Cardiorespiratory Responses of 5HT-Deficient Pet-1 Knockout Mice to Repetitive Anoxic Challenges Following *in Utero* Nicotine Exposure

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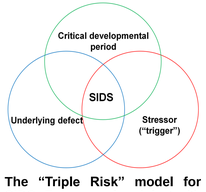
Introduction

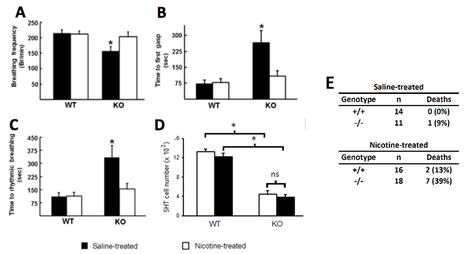
Pet-1 is a transcription factor that is required for normal development of serotonin (5HT) neurons in the mammalian brainstem. Targeted deletion of the *Pet-1* gene results in a 70% loss of central 5HT neurons that is associated with depressed ventilation, an increased incidence of spontaneous apneas, abnormal autoresuscitation responses to prolonged apnea, and high neonatal mortality in newborn mice (Erickson *et al*., 2007). Endogenous 5HT provides excitatory drive to neural circuits in the caudal brainstem that generates respiratory rhythm (Bonham, 1995), and recent studies have correlated brainstem 5HT deficiency with Sudden Infant Death Syndrome (SIDS) in humans (Duncan *et al*., 2010), the leading cause of postnatal infant mortality in the United States. This has led to the "5HT Triple Risk" model for SIDS which postulates that an infant with an underlying defect (deficiency of brainstem 5HT) that is exposed to an environmental stressor (e.g. low environmental 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 brainstem 5-HT system to an exogenous stressor during a critical developmental 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 therefore repeated our autoresuscitation study while measuring breathing and heart rate simultaneously. In addition, we extended this study by exposing saline- and nicotine-treated WT and *Pet-1* KO mice to repeated autoresuscitation challenges.

Initial Findings



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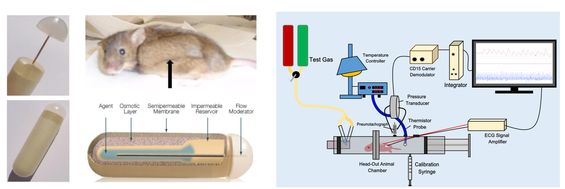
Saline-treated		
Genotype	n	Deaths
+/+	14	0 (0%)
-/-	11	1 (9%)

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

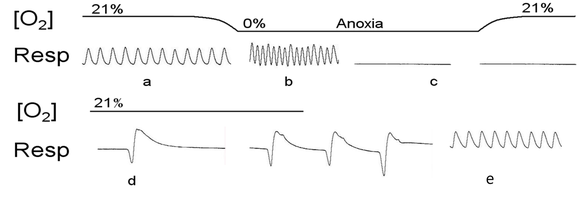
Compromised breathing in the *Pet-1* KO mouse is normalized by developmental nicotine exposure without improving neonatal mortality. Breathing frequency (A), time to first gasp (B), and time to rhythmic breathing (C) during an autoresuscitation challenge. The improved breathing behavior in the nicotine-treated KO mice cannot be due to "rescue" of 5HT neurons since both saline- and nicotine-treated KO mice suffer a similar 70% loss of 5HT neurons (D). Neonatal mortality was greatest in KO mice exposed developmentally to nicotine. Values are means ± SE. *P<0.05, saline-treated WT *vs.* saline-treated KO comparison.

Nicotine Exposure and Plethysmography



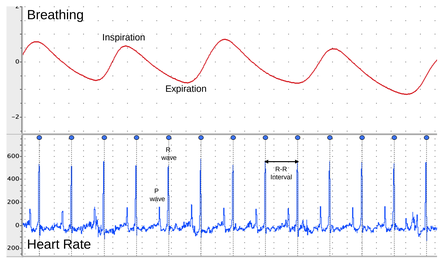
Nicotine delivery and cardiorespiratory measurements. Osmotic mini-pumps were implanted subcutaneously in pregnant *Pet-1* heterozygous dams on embryonic day 5 (left panel). Nicotine concentrations were adjusted to provide a nicotine dose of 60 mg/kg/day for up to 28 days. **Schematic diagram of head-out 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

Autoresuscitation Protocol



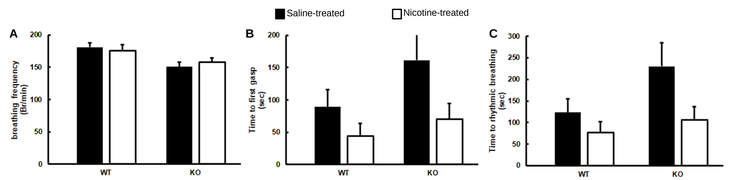
Schematic representation of a single successful autoresuscitation challenge. Top trace: changes in oxygen concentration [O_2] in the breathing gas. Bottom trace: ventilatory responses at each of the four main stages of the autoresuscitation challenge. The animal breathes a normoxic (21% O_2) gas mixture during an initial control period (a) before exposure to an anoxic (0% O_2, 97% N_2/3% CO_2) gas mix (b). Exposure to anoxia results in increased ventilation (b, hyperpnea) followed by primary apnea (c). At this point the gas is switched back to normoxia to allow for autoresuscitation. A series of gasps ensue (d) which results in the restoration of eupneic breathing (d). The trace represents a condensed version of an actual respiratory recording.

Cardiorespiratory Measurements



Simultaneous measurement of ventilation and heart rate. Whole-body plethysmography and non-invasive electrocardiology was used to measure breathing and heart rate simultaneously. Upper trace: Periodic ventilation with the inspiratory and expiratory phases of the respiratory cycle indicated. Lower trace: Heart rate. The p-wave (atrial contraction) and the R wave of the QRS Complex (ventricular contraction) are highlighted. The blue dots represent detection of an R-wave using peak detection algorithm software (Chart, ADInstruments). The main focus for measurement was the R-R interval which allowed measurement of heart rate and heart rate variability.

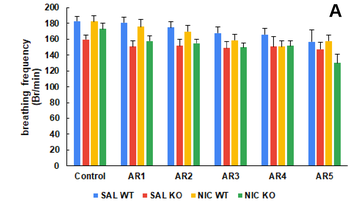
Subsequent Findings

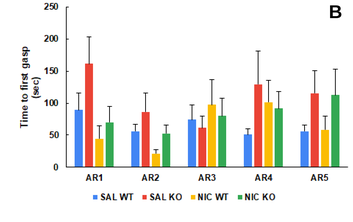


***Pet-1* KO phenotype confirmed in second data set.** An independent data set appears to confirm that *Pet-1* KO neonates have a depressed breathing frequency (A) and abnormal autoresuscitation responses to a single round of experimentally induced apnea (B,D), compared to WT (control) littermates. In addition, developmental exposure to nicotine also appears to reverse the autoresuscitation deficits, although in this data set a normalization of breathing frequency in KO neonates following nicotine exposure was not observed. These data also suggest that exposure to nicotine may improve autoresuscitation responses in WT neonates as well.

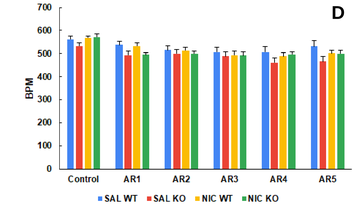
Repetitive Autoresuscitation

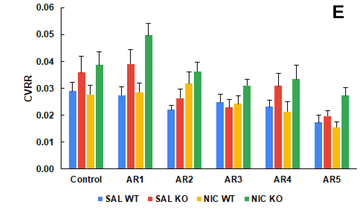
Breathing

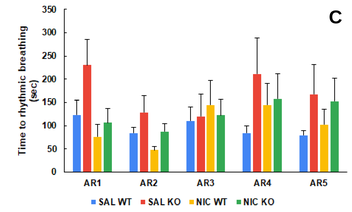




Heart Rate







Comparison of breathing and heart rate parameters of neonatal wild type (WT) and *Pet-1* knockout (KO) mice exposed developmentally to either saline (SAL) or nicotine (NIC) during five sequential autoresuscitation challenges. Each bar represents measurements during the final three minutes of the control period (blue) or the final three minutes of the recovery period following each anoxic challenge (AR1, AR2, AR3, AR4, AR5). Sample sizes: SAL WT, *n*=20; SAL KO, *n*=20; NIC WT, *n*=19; KO NIC KO, *n*=22. Each bar represents the mean ± SE.

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 5HT deficiency has been linked to SIDS in humans.
- Neonatal WT and 5HT-deficient *Pet-1* KO mice were exposed developmentally to either saline (control) or nicotine and subjected to five sequential autoresuscitation challenges while simultaneously measuring breathing and heart rate via plethysmography and non-invasive electrocardiography.
- Compared to previous data obtained using plethysmography alone we confirmed that, relative to saline-treated WT controls, the *Pet-1* KO neonate has a lower resting breathing frequency and takes longer to initiate an initial gasp and recover rhythmic breathing following a single autoresuscitation challenge.
- Based on responses to the first autoresuscitation challenge, the new data indicate that exposure to nicotine during development can reverse the breathing deficits in the *Pet-1* KO mice, also consistent with previous findings.
- Resting heart rate appears to be lower, and heart rate variability higher, in *Pet-1* KO neonates compared to WT controls. Nicotine treatment appears to exacerbate the increased variability in heart rate due to *Pet-1* gene deletion alone, both in control conditions and after each autoresuscitation challenge. In addition, any early "protective" effect conferred by nicotine exposure on breathing behavior in the Pet-1 KO neonate appears to be lost by the fifth autoresuscitation challenge.
- These data suggest that developmental exposure to nicotine weakens the respiratory component of the autoresuscitation response over successive anoxic challenges and exacerbates cardiac abnormalities both at rest and during recovery from anoxic challenge in neonatal 5-HT-deficient *Pet-1* KO mice.

Acknowledgements

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