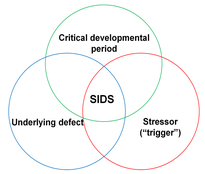
Assessing the Effects of Developmental Nicotine Exposure on Cardiorespiratory Responses to Anoxic Challenge in Serotonin-Deficient *Pet-1* Knockout Mice

Muhammad Siddiqui, Nicole Lester, and Dr. Jeffery Erickson
Biology Department
The College of New Jersey, Ewing, NJ 08628

Introduction

Pet-1 is a transcription factor that is required for normal development of serotonin (5-hydroxytryptamine, 5-HT) neurons in the mammalian brainstem. Targeted deletion of the *Pet-1* gene results in a 70% loss of central 5-HT neurons that is associated with depressed ventilation, an increased incidence of spontaneous apneas, abnormal autoresuscitation responses to prolonged apnea, and high neonatal mortality in newborn mice. Endogenous 5-HT provides excitatory drive to neural circuits in the caudal brainstem that generate respiratory rhythm, and recent studies have correlated brainstem 5-HT deficiency with Sudden Infant Death Syndrome (SIDS) in humans, the leading cause of postnatal infant mortality in the United States. This has led to the "Serotonin Triple Risk" model for SIDS which postulates that an infant with an underlying defect (deficiency of brain-stem 5-HT) that is exposed to an environmental stressor (e.g. low environ-mental oxygen levels) during a critical period of postnatal development is at risk of increased neonatal mortality. The suddenness of death, which often occurs during a sleep period, suggests catastrophic respiratory and/or cardiac failure. Several important risk factors for SIDS have been identified, including a prone sleeping position, elevated environmental temperatures, and developmental exposure to cigarette smoke.



The "Triple Risk" model for SIDS. Exposure of an infant with an underlying defect in the brain-stem 5-HT system to an exogen-ous stressor during a critical dev-elopmental period may result in sudden death. Adapted from Filiano and Kinney, *Biol. Neonate* 65: 194-197, 1994.

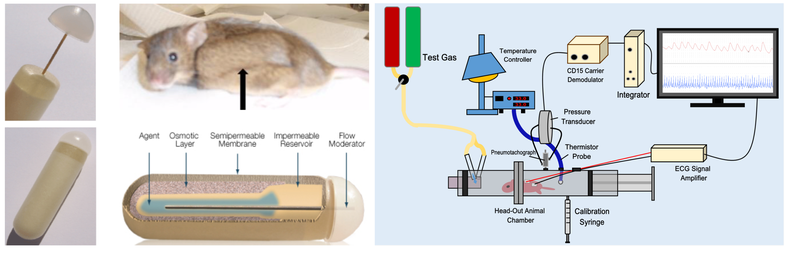
We previously tested the effects of developmental nicotine exposure (DNE) on breathing behavior in intact and unanesthetized neonatal wild type (WT) and *Pet-1* knockout (KO) mice. Nicotine is a neuroteratogen, a major component of cigarette smoke, and is widely believed to be the causative agent underlying the increased risk for SIDS due to maternal smoking. Surprisingly, we found that DNE resulted in a functional recovery of the breathing deficits that are characteristic of the *Pet-1* KO phenotype, but did not decrease neonatal mortality. This led us to consider the effects of DNE on cardiac control since abnormalities in heart rate and/or heart rate variability could result in sudden death. We have developed the ability to record the neonatal mouse electrocardiogram noninvasively and are currently extending our study by challenging saline- and nicotine-treated WT and *Pet-1* KO mice to repeated autoresuscitation challenges while simultaneously recording breathing and heart rate. Concurrently, we are developing methods to analyze our heart rate measurements.

Pet-1 mice are born in normal Mendelian ratios but suffer high neonatal mortality

A. Mendelian frequency			
Genotype	Number born	Frequency (%)	Expected
(+/+)	167	23	25
(+/-)	374	52	50
(-/-)	172	24	25
B. Mortality rate			
Genotype	Number born	Deaths	Mortality (%)
(+/+)	141	6	4
(+/-)	281	19	7
(-/-)	127	36	28

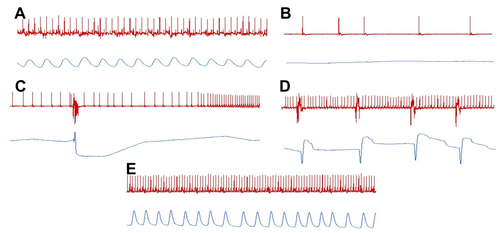
Over a large number of births, the genotypic distribution of pups resulting from heterozygous crosses is 23% wild type (+/+), 52% heterozygous (+/-) and 24% knockout (-/-), approximating expected Mendelian ratios. However, the mortality rate is much higher in knockout, compared to wild type and heterozygous neonates. Death typically occurred overnight and within the first week of birth.

Nicotine Exposure and Plethysmography



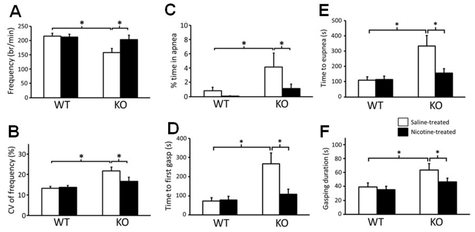
Nicotine delivery and cardiorespiratory measurements. Osmotic mini-pumps were implanted subcutaneously in pregnant *Pet-1* heterozygous dams on embryonic day 5 (left panel). Nicotine concentrations were adjusted to provide a nicotine dose of 60 mg/kg/day for up to 28 days. **Schematic diagram of head-out plethysmography combined with heart rate measurements using superficial ECG recording leads** (right panel). The breathing gas could be changed from room air to an anoxic gas via a gas switch. The pneumotachograph measured tidal airflow produced by ventilatory movements of the animal which was then integrated into tidal volume. Temperature was monitored and maintained constant (33.0+/- 1.0°C) within the animal's thermo-neutral zone by attaching the infrared lamp to a temperature controller. Pump images from: www.alzet.com

The autoresuscitation response



Simultaneous recordings of heart rate (red trace) and ventilation (blue trace) in a neonatal mouse pup during an autoresuscitation challenge. (A) Breathing and heart rate under resting conditions while breathing a normoxic (21% O_2) gas mixture. (B) Apnea and severe bradycardia resulting from breathing an anoxic gas mixture (97% N_2, 3% CO_2). (C) The initial gasp following re-exposure to the normoxic gas. (D) Subsequent gasps leading to (E) the resumption of rhythmic breathing. Note in (C) that the heart rate increased following the first gasp, suggesting that tissue re-oxygenation promotes cardiac resuscitation.

Nicotine exposure normalizes breathing deficits but does not improve survival



G

Saline-treated

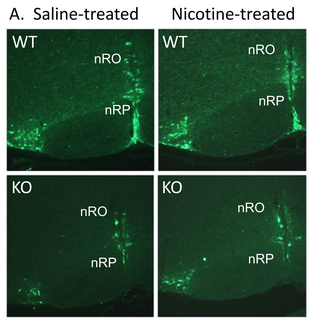
Genotype	n	Deaths
+/+	14	0 (0%)
-/-	11	1 (9%)

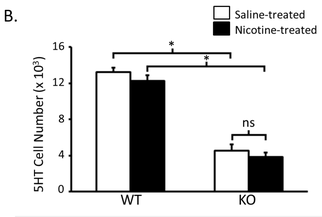
Nicotine-treated

Genotype	n	Deaths
+/+	16	2 (13%)
-/-	18	7 (39%)

Baseline breathing (A-C) and autoresuscitation responses (D-F) in wild type (WT) and *Pet-1* KO mice following developmental exposure to either saline or nicotine. (A) Breathing frequency. (B) Coefficient of variation of breathing frequency. (C) Percent time spent in apnea. (D) Time to first gasp following experimentally induced apnea. (E) Time to rhythmic breathing (eupnea) from the beginning of apnea. (F) Time from the beginning of gasping to resumption of rhythmic breathing (gasping interval). The responses of nicotine-treated P4.5 knockout mice were similar to saline-treated wild-type controls suggesting that developmental nicotine exposure resulted in a functional reversal of the breathing deficits observed in saline-treated KO mice. Despite an improved breathing phenotype, the mortality rate remained high in nicotine-treated *Pet-1* KO mice (G). Values are means ± SE. *P<0.05.

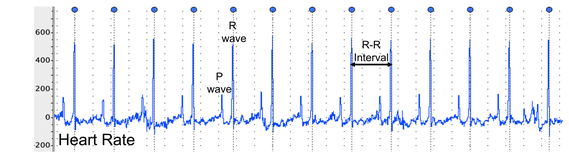
Nicotine exposure does not "rescue" 5HT neurons



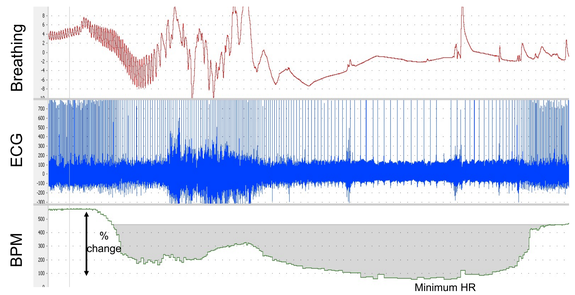


5-HT-immunostained brainstem sections of saline-treated and nicotine-treated wild-type (WT) and *Pet-1* KO mice (A). The 5-HT cell number was reduced by the same amount in saline-treated and nicotine-treated mice (B). Values are means ± SE. *p<0.05. nRO, *nucleus raphe obscurus*; nRP, *nucleus raphe pallidus*. Magnification, 20x.

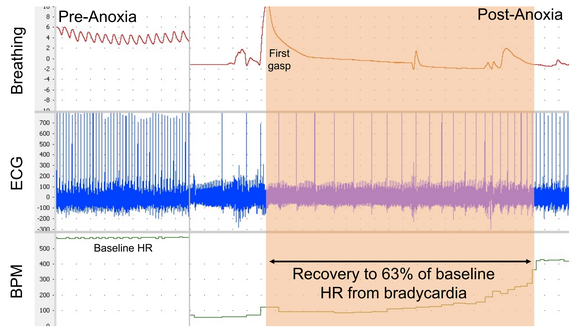
Cardiac Analysis



Heart rate variability (HRV). This analysis will examine variation in the time between each heartbeat (R-to-R interval). Comparisons of heart rate variability between genotypes and treatments groups, both before and after apnea, may reveal deficits in the ability to respond to cardiorespiratory challenges.



Magnitude of bradycardia. This analysis will include comparisons of the lowest heart rate reached during a bradycardic episode, the percent change between the baseline heart rate before apnea and lowest heart rate, and the number of beats lost per minute (represented in grey). Such measures can provide insights into adaptive changes to cardiac output and protective mechanisms against oxygen depletion, or reveal potentially exaggerated and lethal responses to hypoxia.



Recovery from bradycardia. A pro-longed recovery time from a brady-cardic event following an initial gasp can result in myocardial hypoxia and poorer reoxygenation of the brain and body. Inadequate oxygenation of the heart and brain following apneas could lead subsequently to death.

Summary and Conclusions

- This work is part of an ongoing effort to assess the effects of developmental exposure to nicotine on the postnatal maturation of cardiorespiratory control in neonatal mice in a 5HT-deficient context. A 5-HT deficiency has been linked to SIDS in humans.
- Neonatal WT and 5-HT-deficient *Pet-1* KO mice were exposed developmentally to either saline (control) or nicotine and subjected to an autoresuscitation challenge while simultaneously measuring breathing and heart rate via plethysmography and non-invasive electrocardiography.
- These results corroborate past studies that show, relative to saline-treated WT controls, that *Pet-1* KO neonates have a lower resting breathing frequency and take longer to initiate an initial gasp and recover rhythmic breathing following a single autoresuscitation challenge.
- Unexpectedly, developmental nicotine exposure was found to reverse the breathing deficits in the *Pet-1* KO mice. However, despite a recovery of wild type-like breathing behavior, neonatal survival of *Pet-1* KO mice did not improve, suggesting that the higher mortality rates in these animals may be due to impaired cardiac function.
- To explore this possibility, we have developed a range of analytical tools to effectively measure HRV and the magnitude and duration of bradycardia, that will allow us to assess cardiac responses to single or multiple autoresuscitation challenges.

Acknowledgements

This work was supported by National Institutes of Health grant 1R15HL140494-01A1, a TCNJ faculty Support of Scholarly Activity (SOSA) award, and a research grant from the American SIDS Institute.