eNeuro

Research Article: Confirmation | Integrative Systems

Developmental nicotine exposure alters synaptic input to hypoglossal motoneurons, and is associated with altered function of upper airway muscles

https://doi.org/10.1523/ENEURO.0299-19.2019

Cite as: eNeuro 2019; 10.1523/ENEURO.0299-19.2019

Received: 31 July 2019
Revised: 3 October 2019
Accepted: 13 October 2019

1. Manuscript Title (50 word maximum)

Developmental nicotine exposure alters synaptic input to hypoglossal motoneurons, and is associated with altered function of upper airway muscles

2. Abbreviated Title (50 character maximum)

Perinatal nicotine and upper airway motor control

3. List all Author Names and Affiliations in order as they would appear in the published article

Lila Wollman[1]
Jordan Clarke[1]
Claire DeLucia[1]
Richard B. Levine[1,2]
Ralph F. Fregosi[1,2]

[1]Department of Physiology; [2]Department of Neuroscience. The University of Arizona, Tucson, Arizona, USA

4. Author Contributions: LW, RBL & RFF wrote the paper & designed the research; LW, JC & CD Performed Research; LW, JC, CD & RFF analyzed the data

5. Correspondence should be addressed to (include email address)

Ralph F. Fregosi, PhD, Department of Physiology, University of Arizona, College of Medicine,1501 N. Campbell Ave, Tucson, AZ 85724, USA
Email: fregosi@email.arizona.edu

6. Number of Figures **10**
7. Number of Tables 5
8. Number of Multimedia 0
9. Number of words for Abstract 244
10. Number of words for Significance Statement 118
11. Number of words for Introduction 508
12. Number of words for Discussion 2358
13. Acknowledgements

We wish to thank Seres Cross for her expert technical assistance.

14. Conflict of Interest: A. Authors report no conflict of interest
15. Funding sources: Eunice Kennedy Shriver National Institute of Child Health and Human Development; NICHD 5R01HD071302-07.

Abstract

Nicotine exposure during the fetal and neonatal periods (Developmental nicotine exposure, DNE) is associated with ineffective upper airway protective reflexes in infants. This could be explained by desensitized chemoreceptors and/or mechanoreceptors, diminished neuromuscular transmission or altered synaptic transmission among central neurons, as each of these systems depend in part on cholinergic signaling through nicotinic acetylcholine receptors (nAChRs). Here we showed that DNE blunts the response of the genioglossus muscle to nasal airway occlusion in lightly anesthetized rat pups. The genioglossus muscle helps keep the upper airway open and is innervated by hypoglossal motoneurons (XIIMNs). Experiments using the phrenic nerve-diaphragm preparation showed that DNE does not alter transmission across the neuromuscular junction. Accordingly, we used whole cell recordings from XIIMNs in brainstem slices to examine the influence of DNE on glutamatergic synaptic transmission under baseline conditions and in response to an acute nicotine challenge. DNE did not alter excitatory transmission under baseline conditions. Analysis of cumulative probability distributions revealed that acute nicotine challenge of P1-P2 preparations resulted in an increase in the frequency of nicotine-induced glutamatergic inputs to XIIMNs in both control and DNE. By contrast, P3-P5 DNE pups showed a decrease, rather than an increase in frequency. We suggest that this, together with previous studies showing that DNE is associated with a compensatory increase in inhibitory synaptic input to XIIMNs, leads to an excitatory-inhibitory imbalance. This imbalance may contribute to the blunting of airway protective reflexes observed in nicotine exposed animals and human infants.

64

65 **Significance statement**

66 The number one risk factor for sudden infant death (SIDS) is maternal smoking. While
67 the use of nicotine delivery devices such as e-cigarettes is increasing among women of
68 childbearing age, reflecting the belief that the use of nicotine alone is safer than
69 tobacco, SIDS deaths are not decreasing, suggesting that nicotine is the link between
70 maternal smoking and SIDS. Here we show that perinatal nicotine exposure alters a
71 major motor pathway responsible for upper airway patency during sleep. We also
72 introduce an animal model that is well suited to probing the mechanisms underlying the
73 link between maternal nicotine consumption and SIDS, and a phenotype that nicely
74 models key aspects of the events believed to give rise to SIDS.

75

76 **Introduction**

77 Hypoglossal motoneurons (XIIMNs) innervate the muscles of the tongue, which
78 are critically important in the maintenance of airway patency during breathing (Lowe,
79 1980). The breathing-related drive to the tongue muscles relies on the appropriate
80 timing and strength of XIIMN output, which is strongly influenced by the balance of
81 excitatory and inhibitory fast-synaptic inputs that the motoneurons receive (Berger,
82 2011), and a functionally viable neuromuscular junction. Environmental factors, such as
83 exposure to nicotine in the perinatal period, can alter the course of normal nervous
84 system development. This is attributed to nicotine's action on nicotinic acetylcholine
85 receptors (nAChRs), which are well known for their role in modulating synaptic
86 transmission in the brain (Wonnacott et al., 2005) and neuromuscular junction (Wood

and Slater, 2001). In terms of tongue muscle function in nicotine-exposed human neonates, there are limited though interesting observations. These include an increased incidence of obstructive apneas (Kahn et al., 1994) and hypoplasia and immaturity of XIIMNs (Ottaviani et al., 2006; Lavezzi et al., 2010). In contrast with these limited data from human neonates, there is a considerable body of work on the influence of developmental nicotine exposure (prenatal exposure with continued exposure in the first week of life, DNE) on the structure and function of XIIMNs. For example, XIIMNs from DNE animals have a significantly more complex dendritic arbor than cells from control animals on postnatal days 1-2 (P1-P2), but by P3-4 the arbor is less complex suggesting altered neuronal development over the first week of life (Powell et al., 2016). As for intrinsic properties, XIIMNs from DNE animals are hyperexcitable (Pilarski et al., 2011) and show increased GABAergic inhibition (Jaiswal et al., 2016; Wollman et al., 2018a). We believe that the increase in GABAergic inhibition to nicotine-exposed XIIMNs is consistent with a homeostatic mechanism aimed at mitigating the increased intrinsic excitability. However, a homeostatic response to the increased cell excitability may also include reductions in excitatory synaptic input to XIIMNs.

The experimental results reported here were designed to gain a further understanding of how DNE impacts tongue muscle function at rest and in response to a respiratory challenge, the nature of excitatory synaptic inputs to XIIMNs, and the integrity of the neuromuscular junction. First, tongue muscle function was evaluated in vivo by recording the breathing-related tongue muscle EMG before, during and after a period of airway occlusion in lightly anesthetized neonatal rats. Next, since hypoxia and hypercapnia (as occurs during airway occlusion) is associated with increased

acetylcholine release (Metz, 1966; Huckstepp et al., 2016), and DNE is known to alter nAChR function throughout the brain (Wonnacott, 1990; Wonnacott et al., 1990; Gentry and Lukas, 2002), we hypothesized that DNE may change how nAChR activation modulates excitatory fast-synaptic inputs to XIIMNs. Accordingly, we evaluated the amplitude and frequency of AMPA receptor-mediated glutamatergic synaptic inputs to XIIMNs at baseline and in response to acute activation of nAChRs. Finally, using the hemi diaphragm-phrenic nerve preparation as a model, we probed the effects of DNE on the integrity of the neuromuscular junction by measuring neuromuscular transmission failure and susceptibility to fatigue.

Materials and Methods

Animals. We used a total of 195 Sprague-Dawley rat pups of either sex, ranging in age from postnatal day one (P1) through P7, which in terms of comparable brain development in humans corresponds roughly to the 23rd week of gestation through birth (Semple et al., 2013). An equal number of control and nicotine exposed animals were used. All neonates were born via spontaneous vaginal delivery from pregnant adult female rats purchased from Charles River Laboratories (Wilmington, MA). Neonates were housed with their mothers and siblings in the animal care facility at the University of Arizona under a 12:12 hour light/dark cycle (lights on 07:00 h), in a quiet room maintained at 22 °C and 20-30% relative humidity, and with water and food available *ad libitum.* All procedures and protocols described were approved by the University of Arizona Institutional Animal Care and Use Committee, and in accordance with National Institutes of Health guidelines.

Developmental nicotine exposure. Pregnant Sprague-Dawley dams (Charles River Laboratories) were anesthetized with a subcutaneous injection of ketamine (25 mg/kg), xylazine (8.0 mg/kg) and acepromazine (1 mg/kg) and an Alzet 1007D mini-osmotic pump (Alzet Corp., CA, USA) was implanted subcutaneously on gestational day 5. A subcutaneous injection of buprenorphine (0.5 mg/kg) was given to control postoperative pain. The 28-day pump exposes the pup via the placenta throughout the remainder of gestation (approximately 16 days), and via breast milk after birth, and these successive pre and postnatal exposures define DNE. The pump was loaded to deliver an average dose of 6 mg/kg/day of nicotine bitartrate. This dose produces plasma cotinine (a metabolic by-product of nicotine) levels in the pups ranging from 60-92 ng/ml (Powell et al., 2016), which is comparable to that seen in the plasma of human infants born to mothers who are considered moderate smokers (Berlin et al., 2010). Control animals were obtained from pregnant dams implanted with an Alzet pump filled with saline (sham control). Consistent with our previous studies, there were no systematic differences in measured variables between sham control and animals obtained from pregnant dams that did not undergo pump implantation (true control). Pregnant dams were euthanized on postnatal day 7 using institutionally and federally-approved procedures.

In vivo studies. These studies were designed to test the hypothesis that DNE is associated with decreased drive to the tongue muscles in response to airway occlusion (i.e., the model of an external stressor). Neonatal rat pups ranging in age from P3-P7 were lightly anesthetized with a mixture of ketamine (30 mg/mL), xylazine (6 mg/mL) and acepromazine (3 mg/mL), injected subcutaneously at a volume corresponding to

approximately 0.35 µL/g body weight. Pain sensitivity was checked via multiple paw pinches initiated 10 minutes after the time of injection. Supplemental anesthetic was added until paw retraction upon pinching was abolished. Whole muscle electromyographic (EMG) activity of the genioglossus muscle (GG, a tongue protrudor muscle) was recorded using fine wire electrodes inserted into the area underneath the tip of the mandible (Fig. 1A) (Bailey et al., 2005; Rice et al., 2011). An additional hook wire electrode placed into the scruff of the neck near the animal's ear served as an electrical ground. EMG signals were filtered (30-3000 Hz), amplified (Grass P122 AC amplifiers) and sent to an A/D converter (Cambridge Electronic Design (CED); model 1401), which sampled the signal at a rate of 8333 Hz. After the experiment, animals were killed with an overdose of pentobarbital sodium, and electrode location was confirmed by dissection. Data were accepted only if we could confirm that the wires were in the GG muscle. In four animals we also inserted pairs of fine wire electrodes into the diaphragm just beneath the lower ribs, to document that overall respiratory motor drive persisted during airway occlusion (Fig. 1B).

After implantation of the EMG electrodes, the animal was inserted into a 32 ml head-out plethysmograph in the supine position (Fig. 1A). A neck seal was formed with latex, allowing the animal to breathe normally from the room air, with the thorax and abdomen isolated in the sealed chamber. When the animal inhales, its thorax expands, forcing gas out of the sealed chamber. The gas flow entering and leaving the chamber was measured with a pneumotach (Hans-Rudolph) connected to a pressure transducer (Validyne, sensitivity +/- 2 cmH2O), and from this we obtained a recording that is proportional to respiratory airflow (bottom trace in Fig. 1B). This signal was passed to

an analog integrator (Grass) that computed the area under the inspired segment of the curve, providing a measure of the inspired tidal volume (Fig. 1B, third trace from the top). We calibrated tidal volume by injecting known volumes of gas into the chamber with a 50 µL Hamilton syringe. The pressure, tidal volume and EMG signals were sent to an analog-to-digital converter (Cambridge Electronics Design), displayed in real time on a computer monitor (Spike II software) and stored on a hard drive for subsequent offline analysis. The plethysmograph temperature was maintained between 32 and 34°C using a temperature probe and heat lamp. This range is within the thermoneutral zone for neonatal rats (Mortola, 1984; Mortola and Tenney, 1986; Sant'Anna and Mortola, 2003; Mortola, 2004). Though we did not measure body temperature, previous studies show that baseline body temperature is the same in control and DNE rat pups (Ferng and Fregosi, 2015).

After baseline recordings were completed, the animal was challenged with 15 sec of nasal occlusion (Fig. 1B), resulting in strong breathing efforts but an absence of lung inflation, as well as hypoxia, hypercapnia and acidosis. Measurement of peak EMG activity, tidal volume and breathing frequency throughout the period of nasal occlusion were organized into bins corresponding to five, 20% segments of occlusion time. The EMG activity was normalized in each animal by first detecting the largest burst recorded during the experimental procedure and assigning a value of 1.0 to that burst, which represents the peak activity in each experiment. The average burst amplitude within each 20%-time bin was expressed as a fraction of the maximal burst amplitude (e.g., Fig. 1C). We also measured the time between the onset of nasal occlusion and the onset of the first EMG burst during occlusion, and defined this as the response latency

202 (e.g., Fig. 2). Changes in EMG activity during nasal occlusion were analyzed with two-
203 way ANOVA, with time and treatment the main factors. Post hoc analysis was by
204 Tukey's test. The difference in EMG onset latency was tested with the unpaired t-test.
205 $P < 0.05$ was taken as the threshold for statistical significance (Table 4).
206 In the course of working out the *in vivo* techniques, we completed 15-sec nasal
207 occlusion trials in 135 animals. Only a minority of these animals qualified for the main
208 analysis, which required both high quality plethysmographic and EMG recordings (9
209 control, 11 DNE); the remainder were not used in the main analysis. However, we did
210 find that 12 of the 135 animals failed to recover from nasal occlusion, and as shown in
211 Fig. 3., the majority of these were DNE animals.
212 In vitro study A-whole cell voltage clamp recordings of XIIMNs in medullary slice
213 preparations. These experiments were designed to examine the influence of DNE on
214 AMPA-mediated glutamatergic synaptic input to XIIMNs. As indicated below, we studied
215 both spontaneous excitatory post synaptic currents (sEPSCs) and miniature EPSCs
216 (mEPSCs) inputs This dual focus is important, as sEPSCs reflect both action potential-
217 mediated glutamate release, as well as inputs due to the random, quantal release of
218 glutamate from presynaptic terminals. Moreover, nAChRs are located presynaptically
219 on the soma, dendrites and axon end-terminals of glutamatergic neurons as well as
220 postsynaptically on XIIMNs, and exposure to nicotine could alter nAChRs in all of these
221 locations. Therefore, recording both miniature and spontaneous events can help
222 determine whether the actions of DNE on XIIMNs are presynaptic or postsynaptic.
223 Pups of either sex were removed from their cages, weighed, anesthetized on ice
224 and decerebrated at the coronal suture. The vertebral column and ribcage were

exposed and placed in cold (4-8 °C) oxygenated (95% O_2- 5% CO_2) artificial cerebrospinal fluid (aCSF), composed of the following (in mM): 120 NaCl, 26 $NaHCO_3$, 30 glucose, 1 $MgSO_4$, 3 KCl, 1.25 NaH_2PO_4, and 1.2 $CaCl_2$ with pH adjusted to 7.4 and osmolarity to 300-325 mOsm. The brainstem was extracted and glued to an agar block, rostral surface up, and 2-3 transverse medullary slices (300-500mM) containing the hypoglossal motor nucleus were cut in a vibratome (VT1000P, Leica) filled with ice-cold aCSF. The slices were then transferred to an equilibration chamber containing fresh, oxygenated, room temperature aCSF and allowed to recover for 1.5 hours before recording.

Equilibrated slices were transferred to a recording chamber maintained at 27 °C (TC-324B temperature controller, Warner Instrument Corporation) and perfused with oxygenated (95% O_2/5% CO_2) aCSF at a rate of 1.5-2 ml/min. XIIMNs were visualized with an Olympus BX-50WI fixed-stage microscope (40x water-immersion objective, 0.75 N.A.) with differential contrast optics and a video camera (C2741-62, Hamamatsu). Recordings were made with glass pipettes (tip resistance 3-7 MΩ) pulled from thick-walled borosilicate glass capillary tubes (OD: 1.5 mm, ID: 0.75 mm). We used a CsCl based intracellular solution containing (in mM): 130 CsCl, 5 NaCl, 2 $MgCl_2$, 1 CaCl, 10 HEPES, 2 ATP-Mg, 2 Sucrose, with pH adjusted to 7.2 and osmolarity of 250-275 mOsm. Under these conditions, the chloride reversal potential is approximately 0 mV (actual value = -2.8 mV) and therefore both excitatory and inhibitory post-synaptic currents are inward at a holding potential of -75 mV. Filled pipettes were attached to a head stage mounted in a micromanipulator (MP-225, Sutter Instrument Company). The

247 head stage was connected to a Multiclamp 700B amplifier, and the signals were
248 digitized with a Digidata 1440A A/D converter (Molecular Devices).

249 The following procedures pertain to all recordings. First XIIMNs were identified
250 based on their size, shape and location. We targeted the cell soma with the pipette and
251 after a gigaohm seal was achieved the membrane was ruptured by suction. After a 5-
252 minute equilibration period to confirm a stable recording, we pharmacologically
253 isolated AMPA receptor-mediated sEPSCs using D-(-)-2-Amino-5-phosphonopentanoic
254 acid (AP-5), strychnine hydrochloride, and bicuculline methiodide to antagonize the
255 NMDA receptors the glycine receptors, and the $GABA_A$ receptors, respectively (Table
256 1). In the first set of experiments, sEPSCs were recorded for three minutes at baseline,
257 and then for an additional three minutes during bath application of nicotine (acute
258 nicotine challenge). This protocol was followed by five minutes of washout with aCSF.

259 In the second set of experiments we examined the influence of DNE on mEPSCs
260 both before and after an acute nicotine challenge. After a stable recording was
261 achieved, we blocked NMDA, GABAA and glycine receptors, as above, to isolate
262 AMPA-mediated excitatory events. This cocktail was superfused for three minutes,
263 followed by the addition of tetrodotoxin (TTX) for two minutes to block action potential
264 firing (Table 1). AMPA receptor-mediated mEPSCs were recorded at baseline for three
265 minutes, after which nicotine bitartrate was added to the superfusate. mEPSCs were
266 recorded for an additional three minutes in the presence of nicotine, followed by a five-
267 minute washout period.

268 In a third set of experiments, to evaluate the influence of DNE on post-synaptic
269 AMPA receptors, recordings were again made in the presence of AP-5, strychnine

hydrochloride, bicuculline methiodide, and TTX (Table 1). We then bath applied AMPA, to activate post-synaptic AMPA receptors. Under these conditions, activation of the AMPA receptors produces an inward current and the peak of this current was measured. Post-synaptic currents were recorded in voltage clamp with Clampex software (Molecular Devices, Sunnyvale, CA), and analyzed with MiniAnalysis software (Synaptosoft, Decatur, GA, USA).

Drugs: Drugs were purchased from Sigma (St. Louis, MO, USA), except for nicotine bitartrate (MP Biomedicals, LLC, Solon, OH, USA) and TTX (R&D Chemicals, Minneapolis, MN, USA). All drugs were mixed in aCSF on the day of the experiment from previously mixed aliquots that were frozen and stored at 0-2 °C. Antagonists were used at concentrations known to be effective based on our previous studies or the literature. Nicotine was used at the highest dose that produced presynaptic effects (increased frequency of sEPSCs in pilot experiments) without producing a significant inward current, which we found to be 0.5 μM. This is important, as pilot studies showed that higher concentrations of nicotine (1 mM, 10 mM, and 100 mM) activates postsynaptic nAChRs and evokes an inward current, that decreases the ability to discriminate sEPSCs /mEPSCs. AMPA was used at a concentration that produced an approximately half maximal response (2.5 μM), based on dose response experiments previously performed in our lab. The drug solutions were oxygenated and maintained at 27 °C and perfused into the recording chamber at a rate of 1.5-2 ml/min.

Data from a total of 72 cells are reported, with 36 cells from DNE neonates and 36 cells from control neonates. Cell numbers for each experiment are summarized in Table 2. The average number of sEPSCs and mEPSCs evaluated per neuron is shown

in Table 4. At the end of each experiment, the resting membrane potential and input resistance were measured again to confirm the health of the cell and that the gigaohm seal was still intact. Because of the morphological differences seen in neurons from DNE animals aged P1-2 compared to age P3-5 (Powell et al., 2016), we analyzed our data within these two age groups. For all EPSCs, the inter-event interval (IEI) and peak amplitude were measured during the minute before the nicotine challenge, and throughout the second and third minutes of the challenge. For mEPSCs, rise time was measured at these same time points. There were no differences for any of these variables between the second and third minutes of nicotine challenge, so data recorded in the third minute was used for analysis. Lastly, we measured the peak of the whole cell inward current evoked by stimulation of postsynaptic AMPA receptors with bath applied AMPA (e.g., Fig. 9A).

In vitro study B- hemi diaphragm-phrenic nerve preparation. To estimate the influence of DNE on the integrity of the neuromuscular junction, and as a substitute for the hypoglossal nerve-tongue neuromuscular junction (see Discussion), we used neonatal rat (P1-P5) phrenic nerve-hemidiaphragm preparations. Diaphragm muscle was excised and secured in a dish perfused with warmed (37 ^{o}C), oxygenated Krebs solution, containing (in mM): 123 NaCl, 26 $NaHCO^{-}_{3}$, 30 glucose, 1 $MgSO_4$, 3 KCl, 1.25 NaH_2PO_4 and 1.2 CaCl], gassed with 95% O_2/5% CO_2, with pH adjusted to 7.45-7.5. Force was measured with a transducer (Kent Scientific) attached to the central tendon of the hemi diaphragm, and the phrenic nerve was drawn into a suction electrode, which was referenced to a bath ground and connected to a stimulator (Grass S88). Two silver/silver-chloride discs were pinned to the bath on either side of the muscle strip and

316 connected to a second channel on the stimulator for direct depolarization of the muscle
317 fibers, bypassing the neuromuscular junction. Force and the output of the stimulator
318 were digitized and monitored on a computer using Spike II hardware and software
319 (Cambridge Electronics Design, London, UK).

320 To measure muscle twitch force and contraction speed in response to direct
321 muscle stimulation we delivered single, 0.2 ms supramaximal pulses to the bath
322 electrodes. We also measured the decline in force following 5 min of intermittent,
323 supramaximal direct muscle stimulation, while in another set of animals we stimulated
324 the phrenic nerve. Both muscle and nerve were stimulated with 0.2 ms pulses, delivered
325 in 330 ms trains at 40 Hz, with a train delivered every 2 sec. In additional sets of
326 animals, we estimated the contribution of neuromuscular transmission to force loss by
327 applying stimulus trains to the phrenic nerve, as above, while superimposing direct
328 muscle stimulation every 15 sec. Neuromuscular transmission failure was computed by
329 comparing the force loss during nerve stimulation with that evoked by direct muscle
330 stimulation (see Fig. 10C), as described by others (Aldrich et al., 1986; Fournier et al.,
331 1991). Force decline due to neuromuscular transmission failure = (force loss during
332 nerve stimulation - force loss during muscle stimulation) / (1- force loss during muscle
333 stimulation).

334 Statistics. A brief summary of the statistical analysis used in each of the three
335 experimental series is given in Table 2. In vivo experiments. Changes in EMG activity
336 during nasal occlusion were analyzed with two-way ANOVA, with time and treatment
337 the main factors. Post hoc analysis was by Tukey's test. The difference in EMG onset
338 latency was tested with the unpaired t-test. $P < 0.05$ was taken as the threshold for

statistical significance (Table 2). Differences in the number of failed autoresuscitations was compared between groups using a Chi square analysis (Table 2).

In vitro study A-whole cell voltage clamp recordings of XIIMNs in medullary slice preparations. Differences in baseline variables including age, weight, resting membrane potential, and input resistance were evaluated between treatment groups, and between age groups within a treatment group, by comparing the means from each group with a two-way ANOVA, followed by Tukey's post hoc test for multiple comparisons (Table 2).

We analyzed the IEI and amplitude for both sEPSCs and mEPSCs in two ways (see Table 2). First, the IEI and amplitude of all sEPSCs/mEPSCs from all cells within an age or treatment group were used to construct a cumulative probability distribution using Prism (GraphPad Software, Inc., La Jolla, CA) (e.g., Figures 5 and 7). We expressed the cumulative probability as fractions ranging from 0 to 1, such that a value of 0.5 defines the midpoint of the normalized EPSC amplitude or frequency distribution. In these graphs, the y-axis value is the fraction of events that lies at or below the corresponding x-axis value. To compare the IEI and amplitude distributions between baseline conditions and during acute nicotine application, we performed a two-sample Kolmogorov-Smirnov (K-S) test using SPSS (IBM, Armonk, NY).

Second, in each cell we recorded the median value of IEI and the event amplitude for every event, both at baseline and during acute nicotine challenge in both age and treatment groups. We then ran a separate mixed model, three-way factorial ANOVA for each of these variables, with the main factors the treatment group (control vs. DNE), age (P1-P2 vs. P3-P5) and experimental condition (baseline vs. acute nicotine challenge). When the ANOVA was significant, we conducted post hoc

362 analyses using the Holm-Sidak multiple comparisons test, with the alpha level set at $P <$
363 0.05. We used an unpaired t-test to compare the mean peak current in control and
364 nicotine exposed cells (Table 2). We note that this approach is considerably more
365 conservative than the analysis of cumulative probability distributions, which is the most
366 popular technique for analyzing excitatory and inhibitory synaptic potentials (Henze et
367 al., 1997). This is because probability distributions typically pool all events from all cells
368 into a single distribution, which inflates the statistical power.

369 In vitro study B- hemi diaphragm-phrenic nerve preparation. Average values for
370 force loss in response to nerve stimulation, muscle stimulation and estimates of
371 neuromuscular transmission failure in control and DNE animals were compared with the
372 unpaired t-test. As above, $P < 0.05$ was the threshold for statistical significance (Table
373 2). Average data is reported as the mean ± SD throughout the manuscript.

374

375 **Results**

376 ***In vivo studies: Influence of DNE on tongue muscle EMG activity in***
377 ***response to airway obstruction***. We began our study by assessing the impact of
378 DNE on neural drive to the GG muscle of the tongue, which is innervated by XIIMNs
379 and plays a major role in keeping the upper airway open (Remmers et al., 1978). We
380 did this by challenging the system with a 15 sec nasal occlusion, initiated during the
381 expiratory period. Airway occlusion in neonates typically results in a strong increase in
382 drive to the muscles of breathing. The representative recordings in Fig. 1B show that
383 nasal occlusion is associated with a monotonic increase in the EMG activity of both the
384 diaphragm and the GG. The group average data (Fig. 1C) show that the increase in

GG EMG activity during occlusion was significantly blunted in the DNE animals, consistent with a reduced excitation of XIIMNs. In addition to the reduced amplitude of the GG EMG during occlusion, the onset latency was longer in DNE animals ($P < 0.001$), as shown in the representative recordings in Figs. 2A and 2B, with individual and mean values shown in Fig. 2C. This observation is consistent with a DNE-mediated blunting of excitatory drive to hypoglossal motoneurons during airway occlusion.

As indicated in methods, we did the nasal occlusion test in 135 neonates and noted that 12 of the 135 never recovered, and instead entered a state characterized by terminal gasping. The pattern of breathing in animals that did not recover most often approximated that shown in Fig. 3A, in that long periods of apnea were terminated following bursts of GG activity. Fig. 3A also shows that, at times, multiple bursts of GG EMG activity were required to terminate an apnea. These observations are consistent with upper airway obstruction, as the bursting pattern of GG EMG activity suggests that the XIIMNs were receiving drive from the respiratory central pattern generator but airflow was not detected. Interestingly, of the 12 neonates that entered this state of unstable breathing, nine were DNE animals (Fig. 3B). This difference in the ability to survive nasal airway occlusion was statistically significant by Chi square analysis (Chi statistic = 3.594, Z score = 1.896, $P = 0.0290$), with an adjusted odds ratio of 3.5.

In vitro study A-whole cell voltage clamp recordings of XIIMNs in medullary slice preparation.

Influence of DNE on age, weight, Vm and input resistance. The age and weight of the animals used in these experiments are shown in Table 3. The age of the animals

studied in each treatment group was the same, eliminating age bias in the results. The older animals were heavier, as expected, but there were no differences in body weight between control and DNE animals in either age group. There were also no effects of age or treatment for either Vm or input resistance (Table 3). We note that the values for input resistance are higher than values recorded with standard intracellular solutions, but are consistent with values recorded with Cs-based intracellular solutions, as used here.

Influence of DNE on the frequency and amplitude of glutamatergic sEPSCs in brain stem slices. To evaluate the influence of DNE on network level AMPA receptor-mediated synaptic events under baseline conditions, we evaluated differences in the inter-event intervals (IEIs) and the amplitude of sEPSCs in control and DNE cells. The upper traces in Fig. 4A and 4B are example recordings of glutamatergic sEPSCs from one control and one DNE cell under baseline conditions; both recordings were obtained from a P4 animal. There are no obvious differences in the traces from control and DNE cells at baseline, which is consistent with comparisons of the mean data showing no significant differences for baseline sEPSC IEI (Fig. 8A) or amplitude (Table 3) between control and DNE cells, in either of the age groups.

We then studied the influence of an acute nicotine challenge on the frequency and amplitude of sEPSCs. The tracings in Fig. 4 (recordings labeled 0.5μM Nicotine) show a modest increase in sEPSC frequency with nicotine challenge in the control cell, while the DNE cell showed little change in amplitude, but a decrease in frequency. Examination of cumulative probability curves in animals aged P1-P2 (Figs. 5A and 5B) show that the distributions shifted to the left, to lower IEIs (higher frequency) with acute

nicotine challenge in both control (P=0.049, based on 675 and 749 events at baseline and with acute nicotine challenge, respectively) and DNE cells (P<0.0001, 871 events at baseline, 1170 events with nicotine challenge). In animals aged P3-P5, nicotine challenge again shifted the cumulative probability distributions to the left in control animals (Fig. 5C; P<0.0001, based on 570 and 1271 events analyzed at baseline and during nicotine challenge, respectively). However, in the P3-P5 DNE animals acute nicotine challenge shifted the curve to the right, indicating a slowing, rather than an increase in sEPSC frequency (Fig. 5D; P=0.012, based on 678 and 501 events analyzed at baseline and during nicotine challenge, respectively).

We also analyzed the group mean data for all events by first averaging all events in each cell, followed by calculation of the grand mean value within each of the four age and treatment groups (Fig. 8A). The group mean data show that in control cells, acute nicotine challenge had no significant effect on the IEI at either age (Fig. 8A). In contrast, whereas acute nicotine challenge had no significant effect on DNE cells at ages P1-2, it was associated with an increase in sEPSC IEI in cells from animals aged P3-P5, indicating a significant decrease in the frequency of sEPSCs (P = 0.0021, Fig. 8A). In addition, the change in IEI in the DNE cells from P1-2 and P3-5 animals is significantly different (P = 0.0001, Fig. 8A). The IEI in DNE cells from P3-5 pups was also different from the IEI in cells from P3-5 control cells (P = 0.0006, Fig. 8A). As with control cells, nicotine challenge had no effect on sEPSC amplitude in cells from DNE animals at either age, and this was true whether we analyzed the entire population of events with cumulative probability analyses (data not shown), or with ANOVA based on the average data in each age and treatment group (Table 5).

454 Frequency, amplitude and rise time of glutamatergic mEPSCs. We also
455 evaluated how DNE affects the frequency, amplitude and rise time of AMPA receptor-
456 mediated mEPSCs, which represent the random, quantal release of glutamate from
457 presynaptic terminals. The upper traces in Fig. 6A and 6B are mEPSCs recorded under
458 baseline conditions, while the lower traces were recorded during acute nicotine
459 challenges. Figures 6A and 6B are from a control and DNE animal, both studied on P2.
460 Examination of cumulative probability curves in animals aged P1-P2 (Figs. 7A
461 and 7B) show that the distributions shifted to the left, to lower IEIs (higher frequency)
462 with acute nicotine challenge in both control ($P<0.0001$, based on 168 and 221 events
463 at baseline and with acute nicotine challenge, respectively) and DNE cells ($P<0.0001$,
464 169 events at baseline, 361 events with nicotine challenge). In animals aged P3-P5,
465 nicotine challenge again shifted the cumulative probability distributions to the left in
466 control animals (Fig. 7C; $P=0.004$, based on 91 and 164 events analyzed at baseline
467 and during nicotine challenge, respectively). However, in the P3-P5 DNE animals acute
468 nicotine challenge did not affect the cumulative probability distribution of mEPSCs
469 significantly (Fig. 7D; $P=0.066$, based on 186 and 202 events analyzed at baseline and
470 during nicotine challenge, respectively).
471 Analysis of the group mean data did not reveal significant differences in either
472 the frequency (Fig. 8B) or amplitude (Table 5) of mEPSCs in either control or DNE cells,
473 both at baseline and with acute nicotine challenge. Similarly, there were no significant
474 differences for mEPSC rise time either between treatment groups at baseline, or
475 between baseline and nicotine challenge within a treatment group (Table 5).

476 Influence of DNE on postsynaptic AMPA receptors. To gain a more complete
477 understanding of DNE-mediated effects on AMPA receptor function, we obtained
478 recordings from XIIMNs and measured the peak inward current that was produced by
479 bath application of 2.5 μM AMPA in the presence of AP-5, strychnine hydrochloride,
480 bicuculline methiodide, and TTX (Table 1). Figure 6A shows an example trace of the
481 inward current that results from activation of the postsynaptic AMPA receptors with bath
482 application of 2.5 μM AMPA in a control cell from a P1 animal. The average peak
483 amplitude of the inward current was the same in DNE and control cells in both age
484 groups, and there were no differences in current amplitude with age in either control
485 cells or DNE cells (Fig. 9B).
486 ***In vitro study B- Influence of DNE on diaphragm muscle contractile***
487 ***properties and the susceptibility to fatigue.*** Contractile properties (mean ± SD)
488 including peak twitch tension (Control, 2.5 ± 1.2 g; DNE 2.6 ± 1.3 g), time to peak
489 tension (Control, 50 ± 10 ms; DNE 60 ± 10 ms), and ½ relaxation time (Control, 70 ± 20
490 ms; DNE 60 ± 20 ms) were the same in diaphragm strips from control and DNE
491 animals. We note that our values for each of these variables are similar to those
492 reported in 7 day-old neonatal rats (Zhan et al., 1998). Figure 7A is an example of the
493 force decline recorded in diaphragm muscle strips following repetitive trains of phrenic
494 nerve stimulation, while Fig. 10C is an example of the protocol used to estimate the
495 contribution of neuromuscular transmission failure to the decline in force. Note that
496 direct muscle stimulation was superimposed on phrenic nerve stimulation approximately
497 every 15 sec, and the difference in force between nerve and muscle stimulation was
498 used to compute an index of neuromuscular transmission failure (see Methods). The

force loss following repetitive stimulation of muscle directly (Fig. 10D) or the phrenic nerve (Fig. 10E) was the same in control and DNE preparations. Similarly, the percentage of the force decline attributable to neuromuscular transmission failure was the same in both treatment groups (Fig. 10F). Thus, DNE does not impair the physiology of the neuromuscular junction in diaphragm muscle.

Discussion

Inadvertent obstruction of the airway (e.g., an infant's head covered by bedding) is accompanied by an increase in blood CO_2 and a decrease in O_2, leading to an increase in the release of several neurotransmitters, including ACh (Metz, 1966). The increased release of excitatory neurotransmitters helps support the respiratory motor response to chemoreceptor stimulation. Here, we found that nicotine exposed neonatal rats had a delayed and blunted genioglossus muscle motor response to nasal airway occlusion, consistent with an attenuation of excitatory drive to XIIMNs under these conditions. We then used an *in vitro* approach to examine excitatory synaptic input to XIIMNs both under baseline conditions and in response to an acute nicotine challenge, which mimics the stimulation of nAChRs that accompanies the release of endogenous ACh, or an increase in central nicotine levels secondary to tobacco smoking or the use of nicotine delivery devices. The key finding is that in 3-5-day old animals, an acute nicotine challenge evoked a marked increase in excitatory synaptic input to XIIMNs in control animals, while the frequency decreased in the DNE pups. Together with previous work showing that nicotine challenge increased GABAergic inhibitory input to XIIMNs (Neff et al., 2003; Jaiswal et al., 2016; Wollman et al., 2018a), these

observations suggest that DNE shifts the excitatory-inhibitory balance in XIIMNs towards inhibition in response to a nicotine challenge. This is a deviation from the normal response of neural systems, wherein neuronal spiking activity is maintained within homeostatic limits by careful adjustments in the balance of excitatory and inhibitory synaptic inputs to a neuron or network of neurons (Turrigiano, 2012; Wenner, 2014). Below, we discuss how alterations in nAChR function and/or altered development of XIIMNs may underlie the observation that DNE blunts the genioglossus motor response to nasal occlusion and reduces the likelihood of surviving an occlusion.

DNE blunts the genioglossus motor response to airway obstruction. Previous work has mainly utilized in vitro techniques to evaluate how DNE alters neurotransmission and the development of hypoglossal motoneurons, which innervate the tongue muscles. In contrast, we are unaware of any studies on the influence of DNE on the control of tongue muscle activity in-vivo. Here, we show that DNE blunts the magnitude and delays the activation of the GG EMG during imposed nasal occlusions. Although we are unable to establish the mechanisms underlying these observations, there are clues from previous work. First, nasal occlusion prevents lung inflation, and the absence of inflation releases hypoglossal motoneurons from inhibition, leading to increased GG activity, at least in adult rodents (Bailey et al., 2001). Second, the previously described reduction in chemosensitivity with DNE in neonatal rodents (St-John and Leiter, 1999; Fewell et al., 2001; Simakajornboon et al., 2004; Hafstrom et al., 2005; Eugenin et al., 2008; Mahliere et al., 2008; Huang et al., 2010) may explain the reduced GG activity during nasal occlusion, as combined hypoxia and hypercapnia become progressively more severe as the occlusion continues and DNE animals are

not as well equipped to initiate chemoreceptor-mediated activation of hypoglossal motoneurons. The mechanism of the blunted ventilatory response to chemoreceptor stimulation in nicotine-exposed animals is unknown but could be due to effects on peripheral and central chemoreceptor signaling, and/or a reduced respiratory motor response to the chemoreceptor afferent input. Third, in vitro studies show that DNE is associated with various disruptions in the growth, intrinsic membrane properties and synaptic inputs to hypoglossal motoneurons (Robinson et al., 2002; Pilarski et al., 2011; Jaiswal et al., 2013; Powell et al., 2016; Vivekanandarajah et al., 2016). It is likely that each of the factors contribute in some way to the blunted GG response to nasal occlusion in nicotine exposed pups.

We also showed that the majority of pups that failed to recover from nasal occlusion were nicotine exposed. Animals that were used to obtain the main experimental data sets had to meet certain criteria in order for their responses to be analyzed. Criteria included reestablishing inspiratory flow, tidal volume, breathing frequency and EMG activity to baseline levels following nasal occlusions. One hundred and twenty-five animals met these criteria, while 12 did not. Of great interest is that 9 out of the 12 animals that failed to recover were nicotine exposed. These findings suggest that DNE may increase the probability that a neonate experiencing an airway occlusion will fail to reestablish a normal breathing pattern, leading to hypoxia and acidosis. An important caveat that envelopes ventilatory measures in neonatal animals is that the stress of maternal separation may interact with DNE and contribute to the altered GG EMG responses observed. Indeed, impaired volitional motor control in DNE animals is exacerbated by maternal separation (Bassey and Gondre-Lewis, 2019).

568 *Influence of DNE on AMPA receptor-mediated glutamatergic neurotransmission.*

569 Surprisingly, recordings done under baseline conditions did not reveal
570 differences in the frequency or amplitude of sEPSCs or mEPSCs between control cells
571 and DNE cells at either age. We also assessed postsynaptic receptor function with bath
572 application of AMPA in synaptically isolated neurons. There were no differences in peak
573 AMPA current in control or DNE cells in either age group, and there were no age-
574 dependent changes within a treatment group for any of these variables. We note that
575 this approach stimulates both synaptic and extra-synaptic AMPA receptors which may
576 respond differently to DNE. Nevertheless, when taken together with the data showing
577 no change in mESPC amplitude with DNE, the findings indicate that under baseline
578 conditions, DNE does not alter glutamatergic synaptic input to XIIMNs, or the
579 postsynaptic response to AMPA.

580 An important caveat underlying these observations is that previous work showed
581 that the frequency of AMPA receptor-mediated glutamatergic sEPSCs was lower in
582 XIIMNs from DNE cells compared to control cells (Pilarski et al., 2011). However, the
583 latter experiments were done for a different purpose, with recordings made in the setting
584 of elevated extracellular potassium (9 mM) in thick brainstem slices. Moreover,
585 because the cells were bursting under these conditions, the excitatory inputs could only
586 be discerned in the interburst interval, they were not isolated pharmacologically (i.e.,
587 inhibitory transmission was intact) and the response to acute nicotine challenge was not
588 examined. Accordingly, the present results extend these earlier findings.

589 In many physiologic systems underlying pathology is not obvious under baseline
590 conditions but can be revealed when the system is stimulated. It is well known that

591 stimulation of nAChRs on glutamatergic neurons leads to increased release of
592 glutamate (Wonnacott et al., 1990; Wonnacott, 1997; Gentry and Lukas, 2002).
593 Importantly, chronic nicotine exposure is associated with both an upregulation and long-
594 term desnensitization of nAChRs in many neuron types (Wonnacott, 1990; Gentry and
595 Lukas, 2002), including XIIMNs (Pilarski et al., 2012); (Wollman et al., 2016).
596 Accordingly, we also studied glutamatergic synaptic transmission while nAChRs were
597 activated with an acute nicotine challenge. Whereas nicotinic stimulation of
598 glutamatergic synaptic transmission was the same in control and DNE pups studied on
599 P1 and P2, there were significant treatment effects in P3-P5 animals. In P3-P5 control
600 animals, acute nicotine challenge increased the frequency of both sEPSCs and
601 mEPSCs, while the frequency of sEPSCs decreased in the DNE animals. Importantly,
602 the stimulation of mEPSCs by acute nicotine challenge in control preparations indicates
603 that nicotine is likely acting on the presynaptic terminals of glutamatergic neurons that
604 impinge upon the motoneurons. The stimulation of sEPSC frequency could involve
605 other sites of nicotine's action, including the soma or dendrites of glutamatergic neurons
606 or neurons presynaptic to them. Nicotine challenge did not affect the mEPSC frequency
607 of P3-5 DNE preparations. Thus, the stimulatory effect on axon terminals at earlier
608 stages may be blunted and/or the reduction in sEPSC frequency at stages P3-5 reflects
609 sites of action other than the presynaptic terminals. Although the mechanisms
610 responsible for the qualitative change in nicotinic control of glutamatergic synaptic
611 transmission in P3-5 animals is unknown, we hypothesize that this is part of the
612 compensatory response to the increased excitability in XIIMNs from nicotine exposed
613 animals (Pilarski et al., 2011; Jaiswal et al., 2013). The 3-5-day delay may reflect the

614 time needed to establish compensatory mechanisms after birth, which is accompanied
615 by a sudden demand for tongue muscle activation to support suckling, licking and
616 swallowing (Thiels et al., 1990). Strategies that neural systems use to compensate for
617 increased excitability could include reducing dendrite volume, increasing pre and/or
618 postsynaptic inhibitory input, or reducing excitatory input. There is evidence that all
619 three adjustments occur in XIIMNs from DNE animals. First, the dendritic tree in XIIMNs
620 from DNE animals is larger and more complex than that of controls on P1-2, but is
621 smaller and less complex by P3-4 (Powell et al., 2016), indicating changes in the timing
622 of growth and pruning of XIIMNs due to DNE. Similarly, DNE may alter the growth and
623 pruning of specific populations of glutamatergic neurons in this region, which could
624 result in altered responses to acute nicotine challenge as seen here. This is supported
625 by work showing that neuronal development is dependent on the trophic effects of ACh,
626 both in utero and into adolescence, and disruption of nicotinic cholinergic signaling in
627 this developmental window alters brain morphology and function, leading to behavioral
628 abnormalities in both humans and animal models (Slotkin et al., 1987; Navarro et al.,
629 1989; Slotkin, 2004). Additionally, changes in the frequency of inhibitory inputs to
630 XIIMNs are known to occur with DNE (Wollman et al., 2018a, b), and DNE is associated
631 with an increase in the postsynaptic response to muscimol in XIIMNs (Wollman et al.,
632 2018a), consistent with an increase in GABA receptor expression (Jaiswal et al., 2016).
633 Other possibilities include inhibitory effects of nAChR activation, activation of GABAB
634 receptors, which were not blocked, or perhaps nicotine-mediated effects on other
635 neuromodulators that inhibit glutamatergic inputs to XII motoneurons, such as serotonin
636 (Singer et al., 1996).

637 Influence of DNE on the balance of excitatory and inhibitory inputs to XIIMNs. A
638 noteworthy finding from these experiments is that the frequency of glutamatergic
639 sEPSCs invading XIIMNs is lower than the frequency of spontaneous inhibitory
640 postsynaptic currents that have been observed in these cells in previous experiments.
641 For example, glutamatergic sEPSCs in XIIMNs at baseline had an average IEI of 1500
642 ms, while GABAergic inputs to XIIMNs had an average IEI of 400 ms under baseline
643 conditions (Wollman et al., 2018a). These differences carry two important implications.
644 First, the data suggest that XIIMNs are under significant inhibition at rest. And second,
645 it is possible that only small changes in the frequency of glutamatergic inputs have large
646 physiological effects. This could also indicate that the strength of excitatory inputs may
647 be much more relevant during periods of high activity than at rest, and therefore the
648 DNE mediated changes may become more pronounced under conditions where
649 excitatory drive is increased. Interestingly, the reduction in glutamatergic input that is
650 evoked by nicotine challenge in stages P3-5 cannot be due to enhanced GABAergic or
651 glycinergic inhibition because these were blocked in the current experiments. While we
652 can only speculate, it is possible that nicotine evoked a distinct inhibitory effect, either
653 by direct action or by causing the release of inhibitory neuromodulators (Singer et al.,
654 1996; Maggi et al., 2004).
655 DNE and the integrity of the neuromuscular junction. Adult mammals are said to
656 have a high “safety factor” at the neuromuscular junction, which refers to the ability of
657 neuromuscular transmission to remain effective even under stressful conditions such
658 fatiguing contractions which can occur with elevations in airway resistance or sustained
659 hyperpnea. It is believed that the safety factor is a presynaptic phenomenon, such that

660 the amount of ACh released per nerve impulse exceeds the quantity needed to activate
661 nAChRs and depolarize muscle. However, neuromuscular transmission includes
662 postsynaptic mechanisms as well, and it is possible that DNE alters the function of
663 nAChRs at the neuromuscular junction. Moreover, there is evidence that the safety
664 factor in neonates is lower than in adults due to a relatively low quantal content (Kelly,
665 1978) and more axonal branch point failure (Fournier et al., 1991). Accordingly, we
666 examined the integrity of the neuromuscular junction in control and DNE neonates by
667 stimulating the phrenic nerve repetitively, with periodic superimposition of direct
668 stimulation of muscle fibers. Comparison of the force evoked by phrenic nerve and/or
669 direct stimulation of the muscle fibers showed that DNE had no influence on
670 neuromuscular transmission.

671 An important caveat is that we used the diaphragm muscle for these
672 experiments, rather than the tongue muscles, which are innervated by XIIMNs. Pilot
673 studies showed that measuring force in excised tongue muscles from such small
674 animals is difficult and we were not confident that the measured force was accurate or
675 reproducible. The phrenic nerve-diaphragm preparation did not present these
676 challenges, which explains why it has been an archetypal model for studying the
677 neuromuscular junction.

678 Functional Significance. The consensus explanation for sudden infant death
679 (SIDS) and Apparent Life-Threatening Events (ALTE) is the triple risk hypothesis
680 (Filiano and Kinney, 1994), which is based on the convergence of 1) a vulnerable
681 neonate (typically due to risks established in utero); 2) a critical developmental period;
682 3) and an exogenous stressor. Maternal smoking is indeed the number one risk factor

683 for SIDS and apparent life threating events in neonates, but the lack of a suitable animal
684 model to probe the mechanisms underlying this association has been elusive. It is
685 noteworthy that SIDS risk is highest in infants aged 4-5 months, while it has been
686 suggested the first two weeks of life in the rat corresponds to 28-40 weeks of gestation
687 in humans (Dwyer et al., 2009), so although the model mimics the triple risk model of
688 SIDS, the developmental discrepancies between rodents and humans make an exact
689 comparison impossible. Nevertheless, we propose that our animal model replicates, at
690 least in part, the triple risk model. Specifically, DNE results in a decrease in nicotine-
691 mediated glutamatergic drive to XIIMNs (vulnerability) at P3-5, but not P1-2 (critical
692 developmental period), and when we imposed airway occlusion (external stressor), the
693 DNE animals had a markedly blunted genioglossus muscle motor response and were
694 less likely to survive the challenge. In summary, the combination of in vitro and in vivo
695 models used here has proven useful in the quest to understand how, and to what
696 extent, maternal smoking/nicotine exposure impairs the function of hypoglossal
697 motoneurons and protective respiratory reflexes.
698

Bibliography

Aldrich TK, Shander A, Chaudhry I, Nagashima H (1986) Fatigue of isolated rat diaphragm: role of impaired neuromuscular transmission. J Appl Physiol (1985) 61:1077-1083.

Bailey EF, Janssen PL, Fregosi RF (2005) PO2-dependent changes in intrinsic and extrinsic tongue muscle activities in the rat. Am J Respir Crit Care Med 171:1403-1407.

Bailey EF, Jones CL, Reeder JC, Fuller DD, Fregosi RF (2001) Effect of pulmonary stretch receptor feedback and CO(2) on upper airway and respiratory pump muscle activity in the rat. J Physiol 532:525-534.

Bassey RB, Gondre-Lewis MC (2019) Combined early life stressors: Prenatal nicotine and maternal deprivation interact to influence affective and drug seeking behavioral phenotypes in rats. Behav Brain Res 359:814-822.

Berger AJ (2011) Development of synaptic transmission to respiratory motoneurons. Respir Physiol Neurobiol 179:34-42.

Berlin I, Heilbronner C, Georgieu S, Meier C, Spreux-Varoquaux O (2010) Newborns' cord blood plasma cotinine concentrations are similar to that of their delivering smoking mothers. Drug Alcohol Depend 107:250-252.

Dwyer JB, McQuown SC, Leslie FM (2009) The dynamic effects of nicotine on the developing brain. Pharmacol Ther 122:125-139.

Eugenin J, Otarola M, Bravo E, Coddou C, Cerpa V, Reyes-Parada M, Llona I, von Bernhardi R (2008) Prenatal to early postnatal nicotine exposure impairs central chemoreception and modifies breathing pattern in mouse neonates: a probable link to sudden infant death syndrome. J Neurosci 28:13907-13917.

Ferng J, Fregosi RF (2015) Influence of developmental nicotine exposure on the ventilatory and metabolic response to hyperthermia. J Physiol 593:5201-5213.

Fewell JE, Smith FG, Ng VK (2001) Prenatal exposure to nicotine impairs protective responses of rat pups to hypoxia in an age-dependent manner. Respir Physiol 127:61-73.

Filiano JJ, Kinney HC (1994) A perspective on neuropathologic findings in victims of the sudden infant death syndrome: the triple-risk model. Biol Neonate 65:194-197.

Fournier M, Alula M, Sieck GC (1991) Neuromuscular transmission failure during postnatal development. Neurosci Lett 125:34-36.

Gentry CL, Lukas RJ (2002) Regulation of nicotinic acetylcholine receptor numbers and function by chronic nicotine exposure. Curr Drug Targets CNS Neurol Disord 1:359-385.

Hafstrom O, Milerad J, Sandberg KL, Sundell HW (2005) Cardiorespiratory effects of nicotine exposure during development. Respir Physiol Neurobiol 149:325-341.

Henze DA, Card JP, Barrionuevo G, Ben-Ari Y (1997) Large amplitude miniature excitatory postsynaptic currents in hippocampal CA3 pyramidal neurons are of mossy fiber origin. J Neurophysiol 77:1075-1086.

Huang YH, Brown AR, Cross SJ, Cruz J, Rice A, Jaiswal S, Fregosi RF (2010) Influence of prenatal nicotine exposure on development of the ventilatory response to hypoxia and hypercapnia in neonatal rats. J Appl Physiol (1985) 109:149-158.

Huckstepp RT, Llaudet E, Gourine AV (2016) CO2-Induced ATP-Dependent Release of Acetylcholine on the Ventral Surface of the Medulla Oblongata. PLoS One 11:e0167861.

744 Jaiswal SJ, Pilarski JQ, Harrison CM, Fregosi RF (2013) Developmental nicotine exposure
745 alters AMPA neurotransmission in the hypoglossal motor nucleus and pre-Botzinger
746 complex of neonatal rats. J Neurosci 33:2616-2625.
747 Jaiswal SJ, Wollman LB, Harrison CM, Pilarski JQ, Fregosi RF (2016) Developmental nicotine
748 exposure enhances inhibitory synaptic transmission in motor neurons and
749 interneurons critical for normal breathing. Dev Neurobiol 76:337-354.
750 Kahn A, Groswasser J, Sottiaux M, Kelmanson I, Rebuffat E, Franco P, Dramaix M,
751 Wayenberg JL (1994) Prenatal exposure to cigarettes in infants with obstructive
752 sleep apneas. Pediatrics 93:778-783.
753 Kelly SS (1978) The effect of age on neuromuscular transmission. J Physiol 274:51-62.
754 Lavezzi AM, Corna M, Mingrone R, Matturri L (2010) Study of the human hypoglossal
755 nucleus: normal development and morpho-functional alterations in sudden
756 unexplained late fetal and infant death. Brain Dev 32:275-284.
757 Lowe AA (1980) The neural regulation of tongue movements. Prog Neurobiol 15:295-344.
758 Maggi L, Sola E, Minneci F, Le Magueresse C, Changeux JP, Cherubini E (2004) Persistent
759 decrease in synaptic efficacy induced by nicotine at Schaffer collateral-CA1 synapses
760 in the immature rat hippocampus. J Physiol 559:863-874.
761 Mahliere S, Perrin D, Peyronnet J, Boussouar A, Annat G, Viale JP, Pequignot J, Pequignot JM,
762 Dalmaz Y (2008) Prenatal nicotine alters maturation of breathing and neural
763 circuits regulating respiratory control. Respir Physiol Neurobiol 162:32-40.
764 Metz B (1966) Hypercapnia and acetylcholine release from the cerebral cortex and
765 medulla. J Physiol 186:321-332.
766 Mortola JP (1984) Breathing pattern in newborns. J Appl Physiol 56:1533-1540.
767 Mortola JP (2004) Implications of hypoxic hypometabolism during mammalian
768 ontogenesis. Respir Physiol Neurobiol 141:345-356.
769 Mortola JP, Tenney SM (1986) Effects of hyperoxia on ventilatory and metabolic rates of
770 newborn mice. Respiration physiology 63:267-274.
771 Navarro HA, Seidler FJ, Schwartz RD, Baker FE, Dobbins SS, Slotkin TA (1989) Prenatal
772 exposure to nicotine impairs nervous system development at a dose which does not
773 affect viability or growth. Brain Res Bull 23:187-192.
774 Neff RA, Wang J, Baxi S, Evans C, Mendelowitz D (2003) Respiratory sinus arrhythmia:
775 endogenous activation of nicotinic receptors mediates respiratory modulation of
776 brainstem cardioinhibitory parasympathetic neurons. Circ Res 93:565-572.
777 Ottaviani G, Matturri L, Mingrone R, Lavezzi AM (2006) Hypoplasia and neuronal
778 immaturity of the hypoglossal nucleus in sudden infant death. J Clin Pathol 59:497-
779 500.
780 Pilarski JQ, Wakefield HE, Fuglevand AJ, Levine RB, Fregosi RF (2011) Developmental
781 nicotine exposure alters neurotransmission and excitability in hypoglossal
782 motoneurons. J Neurophysiol 105:423-433.
783 Pilarski JQ, Wakefield HE, Fuglevand AJ, Levine RB, Fregosi RF (2012) Increased nicotinic
784 receptor desensitization in hypoglossal motor neurons following chronic
785 developmental nicotine exposure. J Neurophysiol 107:257-264.
786 Powell GL, Gaddy J, Xu F, Fregosi RF, Levine RB (2016) Developmental nicotine exposure
787 disrupts dendritic arborization patterns of hypoglossal motoneurons in the neonatal
788 rat. Dev Neurobiol 76:1125-1137.

789 Remmers JE, deGroot WJ, Sauerland EK, Anch AM (1978) Pathogenesis of upper airway
790 occlusion during sleep. J Appl Physiol Respir Environ Exerc Physiol 44:931-938.
791 Rice A, Fuglevand AJ, Laine CM, Fregosi RF (2011) Synchronization of presynaptic input to
792 motor units of tongue, inspiratory intercostal, and diaphragm muscles. J
793 Neurophysiol 105:2330-2336.
794 Robinson DM, Peebles KC, Kwok H, Adams BM, Clarke LL, Woollard GA, Funk GD (2002)
795 Prenatal nicotine exposure increases apnoea and reduces nicotinic potentiation of
796 hypoglossal inspiratory output in mice. J Physiol 538:957-973.
797 Sant'Anna GM, Mortola JP (2003) Thermal and respiratory control in young rats exposed to
798 cold during postnatal development. Comparative biochemistry and physiology Part
799 A, Molecular & integrative physiology 134:449-459.
800 Semple BD, Blomgren K, Gimlin K, Ferriero DM, Noble-Haeusslein LJ (2013) Brain
801 development in rodents and humans: Identifying benchmarks of maturation and
802 vulnerability to injury across species. Prog Neurobiol 106-107:1-16.
803 Simakajornboon N, Vlasic V, Li H, Sawnani H (2004) Effect of prenatal nicotine exposure on
804 biphasic hypoxic ventilatory response and protein kinase C expression in caudal
805 brain stem of developing rats. J Appl Physiol (1985) 96:2213-2219.
806 Singer JH, Bellingham MC, Berger AJ (1996) Presynaptic inhibition of glutamatergic
807 synaptic transmission to rat motoneurons by serotonin. J Neurophysiol 76:799-807.
808 Slotkin TA (2004) Cholinergic systems in brain development and disruption by
809 neurotoxicants: nicotine, environmental tobacco smoke, organophosphates. Toxicol
810 Appl Pharmacol 198:132-151.
811 Slotkin TA, Orband-Miller L, Queen KL (1987) Development of [3H]nicotine binding sites in
812 brain regions of rats exposed to nicotine prenatally via maternal injections or
813 infusions. J Pharmacol Exp Ther 242:232-237.
814 St-John WM, Leiter JC (1999) Maternal nicotine depresses eupneic ventilation of neonatal
815 rats. Neurosci Lett 267:206-208.
816 Thiels E, Alberts JR, Cramer CP (1990) Weaning in rats: II. Pup behavior patterns. Dev
817 Psychobiol 23:495-510.
818 Turrigiano G (2012) Homeostatic synaptic plasticity: local and global mechanisms for
819 stabilizing neuronal function. Cold Spring Harb Perspect Biol 4:a005736.
820 Vivekanandarajah A, Chan YL, Chen H, Machaalani R (2016) Prenatal cigarette smoke
821 exposure effects on apoptotic and nicotinic acetylcholine receptor expression in the
822 infant mouse brainstem. Neurotoxicology 53:53-63.
823 Wenner P (2014) Homeostatic synaptic plasticity in developing spinal networks driven by
824 excitatory GABAergic currents. Neuropharmacology 78:55-62.
825 Wollman LB, Levine RB, Fregosi RF (2018a) Developmental plasticity of GABAergic
826 neurotransmission to brainstem motoneurons. J Physiol 596:5993-6008.
827 Wollman LB, Levine RB, Fregosi RF (2018b) Developmental nicotine exposure alters
828 glycinergic neurotransmission to hypoglossal motoneurons in neonatal rats. J
829 Neurophysiol 120:1135-1142.
830 Wollman LB, Haggerty J, Pilarski JQ, Levine RB, Fregosi RF (2016) Developmental nicotine
831 exposure alters cholinergic control of respiratory frequency in neonatal rats. Dev
832 Neurobiol 76:1138-1149.
833 Wonnacott S (1990) The paradox of nicotinic acetylcholine receptor upregulation by
834 nicotine. Trends Pharmacol Sci 11:216-219.

Wonnacott S (1997) Presynaptic nicotinic ACh receptors. Trends Neurosci 20:92-98.
Wonnacott S, Sidhpura N, Balfour DJ (2005) Nicotine: from molecular mechanisms to behaviour. Curr Opin Pharmacol 5:53-59.
Wonnacott S, Drasdo A, Sanderson E, Rowell P (1990) Presynaptic nicotinic receptors and the modulation of transmitter release. Ciba Found Symp 152:87-101; discussion 102-105.
Wood SJ, Slater CR (2001) Safety factor at the neuromuscular junction. Prog Neurobiol 64:393-429.
Zhan WZ, Watchko JF, Prakash YS, Sieck GC (1998) Isotonic contractile and fatigue properties of developing rat diaphragm muscle. J Appl Physiol (1985) 84:1260-1268.

Figure and Table legends

Table 1. List of drug cocktails used to isolate AMPA receptor-mediated glutamatergic EPSCs and postsynaptic AMPA receptors, with the number of cells used in each condition. The number of cells studied in each experiment are also shown.

Table 2. Statistical tests and significance threshold used for each experiment in each experimental series.

Table 3. Age, weight, resting membrane potential (mV), and input resistance (Rin) of XIIMNs from control and DNE animals. Values are mean ± SD. There were no differences in any of these variables between control and DNE cells. The only significant differences between P1-P2 and P3-P5 *within* a treatment group was body weight, as expected. ***, $P<0.001$, P1-P2 vs. P3-P5 within a treatment group.

Table 4. Number of sEPSCs and mEPSCs recorded per neuron at baseline and during acute nicotine application. Values are mean ± SD.

Table 5. Mean values for amplitude of glutamatergic sEPSCs, mEPSCs and mEPSC rise time at baseline and during acute nicotine challenge. There were no significant differences in any of these variables.

Figure 1. **In vivo experimental model and EMG response to nasal occlusion.** *Panel A*. Schematic rendition of the in vivo experimental preparation, which combines head-out plethysmography and genioglossus EMG recordings in lightly anesthetized neonatal rats, as described in Methods. *Panel B*. Example trace showing diaphragm and genioglossus EMG and integrated EMG along with volume and flow traces obtained from the plethysmograph. After 5 minutes of uninterrupted baseline recordings, a 10- 15 second nasal occlusion was administered (*rectangle)*. *Panel C*. Genioglossus (GG) EMG amplitude during the nasal occlusion. The duration of the nasal occlusion was normalized by dividing the total duration of each occlusion into equal 20%-time bins. EMG amplitude was normalized as a percent of the largest burst recorded (see Methods). All animals in both groups showed increased GG burst amplitude as the nasal occlusion progressed. Post hoc analysis following 2-way ANOVA revealed that DNE animals had a significantly blunted amplitude response compared to control at the 40%, 60%- and 80%-time bins. *, P< 0.05, **P< 0.01, ***, P< 0.001.

Figure 2. Elapsed time between the onset of nasal occlusion to first discernible genioglossus muscle EMG burst, defined as onset latency. *Panel A* shows recordings from representative control and DNE pups, as indicated. The length of the blue line under the EMG tracing represents the latency, which is prolonged in the DNE animal. following the onset of nasal occlusion. *Panel B* shows the onset latency in 9 control and DNE pups. The horizontal lines represent the mean value. An unpaired t-test revealed a significant difference between the groups (***, P< 0.001).

Figure 3. Failure to recover from nasal occlusion. *Panel A*. An example tracing of one of the 12 animals that exhibited continuous breathing difficulties post

886 nasal occlusion, as explained in Results. *Panel B*. The number of animals in each
887 treatment group that either recovered from nasal occlusion or failed to recover. Note
888 that of the 12 animals that failed to recover, 9 were from the DNE group, which is a
889 significant difference by Chi square analysis (P = 0.0290).

890 **Figure 4. Example traces of AMPA receptor-mediated sEPSCs recorded**
891 **from XIIMNs.** Panels A and B show representative traces of pharmacologically isolated
892 sEPSCs from a control animal (Panel A) and a DNE animal (Panel B), both studied on
893 P4. Each trace shows the entire three-minute recording period at baseline (upper trace
894 in each panel) and during acute nicotine application (bottom trace in each panel). Inset
895 panels are an expanded view, showing 10 seconds of the recording at the end of each
896 trace. Note that the size and frequency of events at baseline is similar in the control and
897 DNE animals. In contrast, with acute nicotine challenge (bottom trace in each panel) the
898 DNE cell shows a decrease in sEPSC frequency, whereas there is a modest increase in
899 the control cell.

900 **Figure 5. DNE alters the modulation of glutamatergic sEPSCs in response**
901 **to an acute nicotine challenge, but only in cells from pups aged P3-P5.**
902 Cumulative probability distributions of glutamatergic sEPSC IEIs in control and DNE
903 cells, at baseline and during acute nicotine challenge. At P1-2 (**Panels A and B)**, acute
904 nicotine challenge with 0.5 μM nicotine (black dashed lines) caused a left shift, toward
905 shorter IEIs, of glutamatergic sEPSCs in both control and DNE cells (Control, P=0.049,
906 **Panel A;** DNE, P<0.0001, **Panel B**). In cells from P3-P5 animals **(Panels C and D)**,
907 acute nicotine challenge caused a left shift, toward shorter IEIs in control cells
908 (P<0.0001, **Panel C**), the distribution shifted to the right, towards longer IEIs in the DNE

cells (P=0.012, **Panel D**). Arrows indicate the direction of the shift with acute nicotine challenge and indicates significant differences with K-S test **(See Methods)**. Dotted gray lines indicate the 95% confidence intervals of each curve.

Figure 6. Influence of DNE on AMPA receptor-mediated mEPSCs recorded from XIIMNs. Panels A and B show representative traces of pharmacologically isolated mEPSCs from a control animal (Panel A) and a DNE animal (Panel B) studied on P4. Each trace shows the entire three minutes of recording at baseline (top trace) and during acute nicotine application (bottom trace). Inset panels show an expanded view of 10 seconds at the end of each trace, as in Fig. 4 Note that the size and frequency of events is similar in the control and DNE cell at baseline. However, during acute nicotine challenge, mEPSC frequency increased in control cells but not in DNE cells.

Figure 7. Cumulative probability distributions of glutamatergic mEPSC IEIs in control and DNE cells, at baseline and during acute nicotine challenge. In cells from animals aged P1-2 (**Panels A and B)**, acute nicotine challenge with 0.5 μM nicotine (black dashed lines) caused a left shift, toward shorter IEIs, of glutamatergic mEPSCs in both control (P<0.0001, **Panel A**) and DNE cells (P<0.0001, **Panel B**). In cells from animals aged P3-5 **(Panels C and D)**, acute nicotine challenge caused a significant left shift of glutamatergic mEPSCs in control cells (P=0.004, **Panel C**), but there was no change in the distribution of IEIs in DNE cells (P=0.066, **Panel D**). Arrows indicate the direction of the shift with acute nicotine challenge and indicates significant differences with K-S test **(See Methods)**. Dotted gray lines indicate the 95% confidence intervals of each curve.

Figure 8. Individual and average IEI of sEPSCs and mEPSCs at baseline and during acute nicotine challenge. Panel A shows the individual and average IEI of AMPA receptor-mediated sEPSCs under baseline conditions and following acute nicotine challenge. Note that in control cells (circles, left of the vertical line) there was a slight though non-significant decline in IEI (i.e., an increase in frequency) on both P1-P2 (filled circles) and P3-P5 (open circles). The IEI in response to an acute nicotine challenge in cells from DNE animals was age-dependent (squares, right of the vertical line). Note that on P1-P2, nicotine challenge decreased the IEI, though as in controls this trend was not significant. Surprisingly, on P3-P4 acute nicotine challenge significantly increased the IEI ($P = 0.0021$). Moreover, the mean value for IEI in P3-5 DNE cells during acute nicotine challenge is significantly different than corresponding data in P1-2 DNE cells ($P = 0.0001$), and in P3-5 cells from control animals. **Panel B** shows the individual and average IEI of AMPA receptor-mediated mEPSCs under baseline conditions and following acute nicotine challenge. Note that in the P1-2 group, both control and DNE cells show a trend toward a decrease in IEI with acute nicotine challenge, however this was not significant. Analysis of average IEI from control and DNE neurons at P3-5 shows no differences at baseline and no change in frequency with acute nicotine challenge.

Figure 9. Activation of postsynaptic AMPA receptors in control cells and DNE cells. Panel A shows a representative trace of the AMPA receptor mediated inward current in XIIMNs from a control animal at P1**. Panel B** shows individual values for the peak inward current in response to bath application of AMPA. Mean values within each treatment group are indicated by the horizontal lines. There were no

differences in the magnitude of the postsynaptic inward current either within or between treatment groups.

Figure 10. Influence of DNE on diaphragm muscle fatigue and neuromuscular transmission failure during repetitive muscle or phrenic nerve stimulation in diaphragm phrenic nerve-muscle strips. Panel A shows representative force recording over a 5-minute period of direct muscle stimulation. Fatigue was quantified as the % of the maximum force remaining at the end of the 5-minute stimulation period (see Methods). **Panel B** shows the muscle twitch produced by a single stimulus pulse, and an expanded view of the muscle force produced by a 330 ms train of stimulation pulses, as described in Methods. An identical protocol was used when phrenic nerve stimulation was used to assess the magnitude of force decline. **Panel C** shows representative force recording during 5 minutes of repeated phrenic nerve stimulation with superimposed direct muscle stimulation every 15 sec. Neuromuscular transmission failure (NTF) was calculated as: Panels D and E show the percent force decline in each control and DNE preparation subjected to either muscle (Panel D) or phrenic nerve (Panel E) stimulation. Panel F shows the percent neuromuscular transmission failure in control and DNE preparations. Horizontal lines in Panels D, E and F indicate the mean. There were no significant differences in the percent force loss between control and DNE preparations with either muscle or nerve stimulation, or the force loss due to neuromuscular transmission failure.

TABLE 1. List of drug cocktails used to isolate AMPA receptor-mediated glutamatergic EPSCs and postsynaptic AMPA receptors, with the number of cells used in each condition. The number of cells studied in each experiment are also shown.

Experiments and drugs used	*Age*	*# Cells (Control:DNE)*
Glutamate sEPSCs 50 μM AP-5, 10 μM bicuculline, 0.4 μM strychnine + 0.5 μM nicotine	P1-2 P3-5	6:6 6:6
Glutamate mEPSCs 50 μM AP-5, 10 μM bicuculline, 0.4 μM strychnine, 1 μM tetrodotoxin (TTX) + 0.5 μM nicotine	P1-2 P3-5	6:6 6:6
Postsynaptic receptors 50 μM AP-5, 10 μM bicuculline, 0.4 μM strychnine, 1 μM TTX + 2.5 μM AMPA	P1-2 P3-5	6:6 6:6

TABLE 2. Statistical tests and significance threshold used for each experiment in each experimental series.

Experiment	*Statistical test, post hoc*	*Significance Threshold*
In vivo series 1. Changes to EMG during nasal occlusion	Two-way mixed-model ANOVA with Tukey's post-hoc analysis	$P < 0.05$
2. Differences in EMG onset latency	Unpaired Student's t-test	$P < 0.05$

3. Autoresuscitation	Chi Squared analysis	$P < 0.05$
In vitro series A 1. Differences in baseline parameters	Two-way mixed model ANOVA with Tukey's post-hoc analysis	$P < 0.05$
2. Differences in IEI and amplitude of EPSCs	Kolomogorov-Smirnoff test of cumulative probability distributions, and Three-way mixed model ANOVA with the Holm-Sidak post-hoc analysis	$P < 0.05$
3. Differences in peak whole cell current	Unpaired Student's t-test	$P < 0.05$
In vitro series B 1. Differences in nerve stimulation, muscle stimulation, and estimates of neuromuscular transmission failure	Unpaired Student's t-test	$P < 0.05$

TABLE 3. Age, weight, resting membrane potential (mV), and input resistance (Rin) of XIIMNs from control and DNE animals.

	Control	*DNE*	*P Value*	*n (Control:DNE)*
Age (days)				
P1-2	1.7±0.2	1.4±0.1	P= n/s	18:18
P3-5	3.4±0.1	3.7±0.2	P= n/s	18:18
Weight (grams)				
P1-2	7.72±0.31	7.4±0.1	P= n/s	18:18
P3-5	10.53±0.44***	10.8±0.3***	P= n/s	18:18
Vm				
P1-2	-49±3	-48±2	P= n/s	18:18
P3-5	-48±2	-47±2	P= n/s	18:18
Rin (MΩ)				
P1-2	221±49	184±23	P= n/s	4:4
P3-5	185±41	189±42	P= n/s	4:4

Table 4. Number of sEPSCs and mEPSCs recorded per neuron at baseline and during acute nicotine application. Values are mean ± SD.

sEPSCs

P1-2		

Baseline	65±23	91±38
Acute nicotine	74±59	187±105
P3-4		
Baseline	145±107	116±84
Acute nicotine	211±211	81±60

mESPCs

P1-2		
Baseline	28±14	28±22
Acute nicotine	38±20.8	61±39
P3-4		
Baseline	18±13	41±38
Acute nicotine	31±15	33±27

TABLE 5. Mean values for amplitude of glutamatergic sEPSCs, mEPSCs and mEPSC rise time at baseline and during acute nicotine challenge. There were no significant differences in any of these variables.

	Baseline	*Acute nicotine challenge*
sEPSC Amplitude (pA)		
Control:		
P1-2	-15.2 ± 2.7	-16.0 ± 3.2
P3-5	-15.5 ± 2.3	-15.7 ± 3.9
DNE:		
P1-2	-18.5 ± 7.4	-18.3 ± 8.9
P3-5	-16.4 ± 2.8	-14.8 ± 2.9
mEPSC Amplitude (pA)		
Control:		
P1-2	-16.2 ± 2.6	-14.6 ± 2.6
P3-5	-14.9 ± 3.0	-16.3 ± 3.7
DNE:		
P1-2	-16.0 ± 2.9	-15.6 ± 3.8
P3-5	-17.5 ± 1.9	-17.4 ± 1.9
mEPSC rise time (msec)		
Control:		
P1-2	2.1 ± 0.2	2.1 ± 0.2
P3-5	2.0 ± 0.1	2.2 ± 0.1
DNE:		
P1-2	2.1 ± 0.2	2.0 ± 0.1
P3-5	1.8 ± 0.1	2.0 ± 0.1