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Developmental nicotine exposure adversely effects respiratory patterning in the barbiturate anesthetized neonatal rat

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ABSTRACT

Neonates at risk for sudden infant death syndrome (SIDS) are hospitalized for cardiorespiratory monitoring however, monitoring is costly and generates large quantities of averaged data that serve as poor predictors of infant risk. In this study we used a traditional autocorrelation function (ACF) testing its suitability as a tool to detect subtle alterations in respiratory patterning in vivo. We applied the ACF to chest wall motion tracings obtained from rat pups in the period corresponding to the mid-to-end of the third trimester of human pregnancy. Pups were drawn from two groups: nicotine-exposed and saline-exposed at each age (i.e., P7, P8, P9, and P10). Respiratory-related motions of the chest wall were recorded in room air and in response to an arousal stimulus (F_IO_2 14%). The autocorrelation function was used to determine measures of breathing rate and respiratory patterning. Unlike alternative tools such as Poincare plots that depict an *averaged* difference in a measure breath to breath, the ACF when applied to a digitized chest wall trace yields an *instantaneous* sample of data points that can be used to compare (data) points at the same time in the next breath or in any subsequent number of breaths. The moment-to-moment evaluation of chest wall motion detected subtle differences in respiratory pattern in rat pups exposed to nicotine in utero and aged matched saline-exposed peers. The ACF can be applied online as well as to existing data sets and requires comparatively short sampling windows (~2 min). As shown here, the ACF could be used to identify factors that precipitate or minimize instability and thus, offers a quantitative measure of risk in vulnerable populations.

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1. Introduction

Sudden infant death syndrome (SIDS) is defined as the sudden sleep related death of an infant <12 months of age that is unexplained despite an autopsy and post-mortem examination (Hilaire et al., 2010; Willinger et al., 1991). Maternal tobacco smoking or exposure to environmental tobacco smoke are major risk factors for SIDS (Mitchell and Milerad, 2006) and infants born to mothers that smoke exhibit increased nicotinic acetylcholine receptor expression in brainstem nuclei that control normal respiration, arousal (Cohen et al., 2005; Duncan et al., 2008; Machaalani et al., 2011) and integrate information from cardiovascular, pulmonary, respiratory tract and gastrointestinal receptor afferents (Machaalani et al., 2011; Machaalani and Waters, 2008; Waters et al., 1999). Despite neuro-pathologic evidence of morphological and receptor binding abnormalities affecting multiple brainstem nuclei (Jaiswal et al., 2013; Machaalani et al., 2011; Slotkin et al., 1987; Smith et al., 2010), there is no consistent phenotype associated with gestational or developmental exposure to cigarette smoke or to nicotine. And, although SIDS victims show a higher incidence of respiratory abnormalities including apneas, delayed arousal responses and diminished ventilatory chemo-reflexes, the frequency of those events is low and of limited diagnostic and therapeutic utility.

There is a pressing need for novel approaches to better identify respiratory patterns that may increase the risk for SIDS. One approach to the problem has been the use of nonlinear analyses such as Poincaré plots (Barrett et al., 2012; Brennan et al., 2002; Dick et al., 2014). Although this approach also can be used to detect respiratory instability, Poincare plots represent an average of the data points within the breath rather than considering individual data points within each breath. In view of this limitation, we applied a standard autocorrelation function (ACF) to digitized respiration-related chest wall motion traces to obtain *unrestricted* estimates of respiratory system stability using this approach to test the hypothesis that rats exposed to nicotine in utero i.e., subject to developmental nicotine exposure (DNE), will exhibit greater respiratory instability relative to age-matched, sham-treated controls.

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2. Materials and methods

2.1. Study animals

Thirty-four Sprague-Dawley rat pups (Charles-River Laboratories) were studied on postnatal days 7–10 (P7–P10). Pups were drawn from two experimental groups: nicotine-exposed ($n = 11$) and saline-exposed ($n = 23$) at each age (i.e. P7, P8, P9, and P10) corresponding to the developmental stage of preterm and term human infants (i.e., mid-to-end of the third trimester of human pregnancy) (Clancy et al., 2007). Animals had access to nutrition via the dam and were housed under a 12 h light/12 h dark cycle. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Arizona and adhered to the Public Health Service Policy on Humane Care and Use of Laboratory Animals.

2.2. Developmental nicotine and saline exposure

Procedures related to nicotine and saline exposure were based on prior reports (Luo et al., 2004; Robinson et al., 2002). Briefly, pregnant dams were delivered to the Animal Care facility at the University of Arizona and given 24 h to acclimate. On the following day, dams were anesthetized via intramuscular injection of 0.75 cc kg^{-1} "rabbit mix" (5 cc ketamine, 8 cc 20 mg ml^{-1} xylazine, 2 cc acepromazine) into the hind limb, followed by a subcutaneous injection of analgesic (Buprenex, 0.5 mg kg^{-1}) and preparation of the surgical site. Absence of limb withdrawal to paw pressure was assessed every 15 min to confirm a sufficient plane of anesthesia.

An incision was made at the base of the neck and an osmotic mini pump (Alzet 2ML4, Durect Corp., Cupertino, CA) was inserted through the incision and sutured shut (nylon). Antibiotics (Combi-Pen-48, Bimedi Inc.) were provided via a subcutaneous injection upon completion of the surgery. Post-operative care included analgesia (Buprenex, 0.5 mg kg^{-1}) every 12 h for a period of 36 h and an antibiotic injection 24 h post-surgery. Surgeries were performed on embryonic day 5 to coincide with embryonic implantation in the uterine wall (Serra et al., 2001) and pumps were charged to deliver saline or nicotine bitartrate at 6 mg kg^{-1} day^{-1} which corresponds to 2.1 mg kg^{-1} day^{-1} of free base nicotine; similar to a moderate to heavy smoker (Stephan-Blanchard et al., 2013). Because the Alzet 2ML4 pump delivers over 28 days, pups were exposed to nicotine or saline through the remainder of gestation to P10 via milk from the dam hence, we use the term developmental nicotine exposure (DNE) to indicate exposure to nicotine that continues after birth.

2.3. Study design

The following experimental conditions applied to *all* pups. Pups were studied in a flow through chamber the details of which have been published elsewhere (Kidder et al., 2014). Pups were sedated via an intraperitoneal injection of barbiturate (35 mg kg^{-1}, Inactin hydrate, Sigma–Aldrich) to minimize movement and to permit recording of respiratory-related motions of the chest wall. Sedation was titrated on the basis of breathing rate and did not drop below 110 ± 10 breaths min^{-1}. Chest wall motions were detected via a force transducer (World Precision Instruments, FORT100) in contact with the chest wall. Pups' respiration was recorded for 2 min in room air and in normocapnic hypoxia (0.14 F_IO_2) to assess the arousal response. Humidified gas mixtures were introduced into the chamber via a rotameter (Matheson Tri-gas, model FM-1050-VO-3T). The composition of the inspired gas was mixed online and monitored with O_2 and CO_2 gas analyzers (VacuMed 17518 and 17515). Rectal temperature was obtained via a thermocouple probe (IT-18, Physitemp Instruments) and maintained at 37 ± 0.9 °C with a heating pad. Temperature and humidity were controlled online and monitored via a thermometer hygrometer (FLUKER'S CF-22642). Experimental sessions were captured on video using a Microsoft LifeCam Studio web camera synchronized with EMG signals via Spike2 (version 7.1) software. All data were recorded using Spike2 software (Cambridge Electronic Design, UK) and analyzed off-line in MATLAB. For each pup, we obtained 2 min of chest wall motion associated with an otherwise stable period of rest breathing uninterrupted by movement or apnea was included in the analysis (see Fig. 1).

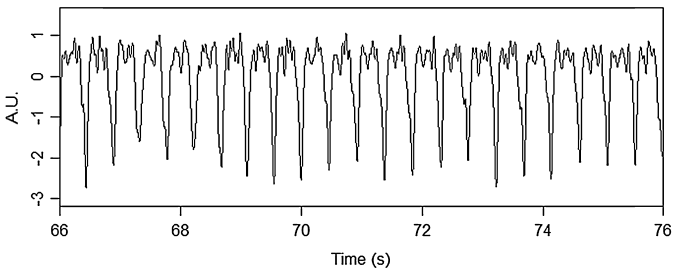

Fig. 1. Representative chest wall motion traces from a saline exposed pup obtained on post-natal day 7. Negative deflections correspond to inspirations (i.e., chest wall expansion). Note periodic oscillations in the magnitude of the chest wall excursion.

Breathing rates were measured by summation of peak chest wall expansions (inspirations) in 1-min epochs for each gas condition. Heart rates were determined from the EKG signal obtained from the intercostal EMG lead and R wave events were discriminated from the lead signal and summed over 1-min epochs for each gas condition. Initially, we used a univariate ANOVA to assess differences in body mass between DNE and saline pups and assessed the effects of experimental group and post-natal age on average heart rate and breathing frequency via a general linear ANOVA and via a negative binomial for apnea frequency. We subsequently obtained an autocorrelation function of each respiratory trace using a 10 s (5001 sample) window that encompassed at least 18 breaths that was devoid of spontaneous apneas and/or movement-related artifact.

3. Analysis

The ACF is the Pearson product-moment correlation of a signal with a copy of itself that is delayed in time. The delay is measured in lags, wherein each lag corresponds to a delay of a *single data point* – an estimate of where a given data point will occur (some) time later based on upon its current location. Representative ACFs for a saline-exposed pup are shown in Fig. 1. Each ACF highlights between-group differences in the regularity of the functions, peak heights and the rates at which the peak heights approach zero as a function of the elapsed time. By contrasting ACFs in this manner it is possible to make statements regarding the stability of a (given) measure for each rat pup over time (see below). Note that in applying the ACF to the respiratory trace directly, the ACF considers the stability of *individual data points* over successive breaths. It does not represent an average of data points across breaths as is the case in a Poincare plot. In this regard, the ACF can be used to quantify stability based on instantaneous samples of the digitized trace, affording a highly sensitive view of system stability over the short, mid-range or long term.

The application of the ACF to a data trace is demonstrated in Fig. 2, which shows a section of a representative respiratory trace obtained from a saline-exposed rat pup. The trace as shown in Panel A when overdrawn with a time lagged copy of itself (Panels B, C and D) can be used to assess instantaneous similarities and differences in chest wall motion as a function of time. The differences in respiratory patterning subsequently were assessed using repeated-measures ANOVA with one between-subjects factor,

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