiratory and heart rates returned to ≥ 63% of baseline values. The duration of a typical experiment for the pups that survived all 15 bouts of anoxia was approximately two hours, including the experiment set-up. For the anoxic bout, gas (97% N_2/3% CO_2) was delivered directly from the tank to the open end of the pneumotachograph and the downstream pump drew this gas into the mask for a rapid anoxia exposure. Gas was applied until the animal became apnoeic and motionless with lack of muscle tone for 5 s (this period of apnoea will be referred to as ‘primary apnoea’), at which point the gas was immediately removed to flush the mask with room air. Litters from Ctr or TD diet dams treated with either saline vehicle or nicotine underwent the autoresuscitation protocol at four ages (P5, P8, P10 and P12) to assess their ability to survive from events of acute asphyxia. Each pup was tested once at one age only. Following the autoresuscitation experiment, the pups were killed by pentobarbital overdose (>75 mg kg^{-1}, Euthasol, Virbac Inc. USA) and their medullas collected, frozen and stored at −80°C for future analysis.

Data and statistical analysis

Baseline respiratory parameters ($\dot{V}_E$, V_T, and f_R), HR, and metabolic rate ($\dot{V}_{O_2}$) of all four treatment groups are summarized in Table 2 (means ± SD). To evaluate the effects of treatments on baseline parameters at different ages, a three-way ANOVA (analysis of variance) was employed, with factor 1 being tryptophan deficiency; factor 2 developmental nicotine exposure, and factor 3 age (P5, P8, P10 and P12). Data were collected from both male and female pups of each treatment group at the four experimental ages, and for all statistical analyses an α level of $P < 0.05$ was used.

Table 2. Baseline physiological parameters at each age in each group

	HR (min^{-1})	$\dot{V}_E$ (ml min^{-1} kg^{-1})	V_T (ml kg^{-1})	f_R (min^{-1})	$\dot{V}_{O_2}$ (ml min^{-1} kg^{-1})
P5					
Ctr diet/vehicle	363.4 ± 29.6	705.7 ± 123.7	5.8 ± 0.9	128.9 ± 21.1	24.9 ± 3.8
TD diet/vehicle	370.7 ± 27.5^{D}	674.9 ± 68.7	5.9 ± 1.4	122.2 ± 16.9	26.2 ± 3.1
Ctr diet/nicotine	367.1 ± 28.0	748.2 ± 103.8	5.7 ± 0.8	137.0 ± 22.3	27.4 ± 4.7
TD diet/nicotine	385.7 ± 20.2^{D}	673.2 ± 89.6	6.0 ± 0.9	120.5 ± 12.6	26.7 ± 2.6
P8					
Ctr diet/vehicle	399.9 ± 25.3	757.0 ± 134.3	5.2 ± 0.9	150.2 ± 13.4	25.6 ± 2.7
TD diet/vehicle	390.2 ± 31.1	720.8 ± 108.5	5.8 ± 1.3	129.1 ± 16.6^{D}	25.3 ± 3.3
Ctr diet/nicotine	391.4 ± 11.9^{T}	704.6 ± 98.2	5.3 ± 0.9	139.5 ± 21.9	28.0 ± 3.7
TD diet/nicotine	380.3 ± 12.1^{T}	771.9 ± 100.8	5.8 ± 0.7	136.8 ± 12.3^{D}	27.3 ± 3.2
P10					
Ctr diet/vehicle	379.8 ± 21.4	782.9 ± 252.6	6.4 ± 3.0	131.6 ± 19.1	27.2 ± 2.6
TD diet/vehicle	374.1 ± 23.5^{D}	802.2 ± 213.4	6.3 ± 1.4	130.5 ± 19.9^{D}	26.0 ± 1.8
Ctr diet/nicotine	394.2 ± 31.2^{T}	845.1 ± 529.2	6.0 ± 1.7	133.3 ± 27.0	26.7 ± 3.6
TD diet/nicotine	401.3 ± 24.1^{D,T}	894.8 ± 372.8	6.3 ± 1.1	138.0 ± 20.7^{D}	28.2 ± 3.1
P12					
Ctr diet/vehicle	415.5 ± 18.2	708.7 ± 198.6	5.7 ± 1.6	124.6 ± 19.0	28.5 ± 2.6
TD diet/vehicle	409.9 ± 26.3^{D}	788.8 ± 191.0	6.6 ± 1.0	119.5 ± 14.0^{D}	30.1 ± 3.8

	HR (min)	$\dot{V}_E$ (ml min kg)	*V* (ml kg)	*f* (min)	$\dot{V}_{O_2}$ (ml min kg)
Ctr diet/nicotine	436.4 ± 33.8[T]	721.0 ± 148.9	5.8 ± 1.5	125.9 ± 11.7	27.9 ± 2.0
TD diet/nicotine	408.9 ± 27.8[D,T]	743.4 ± 208.8	6.2 ± 1.1	120.1 ± 20.8[D]	27.5 ± 4.6

There was a significant difference in HR between TD diet pups and age-matched Ctr diet pups at P5, P10, and P12 ($P < 0.001$; 'D' denotes diet effect), whereas there was a significance difference in HR between nicotine-treated and non-treated pups at P8, P10, and P12 ($P < 0.05$; 'T' denotes nicotine treatment effect). During baseline, HR was significantly affected by age ($P < 0.001$) and treatment ($P = 0.03$), and significant interactions were found between age and diet ($P = 0.028$), as well as between age and treatment ($P = 0.018$). Three-way ANOVA with Holm-Sidak *post hoc* analysis. Data presented as means ± SD.

Mortality or survival rate was analysed using the Kaplan-Meier survival analysis (Gehan-Breslow method) and the Cox Regression–Proportional Hazards Model among the four treatment groups of all ages (P5, P8, P10 and P12). Additionally, at each age, percentage survival was assessed across the 15 anoxic episodes for each treatment group to determine and compare their survival rates. There were no significant differences in survival rates between the treatment groups at P5, P8 and P12; therefore, further detailed analyses and data plots are presented only for P10 groups (Figs 3 and 4).

Breathing and heart rate (HR) indices were defined as gasp latency (duration of primary apnoea), eupnoea recovery (time taken to restore eupnoeic breathing from the primary apnoea elicited by the anoxia to 63% of baseline values), and HR recovery (time taken to restore HR from the bradycardia during primary apnoea elicited by the anoxia to 63% of baseline values). To evaluate whether repetitive anoxia would progressively impair the breathing and HR indices at P10, gasp latency, eupnoea and HR recovery following anoxia episode 1, 5, 10, 15 or last survived anoxic bout were determined and compared among all experimental groups using a repeated-measures (RM) ANOVA followed by Holm-Sidak *post hoc* test (repeat with episode). Animals that died before episode 10 were not included in this analysis, and numbers of animals in each group used for this analysis are shown in Fig. 5. A value of 63% was used since that is the definition of the time constant, tau (Fewell *et al*. 2000).

The effects of tryptophan-deficient diet and developmental nicotine exposure on the medullary tissue monoamine levels were analysed using a two-way ANOVA at each age, followed by Holm-Sidak method *post hoc* test. Since nicotine alone has no effect on the levels of medullary monoamines, the results from nicotine- and saline vehicle-treated pups were combined based on their diet treatments (Fig. 1).

Results

Effect of nicotine on serum cotinine level

Cotinine (a metabolite of nicotine) levels were undetectable in the offspring of saline vehicle-treated dams on either Ctr or TD diet at any age. The offspring of nicotine-treated dams had serum cotinine levels between 70 and 80 ng ml^{-1} (Ctr diet/nicotine pups: 80.4 ± 26.3; TD diet/nicotine: 76.2 ± 22.5; data are means ± SD) between P8 and P12, with no significant difference between the two diet groups. These levels are comparable to those seen in other studies of developmental nicotine exposure in rat pups (Benowitz *et al*. 1982; Lichtensteiger *et al*. 1988; Slotkin *et al*. 1995; Milerad *et al*. 1998; Hafstrom *et al*. 2005).

Effect of tryptophan-deficient diet on 5-HT and 5-HIAA content in medulla

The levels of medullary monoamines, including 5-HT, noradrenaline (NAd), adrenaline (Adr), dopamine (DA) and their major metabolites (5-HIAA, homovanillic acid (HVA), dihydroxyphenylacetic acid (DOPAC or DOP)), were measured in the offspring from Ctr and TD diet dams. There was no significant difference in medullary monoamine levels between nicotine- and vehicle-treated pups in either Ctr or TD diet groups; therefore, the pups were regrouped into two groups based only on their diet, Ctr or TD diet (Fig. 1).

Nicotine treatment alone did not significantly affect levels of catecholamine, dopamine, 5-HT and their metabolites in the medulla (data not shown). TD diet did not significantly affected levels of catecholamines, dopamine and their metabolites in the medulla (Fig. 1*D*, $P > 0.05$) but significantly decreased medullary tissue total 5-HT (5-HT + 5-HIAA) content and 5-HT activity index (ratio of 5-HIAA/5-HT) (Fig. 1*A*–*C*). The levels of 5-HIAA and 5-HIAA/5-HT are highly correlated with 5-HT level in the brain, and therefore the changes of 5-HT, 5-HT and its metabolite 5-HIAA, and the 5-HIAA/5-HT ratio were further analysed to evaluate the total 5-HT activity in the brain in this study. At P10, the total 5-HT (5-HT + 5-HIAA) content, 5-HIAA, and 5-HIAA/5-HT ratio were all significantly lower in TD diet pups than Ctr diet pups (diet effect: $P = 0.035$; 0.03 and 0.04, respectively; two-way ANOVA) (Fig. 1*A*–*C*). At P10, even though the medullary 5-HT only decreased mildly (∼10%) and was not statistical significant ($P = 0.116$, two-way ANOVA) between TD diet and Ctr diet pups, the level of 5-HT metabolite, 5-HIAA, decreased significantly (∼19%; $P = 0.03$, two-way ANOVA), and both 5-HT and 5-HIAA contribute to a significant decrease in total 5-HT (∼16%; $P = 0.035$, two-way ANOVA). At P15 and P25, all 5-HT product levels in the medulla, including 5-HT, 5-HIAA, total 5-HT, and 5-HIAA/5-HT, were significantly lower in TD diet pups than in Ctr diet pups (diet effect: $P \leq 0.05$, two-way ANOVA) (Fig. 1*A*–*C*). An age difference effect on the severity of 5-HT deficiency (total 5-HT content) in TD diet pups was also observed ($P < 0.001$, two-way ANOVA) and this was expected as the pups started to consume more TD diet food themselves as they were ageing.

Effects of tryptophan-deficient diet and nicotine on baseline body weight, respiratory and heart rate parameters

Baseline values, including respiratory parameters, HR, and metabolic rates of all four treatment groups at P5, P8, P10 and P12, have been summarized in Table 2.

A three-way ANOVA analysis showed that body weight was significantly affected by age ($P \leq 0.001$), diet (TD or Ctr diet; $P \leq 0.001$) and treatment (nicotine or vehicle control; $P = 0.005$), and the significant interactions were found between age and diet ($P \leq 0.001$),

diet and treatment (P = 0.003) (three-way ANOVA with Holm-Sidak *post hoc* test). TD diet pups had significantly lower body weights than age-matched Ctr diet pups at P8, P10 and P12 (P < 0.001, three-way ANOVA), and when treated with nicotine, the TD diet/nicotine pups exhibited significantly lower body weights than the other three treatment groups at P8, P10 and P12 ((P < 0.001, three-way ANOVA; Fig. 2). Body weight changes induced by the TD diet are similar to those observed in our previous study (Penatti *et al*. 2011).

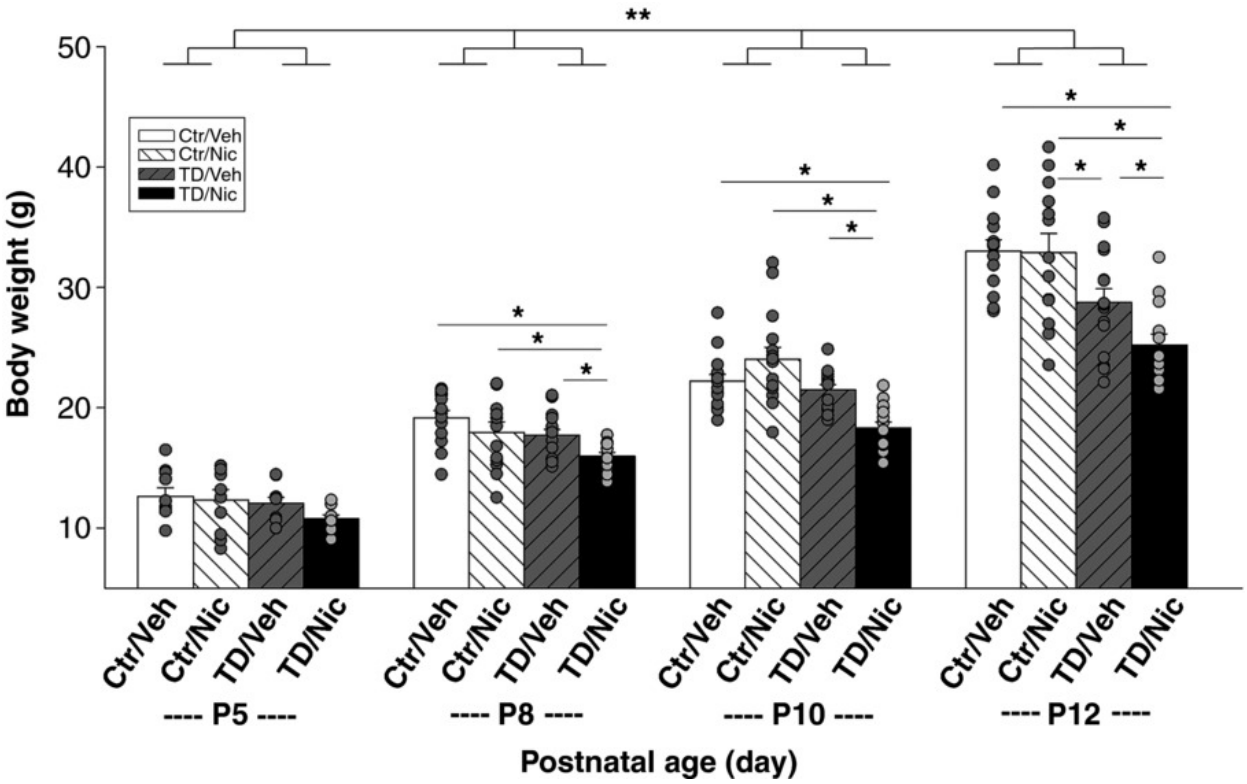

Figure 2. The effects of diet and nicotine on body weights in P5, 8, 10 and 12 neonates

Body weight was significantly affected by age ($P \leq 0.001$), diet ($P \leq 0.001$) and treatment (P = 0.005), and the significant interactions were found between age and diet ($P \leq 0.001$), diet and treatment (P = 0.003) (three-way ANOVA with Holm-Sidak *post hoc* test; **significant interaction between age and diet). At P8, P10 and P12, the TD diet/nicotine (TD/Nic)-treated pups (black bars) were significantly smaller than pups from other treatment groups at P8, P10 and P12 (*statistical significance; $P \leq$ 005). At P12, TD diet/vehicle (TD/Veh) pups (grey hatched bars) were significantly smaller than the Ctr/Veh (white bars) and Ctr/Nic (white hatched bar) pups (P = 0.011). Data presented as means ± SEM and each circle represents an individual animal.

Baseline HR was significantly affected by age (P < 0.001) and treatment (P = 0.03), and significant interactions were found between age and diet (P = 0.028), as well as between age and treatment (P = 0.018) using a three-way ANOVA. In particular, there was a significant difference in HR between TD diet pups and age-matched Ctr diet pups at P5, P10 and P12 (P < 0.001), whereas there was a significance difference in HR between nicotine-treated and non-treated pups at P8, P10 and P12 (P < 0.05) (three-way ANOVA, Holm-Sidak *post hoc* test; Fig. 2).

Baseline respiratory parameters, f_R, V_T and $\dot{V}_E$, were significantly affected by age

($P < 0.001$, $P < 0.001$, and $P = 0.027$, respectively, three-way ANOVA). The treatment and diet had no effect on baseline V_T and $\dot{V}_E$, whereas diet did significantly affect f_R ($P = 0.01$) at P8, P10 and P12 (three-way ANOVA, Holm-Sidak *post hoc* test; Fig. 2).

Effect of tryptophan-deficient diet and nicotine on autoresuscitation

The ability to autoresuscitate from repetitive episodic anoxia was assessed in pups of all treatment groups at each age, P5, P8, P10 and P12. The percentage of pups that survived all 15 anoxic episodes was not significantly different between the treatment groups at P5, P8 and P12 (a Kaplan-Meier survival analysis, Gehan-Breslow method; data not shown), whereas at P10, the pups from the dams treated with both maternal TD diet and nicotine (TD diet/nicotine) exhibited the highest mortality rate when challenged with repeated bouts of anoxia (Fig. 3). The percentage of pups that survived all 15 bouts of anoxia was significantly different among the treatment groups ($P = 0.029$, Gehan-Breslow analysis, Fig. 3). The subsequent all pairwise multiple comparison analysis showed that, compared to the Ctr diet/vehicle pups, tryptophan deficiency or nicotine exposure alone did not significantly change the survival rate; however, when the two factors, tryptophan deficiency and nicotine exposure, were combined, percentage survival decreased significantly ($P = 0.045$, Survival Gehan-Breslow and Holm-Sidak method). P10 TD diet/nicotine-exposed pups also survived fewer anoxic episodes than non-nicotine (Ctr diet/vehicle and TD diet/vehicle; $P = 0.03$ and 0.025 respectively, one-way ANOVA)-treated pups (Fig. 4). In addition, a Cox regression proportional hazards survival model further showed that TD diet/nicotine pups at P10 had a significantly lower survival rate than those at P5 and P8 ($P < 0.001$ and $P = 0.002$, respectively). These data suggest that a mild 5-HT deficiency (induced by a maternal tryptophan-deficient diet) together with pre- and early postnatal nicotine exposure can exacerbate autoresuscitation failure at a specific age.

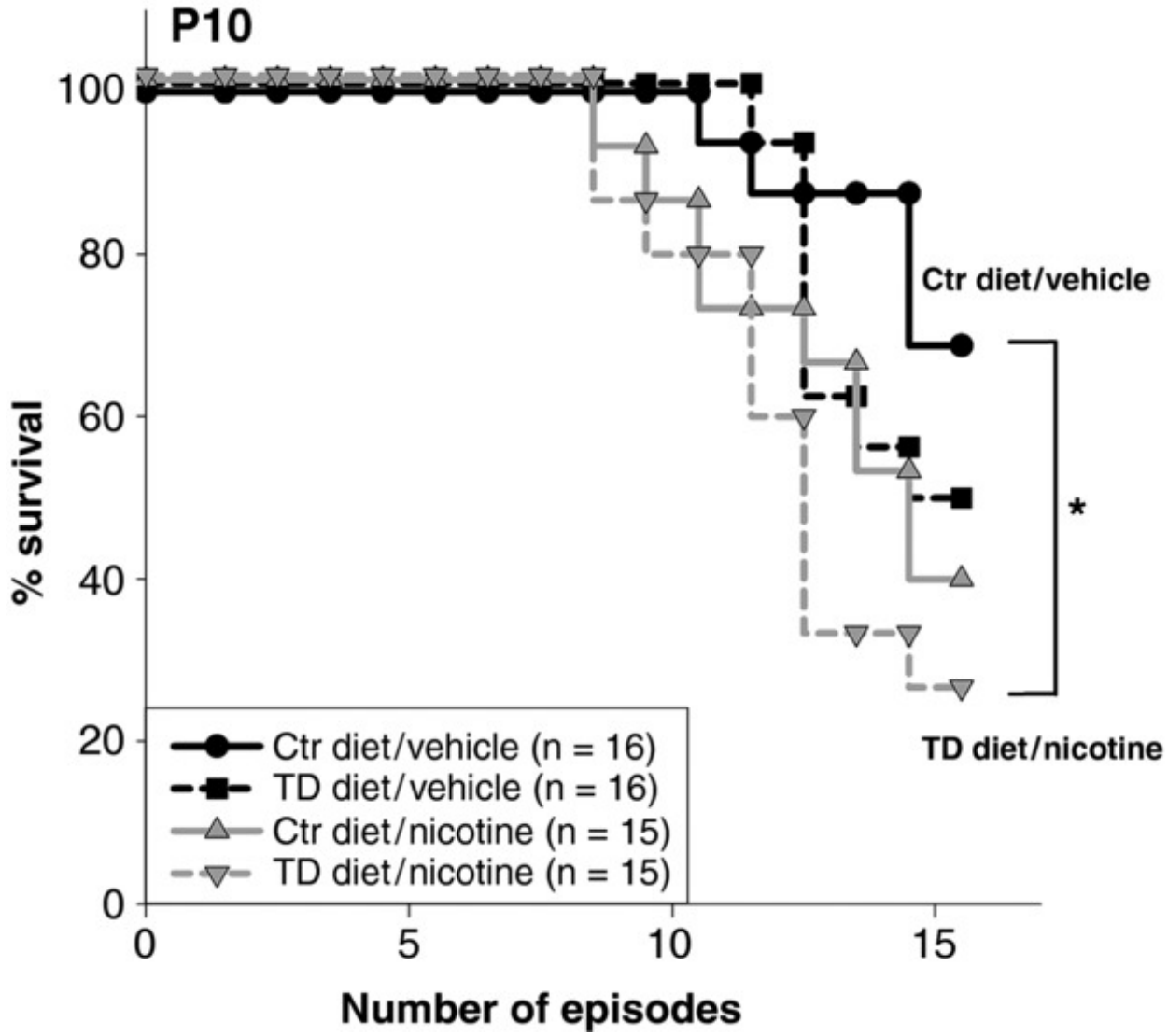

Figure 3. Percentage survival across 15 bouts of anoxia in P10 neonates

At P10, there is a statistically significant difference between survival curves (P = 0.029, Kaplan-Meier survival (Gehan-Breslow) analysis), with 69% of Ctr diet/vehicle-treated pups (11 of 16), 50% of TD diet/vehicle pups (8 of 16), 40% of Ctr diet/nicotine pups (6 of 15), and only 27% of the TD diet/nicotine-exposed pups (4 of 15) surviving all 15 episodes. All pairwise comparisons showed that TD diet/nicotine-treated pups had significant lower surviving rates than Ctr diet and saline (Ctr diet/vehicle)-treated pups ($^{*}P$ = 0.0425, Survival Gehan-Breslow–Holm-Sidak all pairwise analysis).

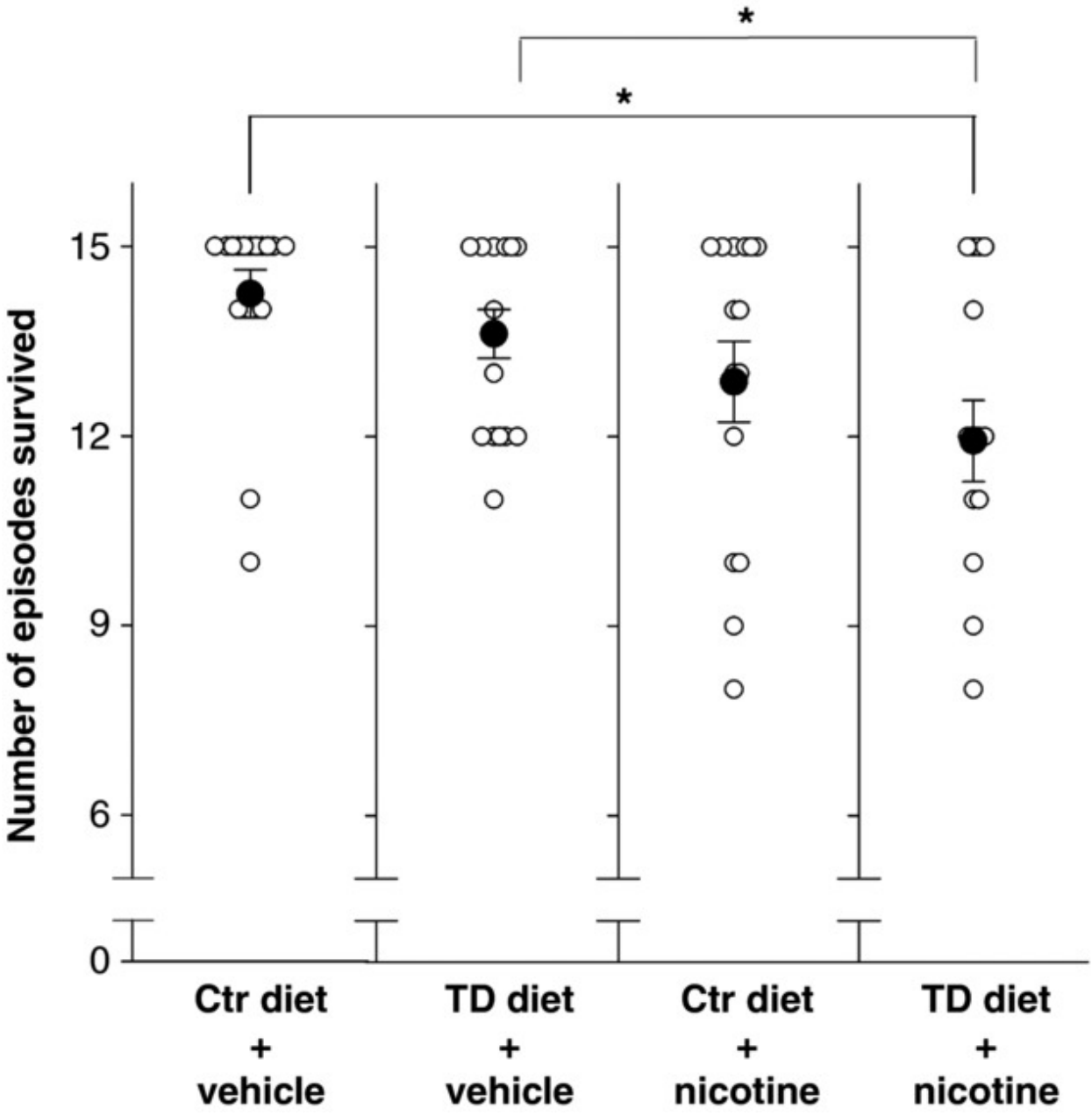

Figure 4. Mean number of anoxic bouts survived in P10 neonates
The mean number of anoxic episodes survived (filled circles) was significantly less in TD diet/nicotine-exposed P10 rats (12 episodes; far right column) than saline vehicle-treated Ctr and TD diet pups ($^{*}P = 0.025$). Neither tryptophan deficiency nor nicotine exposure alone significantly altered the mean number of anoxic episodes survived. Data presented as means ± SEM and each circle represents an individual animal.

Effect of tryptophan-deficient diet and nicotine on cardiorespiratory recovery from apnoea during autoresuscitation

Breathing and heart rate indices (gasp latency, eupnoea recovery and HR recovery) were quantified as possible indicators of successful *vs.* failed autoresuscitation in this study. There were no differences in both breathing and HR indices between treatment groups at P5, P8 and P12 (data not shown).

At P10, gasp latency (the time between the last breath prior to the primary apnoea and the

first gasp post apnoea) did not differ among the four treatment groups at any anoxic episode (Fig. 5*A*). Neither maternal tryptophan deficiency diet nor developmental nicotine exposure on their own or combined altered the offspring's gasp latency.

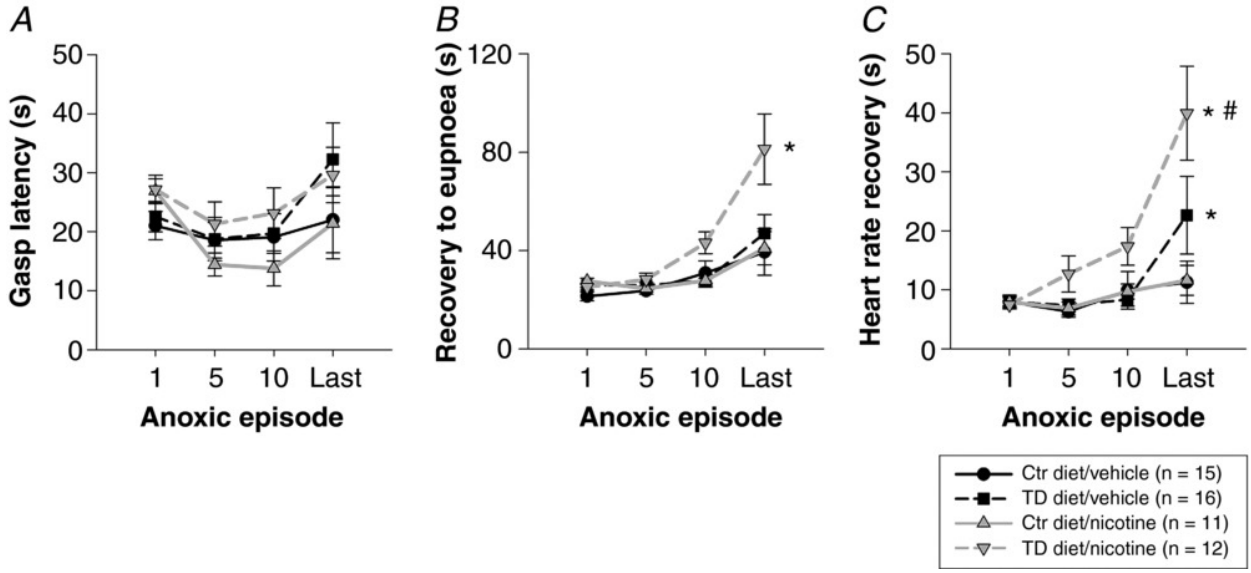

Figure 5. Gasp latency, eupnoea and HR recovery at anoxic episodes 1, 5, 10 and the last survived episode in P10 neonates

A, no significant differences in gasp latency (duration of primary apnoea) were observed amongst the four treatment groups (no treatment effect) across the repeated bouts of anoxia (no episode effect). *B*, recovery to eupnoea was delayed with progressive anoxic bouts (episode effect: $P < 0.0001$,) in all treatment groups, with the longest recovery period observed during the last survived anoxic episode. Eupnoea recovery was significantly delayed in TD diet/nicotine-treated pups at P10 (grey inverted triangles, dashed line; treatment effect: $^{*}P = 0.0053$) compared to all other treatment groups during the last survived anoxic episode. *C*, HR recovery was delayed with progressive anoxic bouts in all treatment groups. The pups treated with TD diet had longer HR recovery periods during the last anoxia episode than the pups treated with Ctr diet ($^{*}P \leq 0.05$). TD diet/nicotine pups had the slowest HR recovery compared to all other treatment groups (grey inverted triangles, dashed line; $^{\#}P < 0.0001$). Two-way RM ANOVA with Holm-Sidak *post hoc* analysis. Data presented as means ± SEM.

Recovery to eupnoea (the time from the first gasp post apnoea to when breathing frequency reaches 63% of baseline values) was delayed with progressive anoxic bouts in all treatment groups with the longest recovery period observed during the last survived anoxic episode (episode effect: $P = < 0.0001$, two-way RM ANOVA) (Fig. 5*B*). In the TD diet/nicotine-treated P10 neonates, eupnoea recovery was significantly delayed (2-fold longer) compared to all other treatment groups (treatment effect: $P = 0.0053$) during the last survived anoxic episode. Eupnoea recovery was prolonged by 4-fold during the last survived episode *vs*. the first in the TD diet/nicotine-exposed pups. In contrast, eupnoea recovery was only 2-fold longer by the last survived anoxic episode in other treatment groups.

HR recovery (the time from the first gasp post apnoea to when HR reaches 63% of baseline) was also delayed with increasing number of repeated anoxic episodes in all

treatment groups, particularly in the TD diet/nicotine-exposed P10 animals (Fig. 5*C*). When compared to the other three treatment groups, HR recovery in TD diet/nicotine pups was notably delayed from episode 5 onward and was significantly longer during the last survived anoxic episode (P = < 0.0001, two-way RM ANOVA). HR recovery in TD diet/nicotine-treated pups was 4-fold longer during the last survived episode compared to the first anoxic episode. HR recovery was also significantly delayed in TD diet/vehicle-treated pups ($P \leq 0.05$) during the last survived anoxic episode compared to both vehicle- and nicotine-treated Ctr diet groups. These data suggest that the 5-HT deficiency induced by the maternal TD diet may exacerbate the delay of HR recovery from repetitive anoxia, which can be even further exacerbated by pre- and perinatal nicotine exposure in neonates at P10.

Discussion

The main findings include the following: (1) maternal tryptophan-deficient diet resulted in a mild brainstem 5-HT deficiency in the developing offspring, (2) autoresuscitation was significantly compromised when perinatal nicotine exposure interacted with the mild 5-HT deficiency at P10, even though the mild 5-HT deficiency or developmental nicotine exposure alone did not significantly alter the cardiorespiratory response to repetitive anoxia, (3) the impaired autoresuscitation was characterized as a progressively delayed restoration of eupnoea and HR over subsequent episodes of anoxia, despite a normal initiation of gasping, and (4) the combination of perinatal nicotine exposure and mild 5-HT deficiency increased resting HR in P10 neonates. These key findings related to the gestational nicotine exposure and 5-HT deficiency with altered cardiorespiratory responses to repetitive anoxia at a specific developmental period are relevant to malnutrition, smoking/nicotine exposure and homeostasis in early life, including SIDS.

Maternal tryptophan-deficient diet and nicotine administration on the medullary 5-HT system in the offspring

Serotonin synthesis in the brain depends on levels of tryptophan (the primary precursor), which in turn depends on blood tryptophan concentrations that are highly affected by diet. Elimination of dietary tryptophan can profoundly lower the brain serotonin levels (Culley *et al*. 1963; Gessa *et al*. 1975; Franklin *et al*. 1995; Gonzalez *et al*. 2008), while increased tryptophan administration can increase plasma tryptophan and whole brain 5-HT and 5-HIAA (Haider *et al*. 2006; Khaliq *et al*. 2006). 5-HIAA is the primary metabolite of 5-HT, and the 5-HIAA/5-HT ratio is an index that measures brain serotonin turnover; the levels of 5-HIAA and 5-HIAA/5-HT are highly correlated with 5-HT levels in the brain. In this study, we showed that treating pregnant dams with a tryptophan-deficient diet (55% of control level) until postnatal day 25 can result in a mild brainstem 5-HT deficiency in neonatal offspring with the total 5-HT (5-HT + 5-HIAA) content in the medulla reduced 16%, 18% and 32% at P10, P15 and P25, respectively, compared to age-matched Ctr diet pups (Fig. 1). The changes of total 5-HT in the medulla are associated with significantly decreased 5-HT turnover (5-HIAA/5-HT) in TD diet pups. In TD diet pups at P10, there was a strong trend towards a significant decrease in medullary 5-HT content alone (−10%), in addition to significant decreases in 5-HIAA, total 5-HT, and 5-HIAA/5-HT ratio (−19%, 16%, and 11%, respectively), indicating a measureable 5-HT deficiency in the medulla from the age of P10.

At P15 and P25, all 5-HT activity, i.e. 5-HT, 5-HIAA and 5-HIAA/5-HT levels, were significantly lower in TD diet pups. The lower levels of all 5-HT parameters in the older TD diet pups may reflect longer exposure to the tryptophan-deficient diet through the dam's milk and the food itself as the pups start to consume solid foods from 2 weeks of age onward. The degradation of monoamines, e.g. catecholamine, serotonin and dopamine, requires the monoamine oxidase enzyme (MAO), and the normal levels of catecholamines and dopamine and their metabolites support the interpretation that the lower medullary 5-HIAA in TD diet pups is due to lower 5-HT content and not abnormal MAO. Unlike other transgenic 5-HT-deficient mice, e.g. pet1, Lmxb or TPH2 knockout, the reduction in the medullary 5-HT levels in these dietary-induced 5-HT-deficient pups was only partial, and appeared to be more closely aligned to those documented in SIDS cases (Duncan *et al*. 2010).

The degree of 5-HT deficiency in the offspring at P25 seen here is less severe than those reported previously (Penatti *et al*. 2011); unfortunately, we could not verify whether there is any difference between the two sets of data in P10 and P15 animals as these data were not available from the earlier study by Penatti *et al*. The discrepancy in the resultant brainstem 5-HT levels from the two studies at P25 is very likely due to the variable sizes and the precise location of the tissue samples used for the HPLC analysis in the two studies. Both studies collected tissue wedge sections enriched with 5-HT neurons from the brainstem for the HPLC analysis; however, the precise region and size of the tissue wedges were heavily dependent on the judgement of the individual investigator at the time. A larger tissue wedge could by chance include nearby regions less concentrated in 5-HT and dilute the 5-HT levels detectable by HPLC. In addition, there is potential variability between the two batches of samples used for the HPLC analysis which were performed 6 years apart. Nevertheless, both studies were internally consistent in terms of who performed the tissue dissection, the diet batch used, and the timing of HPLC. Both studies showed that maternal dietary tryptophan deficiency can lead to 5-HT deficiency in offspring. Here we further demonstrated that the brainstem 5-HT deficiency induced by maternal dietary tryptophan deficiency is measurable as early as P10 in these pups.

Perinatal nicotine exposure can directly or indirectly affect the 5-HT system (Xu *et al*. 2001; Kamendi *et al*. 2009; Blood-Siegfried & Rende, 2010; Slotkin *et al*. 2015), e.g. increased serotonin 5-HT_{1A}R binding in raphe obscurus, and nicotinic receptor binding in the raphe obscurus and vagal complex of baboons (Duncan *et al*. 2009), as well as both decreased and increased 5-HT transporter expression in the cortex and the midbrain/brainstem, respectively, with the midbrain/brainstem being the region that contains the 5-HT cell bodies that project to the cerebral cortex (Xu *et al*. 2001). Cerpa *et al*. (2015) recently further showed that the increased 5-HT_{1A}R expression in raphe 5-HT neurons is accompanied by reduced spontaneous firing frequency and reduced hypercapnia activated c-*Fos*-positive 5-HT neuron numbers in the raphe obscurus of P3–5 mice. Taken together, results of these studies suggest that perinatal nicotine exposure can lead to a dysfunctional 5-HT system in the neonate offspring, and the phenotypes observed in our current study may reflect the results of the combination of nicotine exposure and 5-HT deficiency.

Impaired autoresuscitation in perinatal nicotine-exposed 5-HT-deficient neonates at a critical age

Autoresuscitation is considered as the last protective mechanism to severe hypoxia, anoxia and asphyxia in newborn mammals and successful autoresuscitation from hypoxia-induced apnoea requires integration of several physiological systems including the central nervous, respiratory and cardiovascular systems (Sridhar *et al*. 2003; Fewell, 2005). A typical autoresuscitation from anoxia or asphyxia includes a sequence of events, hyperpnoea, apnoea, gasping, which is then followed by either successful termination of apnoea and restoration of eupnoea and HR or unsuccessful termination of apnoea or HR and death (Fewell *et al*. 2000; Sridhar *et al*. 2003). Autoresuscitation failure has been reported in some SIDS cases (Poets *et al*. 1999; Sridhar *et al*. 2003) and in several severe 5-HT deficiency animal models at specific ages (Cummings *et al*. 2009, 2010; Erickson & Sposato, 2009; Chen *et al*. 2013; Barrett *et al*. 2016), with defects including prolonged apnoea or delayed onset of gasping and delayed restoration of eupnoea and heart rate. The reported deficiency of brainstem 5-HT includes ∼26% in SIDS cases (Duncan *et al*. 2010), 35% in Pet1::Flpe-silenced pups (Barrett *et al*. 2016), and over 80% in *Pet-1*$^{-/-}$ knockout mouse (Cummings *et al*. 2009, 2011) and serotonin lesioned rat pups (Cummings *et al*. 2009). In contrast to these severely 5-HT-deficient animal models, a mild 5-HT deficiency induced by the maternal tryptophan-deficient diet alone did not alter autoresuscitation significantly; however, when such mild 5-HT deficiency interacted with developmental nicotine exposure, the ability to autoresuscitate from repetitive anoxia was significantly compromised and mortality rate was exacerbated at P10 (Fig. 3). The impaired autoresuscitation is characterized by a relatively normal gasp latency, delayed cardiorespiratory (eupnoea and HR) recovery from primary apnoea, and fewer number of hypoxic exposures tolerated in P10 TD diet/nicotine-treated pups. These phenotypes are similar to those previously reported in perinatal nicotine-exposed developing rat pups, piglets and lambs (Fewell & Smith, 1998; Froen *et al*. 2000; Fewell *et al*. 2001; Hafstrom *et al*. 2005). However, there are a few notable differences, e.g. the age and the effect of nicotine alone *vs*. combined nicotine and 5-HT deficiency. Fewell *et al*. showed a similar phenotype of impaired autoresuscitation in P5–6 but not P10–11 perinatal nicotine-exposed rat pups, whereas in the current study, the impaired phenotype was only observed at P10. In our study, the autoresuscitation failure rate is ranked as TD diet/nicotine > Ctr diet/nicotine > TD diet/vehicle > Ctr diet/ vehicle, with the highest mortality rate observed in the perinatal nicotine-treated 5-HT-deficient (TD diet/nicotine) pups (Fig. 3). The differences between our study and Fewell *et al*. (2001) can be attributed to the diet-induced 5-HT deficiency in the pups. Reduced concentrations of central 5-HT are related to failure of autoresuscitation and spontaneous cardiorespiratory recovery from episodes of anoxia and apnoea during the first few weeks of life, especially between the ages of P8 and 13 (Fewell & Smith, 1998; Fewell *et al*. 2001; Cummings *et al*. 2009, 2010, 2011; Erickson & Sposato, 2009; Chen *et al*. 2013; Barrett *et al*. 2016). It is noteworthy that the TD diet/nicotine pups have a relatively normal autoresuscitation at an older age (P12 or older) even when their brainstem 5-HT deficiency persists, and this time-specific effect could be the result of developmental compensatory changes with age. In this study, we have demonstrated that the interaction between a mild 5-HT deficiency and developmental nicotine exposure can significantly increase pups' vulnerability to environmental stress, e.g. repetitive anoxia, and exacerbate defects in cardiorespiratory protective reflexes at a specific age, in this case P10. The age-specific phenotype probably exists due to the changes in neurochemical development and in cardiorespiratory responses that occur within a matter of days or even less.

Altered resting HR and cardiorespiratory recovery from anoxia in perinatal nicotine-exposed 5-HT deficiency neonates

Perinatal nicotine-exposed 5-HT-deficient (TD diet/nicotine) pups have significantly elevated HR at rest (Table 2) at P10, and yet a significantly delayed or reduced HR recovery or response to repetitive anoxia. HR recovery in TD diet/nicotine pups was notably delayed from episode 5 onwards and was significantly longer during the last survived anoxic episode, which was 4-fold longer than the first anoxic episode (Fig. 5). The results are consistent with reports that nicotine exposure results in autonomic imbalance in developing offspring (Slotkin *et al*. 1995; Hafstrom *et al*. 2002b; Fewell, 2005; Hafstrom *et al*. 2005; Zeskind & Gingras, 2006; Slotkin *et al*. 2015). In contrast to the elevated HR during rest, nicotine-exposed 5-day-old lambs did not increase HR in response to hypoxia to the same extent as unexposed controls (Hafstrom *et al*. 2002a). Perinatal nicotine exposure can delay the development of β-adrenergic receptors in the heart (Slotkin *et al*. 1997) and abolish serotonergic neurotransmission to cardiac vagal neurons (Kamendi*et al*. 2009), both of which can lead to an altered neuronal input in cardiac control, imbalanced autonomic function, and reduced HR responsiveness to severe hypoxia and anoxia.

Inability to maintain adequate cardiac output, blood pressure and its associated poor reoxygenation process during autoresuscitation may also contribute to the delayed cardiorespiratory recovery and autoresuscitation failure (Gershan *et al*. 1992; Fewell *et al*. 2000; Fewell, 2005; Yang & Cummings, 2013). In acute 5-HT depleted rat pups, Yang *et al*. showed that a progressive and premature deterioration of blood pressure is associated with delayed HR recovery with successive episodes of anoxia despite normal gasping (Yang & Cummings, 2013), suggesting 5-HT deficiency can also lead to an autonomic imbalance and impair autoresuscitation process.

Developmental nicotine exposure can also functionally reduce the ventilatory response to hypercapnia and hypoxia in mice, rats and lambs (see review by Hafstrom *et al*. 2005), impair autoresuscitation from severe hypoxia and anoxia, and anatomically alter multiple neurotransmitter systems, e.g. catecholamines and serotonin. When nicotine exposure interacts with a mild 5-HT deficiency in developing neonates, autoresuscitation failure is exacerbated.

The underlying mechanism for eupnoea and HR recovery and the involvement of 5-HT neurons and nicotine exposure is still unclear at the present time. Regardless of the origin of the autonomic dysregulation, the autonomic imbalance is amplified in perinatal nicotine-exposed 5-HT-deficient pups at P10.

Perspective and concluding remarks

SIDS persists as a major cause of death in infants under 1 year of age (Kochanek*et al*. 2017). Maternal cigarette smoking remains a major preventable risk factor for SIDS. With increasing numbers of nicotine patch and electronic cigarette users during pregnancy, there is an increasing urgency to better understand the impact of developmental nicotine exposure on the health of neonates, especially those who are more vulnerable with an intrinsic medullary 5-HT defect. SIDS is a complex disorder of homeostasis, and while abnormalities in the brainstem 5-HT system have been consistently found in most SIDS

cases studied, it is likely that SIDS involves the interactions of multiple neurotransmitters and neuromodulators, multiple stressors acting simultaneously, and multiple genetic and environmental factors (Kinney & Thach, 2009; Kinney *et al*. 2009; Duncan *et al*. 2010). In this study, we further investigated the effect of a specific interaction between developmental nicotine exposure and mild brainstem 5-HT deficiency on autoresuscitation in developing animals. Our data suggest that the interaction of developmental nicotine exposure and a mild brainstem 5-HT deficiency can significantly compromise autoresuscitation in response to environmental stressors, e.g. severe hypoxia, anoxia, or asphyxia, at a specific developmental age. The age-specific effect probably exists due to either changes in neurochemical development or changes in cardiorespiratory responses that occur within a matter of days during the development. We speculate that maternal nicotine exposure places infants who have other vulnerabilities, e.g. mild 5-HT deficiency, at high risk for an impairment of protective response to severe hypoxia, anoxia and asphyxia.

Biography

Stella Y. Lee completed her PhD studies in the Department of Physiology and Neurobiology at the Geisel School of Medicine at Dartmouth in Hanover, New Hampshire. Her primary research focus was on the physiological consequences of neurochemical abnormalities in a rodent model of SIDS. She is currently working as a teaching faculty at the University of British Columbia in the Department of Zoology in Vancouver, British Columbia. She integrates her research background into her teaching, specifically animal physiology and developmental neurobiology.

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Additional information

Competing interests

There are no competing interests for the authors of this paper.

Author contributions

S.Y.L., E.N., and A.L. conceived of and designed the work. S.Y.L. and A.L. wrote the manuscript, performed data analysis and interpretation. S.Y.L. performed the whole animal experiments and the tissue sampling. C.M.S. acquired data for the cotinine analysis. All authors contributed to the editing of the manuscript, gave approval for the final version, and agreed to be accountable for all content.

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Original investigation

Perception and Current Use of E-cigarettes Among Youth in China

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Abstract

Introduction: This study provides nationally representative estimates of electronic cigarette (e-cigarette) use among youth in China and explores the factors associated with awareness and use of e-cigarettes and the relationship between e-cigarette and conventional tobacco use.
Methods: This study examined data from the Global Youth Tobacco Survey, which was completed by 155 117 middle school students (51.8% boys and 48.2% girls) in China, and employed a multi-stage stratified cluster sampling design. For data analysis, SAS 9.3 complex survey procedures were used, and logistic regression was used to explore factors associated with e-cigarette use and the relationship between e-cigarette and conventional tobacco use.
Results: About 45.0% of middle school students had heard of e-cigarettes, but only 1.2% reported using e-cigarettes in the last 30 days. Among never-smokers, e-cigarette users were more likely to intend to use a tobacco product in the next 12 months than nonusers (adjusted odds ratio [OR] = 6.970, 95% confidence interval [CI] = 4.474% to 10.857%), and more likely to say that they would enjoy smoking a cigarette (adjusted OR = 14.633, 95% CI = 11.328% to 18.902%). E-cigarette use was associated with previous experimentation with cigarette smoking (OR = 3.2), having noticed tobacco advertising in the past 30 days (OR = 2.7), having close friends who smoke (OR = 1.4), and thinking tobacco helps people feel more comfortable in social situations (OR = 3.3) and makes young people look more attractive (OR = 1.3).
Conclusions: E-cigarette use among youth in China remains low but awareness is high. E-cigarette use was associated with increased intentions to use tobacco. Enhanced prevention efforts are needed targeting e-cigarette use among youth.
Implications: This study is the first nationally representative survey of e-cigarette use among youth in China. It found that among middle school students, prevalence of e-cigarette use is 1.2% and prevalence of e-cigarette awareness is 45.0%. Chinese youths use e-cigarettes as a tobacco product rather than an aid to quitting. Among never-smokers, e-cigarette users were more likely to have intentions to use a tobacco product in the next 12 months, more likely to use a tobacco product offered by their best friends and enjoy smoking a cigarette than nonusers.

Introduction

Recent studies from the United States and other countries have shown a rapid rise in the use of electronic cigarettes (e-cigarettes) among youth.[1,2] For example, national survey data show that between 2013 and 2015, e-cigarette use among US high school students increased by about four times.[3,4] This trend raises concern because e-cigarette use among youth tends to be associated

with the use of other tobacco products, including conventional cigarettes.[5,6] Indeed, studies have shown e-cigarette use to be associated with greater intention to smoke and with subsequent initiation of conventional cigarette smoking.[7–10] Youths who use e-cigarettes are more likely to have lower perceptions of harm and addictiveness from both e-cigarettes and conventional cigarettes, particularly flavored e-cigarettes, compared with their nonusing peers.[11] E-cigarette advertising is also associated with increased use of e-cigarettes and other tobacco products among adolescents.[12,13] Although data on the long-term health effects of e-cigarette use are limited, previous studies have found that e-cigarettes can contain harmful and potentially harmful constituents, including nicotine, carbonyl compounds, and volatile organic compounds, known to have adverse health effects.[14–16]

Marketing of e-cigarettes has expanded rapidly in China in recent years.[17,18] Retail e-cigarette shops appear in rural counties as well as large cities,[18] and e-cigarettes can be easily purchased online.[17] Advertising of e-cigarettes is not regulated in China, and online advertisements are prevalent[19] and frequently include claims about health benefits, promote flavored products, and use celebrity endorsements to entice users.[18,20] In some places, e-cigarette promotions have taken place on school campuses.[21,22]

However, there is limited data on actual use of e-cigarettes in China and attitudes or knowledge about them. This article provides nationally representative estimates of e-cigarette use among youth in mainland of China and explores the factors associated with awareness and use of e-cigarettes and the relationship between e-cigarette use and conventional cigarette tobacco use. The aim of this study is to provide baseline data on e-cigarette use in China to guide subsequent research and tobacco control efforts.

Methods

Data Source

The Global Youth Tobacco Survey (GYTS) China Project was conducted by the Tobacco Control Office of the Chinese Center for Disease Control and Prevention from 2013 to 2014 in 31 provinces of mainland China, using a standardized two-stage sample design. The global standard questionnaire used addressed the following topics: tobacco use (smoking and smokeless), e-cigarette use, cessation, secondhand smoke, access and availability of tobacco products, pro- and antitobacco media and advertising, and knowledge and attitudes regarding tobacco. A detailed description of the methods of the GYTS has been reported by Warren et al.[23] All materials and procedures used in the GYTS China survey were approved by the ethics review committee of the Chinese Center for Disease Control and Prevention.

Participants

The target population for this survey was defined as all middle-school students in mainland China. A total of 155 117 students from 1020 schools in 31 provinces completed the questionnaire, including 80 357 boys and 74 760 girls. The overall response rate was 98.0%. Respondents included 70 461 students in rural areas and 84 656 in urban areas. There were 52 729 respondents in grade 7, 52 084 in grade 8, and 50 304 in grade 9. Because of different policies regarding school age in each province, this survey included 46 641 thirteen-year-old students, 44 795 fourteen-year-old students, and 28 940 fifteen-year-old students. The number of participants for each province ranged from 3094 to 7789. Characteristics of participants are summarized in Table 1.

Table 1. Characteristics of Middle School Students

	Weighted			
	Proportion		Number	
	%	95% CI		Unweighted
Overall			47 192 405	155 117
Gender				
Boy	52.9	52.3% to 53.5%	24 968 958	80 357
Girl	47.1	46.5% to 47.7%	22 223 447	74 760
Age				
11–12 y old	14.2	13.6% to 14.8%	6 686 002	24 229
13 y old	28.3	27.7–29.0	13 357 790	46 641
14 y old	29.0	28.6% to 29.4%	13 687 310	44 795
15 y old	20.6	19.9% to 21.2%	9 702 161	28 940
16–17 y old	7.9	7.2% to 8.7%	3 738 971	10 447
Residence				
Urban	27.9	26.0% to 29.8%	13 174 782	70 461
Rural	72.1	70.2% to 74.0%	34 017 623	84 656
Pocket money (RMB)				
0	11.0	10.4% to 11.6%	5 205 216	17 539
≤10 (Not including 0)	28.7	27.6% to 29.8%	13 523 822	42 335
11–20	22.9	22.2% to 23.6%	10 758 729	34 441
>20	37.3	35.9% to 38.9%	17 579 521	60 455
Smoking status				
Cigarette experimentation	17.9	16.9% to 18.9%	8115115	24643
Current smoking	5.9	5.4% to 6.5%	2735099	8151
Never smoking	82.1	81.1% to 83.1%	37262315	124962

CI = confidence interval; RMB = renminbi.

Sampling Method

Following the GYTS Sample Design Manual, a multistage stratified cluster probability sampling method was used in the GYTS China project. First, 8–16 primary sampling units were assigned to each province according to population size, which located half in rural areas and half in urban areas. Overall, 336 primary sampling units were identified. Second, three schools were selected within each primary sampling unit using a random probability proportionate to size sampling technique. Because the school size was too small in some rural areas, the final selection included 1020 schools. One class was selected randomly in each grade from each school, and all of the students in this class were interviewed.

Measures

Demographic Variables

Gender, school grade (7, 8, or 9), age (11–12, 13, 14, 15, and 16–17 years), and pocket money were recorded in the survey. Pocket money was measured by the question "During a week, on average, how much money do you have that you can spend on yourself, however you want?" Response categories were: no money, less than 10 yuan, 11–20 yuan, and more than 20 yuan.

E-cigarette Awareness

E-cigarette awareness was measured using the question "Before today, had you ever heard of electronic cigarettes or e-cigarettes?" Responses were coded as 1 = yes versus 2 = no.

E-cigarette Use

"During past 30 days, on how many days did you use electronic cigarettes?" was used to measure e-cigarette use. Responses were classified as 0, 1–2, 3–5, 6–9, 10–19, 20–29, and 30 days. If the response was 1 day or more, the respondent was identified as an e-cigarette user.

Smoking Status

In this article, smoking status includes smoking experimentation, current smoking and never smoking. "Have you ever tried or experimented with cigarette smoking, even one or two puffs?" was used to measure smoking experimentation. If the response was "yes", the respondent was classified as positive for smoking experimentation. Current smoking was measured using the question: "During the past 30 days, on how many days did you smoke cigarettes?" Responses were classified as 0, 1–2, 3–5, 6–9, 10–19, 20–29, and 30 days. If the response was 1 day or more, the respondent was identified as a current smoker. Never-smoker refers to those who are not current smokers and never tried or experimented with smoking.

Quit Behavior

Among current smokers, dependence was measured by the question: "Do you ever smoke tobacco or feel like smoking tobacco first thing in the morning? (1 = no; 2 = some time; 3 = always)." Desire to quit was assessed by the question: "Do you want to stop smoking now? (1 = yes; 2 = no)," and quit attempts were assessed by the question: "During the past 12 months, did you ever try to stop smoking? (1 = yes; 2 = no)".

Susceptibility to Tobacco Use

In this study, three questions were used to measure susceptibility to tobacco use among never-smokers: "At any time during the next 12 months do you think you will use any form of tobacco? (1 = yes; 2 = no)," "If one of your best friends offered you a tobacco product, would you use it? (1 = yes; 2 = no)," and "Do you agree or disagree with the following: 'I think I might enjoy smoking a cigarette'? (1 = yes; 2 = no)."

Noticed Tobacco Advertisement and Antitobacco Media Message

Respondents were asked if they saw tobacco advertisements in a variety of venues and media (including points of sale, TV, newspapers and magazines, billboards, Internet, sports events, fairs, concerts, or community events) during the past 30 days. Responses were coded as 1 = yes, 2 = no. to each of the items listed earlier. If the participant answered "yes" to one or more items on the list, they were coded as exposed to tobacco advertising. Similarly, the question "During the past 30 days, did you see or hear any anti-tobacco media messages on television, radio, internet, billboards, posters, newspapers, magazines, or movies?" (1 = yes; 2 = no) was used to measure exposure to antitobacco media messages.

Peer and Parental Tobacco Use

Two questions were used to explore the influence from parents and peers on respondents' tobacco use: "Do any of your closest friends smoke tobacco?" (1 = yes; 2 = no) and "Do your parents smoke tobacco?" (1 = yes; 2 = no).

Perceived Smoking Outcomes

Perceived smoking outcomes were measured by three questions: "Do you think smoking tobacco makes young people look more or less attractive? (1 = more; 2 = no difference; 3 = less)," "Do you think smoking tobacco helps people feel more comfortable or less comfortable at celebrations, parties, or in other social gatherings? (1 = more comfortable; 2 = no difference; 3 = more uncomfortable)," and "Do you think smoke from other people is harmful to you? (1 = yes; 2 = no)."

Statistical Analysis

To take the complex survey sample design into account, all computations were performed using the SAS 9.3 complex survey data analysis procedure. A chi-square test was used for comparing the differences between groups. Logistic regression was used to explore the factors associated with e-cigarette use and the relationship between e-cigarette and tobacco use. A p value less than .05 was considered statistically significant.

Results

Characteristics of Participants

A total of 155 117 participants were representative of a total population of 24 968 958 boys and 22 223 447 girls of middle school age in China. Table 1 shows selected demographic characteristics of the weighted respondent data. The proportion of students in urban areas made up 27.9% of this total, and that of students in rural areas made up 72.1%; students in grade 7 accounted for 33.1%; grade 8, 33.3%; and grade 9, 33.6%; 37.3% of participants reported having more than 20 yuan per week in pocket money, and 22.9% reported 11–20 yuan. In addition, 17.9% of respondents reported that they had tried or experimented with cigarette smoking, 5.9% of the respondents smoked cigarette in the past 30 days (classified as current smokers), and 82.1% of the respondents were never-smokers. Details are reported in Table 1.

Awareness and Perceptions of E-cigarette Use

In this survey, 45.0% of students reported that they had heard of e-cigarettes. Among boys, the proportion was substantially higher (52.3%) than girls (36.8%). Awareness of e-cigarettes was higher among students in urban areas (46.2%) compared with those in rural areas (44.5%). Awareness differed among age groups, increasing from around 36.4% among 11- to 12-year-old students to more than 49.5% among students aged 16 and above. Awareness was also higher among respondents who reported more than 20 yuan per week as pocket money (52.2%) compared with those who reported less (38.1% for those who reported 10 yuan per week or less). Having experimented with cigarette smoking, noticed tobacco advertising in the last 30 days, noticed antitobacco media in the past 30 days, and having parents or close friends who smoke were all associated with awareness of e-cigarettes as well.

Among current smokers, awareness of e-cigarettes was higher among those who always or sometimes smoke tobacco or feel like smoking tobacco first thing in the morning (81.1%) compared with those who do not (71.0%). In addition, those who had tried to stop smoking in the past 12 months were more likely to be aware of e-cigarettes than those who had not made a quit attempt, though desire to quit smoking now was not significantly associated with any difference in awareness.

Results were consistent in the multivariate analysis. The strongest associations with e-cigarette awareness were seen for having close friends who smoke (odds ratio [OR] = 1.66), having experimented with cigarette smoking (OR = 1.72), noticing tobacco advertising (OR = 1.55), and male gender (OR = 1.49). For details, see Table 2.

Current E-cigarette Use

Only 1.2% of students reported that they had used an e-cigarette in the past 30 days. Use among boys (1.8%) was higher than girls (0.5%). There was no significant difference between urban and rural residence or between age groups. Among current smokers, 8.5% had used an e-cigarette in the last 30 days, which was much higher than the rate among non-current smokers (0.6%). Among those who had used an e-cigarette in the past 30 days, about half (49.3%) reported they used it on 1 or 2 days. Only 13.4% of e-cigarette users used it every day.

E-cigarette use also differed by measures of dependence, ranging from 26.5% among those who always smoke tobacco or feel like smoking tobacco first thing in the morning, to 15.3% among those only sometimes feel like smoking first thing, to 6.6% among those who never feel that way. Among cigarette smokers who wanted to quit, prevalence of e-cigarette use was 9.0%, compared with 11.5% among those who did not want to quit. Among those who made a quit attempt in the past 12 months, 7.0% were e-cigarette users, compared with 7.1% for those who did not make a quit attempt.

In the multivariate analysis, the variables most strongly associated with e-cigarette use were having experimented with cigarette smoking (OR = 3.2), thinking that tobacco helps people feel more comfortable in social situations (OR = 3.3), and having noticed tobacco advertising in the past 30 days (OR = 2.7). Respondents were also more likely to use e-cigarettes if they have close friends who smoke (OR = 1.4) or if they intend to use a tobacco product in the next 12 months (OR = 1.5) or would use it if offered by a close friend (OR = 1.7). Boys were more likely to use e-cigarettes than girls (OR = 1.9). Those who think smoking makes young people look more attractive were more likely to use e-cigarettes than those who think smoking makes them less attractive (OR = 1.3). For details, see Table 3.

Susceptibility to Tobacco Use Among Never-Smokers

Among never-smokers, 4.8% of e-cigarette users intended to use a tobacco product in the next 12 months, and 0.7% of non-e-cigarette users had such intentions (adjusted OR = 6.970, 95% confidence interval [CI] = 4.474% to 10.857%). If a best friend offered a tobacco product, 4.2% of e-cigarette users reported that they would use it, whereas the proportion among non-e-cigarette users was 0.9% (adjusted OR = 5.136, 95% CI = 3.229% to 8.17%). In addition, 27.1% of e-cigarette users thought they might enjoy smoking a cigarette compared with only 2.5% of non-e-cigarette users (adjusted OR = 14.633, 95% CI = 11.328% to 18.902%).

Discussion

Although only 1.2% of middle school students reported they used e-cigarette in past 30 days, almost half (45.0%) of students have heard of e-cigarettes. Factors associated with e-cigarette awareness and/or use include having experimented with cigarette smoking, having parents or close friends who smoke, exposure to tobacco advertising and antitobacco messages, positive attitude to smoking, and having more pocket money. These factors are similar to those associated with conventional cigarette smoking in China.[24–26] Although China has strict policies to prevent youth access to tobacco products, there is no regulation of e-cigarette sales or promotion to youth.

Prevalence of e-cigarette use among youth varies widely across countries and may be influenced by a range of factors, including price, availability of products, and regulatory environment.[27,28] For example, although e-cigarette use has risen dramatically among US youth over the past several years,[1] this has not been the case in the United Kingdom[29] and Korea,[30] where e-cigarette sales and marketing are more tightly regulated. Prevalence of past 30-day e-cigarette use among youth in China is relatively low (1.2%) compared with the United States (5.3% among middle school students in 2015).[31] However, given the lack of regulations on e-cigarette sales and marketing in China and widespread tobacco advertising in retail tobacco shops, there is reason for concern that e-cigarette use may increase among Chinese adolescents, as seen in other countries.[29,32,33]

According to this study, students who are aware of or using e-cigarettes have more positive views about tobacco use in general. They are more likely to say they will use a tobacco product in the next 12 months, and they are more likely to say that tobacco helps people feel comfortable in social situations. Those who have used an e-cigarette are also more likely to say they would use a tobacco product if offered by a friend and to say that smoking makes young people look more attractive. Among never-smokers, those who used e-cigarettes were more likely to use a tobacco product in the next 12 months (OR = 7.0), to use a tobacco product offered by their best friends (OR = 5.1), and to say that they might enjoy smoking a cigarette (OR = 14.6). These findings suggest that awareness and use of e-cigarettes are associated with susceptibility to tobacco use. Interestingly, in this study exposure to tobacco product advertising and exposure to antitobacco messages were both associated with e-cigarette awareness. This suggests that students who are aware of or using e-cigarettes may be more attentive to or have greater exposure to tobacco-related messages of all kinds, both positive and negative.

Table 2. Awareness of E-cigarette Among Middle School Students

	%	95% CI	OR	95% CI
Overall	45.0	43.5% to 46.5%		
Gender				
Boys	52.3	50.8% to 53.9%	1.493	1.443% to 1.545%
Girls	36.8	35.3% to 38.3%	reference	—
Age				
11–12 y old	36.4	35.1% to 37.6%	reference	—
13 yold	42.1	40.8% to 43.3%	1.154	1.103% to 1.208%
14 y old	47.5	46.0% to 49.1%	1.291	1.217% to 1.369%
15 y old	49.7	47.2% to 52.2%	1.293	1.170% to 1.428%
16–17 y old	49.5	44.9% to 54.1%	1.220	1.041% to 1.429%
Residence				
Urban	46.2	44.7% to 47.7%	1.147	1.042% to 1.262%
Rural	44.5	42.6% to 46.5%	reference	—
Pocket money (RMB)				
0	39.6	37.1% to 42.1%	reference	—
≤10 (Not including 0)	38.1	36.3% to 39.9%	0.994	0.929% to 1.065%
11–20	44.5	42.9% to 46.0%	1.190	1.093% to 1.296%
>20	52.2	50.6% to 53.8%	1.436	1.302% to 1.583%
Ever experimented with cigarette smoking				
Yes	65.8	64.2% to 67.3%	1.723	1.626% to 1.824%
No	40.2	38.7% to 41.7%	reference	—
Exposed to tobacco advertisement in last 30 d				
Yes	53.6	52.2% to 55.1%	1.550	1.504% to 1.598%
No	37.0	35.5% to 38.5%	reference	—
Exposed to antitobacco media messages in past 30 d				
Yes	47.3	45.9% to 48.7%	1.223	1.184% to 1.263%
No	40.8	39.1% to 42.5%	reference	—
Parents smoke				
Yes (mother or father or both)	48.8	47.3% to 50.4%	1.250	1.206% to 1.296%
No	40.0	38.5% to 41.5%	reference	—
Closest friends smoke				
Yes (some or all)	59.1	57.6% to 60.6%	1.660	1.579% to 1.745%
No	36.4	35.0% to 37.9%	reference	—
Think smoke from other people is harmful to you				
Yes	46.3	44.8% to 47.7%	1.216	1.177% to 1.257%
No	41.4	39.7% to 43.0%	reference	—
Ever smoke tobacco or feel like smoking tobacco is the first thing in the morning (smokers)				
Never	71.0	68.8% to 73.2%	—	—
Sometimes	81.1	77.6% to 84.6%	—	—
Always	81.4	74.4% to 88.3%	—	—
Want to stop smoking now (smokers)				
Yes	74.9	72.8% to 77.1%	—	—
No	72.7	69.5% to 75.9%	—	—
Ever try to stop smoking in past 12 mo (smokers)				
Yes	73.4	71.3% to 75.4%	—	—
No	67.3	64.6% to 69.9%	—	—

CI = confidence interval; OR = odds ratio; RMB = renminbi.

Although some studies have suggested that people use e-cigarettes to help them quit smoking,[34,35] no significant relationship was seen in this study between use of e-cigarettes and wanting to stop smoking or having tried stopping in the past 12 months. Thus, the data do not show any indication that e-cigarettes are being used as smoking cessation devices among Chinese youth.

It is important to acknowledge some limitations of this study. Because the GYTS is a cross-sectional survey, it was not possible to assess changes in e-cigarette or conventional cigarette use over time or to directly measure the impact of e-cigarette use on initiation of tobacco smoking. However, prospective studies in other countries have found that youths who use e-cigarettes are more likely to go on to initiate conventional cigarette use.[36] In addition, because this study involved secondary data analysis, we were limited to questions that appeared in the GYTS China survey. Notably, data on e-cigarette use history over time and information on exposure to e-cigarette advertising were not available. However, this dataset represents the first and only nationally representative survey of e-cigarette use among youth in China.

China is the largest consumer of tobacco in the world, with 3.16 million current smokers and an annual death toll of more than 1 million people attributed to tobacco use. Owing to great efforts, prevalence of tobacco use remains relatively low among middle school students (6.9%)[24] and only 32.2% of daily smokers start smoking

Table 3. Current Use of E-cigarette Among Middle School Students

	%	95% CI	OR	95% CI
Overall	1.2	1.1% to 1.3%		
Gender				
Boys	1.8	1.7% to 2.0%	1.881	1.559% to 2.269%
Girls	0.5	0.4% to 0.5%	reference	—
Age				
11–12 y old	0.7	0.6% to 0.8%	reference	—
13 y old	0.9	0.8% to 1.0%	0.970	0.772% to 1.219%
14 y old	1.2	1.0% to 1.3%	1.007	0.794% to 1.275%
15 y old	1.5	1.3% to 1.8%	1.172	0.924% to 1.487%
16–17 y old	2.1	1.6% to 2.6%	1.236	0.921% to 1.658%
Residence				
Urban	1.0	0.8% to 1.1%	0.925	0.785% to 1.089%
Rural	1.3	1.1% to 1.4%	reference	—
Pocket money (RMB)				
0	1.1	0.9% to 1.3%	reference	—
≤10 (Not including 0)	0.9	0.8% to 1.1%	0.946	0.713% to 1.254%
11–20	1.0	0.9% to 1.2%	0.915	0.695% to 1.204%
>20	1.5	1.3% to 1.6%	0.949	0.757% to 1.190%
Ever experimented with cigarette smoking				
Yes	4.0	3.6% to 4.3%	3.217	2.707% to 3.823%
No	0.5	0.4% to 0.5%	reference	—
Exposed to tobacco advertisement in last 30 d				
Yes	2.0	1.8% to 2.2%	2.747	2.326% to 3.244%
No	0.4	0.4% to 0.5%	reference	—
Close friends smoke				
Yes (some or all)	2.3	2.1% to 2.5%	1.439	1.199% to 1.727%
No	0.5	0.4% to 0.6%	reference	—
Think smoking makes young people look more or less attractive				
More	2.9	2.5% to 3.3%	1.291	1.065% to 1.566%
No difference	1.4	1.2% to 1.5%	0.938	0.785% to 1.121%
Less	0.7	0.7% to 0.8%	reference	—
Will use a tobacco product, if best friends offered				
Yes	8.4	7.0% to 9.7%	1.694	1.321% to 2.172%
No	1.0	0.9% to 1.1%	reference	—
Next 12 mo, do you think you will use any form of tobacco				
Yes	8.2	6.6% to 9.8%	1.469	1.121% to 1.926%
No	1.1	1.0% to 1.1%	reference	—
Think smoke from other people is harmful to you				
Yes	1.0	0.9% to 1.0%	0.651	0.566% to 0.748%
No	1.8	1.6% to 2.1%	reference	—
Think tobacco helps people feel more comfortable in social situations				
More comfortable	6.2	5.5% to 6.9%	3.327	2.729% to 4.058%
No difference	2.4	2.1% to 2.8%	2.031	1.693% to 2.436%
More uncomfortable	0.6	0.6% to 0.7%	reference	—
Ever smoke tobacco or feel like smoking tobacco is the first thing in the morning (smokers)				
Never	6.6	5.8% to 7.4%	—	—
Sometimes	15.3	12.3% to 18.4%	—	—
Always	26.5	20.2% to 32.8%	—	—
Want to stop smoking now (smokers)				
Yes	9.0	7.9% to 10.2%	—	—
No	11.5	9.4% to 13.6%	—	—
Ever try to stop smoking in past 12 mo (smokers)				
Yes	7.0	6.2% to 7.7%	—	—
No	7.1	5.8% to 8.3%	—	—

CI = confidence interval; OR = odds ratio; RMB = renminbi.

before age 18 in 2010[37]. However, an increase in use of e-cigarettes among youth in China could challenge this picture. Given the strong relationship observed between awareness and use of e-cigarettes and susceptibility to tobacco use, there is reason for concern that increased promotion and use of e-cigarettes could impact smoking prevalence among Chinese youth. Thus, continued monitoring of e-cigarette and conventional cigarette use among youth in China is needed. In addition, tobacco control efforts in China should consider e-cigarette use among youth and could include actions such as stronger regulation of sales and marketing and media campaigns targeting youth.

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Declaration of Interests

None declared.

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