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Activity-dependent plasticity in the isolated embryonic avian brainstem following manipulations of rhythmic spontaneous neural activity

Michael A. Vincen-Brown^a, Ann L. Revill^c, and Jason Q. Pilarski^{a,b,*}

^aDepartment of Biological Sciences, Idaho State University, Pocatello, ID, United states

^bDepartment of Dental Sciences, Idaho State University, Pocatello, ID, United states

^cDepartment of Physiology, University of Alberta, Edmonton, AB, Canada

Abstract

When rhythmic spontaneous neural activity (rSNA) first appears in the embryonic chick brainstem and cranial nerve motor axons it is principally driven by nicotinic neurotransmission (NT). At this early age, the nicotinic acetylcholine receptor (nAChR) agonist nicotine is known to critically disrupt rSNA at low concentrations (0.1–0.5 μM), which are levels that mimic the blood plasma levels of a fetus following maternal cigarette smoking. Thus, we quantified the effect of persistent exposure to exogenous nicotine on rSNA using an *in vitro* developmental model. We found that rSNA was eliminated by continuous bath application of exogenous nicotine, but rSNA recovered activity within 6–12 h despite the persistent activation and desensitization of nAChRs. During the recovery period rSNA was critically driven by chloride-mediated membrane depolarization instead of nicotinic NT. To test whether this observed compensation was unique to the antagonism of nicotinic NT or whether the loss of spiking behavior also played a role, we eliminated rSNA by lowering overall excitatory drive with a low $[\text{K}^+]_o$ superfusate. In this context, rSNA again recovered, although the recovery time was much quicker, and exhibited a lower frequency, higher duration, and an increase in the number of bursts per episode when compared to control embryos. Importantly, we show that the main compensatory response to lower overall excitatory drive, similar to nicotinergeric block, is a result of potentiated chloride mediated membrane depolarization. These results support increasing evidence that early neural circuits sense spiking behavior to maintain primordial bioelectric rhythms. Understanding the nature of developmental plasticity in the nervous system, especially versions that preserve rhythmic behaviors following clinically meaningful environmental stimuli, both normal and pathological, will require similar studies to determine the consequences of feedback compensation at more mature chronological ages.

*Corresponding author at: Memorial Drive, Building 65, Stop 8007, Pocatello, ID, United states. pilajaso@isu.edu (J.Q. Pilarski).

Author contributions

Michael Vincen-Brown performed the experiments, wrote portions of the manuscript, analyzed the data and helped interpret these findings. MVB was integral to all phases of these experiments and the preparation of the manuscript. Dr. Ann Revill helped interpret the findings, edited the manuscript and wrote custom software for waveform analyses. Dr. Jason Q. Pilarski performed the experiments, interpreted the findings, and wrote the majority of the manuscript.

Keywords

Developmental nicotine exposure; Chick brainstem; Avian; Activity-dependent plasticity; In vitro

1. Introduction

One of the most striking and consistent features of the developing central nervous system is its innate ability to generate spontaneous bioelectric rhythms among spinal and cranial motor pools. Sometimes referred to as rhythmic spontaneous neural activity (rSNA), rSNA can be measured *in vivo* and/or *in vitro* at a time when the formation of central motor circuitry and muscle innervation is just beginning (Provine 1972; Bekoff et al., 1975; O'Donovan and Landmesser, 1987; Hanson and Landmesser, 2006; Wang et al., 2009; for reviews see Bekoff, 2001; Momose-Sato and Sato, 2013; Moody and Bosma, 2005). While rSNA is considered a relatively generic multisynaptic depolarization wave synaptically shared by many disparate regions of the embryonic neuraxis, evidence suggests that it is important for proper network construction. For example, both the presence and pattern of rSNA has been shown to influence neuronal migration, axon guidance, expression of individual ion channels, and neurotransmitter phenotypes in the CNS (Borodinsky et al., 2013; Chub and O'Donovan, 1998; Hanson and Landmesser, 2006; Spitzer, 2012; Wenner, 2013; Yoon et al., 2010, 2008). When examining the isolated neuraxis *in vitro*, rSNA is expressed as periodic episodes of recurrent spike train activity initiated in the hindbrain and or the spinal cord, depending on developmental stage, which then propagates from these initiation zones both rostrally and caudally to the forebrain and the sacral spinal cord, respectively (Hughes et al., 2009; Momose-Sato and Sato, 2014). In the chick brainstem, rSNA is first revealed by synchronous cranial motor output immediately following rhombomeric segmentation near embryonic day four (Lumsden and Keynes, 1989; Fortin et al., 1995, 1994). Once rSNA begins in the hindbrain, data show that excitation is generated as single electrical episodes (Fortin et al., 1994, 1995, 1999) and is critically dependent on nicotinic neurotransmission (NT) with weaker modulatory contributions from neurons that release GABA (γ -aminobutyric acid) and glycine (Hughes et al., 2009; Mochida et al., 2009). As development proceeds, the central episodes become more complex, *i.e.*, single episodes with multiple bursts, and the dominant neurotransmitter system that drives rSNA transforms from chiefly nicotinic NT to a combination of GABA, glycine and glutamate NT (Mochida et al., 2009). Despite these data, the cellular signals that govern the early specification of NT systems and the role of spiking behavior in this process are still incomplete. Moreover, a hallmark of rSNA, as well as other forms of correlated neuronal activity, is the ability of the underlying interneuronal circuits to adapt to changes in synaptic input (*e.g.*, synaptic depression/augmentation) to maintain appropriate levels of excitability (Momose-Sato and Sato, 2013). While several studies show that early neuronal circuits can regain function in the face of synaptic imbalance, there is a poverty of information on the mechanisms that provide neurotransmitter phenotypic stability in the context of potentially dangerous environmental influences experienced by human embryos. Therefore, our goal was to test how the prolonged or persistent *in vitro* exposure to nicotine, a clinically relevant acetylcholine receptor (nAChR) agonist, may alter the expression of rSNA in the brainstem. The *in utero* exposure to nicotine in human embryos contributes to significant neonatal

mortality and leads to substantial medical expenses worldwide each year (Aligne and Stoddard, 1997; Bank, 1999; Wickström, 2007). Previous studies examining the effects of nicotinic neurotransmitter signaling on the embryonic chick lumbar spinal cord show that low doses of nicotine (0.1–1 μ M) eliminates rSNA by overexciting the network and desensitizing nAChRs (Hughes et al., 2009; Milner and Landmesser, 1999). Moreover, several experiments suggest that nicotinic NT can increase GABA and glycine release onto spinal motoneurons via Renshaw cell activity (Gonzalez-Islas et al., 2016) and can even drive rSNA in the absence of normal excitatory NT, for example, following the bath application of a glutamate and cholinergic blocker cocktail (Chub and O'Donovan, 1998). Yet, a direct connection between nicotine-induced elimination of spontaneous bursting and the potentiation of GABAergic and glycinergic NT has not been established. Moreover, how early brainstem circuits respond to persistent nicotine insults is completely unknown, especially at a chronological stage where nicotinic NT is a principal driver of rSNA (Mochida et al., 2009). In addition, while early rSNA is considered a widespread intersegmental wave, data show that different regions of the CNS exhibit unique patterns of rSNA activity (Fortin et al., 1995) and nicotinic receptor expression (Hughes et al., 2009). Therefore, understanding the control of rSNA in the developing brainstem, where critical breathing circuits reside, is particularly important for understanding respiratory-related homeostasis in neonatal health and disease. In the present study, we aim to determine how nicotine may alter brainstem rSNA waveform characteristics, and the degree to which rSNA may adapt to this clinically realistic situation. We also aim to test whether chloride-mediated ionic conductance, which are depolarizing in the embryonic chick brainstem (Hughes et al., 2009; Mochida et al., 2009), can substitute for the complete loss of nicotinic NT. Specifically, we tested the hypothesis that following persistent developmental nicotine exposure, brainstem nAChRs are desensitized, which eliminates rSNA transiently, and that rSNA recovers by an increase in GABAergic and glycinergic NT. Additionally, because blocking nicotinic NT also blocks spiking behavior, we tested whether the motor circuits in the brainstem can adapt—via increases in GABAergic/glycinergic NT—when spiking behavior is experimentally eliminated by reducing overall excitatory input to these oscillatory circuits.

We show that brainstem circuits that participate in rSNA, measured through cranial nerve IX (glossopharyngeal), can self-regulate and recover its activity regardless of whether rSNA is blocked by nAChR antagonism or by lowering excitatory drive. In both experimental manipulations, rSNA returns via increases in GABAergic/glycinergic transmission. Importantly, the time course of recovery and the shape of the rSNA waveform (*e.g.*, duration, burst number) are unique to the nature of the insult. These data confirm that spiking activity is an important, regulated variable and suggests that decreases in brainstem neural activity plays an important role in the strength and timing of Cl^- -mediated membrane conductance, especially when GABA/glycine currents are depolarizing. Future studies will need to address whether this type of activity-dependent plasticity, while seemingly beneficial in the short-term, may adversely affect the stability of neural drive over the long-term (*e.g.*, mature breathing rhythms).

2. Methods

2.1. Research animals

For all experiments we used White Leghorn chicken embryos (*Gallus gallus domesticus*) and restricted our studies to stage 26–27, *i.e.*, embryonic day 5–5.5 (E5–5.5) according to H&H staging (Hamburger and Hamilton, 1951). We studied a total of 45 embryos of either sex. At these early embryonic stages, large-scale multisynaptic depolarization waves occur in the brainstem and exit via cranial nerves (Fortin et al., 1999, 1995, 1994). Chicken embryos were purchased as fertilized eggs from a local distributor (Merrill Farms, Paul, ID.) and were compelled to develop using a forced-draft cabinet incubator maintained at 38 °C, 60–70% humidity and turned automatically every hour. Embryonic day 0 (E0) was defined as the day the fertilized eggs were placed into the incubator.

2.2. Preparation

Each surgery and electrophysiological recording was performed in an artificial cerebrospinal fluid (aCSF). The aCSF was kept at 2–4 °C during brainstem removal and then the temperature was raised to 27 ± 2 °C during suction electrode recordings. In between, *i.e.*, during recovery from surgery in the control group and embryos participating in the *in vitro* model of developmental nicotine exposure (treatment group), the isolated tissue was kept at room temperature (21–23 °C) for 6–12 h (see Protocols below). The aCSF included (in mM) 120 NaCl, 12 D-glucose, 1.15 MgCl₂, 1.26CaCl₂, 8 KCl, 26 NaHCO₃, 0.58 NaH₂PO₄ bubbled with a 95% O₂, 5% CO₂ gas mixture producing a final pH = 7.4 ± 0.1 , as described previously (Fortin et al., 1995). Briefly, we used an isolated *in vitro* brainstem spinal cord preparation *en bloc* for all experiments, which is an established experimental model to probe brainstem circuits during development in both birds and mammals (Ballanyi et al., 1999; Sholomenko and O'Donovan, 1995; Smith and Feldman, 1987; Suzue, 1984). The isolation of the hindbrain was done via a ventral approach after rapid decerebration near the intersection of the coronal and sagittal sutures. Next, the mesenchyme was cleared from the ventral and lateral surface, and cranial nerves (CN) V–XII were freed as rootlets. The targeted cranial nerve was the glossopharyngeal (IX) nerve. Cranial nerve IX harbors both sensory and motor pathways and so was transected proximal to the sensory ganglion to achieve the highest-quality electrophysiological recordings possible. Cranial nerve IX contains axons that transmit inspiratory motor outflow to the larynx and glottis (White and Chubb, 1967; Hammond, 2009) in continuously breathing birds and has been shown to carry rhythmic spontaneous nerve activity (rSNA) beginning near E4 (Fortin et al., 1995). The physical boundaries of the isolated brainstem tissue consisted of the isthmus (mesencephalic-rhombomeric junction) rostrally as well as a small section of the cervical spinal cord (C1–2) caudally. These boundaries include the previously described boundaries for the caudal rhythm generators at E5–5.5 (Momose-Sato et al., 2009). A region of the suprahyoid musculature was retained for visual confirmation of rhythmic muscle activation originating from cranial nerve IX (laryngopharyngeal branches). Throughout the remainder of the manuscript the use of the abbreviation rSNA (rhythmic spontaneous neural activity) is analogous to cranial nerve IX rSNA as this was always our target nerve for each experiment.

2.3. Electrophysiological recordings

rSNA was measured from cranial nerve IX by using back pressure to suction the transected nerve ending into a glass micropipette filled with aCSF and a Ag/AgCl wire. The activity was referenced to an indifferent electrode present within the bath chamber. Signals were augmented using a HI-Z differential pre-amplifier (HZP, Grass Instruments, Quincy, MA). The HI-Z preamplifier was fed into a P511 alternating current (AC) amplifier (Grass Instruments, Quincy, MA) and the signal was amplified (gain = 1000–10000) and band pass filtered (0.1–1 kHz). The amplified electrical activity was processed by an analog to digital converter (Power1401, Cambridge Electronic Design, Cambridge, UK) at 5 kHz and then rectified and integrated at a time constant of 200 ms with a moving average using Spike II software (Cambridge Electronic Design, Cambridge, UK). Files were stored for offline data analysis.

2.4. Drugs

Stock solutions of drugs were prepared as needed by dilution in aCSF. We used 10 μ M bicuculline methiodide (Tocris Bioscience, Ellisville, MO) to block GABA_A receptors and 0.4 μ M strychnine (Sigma, St. Louis, MO) to block glycine receptors. We also used atropine (Sigma, St. Louis, MO) to block muscarinic-AChRs and acetylcholinesterase inhibitor physostigmine hemisulfate (Tocris Bioscience, Ellisville, MO) to stimulate neuronal nAChRs. For developmental nicotine exposures, we used 0.5 μ M (–)-nicotine ditartate (Sigma, St. Louis, MO). The effects of low $[K^+]_o$ (0–1 mM KCl) aCSF were also tested to determine the effect of excitatory drive to the circuits that produce brainstem rSNA.

2.5. Experimental protocols

With the exception of long term nicotine exposure (see below), pharmacological agents were bath applied to the superfusate following at least 15 min of stable baseline measurements following the recovery period. The volume of the recording chamber was 3 ml with a flow rate of nearly 3 ml/min. We determined that it requires approximately 2 min for the addition of drugs to the superfusate reservoir to reach the recording chamber.

2.5.1. Experimental series 1—For this set of experiments our aim was twofold: The first aim was to compare normal (control) in vitro rSNA frequency, area, duration and amplitude with treatment rSNA characteristics following the recovery of electrical activity from nicotine exposure. Please note that ‘recovery’ always refers to rSNA produced in the continuous presence of nicotine unless otherwise stated. The second aim was to determine how rSNA adapted to the loss of nAChR function (i.e., desensitization) by comparing the effects of GABA_A and glycine receptor blockers, bicuculline (10 μ M) and strychnine (0.4 μ M), on control rSNA versus rSNA following recovery.

Developmental nicotine exposure was initially achieved by recording baseline (control) rSNA and then adding 0.5 μ M nicotine continuously for > 6 h (n = 3). Fig. 1 shows an early experiment in this context that lasted > 12 h. However, due to need to constantly monitor the fidelity of the recording and the potential nerve damage caused by long duration suction electrode back pressure on delicate embryonic rootlets we modified our developmental nicotine exposure protocol as follows: Instead of “around the clock” electrical recording

sessions, immediately following the isolation of the brainstem, the tissue was allowed to recover in a custom recirculating flow-through superfusion chamber that mimicked all aspects of our electrophysiological recording chamber. For some other preparations, we also used a standard non-recirculating recovery chamber fitted with a mesh basket and an air stone beneath the tissue to provide proper oxygenation (Scientific Systems Design, Ontario, Canada). In all cases, the brainstems were continuously exposed to nicotine (0.5 μM) for at least 6 h (range = 6–12 h), which we determined was the minimum time required for rSNA recovery (see Results). To account for variation resulting from this extended recovery time and effects of small differences in developmental stage on rSNA, control embryos were isolated and allowed to recover using exactly the same protocol as described above for the treatment group prior to baseline (control) electrophysiological recording.

For the second aim of Experimental Series 1, we sought to determine if brainstem rSNA could recover its activity following in vitro nicotine exposure, and, if so, whether a potentiation of excitatory chloride mediated neurotransmission was involved. In the same embryos as described above (Aim 1), once a stable rSNA was observed, we added bicuculline (10 μM) and strychnine (0.4 μM) for a period of 15 min followed by a drug free washout period. If drug washout did not result in a recovery of rSNA the record was discarded.

2.5.2. Experimental series 2—Similar to Experimental Series 1, we aimed to compare normal (control) rSNA waveform characteristics with rSNA following the recovery of activity. However, unlike the above experiments, we designed these experiments to determine if embryonic brainstem neural circuits can recover from the elimination of episodic spike train behavior without altering nicotinic NT and to test whether similar mechanisms are involved. Therefore, we eliminated rSNA following baseline measurements by lowering overall excitatory drive using aCSF with low extracellular $[\text{K}^+]_o$ (0–1 mM KCl) and then quantified the degree of rSNA recovery. Resembling the above experiments, following the stable recovery of cranial nerve IX rSNA, GABA_A and glycine receptor blockers, bicuculline (10 μM) and strychnine (0.4 μM), were added to the bath of control and treatment embryos for 15 min, which was followed by a washout period.

2.6. Data analysis and statistics

For all protocols, the average frequency, amplitude, area, duration and burst number of the rectified and integrated cranial nerve IX motor output generating rSNA was quantified off-line using sets of stable 10 min windows derived from baseline, drug infusion, and the recovery periods. rSNA was analyzed using a custom software program written in Spike2 software (CED, Cambridge, UK). To be detected, the individual episodes had to cross a baseline threshold value defined by the user, who was blind to treatment. Detection of an episode then triggers a duration and amplitude calculation. Each rSNA signal detection was visually checked for quality assurance. In some records, episodes were complex and divided by clear individual bursts. When this occurred, if the individual bursts were separated by less than a preset delay, then bursts were detected counted together and included as a contributor to a single episode. Thus, the total duration of an episode sometimes included more than one burst if present. To quantify differences between control and treatment groups, means of

rSNA discharge parameters were analyzed using paired Student's *t*-tests (frequency, amplitude, episode area and duration, and burst number) or one-way ANOVA with repeated measures (main effect: treatment group) if multiple comparisons were required. Tukey's post-hoc comparisons were performed if a significant ANOVA model effect was detected. A probability value ≤ 0.05 was considered significant in all of our analyses. Reported values are means $\pm$ standard error of the mean ($\pm$ SEM) unless stated otherwise. All statistical analyses were performed with JMP-IN software (SAS, Cary, SC).

3. Results

Previous studies have demonstrated that the generation and maintenance of early rSNA (embryonic day 4–6 or E4–E6) is critically dependent on nicotinic NT in both the brainstem and spinal cord (Hughes et al., 2009; Milner and Landmesser, 1999; Mochida et al., 2009). Additionally, data show that weaker excitatory inputs are generated from outward chloride conductance at this age, which serve to modulate rSNA (Chub and O'Donovan, 1998; Hughes et al., 2009; Milner and Landmesser, 1999; Mochida et al., 2009). We confirmed these findings as shown in Fig. 2. Between E5–E5.5, rSNA was generated in single episodes with a mean baseline frequency of $1.02 \pm 0.05 \text{ min}^{-1}$ and a mean coefficient of variation (standard deviation/mean) for interspike interval of 0.27 ($n = 25$). Fig. 2A shows an experiment using nicotinic cholinergic antagonists mecamylamine (50 μM and dihydro- β -erythroidine or DH β E (20 μM), which fully blocked rSNA at E5, as expected, and also tended to increase tonic baseline activity. We were similarly able to eliminate rSNA in control animals at E5 by using nAChR blocker d-tubocurarine (10 μM) (data not shown), as others have done (Hughes et al., 2009; Milner and Landmesser, 1999; Mochida et al., 2009). However, we decided to use mecamylamine (50 μM and DH β E (20 μM) instead of d-tubocurarine because d-tubocurarine is not an ideal tool to precisely target nAChRs as elevated concentrations of d-tubocurarine can also inhibit GABA_A receptor-mediated currents and serotonergic-mediated currents (*e.g.* 5HT₃), which are both known to contribute to rSNA at this age (Hughes et al., 2009; Mochida et al., 2009). Thus, we felt that d-tubocurarine would not be discriminating enough to separate the identity and timing of the critical neurotransmitter systems that maintain rSNA drive at this age (E5–5.5), especially following nicotine exposure (see below). Figs. 2B and Bb show the modulatory effect of bicuculline (10 μM) and strychnine (0.4 μM) on the frequency of rSNA in E5.5 control animals, which was used to block GABA_A and glycine receptors, respectively. Because ionotropic GABA_A and glycine receptors mediate outward Cl[−] currents at this developmental age they are excitatory (Milner and Landmesser, 1999). Thus, bath addition of bicuculline and strychnine predictably slowed rSNA frequency by $22.9 \pm 5.2 \%$ ($P < 0.0002$, $n = 14$) compared to control. Summary data shown in Fig. 2C ($n = 14$) further reveals that the amplitude of rSNA, while trending down, did not reach significance, and thus was not different than baseline after GABA_A and glycine receptor block. However, rSNA area was decreased by $17.7 \pm 6.5 \%$ ($P < 0.019$, $n = 14$) and episode duration was decreased by $12.9 \pm 5.5 \%$ ($P < 0.038$; $n = 14$) consistent with a minor decrease in overall excitatory drive mediated by Cl[−] currents. These data indicate that Cl[−]-mediated conductance contributes to the maintenance of rSNA at E5.5 in control animals, but is not a primary mechanism for burst generation. Please note that E5.5 was specifically targeted for

the control experiments to compare these data to the effects of GABA_A and glycine inhibition following persistent developmental nicotine exposure protocol at the same chronological age (see below).

3.1. Effects of persistent *in vitro* nicotine exposure on brainstem rSNA and recovery

In a total of 38 embryos ($n = 25$, control; $n = 13$, nicotine exposed) we tested the hypothesis that following chronic developmental nicotine exposure, nAChRs are desensitized, which eliminates rSNA, and, in turn, results in an increase in the strength of chloride-mediated membrane depolarization that compensates for the loss of nicotinic NT. Fig. 3A shows an example suction electrode recording from cranial nerve IX on E5 in which rSNA was initially measured under control baseline conditions followed by bath application of nicotine ($0.5 \mu\text{M}$). Similar to the record shown in Fig. 1, the addition of nicotine to the superfusate rapidly eliminated rSNA. Fig. 3A demonstrates that the initial early recovery (≈ 6 h after initial nicotine application) was slower and often exhibited a heightened tonic discharge. Note that recovery is defined as the ability to generate rSNA in the continued presence of $0.5 \mu\text{M}$ nicotine. Fig. 3B shows late recovery (≈ 12 h after initial nicotine application). In the embryos we studied in this manner ($n = 13$), the time range for the re-establishment of rSNA was between 6 and 12 h with a non-statistically significant trend toward higher recovery baseline frequencies during longer exposure-recovery durations (total frequency range = $0.3\text{--}1.5$ bursts min^{-1}). These data were therefore pooled. To confirm that nAChRs remained functionally desensitized to nicotine following our chronic exposure model we always added newly mixed nicotine ($0.5 \mu\text{M}$) from stock mixtures once recovery had stabilized. In every instance, there was no additional nicotinic effect on rSNA. As an additional test for the presence of nAChR function in this context, we bath applied physostigmine ($10 \mu\text{M}$) following rSNA recovery and observed zero effects on the recovery waveform (Fig. 3B). Bath application of atropine ($10 \mu\text{M}$) was included with physostigmine to block the potential effects of m-type AChR on rSNA. These tests for nAChR function during developmental nicotine exposure showed that nAChRs were functionally desensitized, despite the full recovery of rSNA. To determine whether the recovery of rSNA can be explained by an increase in Cl^- -mediated membrane conductance, we used bicuculline ($10 \mu\text{M}$) and strychnine ($0.4 \mu\text{M}$) to block GABA_A and glycine receptors following rSNA recovery near E5.5, when GABAergic and glycinergic NT have a modulatory effect on rSNA in control animals (see Fig. 2B and C for control data). Fig. 3C shows that rSNA recovery was fully blocked when GABAergic and glycinergic NT were inhibited ($n = 5$; summary data shown in Fig. 3D). As shown in Fig. 3D please note that the mean frequency of recovery rSNA is not different from mean control baseline rSNA (hatched bar).

3.2. Effects of low $[\text{K}^+]_o$ on brainstem rSNA and recovery

The previous set of experiments showed that rSNA adapted and fully recovered relatively normal oscillations when nicotinic NT is blocked for extended lengths of time *in vitro*. This self-regulation is critically dependent on an increase in the strength of GABAergic/glycinergic NT. The aim of these low $[\text{K}^+]_o$ experiments ($n = 7$) was to test the hypothesis that lowering overall excitatory drive to a level that eliminated rSNA would similarly evoke GABAergic/glycinergic compensatory regulatory mechanisms and preserve rSNA. Previous studies have shown that Cl^- -mediated depolarizations during early development are dynamic

and that $[Cl^-]_{in}$ is altered by changes in spiking behavior (Gonzalez-Islas et al., 2010). Fig. 4A shows that when aCSF $[K^+]_o$ was lowered from 8 mM to 0 mM in E5 embryos, rSNA was eliminated, but was followed by a rapid recovery (range = 5–10 min) of rhythmic activity while still in the presence of low $[K^+]_o$. The initial recovery of rSNA produced a frequency that was only $28.1 \pm 3.8\%$ of baseline ($P < 0.0001$) with an average amplitude that tended to be higher ($196.4 \pm 46.5\%$; $P = 0.0833$) but was not significantly different from controls. rSNA duration was more than six times longer than control values during recovery ($630.2 \pm 122.3\%$, $P = 0.0049$; Fig. 4B). Unlike embryos exposed to nicotine, once recovery from low $[K^+]_o$ stabilized, rSNA was changed from single episodes, characteristic of rSNA at E5–5.5, into episodes that consisted of multiple bursts (see expanded record in Fig. 4A). A single episode with multiple bursts is typical of rSNA in animals older than E7 and has been attributed to the development of I_h currents (Fortin et al., 1999). These data suggests that rSNA elimination followed by compensation and recovery may induce the early maturation of specific biophysical properties in the underlying oscillatory network. Nevertheless, the bath application of bicuculline (10 μ M) and strychnine (0.4 μ M) to block GABA_A and glycine conductance, respectively, following the recovery of rSNA, abolished rSNA (Fig. 4C), similar to embryos exposed to nicotine. These results suggest that the mechanisms that permitted rSNA to recovery, both in the context nicotinic NT antagonism and when drive is lowered with low $[K^+]_o$, were similar but not identical.

4. Discussion

In the present study we examined the effects of experimentally eliminating rhythmic spontaneous neural activity (rSNA) on neurotransmitter signals that normally support this behavior. In the first set of experiments, we used an *in vitro* model that produced a persistent, long duration developmental nicotine exposure directly to the hindbrain. Using this preparation it was possible to continuously measure rSNA from a specific developing motoneuronal population while also carefully controlling external drug concentrations. Our goal was to better understand how the presence of a clinically relevant dose of nicotine (Gentry and Lukas, 2002; Wonnacott et al., 1990) disturbs the rhythm and maturation of early motor-related neural networks in the brainstem. Human prenatal exposure to nicotine is relatively common and leads to breathing problems that involve abnormal brainstem motor development, such as the efficacy of neurotransmission to motoneurons that normally stiffen and open upper airway musculature during a typical breath cycle (Fuller et al., 2009; Jaiswal et al., 2016; Nachmanoff et al., 1998; Pilarski et al., 2011). In the second set of experiments, we eliminated rSNA by lowering overall excitatory drive with a low $[K^+]_o$ aCSF and examined whether the embryonic brainstem could also recover rSNA. The latter experiments when compared to the former permitted us to assess whether modifications to rSNA following nicotine exposure are linked to neuronal spiking behavior or whether alterations in nicotinic NT provokes a unique signal.

Overall, we found that: (1) E5 rSNA measured via cranial nerve IX is eliminated by chronic *in vitro* developmental nicotine exposure for at least six hours. These results confirm previous work showing that nicotinic cholinergic chemical transmission is critically important for normal rSNA at this age (Milner and Landmesser, 1999; Mochida et al., 2009); (2) After at least 6 h, rSNA recovered to control levels despite the continued presence of

nicotine in the superfusate. We further show that nAChRs are not involved in the recovery of rhythmic activity in this context, because the bath application of “freshly” hydrated nicotine-bitartate or the acetylcholinesterase inhibitor, physostigmine, did not change recovery rSNA; (3) The mechanism of rSNA recovery in the presence of nicotine occurs by potentiating GABA_A or glycine NT because bath application of bicuculline and strychnine abolished rSNA when applied to nicotine treated embryos during recovery. In control embryos at E5.5, blocking GABA_A and glycine NT had a minor effect of rSNA. In the nicotine treated embryos, recovery rSNA did not return again until bicuculline and strychnine were cleared from the bath; (4) To address whether nAChR block had unique effects on GABAergic/glycinergic NT, and thus rSNA, or whether spiking activity was an important regulated variable, we blocked rSNA by lowering excitatory drive using low [K⁺]_o aCSF (0–0.1 mM). Following the elimination of rSNA, which usually lasted 10–15 min, activity recovered, but, compared to control animals, recovery rSNA was more dependent on GABAergic/glycinergic NT.

On one hand, the latter results are similar to the embryos exposed to developmental nicotine, *i.e.*, where GABA_A and glycine receptor inhibition eliminated rSNA, but importantly these data also show that the elimination of spiking behavior alone can potentiate GABAergic/glycinergic NT. On the other hand, the time to recovery was considerably shorter when rSNA was eliminated by low [K⁺]_o aCSF compared to developmental nicotine exposure (10–15 min *vs.* 6 h). This is likely due to the continued excitatory contribution of nicotinic cholinergic NT during low [K⁺]_o aCSF experiments. For example, when we blocked nAChRs using mecamylamine and DHβE following the low [K⁺]_o aCSF perturbation and subsequent recovery of rSNA, recovery rSNA was eliminated, but only for 10–15 min (*n* = 3; data not shown). These data are consistent with an overall increase in excitability induced by depolarizing tonic and or phasic outward chloride currents and suggest that developmental nicotine exposure may be less disruptive to rSNA if chloride conductance is boosted in advance of or during exposure. Future studies will attempt to rescue or reverse the acute effects of developmental nicotine exposure on rSNA.

Interestingly, eliminating rSNA by lowering overall drive consistently produced an increase in rSNA burst complexity during recovery, such that rSNA can be described as having a lower frequency, more individual bursts per episode, and much longer episode durations. The latter result is consistent with previous data showing that the number of bursts per episode is related to chloride conductance and suggests that recovery rSNA was qualitatively similar to control rSNA at more mature time points (see Fig. 2b from Fortin et al., 1999). By E7, GABAergic and glycinergic NT play essential roles in driving rSNA (Mochida et al., 2009).

4.1. Comparison with other similar studies

It has long been observed that spontaneously active cells cultured from the brain and spinal cord have the ability to adaptively regulate specific ionic conductance that preserve innate patterns of spiking behavior (Turrigiano et al., 1998, 1995; and for a review see Turrigiano, 2012). Although less studied, activity-dependent plasticity also plays a role in more intact embryonic spinal cord preparations *in vitro* at a time when GABAergic and glycinergic NT

evoked currents and are considered depolarizing and excitatory (for reviews see Wenner, 2013; Momose-Sato and Sato, 2013). One of the first experiments to explore the pharmacological details of rSNA, as well as the potential for activity dependent plasticity, was Chub and O'Donovan (1998), who showed that the combined inhibition of glutamatergic and cholinergic NT in the E10–11 chick lumbar spinal cord produced a strengthening of GABAergic and glycinergic NT. While Chub and O'Donovan's thorough study (1998) is related to the present study, they did not isolate the role of the nicotinic cholinergic signal specifically and their target age range was considerably older, *i.e.*, E10–11, which is a time when cholinergic NT in the spinal cord plays only a secondary or supportive role at central motoneuron synapses and cannot independently drive rSNA as it does in younger embryos. In similar studies involving younger embryos (E4–7), activity-dependent plasticity was also observed when a complicated mixture of receptor blockers were added sequentially to the superfusate bathing the embryonic lumbar spinal cord, including those responsible for glutamate, GABAergic and cholinergic NT (Milner and Landmesser, 1999). In this scenario, following glutamatergic, GABAergic and nicotinic cholinergic NT blockade, the maintenance of rSNA was shown to be dependent on muscarinic acetylcholine receptor function. While these early studies are notable and show that neural circuits of the early embryonic spinal cord also adapt to generalized synaptic insults, the unambiguous role of individual neurotransmitter systems, and or the spiking behavior itself, remained unclear. Thus, the present study adds to these data because a direct role of nicotinic cholinergic NT in brainstem motor circuits had not previously been addressed in the context of activity-dependent plasticity. Our experiments were designed with a focus on stage 26 (E5). This is because it is clear that rSNA is critically dependent on nAChRs at this time and chloride conductance is excitatory (Hughes et al., 2009; Mochida et al., 2009). Moreover, we used a clinically relevant insult to the normal development of central nicotinic NT in the context of early rhythm generation. Milner and Landmesser (1999) and Hughes and Bosma (2009) first noted that low concentrations of nicotine (0.1–1 μ M) transiently increased tonic and phasic rSNA in the lumbar cord and the brainstem, respectively, and then blocked it, but these investigations revealed little information on the recovery waveform. Our data support this earlier work in many respects, *i.e.*, a low concentration of nicotine eliminates rSNA by nAChR hyperstimulation and desensitization, and we also present data on the recovery of the rSNA in this context. We conclude that nicotinic receptor signaling per se is not the critical variable in this type of relatively rapid compensatory plasticity. Instead, our data suggest that activity itself is the critically regulated variable that maintains appropriate excitatory drive for rSNA. These results are consistent with other similar studies focused on more mature spinal circuits in which lidocaine was used to block or reduce action potential generation, and thus rSNA, over longer durations (Lindsly et al., 2014).

4.2. The role of rSNA in NT respecification

The myriad of potential mechanisms responsible for the self-regulation of embryonic rSNA can be broadly grouped as increases and/or decreases in cellular/network excitability and or alterations in synaptic strength (Lindsly et al., 2014; Gonzalez-Islas et al., 2009; for review see Wenner, 2013). Although we have not determined mechanism by which GABAergic/glycinergic NT is potentiated by the loss of rSNA, we speculate that both the absence of

nicotinic-evoked inward currents and the low $[K^+]_o$ perturbation increased the strength of GABAergic/glycinergic NT in the following way: we propose that the observed strengthening of Cl^- -mediated conductance and depolarization is likely a straightforward result of the prolonged cessation of action potential generation, as others have previously shown in the spinal cord (Lindsly et al., 2014). In this scenario, when periodic spike trains are suppressed, Cl^- accumulates intracellularly (via the embryonic chloride co-transporter, NKCC1), and this results in a shift in chloride's equilibrium potential and a strengthening of outward (depolarizing) GABA_A and/or glycine-mediated Cl^- currents. This dynamic shift enables GABA_A and/or glycine to rapidly become the primary NT driving rSNA following the absence of activity without the immediate need to evoke second messenger systems or gene expression. As mentioned above, low $[K^+]_o$ and the resulting recovery of rSNA produce an episodic burst pattern that is reminiscent of chronologically older embryos, *i.e.*, multiple bursts per episode. This cranial nerve pattern is typically seen in chick embryos approximately 48 h older (E7) than we studied, *i.e.*, when GABAergic neurons begin to play a more essential role rSNA (Fortin et al., 1999).

It is also important to note that while data show brainstem motoneurons express postsynaptic nAChRs at a time when rSNA first begins (Hughes et al., 2009), the source of GABA and or glycine excitatory input is not clear. This is in contrast to embryonic spinal circuitry, where Renshaw cells produce recurrent GABAergic/glycinergic effects on motoneurons that are influenced by activation of presynaptic nAChRs. In the spinal cord, unlike the brainstem, there is little evidence of postsynaptic nAChRs on motoneurons. Thus, our data should encourage future investigations as to the nature of GABAergic and or glycinergic connectivity in the brainstem. It is intriguing to speculate that the brainstem motoneuron cell bodies may also be contacted by Renshaw-like GABAergic and or glycinergic interneurons under the influenced of nicotinic cholinergic axon collaterals similar to spinal circuitry (Gonzalez-Islas et al., 2015). In this way, the brainstem may have additional mechanisms to modulate motor unit recruitment.

Alternately, we cannot rule out other activity dependent compensatory mechanisms that may contribute to our results, especially considering that nicotine exposure produced a recovery rSNA that was comparatively distinct from rSNA following low $[K^+]_o$. The potential for nicotine exposure and nAChR desensitization to produce unique and perhaps long term compensatory mechanisms is likely given that nAChRs are non-specific cation channels that pass Ca^{2+} . Data show that neuronal nAChRs are important players in early activity-dependent Ca^{2+} signaling characteristic of developing rSNA (Wang et al., 2009). Although the specific nicotinic subtype expression pattern in the avian brainstem has not yet been described, Milner and Landmesser (1999) demonstrated that the non- $\alpha 7$ isoforms are the most significant in the avian spinal cord. We conjecture that persistent exposure to nicotine may lower the expected intracellular Ca^{2+} signal, which then leads to compensatory mechanisms to regain it. Future studies will need to test how nAChR-evoked Ca^{2+} currents may be linked to the feedback stabilization of rSNA through depolarizing GABAergic/glycinergic NT as mentioned above. Interestingly, Liu et al. (2006) showed that nicotinic cholinergic synaptic activity in the chick ciliary ganglion is responsible for the timing of the maturational transition of GABAergic NT from excitation to inhibition via the embryonic chloride transporters—NKCC1 and KCC2. Blocking nAChR activation delayed the

expression of KCC2 and prolonged the expression of NKCC1. Moreover, nAChRs are known to be present on presynaptic terminals of GABAergic neurons in the newborn mammalian brain that function to modulate neurotransmitter release onto postsynaptic cells (Dani and Bertrand, 2007).

5. Conclusions and clinical considerations

Historically, the importance of embryonic nicotinic NT in the brain and spinal cord has focused on its role in cell proliferation, migration, differentiation, and apoptosis (for a review see Resende and Adhikari, 2009). Clearly, embryonic rhythms also plays an important role in determination of neurotransmitter phenotype (Liu et al., 2006; Myers et al., 2005). The observation that GABAergic/glycinergic NT adjusts for the loss of nicotinic signaling in particular may have important long-term consequences for clinical disease associated with exogenous exposure to nicotine, which is still far too common around the world (Hammond, 2009). We also need to understand how boosting GABAergic/glycinergic NT early in development, during the critical period when Cl^- currents are depolarizing and excitatory, may adversely affect mature neural circuits. It is well known that GABAergic/glycinergic inhibitory NT is important for proper phase transitions in neurons that participate in CPG circuits for both locomotion and breathing in newborn and adult animals (Daun et al., 2009; Dutschmann et al., 2009; McCrea and Rybak, 2008). Moreover, data show that GABAergic NT is involved in the brainstem response to chronic hypoxia and acute intermittent hypoxia (Chung et al., 2006; Darnall et al., 2012). In other words, it will be necessary to understand how early manipulations of neurotransmitter systems translate into appropriate or inappropriate motor behaviors following birth. We also lack information about how to correct these problems when they arise. Details about how active cells and networks respond to environmental stimuli during prenatal and postnatal development could open many doors to medical interventions yet unrealized, such as the application of motor circuit-directed afferent stimuli in humans, which has the potential to harness activity dependent plasticity in a goal direct manner (Spitzer, 2012). New information on the plasticity of critical periods themselves, *i.e.*, their onset and termination, would provide for additional clinical applications.

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Fig. 1.

Persistent *in vitro* nicotine exposure.

Example rectified and integrated suction electrode recording from cranial nerve IX during control baseline conditions (1st half of upper panel) in an embryonic day 5 (E5) chicken embryo. Exogenous nicotine (0.5 μ M) was added to the bath at approximately 15 min of stable baseline rDNA as indicated by the solid hatched bar. The addition of nicotine eliminated episodic activity. Note that an elevated tonic baseline activity accompanies the disappearance of rDNA suggesting a transient overstimulation of the network. The middle panel shows the beginning of rDNA recovery (after 3 h of continuous suction electrode recording) despite the continued presence of 0.5 μ M nicotine. The small arrowheads highlight the beginning of recovery rDNA, which is irregular at its onset but increases in intensity thereafter. The lower panel shows the full recovery of cranial nerve IX activity following 12 h of continuous suction electrode recording also in the continued presence of 0.5 μ M nicotine. $\int$ CN IX = rectified and integrated cranial nerve IX; rDNA = rhythmic spontaneous neural activity. The upper, middle and lower panels display continuous data from one animal. The right panel with higher resolution episodes is taken from areas highlighted with gray boxes.

Fig. 2.

Properties of the rSNA waveform in control conditions at E5 and E5.5.

A. Example rectified and integrated suction electrode recording from cranial nerve IX of an unexposed control E5 embryo during control baseline conditions and following bath application of mecamylamine (50 μ M) and dihydro- β -erythroidine (DH β E, 20 μ M). rSNA is generated largely by nicotinic neurotransmission at this early stage. B. Control baseline rSNA from an unexposed embryo at E5.5 and the effect of applied bicuculline (GABA receptor antagonist) and strychnine (glycine receptor antagonist) showing a clear modulation of rSNA without blocking it. rSNA is modulated by GABAergic/glycinergic

neurotransmission at this early stage. Note the inset labelled Bb showing single higher resolution traces of rSNA in control conditions and in the presence of bicuculline (bic-10 μM) and strychnine (strych-0.4 μM). Black and grey arrows indicate the specific episodes used for the inset during each condition. C. Summary statistics for rSNA bursts characteristics following bicuculline (10 μM) and strychnine (0.4 μM) relative to baseline of unexposed control embryos. * indicates $P < 0.05$.

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Fig. 3.

Effect of persistent nicotine exposure on rSNA at E5–5.5.

A. Baseline electrical recording from cranial nerve IX in control (unexposed) conditions at E5 followed by bath applied 0.5 μ M nicotine, which abolished rSNA (hatched bar). Recovery of rSNA occurred about 6 h later in this embryo while in the continued presence of nicotine (note broken baseline). Recovery rSNA was initially slower than control frequency and tonic activity was increased. All records in panels in A. are from the same embryo. B. Electrical recording of rSNA from an E5.5 brainstem exposed to nicotine for 12 h. Compared to panel (A.) this record shows an example of late rSNA recovery while still in the presence of nicotine. Unlike unexposed (control) brainstems, atropine and physostigmine (physo) had no effect on the recovered rSNA suggesting a functional desensitization of nAChRs. C. Effect of bicuculline (bic-GABA_A receptor antagonist) and strychnine (strych-glycine receptor antagonist) on rSNA following rSNA recovery in the continued presence of nicotine. Unlike control (unexposed) embryos, bic and strychnine eliminated rSNA during recovery. Note rSNA returned following a wash period with 0.5 μ M nicotine still present in the superfusate. D. Summary statistics for the frequency of rSNA as a function of treatment (control = white bars and nicotine treated = cross hatched bar). * indicates $P < 0.05$.

Fig. 4.

Effect of low $[K^+]_o$ on rSNA at E5.

A. Suction electrical recording from cranial nerve IX as the aCSF is varied from a high KCl concentration (8 mM) to low KCl concentration (0 mM). In this example low $[K^+]_o$ transiently slowed rSNA and resulted in a more complex burst envelope and a higher burst duration and amplitude following recovery. Note the expanded traces reflecting rSNA during baseline conditions (high $[K^+]_o$) and during low $[K^+]_o$. B. Summary statistics comparing rSNA properties following the low $[K^+]_o$ manipulation with rSNA baseline properties that were present in control conditions. C. Suction electrical recording from cranial nerve IX when aCSF is varied from a high KCl concentration (8 mM) to low KCl concentration (0 mM). In this example low $[K^+]_o$ transiently reduced rSNA and then it recovered. When bicuculline and strychnine were added to the bath rSNA was eliminated until bicuculline and strychnine were removed from the superfusate. * indicates $P < 0.05$.