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Tonic nicotinic transmission enhances spinal GABAergic presynaptic release and the frequency of spontaneous network activity

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Abstract

Synaptically driven spontaneous network activity (SNA) is observed in virtually all developing networks. Recurrently connected spinal circuits express SNA, which drives fetal movements during a period of development when GABA is depolarizing and excitatory. Blockade of nicotinic acetylcholine receptor (nAChR) activation impairs the expression of SNA and the development of the motor system. It is mechanistically unclear how nicotinic transmission influences SNA, and in this study we tested several mechanisms that could underlie the regulation of SNA by nAChRs. We find evidence that is consistent with our previous work suggesting that cholinergically-driven Renshaw cells can initiate episodes of SNA. While Renshaw cells receive strong nicotinic synaptic input, we see very little evidence suggesting other spinal interneurons or motoneurons receive nicotinic input. Rather, we found that nAChR activation tonically enhanced evoked and spontaneous presynaptic release of GABA in the embryonic spinal cord. Enhanced spontaneous and/or evoked release could contribute to increased SNA frequency. Finally, our study suggests that blockade of nAChRs can reduce the frequency of SNA by reducing probability of GABAergic release. This result suggests that the baseline frequency of SNA is maintained through elevated GABA release driven by tonically active nAChRs. Nicotinic receptors regulate GABAergic transmission and SNA, which are critically important for the proper development of the embryonic network. Therefore, our results provide a better mechanistic framework for understanding the motor consequences of fetal nicotine exposure.

Keywords

nicotinic receptor; spontaneous network activity; chick embryo; spinal cord; GABA_A receptor

INTRODUCTION

Developing neural circuits display episodic patterned spontaneous activity generated by a recurrently connected network in which both GABA- and glutamate-mediated

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neurotransmission are excitatory (O'Donovan, 1999; Ben-Ari, 2001; Blankenship and Feller, 2010). In the embryonic spinal cord spontaneous network activity is involved in multiple aspects of motor maturation, such as muscle and joint development (Ruano-Gil et al., 1978; Roufa and Martonosi, 1981), axonal pathfinding (Hanson and Landmesser, 2004; Hanson et al., 2008; Kastanenka and Landmesser, 2013), and regulation of the strength of the synaptic inputs to motoneurons (Gonzalez-Islas and Wenner, 2006).

SNA is a network-driven phenomenon that depends on chemical neurotransmission between spinal neurons (Chub and O'Donovan, 1998; O'Donovan et al., 1998). The neurotransmitter receptors that SNA is dependent upon change during development (Ladle et al., 2007; Momose-Sato and Sato, 2013). At early stages in chick, rat and mouse spinal development, nicotinic cholinergic transmission is thought to drive the expression of SNA, while excitatory GABA_A receptor activation also contributes; later in embryonic development, glutamatergic transmission becomes increasingly important while nicotinic transmission contributes, but is less critical for the expression of spinal SNA (Sernagor et al., 1995; Chub and O'Donovan, 1998; Milner and Landmesser, 1999; Wenner and O'Donovan, 2001; Hanson and Landmesser, 2003; Ren and Greer, 2003; Mochida et al., 2009; Momose-Sato et al., 2012; Czarnecki et al., 2014). Further, cholinergic transmission in the embryo has been shown to be required for the proper development of spinal locomotor behavior (Myers et al., 2005). Mechanistically, it is unclear how cholinergic transmission influences spinal development or regulates SNA at early and later stages of embryonic development.

We have shown previously that episodes of SNA are triggered at later embryonic stages via motoneuron-driven nicotinic activation of Renshaw cells (Wenner and O'Donovan, 1999; Wenner and O'Donovan, 2001). In the chick embryo, these Renshaw cells activate the rest of the network through their excitatory GABAergic projections. When nicotinic antagonists block the activation of this pathway, SNA is then generated through a different pathway, and the frequency of this activity is reduced. Therefore nicotinic regulation of SNA frequency could occur by blocking the Renshaw pathway. However, it remains possible that nicotinic receptor antagonists exert their influence on SNA frequency through distinct mechanisms. It has been shown at earlier stages of spinal cord development in the mouse that acetylcholine can directly evoke the release of vesicles of GABA and glutamate (Czarnecki et al., 2014). Therefore, nicotinic transmission could influence SNA frequency through modulation of presynaptic release; such presynaptic regulation has been demonstrated in other systems (Wonnacott, 1997; Gotti and Clementi, 2004; Barik and Wonnacott, 2009).

While nicotinic transmission clearly can drive the activation of Renshaw cells, we find additional mechanisms for nicotinic control of SNA at the later embryonic stages of spinal cord development. We also have results that suggest that SNA is significantly influenced by nicotinic regulation of presynaptic release of GABAergic, but not glutamatergic vesicles. We report that nicotine increased and nicotinic antagonists decreased GABAergic mPSC frequency, evoked GABAergic responses, and SNA frequency.

METHODS

Dissection

Experiments were performed on isolated spinal cords of stage 36 (Hamburger and Hamilton, 1951) White Leghorn chicken embryos. Eggs were placed in a 1550 Digital Hatcher incubator (GQF, Manufacturing Co.) for 10 days (embryonic day 10, or E10) at 38°C. The lumbosacral spinal cord, with attached spinal nerves, was isolated under cooled (15°C) oxygenated Tyrode's solution containing (in mM): 139 NaCl, 12 D-glucose, 17 NaHCO₃, 3 KCl, 1 MgCl₂, and 3 CaCl₂. Tyrode's solution was constantly bubbled with a mixture of 95% O₂-5% CO₂ to maintain pH at ~7.3. After dissection, the cord was allowed to recover for at least 6 hours in Tyrode's at 18°C. The cord was then transferred to a recording chamber, continuously perfused with oxygenated Tyrode's solution that was slowly heated to recording temperature (28°C). Stable spontaneous network activity (constant burst frequency, every 8-10 min) was established before starting the experiments.

Immunoblots

The ventral half of the spinal cord from the lumbosacral region was isolated, and then homogenized in RIPA buffer supplemented with protease and phosphatase inhibitors (Sigma-Aldrich). Samples were then centrifuged at 16,000 g for 5 min to remove cell debris. Protein concentration was quantitated using BCA reagent (Pierce). Samples were separated on 4–15% SDS-PAGE, and blotted to a nitrocellulose membrane. $\alpha 4$ nicotinic receptor subunit primary antibody was acquired from Alomone Labs, while primary antibodies for $\alpha 3$ and $\alpha 6$ nicotinic receptor subunits were from Novus Biologicals. The secondary antibody was an anti-rabbit IgG from Jackson ImmunoResearch. The blot was visualized by ECL chemiluminescence (GE Healthcare). Blots were done in duplicate, and each sample represents a lysate of 3 different cords.

Whole cell recordings

Whole cell patch-clamp recordings were made from motoneurons localized between lumbosacral segments 1 and 3 held at -70°C in order to acquire miniature postsynaptic currents. TTX (0.5 μM), CNQX (10 μM) and APV (50 μM) were used to isolate GABAergic mPSCs, while TTX and gabazine (5 μM) were used to isolate AMPAergic mPSCs. Glycine receptor antagonists were not used since glycinergic mPSCs were not observed in the chick embryo motoneurons at the stages studied (Gonzalez-Islas and Wenner, 2006). For paired-pulse experiments, evoked GABAergic postsynaptic currents were produced in motoneurons by stimulating the ventrolateral funiculus (VLF) via a tight fitting glass suction electrode in the presence of CNQX and APV. Motoneurons were identified by their lateral position in the ventral cord and interneurons were recorded from a more central region (middle third of the cord). Patch Clamp tight seals ($>2\text{ G}\Omega$) were obtained using electrodes pulled from thin-walled glass (World Precision Instruments, Inc) in two stages, using a P-87 Flaming/Brown micropipette puller (Sutter Instruments) to obtain resistances between 5 and 10 M Ω . Series resistance during recording varied from 15 to 20 M Ω among different motoneurons and was not compensated. Recordings were terminated whenever significant increases in series resistance ($>20\%$) occurred. Tyrode's solution was used as the extracellular recording solution for mPSCs. The intracellular patch solution for mPSC recordings contained the

following (in mM): 5 NaCl, 100 K-gluconate, 36 KCl, 10 HEPES, 1.1 EGTA, 1 MgCl₂, 0.1 CaCl₂, 1 Na₂ATP, and 0.1 MgGTP; pH was adjusted to 7.3 with KOH. mPSCs were acquired on an Axopatch 200B patch clamp amplifier (Molecular Devices), digitized on-line using PClamp 10 (Molecular Devices), and analyzed off-line with MiniAnalysis (Synaptosoft, Inc).

Recording of Ventral Root and Ventrolateral Funiculus Potentials

Tight-fitting glass suction electrodes were used to record and stimulate the proximal part of the cut ventral root (VR, cut away from the underlying dorsal root ganglion) and VLF as described previously (O'Donovan 1989; O'Donovan and Landmesser 1987; Xu et al., 2005). For monitoring Spontaneous Network Activity, VLF signals were amplified (1000X), filtered (0.1 Hz to 1 kHz) by an extracellular amplifier (A-M Systems Inc.) and acquired using PClamp 10 (Molecular Devices). VR potentials were evoked by electrical stimulation (ISO-Flex/Master 8 - A.M.P.I.) of the VLF or VR through the suction electrode, amplified 1000X and were not filtered. The amplitude of the VR response was measured following stimulation of the adjacent VR or VLF in the absence or presence of nicotine or the combination of DH β E and Mecamylamine - recordings started 20 minutes after bath addition. Analyses of the data were performed off-line.

Statistics

Data from averages are expressed as mean $\pm$ SEM. Student's t-test ($\alpha = 0.05$) was used to perform the statistical analyses of mPSC amplitudes, frequencies and decay times. Statistical analyses of evoked responses (Figure 4-6) were carried out using a paired t-test.

Drugs

Nicotine ((S)-(-)-1-Methyl-2-(3-pyridyl) pyrrolidine (+)-ditartrate salt), *α -bungarotoxin* (α -BTX), *DH β E* ((2S,13bS)-2-Methoxy-2,3,5,6,8,9,10,-13-octahydro-1H,12H-benzo[i]pyrano[3,4-g]indolizin--12-one hydrobromide), *MLA* ([1 α ,4(S),6 β ,14 α ,16 β]-20-Ethyl-1,6,14,16--tetramethoxy-4-[[[2-(3-methyl-2,5-dioxo-1-pyrroli-dinyl)benzoyl]oxy]methyl] aconitane- 7,8-diol citrate), *Bicuculline* ([R-(R*,S*)]-5-(6,8-Dihydro-8-oxofur-o[3,4-e]-1,3-benzodioxol-6-yl)-5,6,7,8-tetrahydro--6,6-dimethyl-1,3-dioxolo[4,5-g] isoquinolinium iodide), *CNQX* (6-Cyano-7-nitroquinoxaline-2,3-dione disodium), *APV* (D-(-)-2-Amino-5-phosphonopentanoic acid), *isoguvacine* (1,2,3,6-Tetrahydro-4-pyridinecarboxylic acid hydrochloride) and *Neostigmine* (3-(N,N-Dimethylcarbamoyloxy)-N,N,N-trimethylanilinium bromide) were purchased from Tocris Bioscience. *Mecamylamine* (2-(Methylamino) isocamphane hydrochloride, Inversine, N, 2,3,3-Tetramethylbicyclo [2.2.1] heptan-2-amine hydrochloride) and *Choline* ((2-Hydroxyethyl) trimethylammonium chloride) were purchased from Sigma-Aldrich.

RESULTS

Nicotine increased the frequency of SNA in control and GABA-driven embryonic spinal networks

Spontaneous network activity (SNA) occurs as episodes of synchronous spiking activity through the spinal network that last seconds, followed by longer-lasting inactive periods

(inter-episode intervals, IEI). Previous studies have shown that nicotinic antagonists dramatically reduce SNA at E6 but only slow its frequency at E10 (Milner and Landmesser, 1999; Wenner and O'Donovan, 2001). We found that nicotine (10 μ M) increased the frequency of SNA in the isolated spinal cord of the E10 chick embryo (10.2 ± 0.8 min; nicotine 8.0 ± 0.6 min, $n=3$, $p<0.05$; Fig. 1A-B). Previously, we had shown that the GABAergic Renshaw cell, formerly referred to as the R-interneuron (Wenner and O'Donovan, 1999), was activated by cholinergic input from motoneurons, and could initiate episodes of SNA (Wenner and O'Donovan, 2001). Therefore, we expected that nicotine could act directly on Renshaw cells and reduce the IEI.

In the E10 chick spinal cord preparation, it has been shown that SNA is homeostatically regulated such that the activity recovers following blockade of either glutamate receptors or GABA_A/glycine receptors, which are excitatory at this stage (Chub and O'Donovan, 1998). However, when glutamatergic, nicotinic, and GABAergic transmission was concurrently blocked SNA did not recover. In the current study we found that simply blocking GABA_A receptors (gabazine 5 μ M) and glutamate receptors (10 μ M CNQX, 50 μ M APV) prevented the recovery of SNA in the period assessed (3-5 hours, $n=3$). The results suggest that the dominant neurotransmitters are glutamate and GABA at this stage, and are consistent with earlier studies in that either GABAergic or glutamatergic networks could express SNA. We reasoned that if the GABAergic Renshaw cell was responsible for the nicotine-induced increase in SNA frequency, then nicotine should be effective in an isolated GABAergic network (in the presence of glutamatergic antagonists), but not in an isolated glutamatergic network (in the presence of GABA_A receptor antagonist). Consistent with this idea, the inter-episode interval of SNA in a GABAergic network (CNQX/APV) decreased following bath application of nicotine (CNQX/APV 9.8 ± 0.9 min to CNQX/APV + nicotine 6.8 ± 0.7 min, $p < 0.05$; Fig. 1A-B). Alternatively, the IEI of SNA in a glutamatergic network (bicuculline) was unaltered following bath application of nicotine (bicuculline 30.4 ± 2.5 min, bicuculline + nicotine 31.5 ± 5.6 ; Fig. 1A-B). As described previously (Chub and O'Donovan, 1998), GABA_A receptor blockade (bicuculline) transiently blocked SNA, which then recovered in the presence of the antagonist, but with a slower frequency.

In a separate set of experiments we followed the IEI as we added nicotine to the control cord (no receptor antagonists). We found that addition of nicotine evoked an episode acutely upon bath application, which may have been due to recruitment of the Renshaw cell (Wenner and O'Donovan, 1999). This was followed by a quiescent period (55.6 ± 4.7 min; Fig. 1C), but when SNA reappeared the average IEI was shorter (control 11.8 ± 0.4 min; nicotine 8.1 ± 0.3 min, $n=3$ cords, $p<0.0001$; Fig. 1C). The ability of nicotine to trigger SNA upon bath application was dependent on non- $\alpha 7$ nAChRs as it was prevented in the presence of a non- $\alpha 7$ nAChR antagonist (DH β E 5 μ M, 3 of 3 cords), but not an $\alpha 7$ nAChR antagonist (α -bungarotoxin 100nM, 2 of 2 cords). These results suggest that continuous nicotine exposure increased the frequency of SNA by increasing the excitability of the GABAergic circuitry through non- $\alpha 7$ nAChRs.

Nicotine does not increase SNA frequency through the Renshaw circuitry

Motoneurons in the embryonic chick are thought to initiate episodes of SNA by firing action potentials, which then drive Renshaw cell activity via their cholinergic recurrent collaterals (Wenner and O'Donovan, 1999). Once activated, these interneurons recruit the rest of the network through their excitatory GABAergic projections (Fig. 2 schematic) (Wenner and O'Donovan, 1999; O'Donovan et al., 2008). When nicotinic transmission was blocked in order to prevent Renshaw cell recruitment, the normal order of interneuron recruitment during the initiation of the episode was changed and SNA frequency was reduced (Wenner and O'Donovan, 2001). Therefore, it was possible that activation of nicotinic acetylcholine receptors could increase SNA frequency by increasing Renshaw cell spiking. We tested the Renshaw pathway by stimulating one ventral root (VR) while recording an adjacent VR. This pathway was also dependent on non- $\alpha 7$ nAChRs (Fig. 2A) as it was completely abolished by DH β E (3 of 3 cords), while blocking $\alpha 7$ nAChRs had no effect on the response (α -bungarotoxin, 2 of 2 cords). In order to test whether the Renshaw circuit was more excitable in the continuous presence of nicotine, we bath applied 10 μ M nicotine and tested the Renshaw cell pathway. The VR-evoked potential in the adjacent VR was abolished in the presence of nicotine, even after SNA had recovered (3 of 3 cords, Fig. 2B). This result suggests that bath application of nicotine prevents the activation of Renshaw cells and therefore these neurons did not appear to mediate the nicotine-induced increase in SNA frequency.

Nicotinic postsynaptic currents do not appear to mediate the ongoing increase in SNA frequency

If the Renshaw circuit was not responsible for the effect of nicotine, then how did nicotine maintain an increase in the rate of SNA? We considered the possibility that nicotine might act directly on other neurons by activating depolarizing currents at the postsynaptic membrane. Accordingly, we bath applied fast pulses of nicotine or acetylcholine (50 μ l of 10 mM stock) in the recording chamber perfusate (Tyrode's solution containing TTX), which was not recirculated for 2 minutes (Fig. 3A, see methods). Motoneuron responses were measured using whole cell recordings (Fig. 3B, see methods). No detectable postsynaptic current was observed during nicotine or acetylcholine injections. The average holding current measured one minute before and after the nicotine pulse was no different (74.9 ± 28.0 pA in control vs. 74.3 ± 32.8 pA, $n=4$), nor did we see any change in spontaneous currents; however, puffing the GABA_A agonist isoguvacine evoked a strong long-lasting current in these motoneurons (Fig. 3B). Together, these results argue that the nicotine injections did not produce a significant postsynaptic activation of nicotinic receptors at the dendrites or soma of motoneurons.

Alternatively, the nicotine-dependent increase of SNA frequency could arise from the depolarization of spinal interneurons other than Renshaw cells. To explore this possibility we recorded whole cell from ventral interneurons while bath applying fast pulses of nicotine. We observed almost no detectable response to nicotine (Fig. 3C). Of 12 interneurons we recorded, 10 showed no response, while 2 showed very small currents (22 and 40 pA). These findings are consistent with previous work that demonstrated a strong response to acetylcholine application in Renshaw cells, but not other interneurons in the

neonatal rat spinal cord (Alvarez and Fyffe, 2007). Together the findings suggest that continuous nicotine exposure may influence SNA frequency by some means other than depolarization of the dendrites or soma of either motoneurons or interneurons.

Nicotine modulates evoked presynaptic GABA release

We considered the possibility that nicotine influenced SNA frequency through presynaptic modulation of transmitter release. It has been widely described that presynaptic nicotinic receptors are involved in the modulation of the release of several neurotransmitters (Dani, 2001) and this has recently been suggested in the early embryonic spinal cord (Czarnecki et al., 2014). We considered the possibility that nicotinic transmission altered presynaptic release of vesicles. Since we had previously demonstrated a nicotinic regulation of GABA-driven SNA, but not glutamate-driven SNA (Fig. 1), we decided to explore if nicotinic transmission was involved in the modulation of evoked GABAergic responses onto motoneurons. Accordingly, we examined the effect of nicotine on ventral root (VR) responses evoked by electrical stimulation of the ventrolateral funiculus (VLF). These VR potentials were predominantly GABAergic as CNQX (10 μ M) and APV (50 μ M) were present during the experiment, and there is little evidence for synaptic currents other than those mediated by GABA_A and glutamate receptors at this stage (Gonzalez-Islas and Wenner, 2006). The amplitude of VLF-evoked VR potentials was enhanced by 11.4% in the presence of nicotine (30 min) relative to controls (Fig. 4B, D). Further, the nicotinic antagonists DH β E (5 μ M) and Mecamylamine (20 μ M) reduced the average VR potential amplitude by 14.6 % suggesting that nAChRs were tonically enhancing evoked release at baseline (Fig. 4C-D).

Next, we wanted to rule out the possibility that nAChR agonists and antagonists had a non-specific direct effect on GABA_A receptors. GABA and nicotinic receptors belong to a homologous superfamily of ligand-activated membrane receptors, and thus it was possible that pharmacologically non-specific effects could occur. Therefore, we assessed the effect of nicotine and nicotinic antagonists on GABA_A-mediated responses. We measured the potentials produced in VLF and VR recordings following isoguvacine puffs (isolated with TTX and glutamatergic antagonists - CNQX 10 μ M and APV 50 μ M). Continuous bath application of Nicotine (10 μ M) had no effect on the isoguvacine-evoked GABAergic VR or VLF potentials (VR – $643 \pm 210 \mu$ V in control vs. $605 \pm 223 \mu$ V in nicotine, $p=0.15$; VLF – $631 \pm 51 \mu$ V in control vs. $642 \pm 61 \mu$ V in nicotine, $p=0.70$, $n=5$; Fig. 5A, C). Similarly, isoguvacine-evoked VR and VLF potentials were unaffected by the nicotinic antagonists (VR – $1498 \pm 156 \mu$ V in control vs. $1478 \pm 114 \mu$ V in DH β E/mecamylamine, $p=0.92$; VLF – $2725 \pm 353 \mu$ V in control vs. $2234 \pm 208 \mu$ V in DH β E/mecamylamine, $p=0.24$, $n=6$; Fig. 5B, C). The results suggest that nicotinic modulators were not directly interfering with GABA_A receptors. Further, the findings suggested that modulation of nAChR activation did not alter postsynaptic sensitivity to GABA.

To further demonstrate the presynaptic nature of the nicotinic control of GABA release, we recorded motoneuron whole cell voltage clamp responses to paired pulse stimulation. A section of the VLF was brought into a suction electrode and paired stimulation separated by 50 ms was delivered while recording motoneuron current responses. GABAergic currents

were isolated by application of glutamatergic antagonists, CNQX (10 μ M) and APV (50 μ M). Before addition of nicotine, the paired pulse ratio (2nd pulse / 1st pulse) was greater than 1, as the response to the second pulse was facilitated (paired pulse ratio 1.70 ± 0.18 , $n=10$; Fig. 6). Bath application of nicotine (10 μ M) for 10 minutes increased the response to the first stimulus with no significant change in the amplitude of the current evoked by the second stimuli (paired pulse ratio 1.18 ± 0.10 , $p=0.025$, $n=10$; Fig. 6). This result was consistent with the above observation that nicotine increases evoked GABA release, and demonstrates a presynaptic action of the drugs. Taken together, our results show that the activation of presynaptic nicotinic receptors enhanced evoked release of GABAergic vesicles onto embryonic motoneurons.

Nicotinic receptor activation increased the frequency of GABAergic mPSCs, but not AMPAergic mPSCs

Since presynaptic activation of nicotinic receptors enhances action potential-dependent GABA release, nicotine could increase SNA frequency through presynaptic increases in probability of GABAergic release. Previously we reported findings consistent with the idea that action potential-independent spontaneous release could influence the expression of SNA (Wenner and O'Donovan, 2001; Gonzalez-Islas et al., 2012). To test the idea that nicotine could influence SNA frequency by altering spontaneous GABA release, we assessed the effect of nicotine on GABAergic mPSCs recorded from embryonic motoneurons. In Figure 7A, representative traces of GABAergic mPSCs are shown before and after bath application of nicotine (10 μ M). In the presence of nicotine (30 min), GABAergic mPSC frequency increased significantly compared to controls (0.46 ± 0.10 Hz in control, $n=12$ vs. 1.44 ± 0.31 Hz in nicotine, $n=9$, $p<0.001$; Fig. 7A, C). This effect took several minutes to develop, as acute nicotine injections in the bath did not trigger vesicle release immediately (Fig. 3C). Nicotine application produced no significant difference in GABAergic mPSC amplitude (13.2 ± 1.0 pA in control, $n=12$ vs. 13.5 ± 0.9 Hz in nicotine, $n=9$; Fig. 7D). To test whether acetylcholine is endogenously released in the embryonic spinal cord, we applied the acetylcholinesterase inhibitor neostigmine (30 μ M). Bath application of neostigmine increased GABAergic mPSC frequency (0.46 ± 0.10 Hz control, $n=12$ vs. 0.92 ± 0.28 Hz in neostigmine, $n=6$, $p<0.001$; Fig. 7B-C) with no change in the amplitude of the GABAergic mPSCs (13.2 ± 1.0 pA in control, $n=12$ vs. 13.9 ± 2.7 Hz in neostigmine, $n=6$; Fig. 7D). The result suggests there is an ongoing endogenous production and release of acetylcholine in the embryonic ventral spinal cord. On the other hand, bath application of nicotine had no effect on AMPA mPSC frequency or amplitude (frequency – 1.1 ± 0.4 Hz control vs. 0.9 ± 0.3 Hz in nicotine; $n=6$; amplitude – 9.8 ± 3.1 pA control vs. 9.3 ± 1.6 pA nicotine, $n=9$). These results further demonstrated that GABA vesicle release is presynaptically modulated by nAChRs, and suggested that nicotinic enhancement of spontaneous release could contribute to the nicotine-induced increase in SNA frequency.

Tonic nicotinic control of presynaptic GABA release is mediated by non- $\alpha 7$ nicotinic receptors

Next we tested whether nAChRs were tonically active at baseline in the control, and determined the class of receptors that mediated changes in GABAergic mPSC frequency. We first bath applied the non- $\alpha 7$ antagonist DH β E (5 μ M) and found that this antagonist

reduced the frequency of GABAergic mPSCs (0.43 ± 0.10 Hz, $n=20$ in control vs. 0.29 ± 0.06 Hz, $n=8$ in DH β E, $p<0.05$; Fig. 8A-B). In order to determine if the α -7 nicotinic receptor also contributed to the presynaptic control of GABAergic release we bath applied 3 different α 7 nAChR modulators. In Figure 8A representative traces of GABAergic mPSCs are shown before and after the application of the α 7 specific agonist choline (10 mM), or the α 7 antagonists MLA (20nM) or α -BTX (100nM). No significant differences in GABAergic mPSC frequency were observed compared to controls (choline - 0.53 ± 0.15 Hz, $n=9$; MLA - 0.47 ± 0.10 Hz, $n=10$; α -BTX - 0.62 ± 0.10 Hz, $n=7$; Fig. 8B), suggesting that nicotinic modulation of GABAergic mPSC frequency is mediated by non- α 7 nicotinic receptors. Moreover, bath addition of nicotine (10 μ M) in the presence of α -BTX was still able to increase GABAergic mPSC frequency (0.62 ± 0.10 Hz, $n=7$, before nicotine vs. 0.97 ± 0.11 Hz, $n=6$, after nicotine, $p<0.01$; Fig. 8B), strongly supporting the conclusion that modulation of GABAergic mPSC frequency by nicotine is specifically mediated by non- α 7 nicotinic receptors. As expected, none of the nicotinic modulators had a significant effect on GABA mPSC amplitude compared to controls (13.4 ± 0.7 pA, $n=20$ for control; 14.0 ± 1.7 pA, $n=8$ for DH β E; 13.6 ± 0.85 pA, $n=9$ for choline; 13.8 ± 1.6 pA, $n=7$ for α -BTX; Fig. 8C).

Bath application of 5 μ M DH β E also reduced the frequency of episodes of SNA in GABA-driven networks (CNQX+APV treated cords). IEIs were increased from 10.3 ± 2.3 min to 15.3 ± 1.8 min ($n=4$ cords, $p<0.05$) suggesting that cholinergic modulation of SNA, like GABA mPSC frequency, was mediated by non- α 7 nicotinic receptors.

Finally we used Western blots of E10 embryonic chick ventral half spinal cords to test for non- α 7 subunits that could be expressed at this stage. The E10 spinal cord expresses at least α 3, α 4 and α 6 nicotinic receptor subunits (Supplemental Fig. 1).

DISCUSSION

The main goal of this study was to explore the role of nicotinic transmission in the expression of SNA in the embryonic spinal cord. We investigated the role of nicotinic receptors on SNA at a stage where glutamatergic and excitatory GABAergic transmission are the major contributors to the expression of SNA. We find that nicotinic modulation of SNA frequency occurs through a regulation of the GABAergic circuitry. We show that activation of nAChRs increases evoked and spontaneous GABAergic release, while nicotinic antagonists reduce GABAergic release. Together our findings suggest that presynaptic nAChRs regulate GABA release and in this way contribute to the expression of SNA in a tonic fashion. Our results have important implications for chronic fetal exposure to drugs that alter nicotinic transmission, and therefore the developmental expression of SNA, which will likely have long-term consequences on network behavior (Dani, 2001).

Nicotinic receptors tonically control presynaptic release of GABA in the embryonic spinal network

Nicotinic receptors have a clear influence on SNA in embryonic spinal circuits, but it is not clear how this influence is manifest. Although it appears that activation of postsynaptic nicotinic receptors does occur for Renshaw cells (Wenner and O'Donovan, 2001), there was less evidence for dendritic or somatic nicotinic currents in motoneurons or non-Renshaw

interneurons (Alvarez and Fyffe, 2007). Nicotine injections did not produce a response in whole cell recordings of motoneurons or many interneurons, despite the expression of nAChRs postsynaptically in chick spinal neurons (Roth and Berg, 2003). Previous reports have demonstrated that nicotine agonist microinjections directly onto motoneurons in neonatal rat slices can produce postsynaptic currents (Zaninetti et al., 1999; Chamberlin et al., 2002). It is possible that we and others (Czarnecki et al., 2014) do not see a nicotinic response in motoneurons as nicotine concentrations at motoneuron nicotinic receptors within the spinal preparation may not achieve high enough levels to activate these receptors.

Here, we show that part of the nicotinic regulation of SNA is dependent on a continuous presynaptic modulation of GABAergic release. The observation that nicotine did not produce a significant postsynaptic response in motoneurons or interneurons is consistent with the possibility that nicotine predominantly activated receptors on the terminals or axons. Bath application of nicotine in the presence of TTX increased the frequency of GABAergic mPSCs in embryonic spinal motoneurons after several minutes, likely through presynaptic nAChRs. Further, nicotinic antagonists (non- $\alpha 7$) decreased GABAergic mPSC frequency. Likewise, evoked GABAergic currents also appeared to be presynaptically regulated; the amplitude of VLF-evoked GABAergic potentials in motoneurons were enhanced by nicotine and inhibited in the presence of nicotinic antagonists (Fig 4). The effects of nicotinic modulators on the evoked currents are likely to be presynaptic. First, the modulators had no effect on GABAergic mPSC amplitude. Second, paired-pulse experiments carried out using whole cell recordings in motoneurons also suggested an enhancement of presynaptic GABA release by nicotine (Figure 6). Finally, nicotine and nAChR antagonists had no influence on the postsynaptic sensitivity to a GABA_A agonist in the presence of TTX (Figure 5).

This nicotinic regulation of presynaptic release appears to be ongoing or tonic. VLF-evoked GABAergic responses and GABAergic mPSCs were reduced upon application of nAChR antagonists, suggesting an ongoing activation of nAChRs at baseline. Further, it is clear that acetylcholine is endogenously produced and released in the embryonic cord as GABAergic mPSC frequency was increased in the presence of the acetylcholinesterase inhibitor, neostigmine. In addition, this nicotinic modulation of mPSC frequency was slow; adding nicotine to the recirculating bath took several minutes to increase mPSC frequency and did not cause an immediate release of GABA vesicles. The observation that the nicotinic modulation of presynaptic release was on the order of minutes suggests a slow constant activation of receptors is necessary, possibly activating calcium dependent second messenger systems. It is unlikely that the delay of the response is due to tissue penetration as episodes were triggered in less than a minute after adding nicotine (10 μ M) to the bath (Figure 1C).

The importance of nicotinic receptors at different developmental stages in the spinal cord

Nicotinic enhancement of presynaptic release has been described at noradrenergic, dopaminergic, cholinergic, glutamatergic and GABAergic synapses (Grady et al., 1992; Wilkie et al., 1993; McGehee et al., 1995; Clarke and Reuben, 1996; Gray et al., 1996; Yang et al., 1996). Anatomical and physiological experiments have demonstrated that presynaptic

nAChRs can enhance presynaptic vesicle release during development as well (Role and Berg, 1996). Nicotinic modulation of presynaptic release has also been identified in the neonatal and adult rodent spinal cord (Umana et al., 2013). In the dorsal horn, nicotine is known to have analgesic effects, and this is in part due to regulation of presynaptic release. Several studies show that non- $\alpha 7$ nicotinic receptors can enhance inhibitory presynaptic release in the substantia gelatinosa (Kiyosawa et al., 2001; Takeda et al., 2003; Genzen and McGehee, 2005; Rashid et al., 2006). In addition, nicotinic regulation of primary afferent glutamatergic release has been described in the dorsal horn (Genzen and McGehee, 2003; Khan et al., 2004). Although far less is known about nicotinic control of presynaptic release in the motor pathways of the ventral part of the cord, nicotinic regulation of presynaptic inputs to sympathetic preganglionic neurons has been demonstrated in the neonatal rat (Bordey et al., 1996; Seddik et al., 2006). Finally, in cultured embryonic rat spinal interneurons, nicotine was found to regulate the release of both GABA and glutamate (Fucile et al., 2002).

It has been unclear whether such a role for presynaptic nicotinic receptors exists in the intact embryonic spinal cord. A recent report in the mouse spinal cord has demonstrated that fast application of acetylcholine can drive the presynaptic release of glutamate, GABA, and glycine (Czarnecki et al., 2014). This work was critical because it was done at a stage when SNA is first expressed and when blockade of nicotinic receptors can abolish the expression of SNA. The study raised the possibility that spontaneous network activity could be regulated by a tonically active control of presynaptic transmitter release. Here we show at a later embryonic stage that, indeed this is the case. Our findings that nicotinic receptor antagonists reduce the frequency of spontaneous network activity and reduce evoked and spontaneous presynaptic release suggest a tonic nicotinic transmission that elevates the baseline release of GABAergic vesicles. We did not observe a presynaptic regulation of glutamatergic release by nicotinic receptors as had been reported at an earlier stage in the mouse cord (Czarnecki et al., 2014), which may represent a developmental restriction in the scope of nicotinic influence.

Blockade of nAChRs reduces spinal SNA frequency at later embryonic stages (E10 in chick, E17.5 in rat - (Wenner and O'Donovan, 2001; Ren and Greer, 2003)). It is likely that reduced nAChR activation reduces GABA release, which slows the occurrence of SNA at these later stages. Nicotinic antagonists, particularly DH β E, have an even more profound influence on SNA frequency at earlier embryonic stages (E4-6 in chick, E12.5-14.5 in mouse, E13.5 in rat - (Milner and Landmesser, 1999; Hanson and Landmesser, 2003; Ren and Greer, 2003; Myers et al., 2005; Mochida et al., 2009; Momose-Sato et al., 2012; Czarnecki et al., 2014)). Acetylcholine application can cause release of glutamatergic, GABAergic, and glycinergic vesicles in the E12 mouse spinal cord (Czarnecki et al., 2014). If nAChRs are tonically active at this earlier stage, then the profound impact of nAChR blockade could be due to the inhibition of presynaptic release of all 3 transmitters.

Working Model of nicotinic influence on the expression of spinal SNA

Our previous work demonstrated that SNA is normally initiated through motoneuron nicotinic activation of Renshaw cells, which recruit other interneurons through their

excitatory GABAergic projections (Wenner and O'Donovan, 2001). The current study is consistent with our previous finding in the following 2 ways. First, when nicotine was bath applied, an episode of SNA was quickly triggered, consistent with a nicotinic activation of Renshaw cells leading to the recruitment of the spinal network, as occurs normally. Both the recurrent activation of Renshaw cells and network recruitment by nicotine was dependent on non- $\alpha 7$ nAChRs. Second, once nicotine was in the bath for a few minutes and the Renshaw pathway was blocked (Figure 2), the following episode was delayed, as would be expected if the normal Renshaw pathway was no longer triggering episodes. As described in our previous study (Wenner and O'Donovan, 2001), SNA recovers after blocking the Renshaw pathway, but with a slower frequency. We argued that the activity was slower due to blockade of the Renshaw circuitry. It was then surprising to us in the current study that nicotine blocked the Renshaw pathway, yet led to an eventual increase in the frequency of SNA. Blockade of the Renshaw pathway may be due to desensitization of nicotinic receptors or depolarizing block at the level of the Renshaw cell. The nicotine-mediated increase of SNA frequency is likely caused by an increase in GABAergic release due to nicotinic receptor activation at presynaptic terminals. We favor the possibility that nAChRs involved in Renshaw activation and changes in probability of release are 2 different classes of non- $\alpha 7$ nicotinic receptors as Renshaw motoneuron inputs are phasic (Wenner and O'Donovan, 1999), while those mediating GABAergic release are tonically active. The observations that spontaneous GABAergic release was influenced by nicotine and DH β E, but not choline, MLA, or α -BTX, that the cord has clear of expression of $\alpha 4$ nAChR subunit, and that the currents were tonically active were all consistent with the possibility that the receptors mediating GABA release are $\alpha 4\beta 2$ nAChRs, (Albuquerque et al., 2009). This is the most abundant nicotinic receptor type.

Regulation of action potential-dependent GABA release by nAChRs could influence SNA through changes in the effective connectivity of the network by determining how faithfully neurotransmission is conveyed. In addition, regulation of action potential-independent spontaneous release can influence SNA frequency. We have shown previously that it is possible that SNA can be triggered off of spontaneous release events (Wenner and O'Donovan, 2001). Further, GABAergic mPSCs get progressively larger along the IEL, leading to stronger depolarizations in motoneurons, which begin to fire action potentials off these events and eventually trigger SNA (Wenner and O'Donovan, 2001; Gonzalez-Islas and Wenner, 2006). Finally, we have shown previously that SNA frequency can be altered by modulating glutamatergic spontaneous release, independently of evoked release, using endocannabinoid receptor modulators (Gonzalez-Islas et al., 2012). Our results here add to the mounting evidence that spontaneous release is an important contributor to the expression of SNA. While we cannot rule out other potential effects of nicotinic modulators on SNA, our results do suggest that one mechanism of influence is through changes in GABA release (spontaneous and/or action potential dependent). Therefore, the influence of nicotinic transmission on SNA extends beyond the Renshaw pathway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Nicotine increases SNA frequency in GABA-driven embryonic spinal networks

A. Representative traces of spinal nerve recordings showing the interval between episodes of SNA before and after the addition of nicotine 10 μ M in control networks, GABAergic-driven networks (10 μ M CNQX + 50 μ M APV), or glutamatergic networks (20 μ M bicuculline). **B.** Summary bar graph showing the average values for SNA inter-episode interval before and after the addition of nicotine in control (n=3), GABAergic (n=3) or glutamatergic (n=3) spinal networks. Error bars indicate standard errors, * p < 0.05. **C.** IEI shown for successive episodes of SNA before, during and after adding nicotine to the bath for 3 different spinal cord preparations. Upon addition of nicotine an episode of SNA is evoked, but then the next episode is significantly delayed before episodes recover to a higher baseline frequency.

Figure 2. Renshaw pathway inhibited by nicotine

A. Schematic showing the Renshaw circuitry that is used to test the pathway. A ventral root is stimulated, which activates Renshaw cells through the motoneuron recurrent collateral. Renshaw cells then provide input to motoneurons in the adjacent segment, which can be recorded in the adjacent ventral root. This ventral root-evoked response is shown before the addition of nicotinic modulators (control, top trace), 20 minutes after the addition of α -BTX (middle trace), and is blocked 20 minutes after adding DH β E to bath (lower trace). **B.** The ventral root-evoked response is shown before the addition of nicotine (control, top trace), and is blocked 30 minutes after adding nicotine to bath (lower trace).

Figure 3. Apparent absence of postsynaptic phasic currents mediated by nicotinic receptors
A. Schematic of perfusion system for pulsing drugs. **B.** Whole cell recording of a motoneuron that shows a strong response to isoguvacine, but no response to nicotine or acetylcholine injection. **C.** Whole cell recording from a spinal interneuron during isoguvacine and nicotine injections. During isoguvacine injection mPSCs could no longer be detected during the inward current that was evoked, but then began to recover as the inward current abated. This may be due to the depletion of vesicles or due to a weakening of the Cl^- gradient.

Figure 4. Nicotinic modulation of the amplitude of motor nerve potentials evoked by stimulation of the ventrolateral funiculus

A. Schematic of suction electrode stimulation of the VLF while recording motoneuron responses in the ventral root. **B.** Representative GABAergic potential in the VR (in presence of glutamate receptor antagonists) evoked by electrical stimulation of the VLF before (black trace) and after bath application of nicotine 10 μM (gray trace). **C.** Representative GABAergic potential in the VR (in presence of glutamate receptor antagonists) evoked by electrical stimulation of the VLF before (black trace) and after bath application of nicotinic antagonists DHβE (5 μM) and Mecamylamine (20 μM). Glutamate receptor antagonists CNQX 10 μM and APV 50 μM were present throughout these experiments. **D.** Bar chart summarizing results. Error bars are S.E..

Figure 5. Nicotine does not affect the amplitudes of the ventrolateral funiculus or the ventral root GABAergic potentials evoked by fast pulses of isoguvacine

A. Schematics showing the experimental setup, where 25 μ l of Isoguvacine (10 mM) solution is delivered to a prepool in the presence of CNQX (10 μ M) and APV (50 μ M). On right, representative traces of the VR (top two traces) and VLF (bottom two traces) are shown in control (black traces) and in the presence of nicotine (10 μ M, gray traces). **B.** Bath addition of nicotinic antagonists DH β E (5 μ M) and Mecamylamine (20 μ M) did not affect the amplitude of the isoguvacine-evoked GABAergic potentials. **C.** Bar graph showing the normalized value of the VLF and VR GABAergic potentials in the presence or absence of nicotine (gray) and in the presence or absence of DH β E and Mecamylamine (black). No significant differences were found between responses before and after drug additions. Error bars indicate S.E..

Figure 6. Nicotine reduces paired-pulse ratio of the evoked GABAergic synaptic currents

A. Representative traces of a whole-cell voltage clamped motoneuron in response to paired stimuli (50 ms interval) applied to a slip of the ventrolateral funiculus (VLF) in control (black trace) and in the presence of nicotine 10 μM (gray trace). **B.** Bar graph summarizing the results of 5 experiments. The stimulation protocol induced facilitation in control conditions but not in the presence of 10 μM nicotine, which significantly increased the amplitude of the response to the first stimulus with no significant effect on the response to the second stimulus. **C.** As a consequence, the paired-pulse ratio is significantly reduced after the bath addition of nicotine. Error bars represent S.E., * $p < 0.05$.

Figure 7. Nicotinic receptor activation enhances GABA mPSC frequency in embryonic spinal motoneurons

A-B. Representative trace of a whole-cell voltage-clamp recording of GABA mPSCs from two representative motoneurons before and after the addition of nicotine 10 μ M (**A**) or neostigmine 30 μ M (**B**). **C.** Bar graph showing a summary of average values for GABAergic mPSC frequency in control conditions or after addition of nicotine or neostigmine. **D.** Bar graph showing a summary of average values for GABA mPSC amplitude in control conditions or after addition of nicotine or neostigmine. Error bars indicate S.E., * $p < 0.001$.

Figure 8. Nicotine enhancement of GABAergic mPSC frequency is mediated by non-α7 nicotinic receptors

A. Whole-cell voltage-clamp recording of GABAergic mPSCs in the presence of CNQX from motoneurons before and after the addition of different nicotinic modulators. **B-C.** Average values for GABA mPSC frequency (**B**) or amplitude (**C**) before and after addition of the indicated drugs; additionally values are shown after addition of nicotine in the presence of α-BTX (stripped bars). Error bars indicate S.E., * $p < 0.05$, ** $p < 0.01$.