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

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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 $[K^+]_o$ 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 nicotinergic 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.

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

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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 nicotinergic 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 nicotinergic 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 nicotinic neurotransmitter signaling on the embryonic chick lumbar spinal cord show that low doses of nicotine (0.1–1 μM) 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 nicotinergic 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 nicotinergic 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. Futures 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 distributer (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 $MgCl_2$, 1.26$CaCl_2$, 8 KCl, 26 $NaHCO_3$, 0.58 NaH_2PO_4 bubbled with a 95% O_2, 5% CO_2 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