Properties and Mechanisms of Spontaneous Activity in the Embryonic Chick Hindbrain

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ABSTRACT: **Spontaneous activity regulates many aspects of central nervous system development. We demonstrate that in the embryonic chick hindbrain, spontaneous activity is expressed between embryonic days (E) 6–9. Over this period the frequency of activity decreases significantly, although the events maintain a consistent rhythm on the timescale of minutes. At E6, the activity is pharmacologically dependent on serotonin, nACh, $GABA_A$, and glycine input, but not on muscarinic, glutamatergic, or $GABA_B$ receptor activation. It also depends on gap junctions, t-type calcium channels and TTX-sensitive ion channels. In intact spinal cord-hindbrain preparations, E6 spontaneous events originate in the spinal cord and propagate into lateral hindbrain tissue; midline activity follows the appearance of lateral activity. However, the spinal cord is not required for hindbrain activity. There are two invariant points of origin of activity along the midline, both within the caudal group of serotonin-expressing cell bodies; one point is caudal to the nV exit point while the other is caudal to the nVII exit point. Additional caudal midline points of origin are seen in a minority of cases. Using immunohistochemistry, we show robust differentiation of the serotonergic raphe near the midline at E6, and extensive fiber tracts expressing GAD65/67 and the nAChR in lateral areas; this suggests that the medial activity is dependent on serotonergic neuron activation, while lateral activity requires other transmitters. Although there are differences between species, this activity is highly conserved between mouse and chick, suggesting that developmental event(s) within the hindbrain are dependent on expression of this spontaneous activity.** © 2009 Wiley Periodicals, Inc. Develop Neurobiol 69: 477–490, 2009

Keywords: **chick hindbrain; spontaneous activity; development; serotonin; pacemaker**

INTRODUCTION

Spontaneous activity (SA) in the developing nervous system is an important mechanism mediating appropriate neuron number, neuronal phenotype, synapse formation, and network properties. In many developing neuronal networks, spontaneous activity is caused by intrinsic electrophysiological properties of equivalent interacting excitatory cells that generate depolarizing events; events can initiate from any one of the neurons in the network and the interval between events is regulated by postevent depression (Tabak et al., 2006). However, it has recently been demonstrated that SA can be driven by a population of neurons acting as a pacemaker; this population initiates activity, which then propagates to other neuronal groups. This pacemaker-driven SA propagates along a stereotyped pathway, as recorded in the mouse embryonic hindbrain (Hunt et al., 2005). In this work, we show that SA in the chick embryonic hindbrain shares many features with that of the mouse hindbrain but that it differs in several important ways.

Unique among developing brain regions, the hindbrain is a transiently segmented structure organized into developmental compartments (rhombomeres)

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along the rostral-caudal axis during early embryonic stages (embryonic day [E]8–11.5 in mouse, E2–5 in chick). Within each rhombomere, cells are fated by the axial patterns of gene expression. Derivatives of the hindbrain include the medulla and pons of the adult brainstem, and the most rostral regions become the cerebellum. Major brainstem structures include motor neuron groups that innervate facial and neck regions, sensory nuclei, and longitudinal tracts that mediate communication between more rostral brain regions and the spinal cord. In addition, the brainstem includes the reticular formation (RF), which mediates brain arousal and awareness. A major component of the RF is the serotonin (5HT)-expressing raphe nuclei, which are organized into two groups that innervate much of the rostral brain and spinal cord. In the E4 chick embryo, the rostral group is 5HT-positive, while the caudal group has only slight expression (Sako et al., 1986; Okado et al., 1992). In mouse, the rostral (B4–B9) raphe nuclei develop starting at E11.5 in rhombomeres 1–3, while the more caudal (B1–B3) group (Jensen et al., 2008) begins 5HT expression at E12.5 in rhombomeres 5–7.

Using $[Ca^{2+}]_i$ imaging to record activity in neuronal cell bodies and axons, we have shown that SA in the mouse hindbrain is driven by a subset of the rostral raphe group, specifically a group of serotonergic cells situated in the former r2 (Hunt et al., 2005). Pharmacological experiments and isolation of the pacemaker region showed that it is the only area that can drive SA, and that the activity spreads rostrally, caudally, and laterally through the activation of $5HT_2$ receptors. In examining the developmental time-course of the SA, we showed that the activity begins at E11.5 with hindbrain-wide activity, recedes to the midline at E12.5, and gradually retracts to only the initiator region by E13.5, ceasing completely by E14.5 (Hunt et al., 2006a,b). During the period of SA expression, 5HT-positive neurons migrate to the adult position to form the median and dorsal raphe rostrally, and the B1–B3 groups caudally. Although extensive migration and maturation of the raphe neurons is taking place, pharmacological investigation demonstrated that activation of the 5HT system is the only mechanism by which SA occurs (Hunt et al., 2006a).

In the chick hindbrain, soon after the period of segmentation, spontaneous firing is synchronized between homologous motor roots on both sides of the hindbrain, and between different motor roots along the rostrocaudal axis. This implies a widely distributed mechanism of coordination within the hindbrain (Fortin et al., 1994, 1995; Champagnat and Fortin, 1997). Transection of the chick hindbrain does not block nerve root firing in the isolated segments, implying that each rhombomere (or possibly pairs of rhombomeres) is capable of independent rhythms, which are dominated by the trigeminal in the intact hindbrain (Fortin et al., 1995). This is strikingly different from the mouse hindbrain, where greater than 90% of $[Ca^{2+}]_i$ events initiate in the former r2 (Hunt et al., 2006b), and isolation of that former r2 pacemaker zone causes cessation of activity in other regions (Hunt et al., 2005). Because of the earlier maturation of the major longitudinal tracts in chick hindbrain (Glover and Petursdottir, 1991) during the embryonic stages when spontaneous nerve firing occurs, we hypothesize that a more differentiated network of neurons may mediate activity throughout the chick hindbrain, with independent sites capable of activity.

To test the above hypothesis, we used $[Ca^{2+}]_i$ imaging to examine the expression of SA in the embryonic chick hindbrain. We find that although the chick brain is ontogenetically more mature, SA there shares many similarities with SA found in mouse: midline activity travels in both rostral and caudal directions from discrete intiation points; it appears soon after segmentation ends; and the 5HT-positive raphe plays a major role in its expression. However, while mouse SA utilizes one pacemaker, chick SA has at least two points of origin; both are situated within the caudal serotonergic group which is not yet expressed in mouse hindbrain. Moreover, activity in the lateral regions precedes initiation of activity along the midline, whereas in mouse the lateral activity derives from the midline. This lateral activity is contiguous with activity observed in the rostralmost spinal cord. Interestingly, SA in the chick hindbrain occurs with greater temporal regularity than in the mouse and the frequency of events decreases dramatically over the developmental time where SA is recorded. In addition, we find that the cholinergic, GABAergic, and glycinergic, as well as the serotonergic, neurotransmitter systems participate in initiation and modulation of SA in chick. Thus, the chick model serves as a comparison to the mouse system, allowing us to ascertain that the robust phenomenon of hindbrain SA observed in the mouse is also expressed in another vertebrate system. Ultimately, we conclude that the SA must play an important role in development and maturation of hindbrain neuronal networks, because its essential nature is conserved between mammals and birds.

METHODS

Fertilized White Leghorn eggs were raised at 37.6°C until the desired stage (E6 = HH28–29, E7 = 30–32, E8 = 33–

34, and E9 = 35). All procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the University of Washington Institutional Animal Care and Use Committee. All efforts were made to minimize the number of animals involved. Embryos were removed from eggs into room temperature carbogen (95%O_2, 5% CO_2)-bubbled artificial cerebrospinal fluid (ACSF; containing, in mM: 119 NaCl, 2.5 KCl, 1.3 $MgCl_2$, 2.5 $CaCl_2$, 1.0 NaH_2PO_4, 26.2 $NaHCO_3$, 11 glucose). Hindbrains were dissected out, severed rostrally at the level of the midbrain and either caudally at the level of the cervical spinal cord (HB only), or at the level of the lumbar spinal cord (low power experiments, HB-SC). Tissue was loaded with 1.75 μM fluo-4AM and 0.07% pluronic (Molecular Probes/Invitrogen, Carlsbad, CA, USA) at room temperature for 15 min (30 min for rapid acquisition).

Once loaded, the hindbrains were positioned marginal side down in the open-book preparation in a microscope chamber and imaged on a Nikon Diaphot inverted microscope (Nikon Instruments, Melville, NY, USA) with a cooled CCD camera (Photometrics, Pleasanton, CA) at 0.3 Hz (10 Hz for rapid acquisition). The chamber was constantly perfused with carbogen-bubbled ACSF at a rate of 1 mL/min. Pharmacological agents were diluted therein as indicated elsewhere. Baclofen, bicuculline, bumetanide, DOI hydrochloride, d-tubocurarine, E-4031, ketanserin, mefloquine, methiothepin, mibefradil, muscimol, N-desmethylclozapine, phaclofen, physostigmine, R-96544, scopolamine, strychnine, and tacrine were from Tocris Bioscience, Ellisville, MO; ammonium chloride, barium chloride, cesium chloride, nicotine, nickel chloride, and yohimbine from Sigma, St. Louis, MO. All pharmacology experiments were done at E6, except where noted.

Images and measurements were recorded using MetaFluor (Universal Imaging/Molecular Devices, Downington, PA) and subsequently analyzed using MetaFluor Analyst, SigmaPlot (Systat, San Jose, CA), Excel 2003 (Microsoft, Redmond, WA ImageJ (Wayne Rasband, National Institute of Mental Health, Bethesda, MD), and Photoshop (Adobe, San Jose, CA). The gradual baseline decline in calcium imaging traces is due to photobleaching. For rapid imaging experiments 2 × 2 or 3 × 3 pixel binning was used. The resulting image stacks were processed and converted into movies using MATLAB (Mathworks, Natick, MA). The first image was subtracted from every subsequent image and a 5–10 pixel linear spatial filter was applied to enhance the signal. For supplemental videos, the delta F signal was thresholded, assigned a colormap, and superimposed onto a grayscale fluorescence image of the tissue preparation.

Immunohistochemistry was performed on E6 hindbrains. Hindbrains were dissected in ACSF and then fixed at 4°C in freshly made 4% paraformaldehyde in phosphate buffered saline (PBS) containing (mM): 3.16 NaH_2PO_4, 6.84 Na_2HPO_4, 150 NaCl, pH 7.2. Embryos were cryoprotected in 20% sucrose in PBS overnight, embedded and frozen in OCT medium (Electron Microscopy Sciences, Hatfield, PA), and sectioned at 14–30 μm in a cryostat at −22°C. IHC was performed in PBS containing 0.3% Triton-X. Tissue was blocked at 4°C for 1 h with 10% normal goat serum (Jackson ImmunoResearch Laboratories, West Grove, PA) and incubated with primary antibodies at 4°C for 72 h. Primary antibodies were: Rabbit anti-GAD65/67 (United States Biological, Swampscott, MA), rat anti-brain nAChR (mAb 270; Jon Lindstrom/Developmental Studies Hybridoma Bank, and University of Iowa), rabbit anti-5HT (ImmunoStar, Hudson, WI). Negative controls without antibody showed no signal when examined in the confocal microscope. Other specificity controls were not performed, thus immunohistochemistry signals are assumed to be "presumptive." Appropriate secondary antibodies (Alexa 488 or 594, Molecular Probes/Invitrogen, Carlsbad, CA) were applied for 1 h at 4°C. Sections were examined and photographed in a Bio-Rad confocal (Bio-Rad, Hercules, CA) and images displayed with Photoshop and ImageJ.

Intertransient intervals (ITI) were determined by subtraction of the absolute time values of the peaks of two spikes. Mean ITIs were determined by compilation of every ITI (under control conditions) from every hindbrain of appropriate age. Standard deviations were determined, as stated below, based on the mean of SDs as determined individually for each hindbrain. Percent reduction in frequencies upon application of pharmacological agents were determined by dividing the stable frequency following drug application by the predrug frequency (at least a 20-min predrug window) and subtracting the value from one. *P*-values were determined by single factor ANOVA, comparing pre- and postapplication frequencies.

RESULTS

Expression and Timeline of Spontaneous, Synchronous Calcium Transients in Chick Hindbrain

Examination of the chick hindbrain showed spontaneous activity. When events within larger regions were compared temporally, we observed spontaneous widespread $[Ca^{2+}]_i$ transients propagating as waves across large areas of the chick hindbrain. To determine the extent and nature of these transients, we examined hindbrains staged between E4 and E10. We found transients occurring between E6 and E9 (see Fig. 1), but not at E4, E5, or E10. By adding 5 mM tetraethylammonium (TEA), we could induce the transients at E5, but not at E4 or E10 (data not shown). This four-day interval exceeds the three-day interval seen in mouse hindbrain.

The time between transients, the intertransient intervals (ITIs), increases significantly, both between individual days and across all days (ANOVA, $p < 0.05$) when activity is present [Fig. 1(E)]. At E6 the ITI is (mean ± s.e.m. minutes) 3.97 ± 0.05 while by E7 it becomes 5.59 ± 0.15. This decrease in fre-

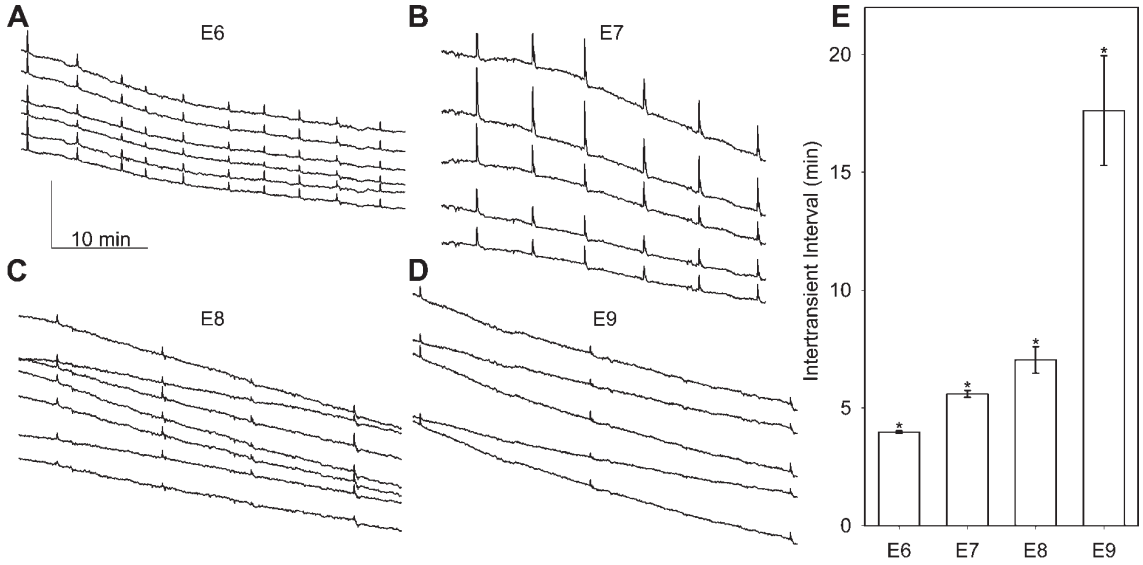

Figure 1 Spontaneous, synchronous calcium transients decrease in frequency between E6 and E9. (A–D) Representative $[Ca^{2+}]_i$ traces from E6, E7, E8, and E9. (E) Mean intertransient interval (ITI) at E6, (mean ± s.e.m min) 3.97 ± 0.05 ($n = 162$ embryos); at E7, 5.59 ± 0.15 (11); at E8, 7.03 ± 0.56 (6); and at E9, 17.6 ± 2.32 (2). Error bars represent the standard error. *$p < 0.05$, relative to all other embryonic days (i.e., E6 separately relative to E7, E8, and E9), single factor ANOVA. Vertical scale bar: 2 ΔF/F.

quency as the embryo ages is most dramatic between E8 and E9, when the ITI more than doubles, from 7.03 ± 0.56 to 17.6 ± 2.32 min.

Though the ITIs increase over developmental time, within each embryonic day the transients occur with tight temporal regularity, so that the ITI is very consistent spike to spike. One way to measure this is to divide ITI standard deviation within a given hindbrain by the frequency. This measure, in effect the fractional variability around the frequency, is 0.168 at E6. At E7, it is 0.126, E8 0.213, and E9 0.115. This implies that the system precisely regulates the interval between events at all developmental stages.

EVENTS PROPAGATE BY TWO DISTINCT PATTERNS, ONE LATERAL AND ONE ALONG THE MIDLINE

Midline Propagation

To determine the pattern of propagation within the hindbrain we acquired images at 10 Hz to produce videos. Along the midline, events usually begin at two points of origin: just caudal to the level of the nV exit point, and in a slightly more variable region between nVII and nIX (Fig. 2; also see Supporting Figure 1a and b, and accompanying videos). In 9 of 10 animals imaged with a low power objective capable of capturing both initation points in the same field

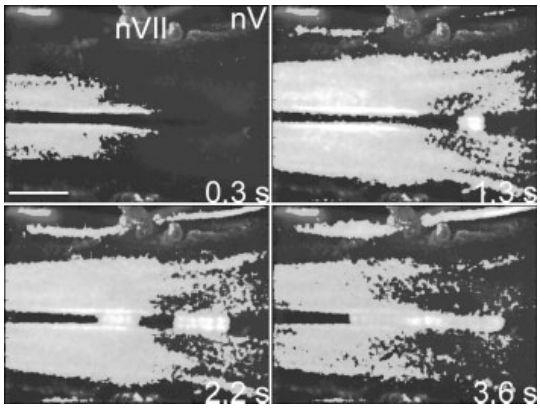

Figure 2 Lateral activity precedes medial event propagation from two invariant initiation points. Selected frames visualizing propagation of SA in E6 chick hindbrain. At 0.3 s, a wave of activity propagates from the spinal cord in the lateral regions before any activity in the hindbrain midline. At 1.3 s, the rostral initiation site becomes active. At 2.2 s, events in the midline propagating from both the caudal and rostral points of origin are seen. At 3.6 s, events originating from the two sites collide in the midline. Rostral is to the right, caudal to the left in all frames; the location of nV and nVII are indicated. Horizontal scale bar: 500 μm.

of view, the rostral midline initiation point led the caudal (Supporting Figure 1a). However, in the remaining embryo, the caudal initiation point led the midline activity (Supporting Figure 1b). Only two initiation points were observed in seven of these animals, but in the other three animals, one or more additional initiation points were observed, with the latter points existing caudal to the usual points of initiation (Supporting Figure 1c). Thus, two initiation points (caudal to the level of the nV and caudal to the level of the nVII) are invariant, while in some cases additional, more caudal initiation points appear. Propagation patterns vary slightly between animals, but are highly stereotyped within the same animal.

From both of the invariant points of origin, the events initiate in close synchrony to each other and each propagates rostrally and caudally. However, they are restricted to the commissural region and do not propagate laterally. In Figure 2, where both initiation points are imaged, waves originating from the two points of origin ($t = 2.2$ s) collide in the midline ($t = 3.6$ s).

To ascertain whether one initiation point could be active in the absence of the other, we transected between them. In each of four experiments, transection between the two invariant midline initiation points, at a level just rostral to the nVII exit point, resulted in the cessation of activity in regions rostral to the cut, including the rostral initiation point. Activity in regions caudal to the transection, including the caudal initiation point, continued normally. Rostral regions did not regain activity in sixty minutes of recording following transection. In Supporting Figure 2, we show a representative example of this phenomenon, with the vertical white line representing the position of the cut (which completely transected the tissue). This demonstrates that the invariant caudal initiation point can be active without corresponding activity in the rostral point, suggesting that input from lateral or medial regions of the caudal hindbrain may be required to initiate activity in the rostral site.

From both invariant medial initiation sites, events propagate both rostrally and caudally within the commissural midline area, colliding approximately between the nV and nVII. The events appear to move in a saltatory fashion, jumping between points that span the width of the commissural region, as if moving on rungs of a ladder. These saltatory events can be clearly observed in Supporting Figure 1c. The propagation rate is not consistent in either direction or at any site within the midline region; the range of speeds was 136–453 μm/sec ($n = 13$ hindbrains).

Lateral Propagation

In lateral regions, events appear just prior to those along the midline, but they do not share the same rostrocaudal propagation pattern, nor do they propagate outward from the midline activity as in the case of mouse (see Fig. 2). Moreover, the activity in the lateral regions is present at levels rostral and caudal to the regions of midline propagation (Supporting Figure 1d). These distinct patterns of propagation suggest the possibility that the activity has a different initiation mechanism and/or a distinct developmental role in the lateral tissue than in the medial tissue.

In experiments done at lower magnification, the region between nV and nVII could be imaged in the same frame as nIX and rostral spinal cord. Preparations containing spinal cord demonstrated that immediately preceding the events in the hindbrain midline, large waves of activity occurred in the spinal cord and traveled rostrally into the hindbrain (Supporting Figure 1d). These waves did not occupy the commissural zone of the hindbrain at all. Upon entry into the wider hindbrain, the waves spread more laterally and propagated to the lateral regions immediately surrounding the midline initiation zones.

The Serotonergic, Cholinergic, GABAergic, and Glycinergic Systems are Necessary in Generating SA at E6

As the following sections will demonstrate, four of the neurotransmitter systems we assayed (5HT, ACh, GABA and glycine) are necessary in the production of spontaneous $[Ca^{2+}]_i$ transients. All of these experiments were done at the E6 stage, as the higher frequency of activity at this stage allows the data to be acquired more efficiently. In each experiment, we focused on the midline region just caudal to the nVII in an E6 embryo. In the following figures, drug is applied for the duration of the time shown by the black bar, followed by washout.

Serotonin

As activation of serotonergic inputs is required for spontaneous activity in mouse hindbrain, we examined the role of this transmitter system in generating spontaneous activity in the chick (see Fig. 3). Methiothepin, a blocker of a range of serotonergic receptors, reduced activity significantly at 100 μM [Fig. 3(A)]. To characterize the receptors involved, we used more specific antagonists. An antagonist specific to $5HT_{2A/C}$ receptors, ketanserin, blocked activity [Fig. 3(A,B)]. At 50 μM R96544, an antagonist specific to the $5HT_{2A}$

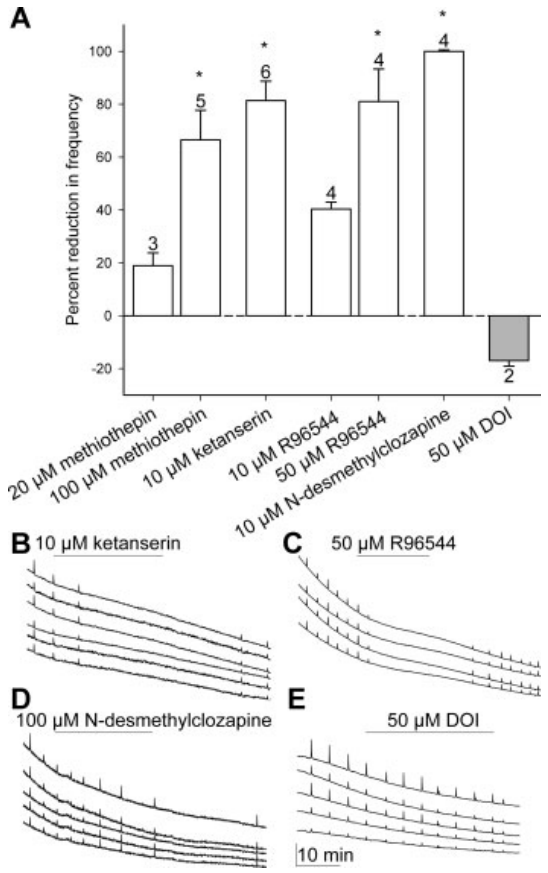

Figure 3 $5HT_{2A}$ and $5HT_{2C}$ receptors are necessary for spontaneous $[Ca^{2+}]_i$ calcium transients. (A) Percent reduction in frequency (percent ± s.e.m.) after application of 20 *μM* methiothepin (general $5HT_R$ antagonist), 18.98 ± 4.85; 100 *μM* methiothepin, 66.55 ± 11.18; ketanserin ($5HT_{2A/CR}$ antagonist), 81.43 ± 17.90; 10 *μM* R96544 ($5HT_{2AR}$ antagonist), 40.42 ± 2.64; 50 *μM* R96544, 81.11 ± 12.24; *N*-desmethylclozapine ($5HT_{2CR}$ antagonist), 100; and DOI ($5HT_{2A/CR}$ agonist), −16.97 ± 2.08. White bars are antagonists, gray bars are agonists. Error bars represent the standard error. Number above or below error bars represents the number of embryos. $*p < 0.05$, relative to preapplication frequency, single factor ANOVA. (B–E) Representative traces with application of ketanserin (B), R96544 (C), N-desmethylclozapine (D), and DOI (E). Vertical scale bar (ΔF/F): Ketanserin: 12, R96544: 20, N-desmethylclozapine: 6, and DOI: 2.

receptor, reduced the frequency of the activity to 81% of control with statistical significance [Fig. 3(A,C)], suggesting that the $5HT_{2A}$ receptor is necessary. *N*-desmethylclozapine, an antagonist specific to the $5HT_{2C}$ receptor, blocks the activity [Fig. 3(A,D)], indicating that this receptor subtype is also necessary. However, addition of an agonist (DOI) of $5HT_2$ receptors [Fig. 3(E)] did not significantly modulate the activity, implying that 5HT receptors are maximally activated under normal conditions.

Acetylcholine

Our results suggest that nicotinic cholinergic inputs are also required to activate transients in chick hindbrain. The nAChR antagonist d-tubocurarine completely stops transients from occurring [Fig. 4(A)], indicating that this receptor is necessary. The muscarinic AChR antagonist scopoloamine, however, had an insignificant effect, indicating that the muscarinic system is not involved in SA in chick hindbrain [Fig. 4(A)].

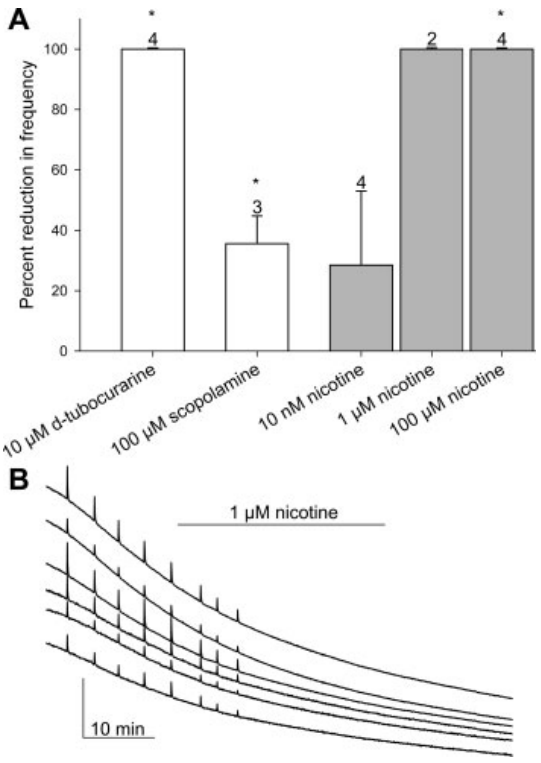

Figure 4 Nicotinic but not muscarinic acetylcholine receptors are necessary for spontaneous, synchronous calcium transients to occur. (A) Percent reduction in frequency (percent ± s.e.m.) after application of d-tubocurarine (nAChR antagonist), 100; scopolamine (mAChR antagonist), 35.56 ± 9.20; 10 *μM* nicotine (nAChR agonist), 28.46 ± 24.52 (one complete block, one increase in frequency, two small decreases in frequency); 1 *μM* nicotine, 100; and 100 *μM* nicotine, 100. White bars are antagonists, gray bars are agonists. Error bars represent the standard error. Number above error bars represents the number of embryos. $*p < 0.05$, relative to preapplication frequency, single factor ANOVA. (B) Example calcium imaging trace with application of nicotine. Vertical scale bar: 50 ΔF/F.

Nicotine, an agonist specific to the nAChR, prevents transients from occurring [Fig. 4(A,B)]. We suggest that the excitatory nicotinic cholinergic system is finely tuned in the developing hindbrain. Increase in nAChR drive either by agonists of the receptor (nicotine) or blocking AChE (physostigmine, 25 μM, $n = 3$; tacrine, 50 μM, $n = 3$; data not shown) results in blockade of activity after an increased excitatory response. With nicotine, several transients occurred at an increased frequency following the application of nicotine before the transients cease completely [Fig. 4(B)]. A similar phenomenon was seen with applications of AChE blockers.

GABA and Glycine

GABA and glycine are widely distributed major inhibitory neurotransmitters in the adult brain. At this early stage in development, however, the chloride reversal potential is relatively positive, such that activation of those receptors is excitatory.

The $GABA_A$ receptor antagonist bicuculline completely blocks SA [Fig. 5(A,B)], indicating that this receptor is necessary. However, picrotoxin, another $GABA_A$ (and $GABA_C$) receptor antagonist, incompletely blocks the activity, reducing it, with statistical significance, to 48.78% of control [Fig. 5(A)]. In three preparations with spinal cord attached, 150 μM bicuculline completely blocked activity. $GABA_A$ receptor agonist muscimol prevents activity from occurring [Fig. 5(A,C)]. We propose that this cessation results from over-excitation of the system as in the case of nicotine application. The $GABA_B$ receptor is not necessary as its antagonist phaclofen does not block the activity [Fig. 5(A)]. It is present, however, given that the agonist, baclofen, appears to over-excite the tissue [Fig. 5(D)].

Blocking the glycine receptor with its antagonist strychnine also blocks the transients, indicating that this receptor is necessary [Fig. 5(A,E)], although as strychnine acts on nAChR, this result may be due to some block of those receptors. Glycine itself has only a minor effect on the frequency of the activity.

We tested the role of the depolarizing drive of the Cl^- gradient in generating SA by application of bumetanide, a blocker of the chloride-extruding KCC2 K^+/Cl^- cotransporter. This protein is developmentally regulated, and the onset of expression generally switches the action of both $GABA_A$ and glycine receptor activation from excitatory to inhibitory (Rivera et al., 2005). Bumetanide, at 20 μM, had no effect on either the frequency or extent of activity ($n = 3$), suggesting that at E6 KCC2 is not yet expressed at a level high enough to change E_{Cl}.

The Glutamatergic, Substance P, Histaminergic, and α-2-Adrenergic Systems are Superfluous

The glutamatergic system, while present at this developmental stage, is not necessary for the activity to occur, though it can modulate it. CNQX (30 μM), an

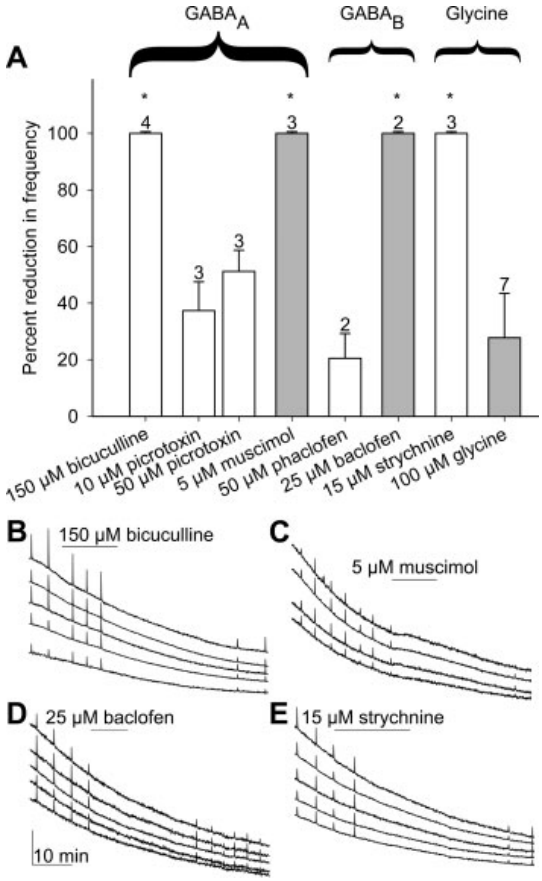

Figure 5 Glycine and $GAB_{A\text{-}A}$ but not $GAB_{A\text{-}B}$ receptors are necessary for spontaneous, synchronous calcium transients to occur. (A) Percent reduction in frequency (percent ± s.e.m.) after application of bicuculline ($GABA_AR$ antagonist), 100; 10 μM picrotoxin ($GAB_{A\text{-}AR}$ antagonist), 37.38 ± 10.15; muscimol ($GABA_AR$ agonist), 100; of 50 μM picrotoxin, 51.22 ± 7.46; phaclofen ($GABA_BR$ antagonist), 20.52 ± 8.73; baclofen ($GABA_BR$ agonist), 100; strychnine (glycineR antagonist), 100; and glycine (glycineR agonist), 27.78 ± 15.71. White bars are antagonists, gray bars are agonists. Error bars represent the standard error. Number above error bars represents the number of embryos. $*p < 0.05$, relative to preapplication frequency, single factor ANOVA. (B–D) Representative $[Ca^{2+}]_i$ calcium imaging traces with application of muscimol (B), bicuculline (C), baclofen (D), and strychnine (E). Vertical scale bar (ΔF/F): Muscimol: 5.6, bicuculline: 10, baclofen: 5, and strychnine: 8.

antagonist to the AMPA and kainate glutamate receptor subtypes, reduces transient frequency by 39.33 ± 10.56% ($n = 5$), but the large variation in response made the result insignificant. In two preparations with spinal cord attached, 30 μM CNQX did not block activity. Ten micromolar of AMPA ($n = 3$) and 10 μM kainate ($n = 3$) both block transients from occurring, indicating the presence of those glutamate receptor subtypes. Applying 10 μM NMDA ($n = 5$) insignificantly reduces transient frequency, suggesting presence of this receptor subtype, but does not block it. This suggests that the AMPA and kainate receptors can influence the activity, but that they are not necessary. The NMDA antagonists AP5 and MK801 have small, insignificant effects on the activity.

Antagonists to substance P (substance P-Arg, $n = 2$), α-2-adrenergic (yohimbine, $n = 1$), and histamine (ranitidine, $n = 2$) receptors have small, insignificant effects on transient frequency, indicating that these systems do not contribute to the activity.

Gap Junctions, Including Connexin-36, and the T-Type Calcium Channel, among Other Ion Channels, are Required for Activity

Mefloquine (50 μM), a Cx36 blocker, confirms that gap junctions are necessary for SA by dramatically reducing transient frequency by 77.19 ± 7.98% ($p = 0.02$, $n = 4$). Gap junctions are opened and then closed by the pH change effected by addition of ammonium chloride. When applied at 10 mM, ammonium chloride transiently increases the frequency of activity (when the gap junctions open) by 86.52 ± 13.64% ($p = 0.003$, $n = 4$) before stopping it entirely (when the gap junctions close). Thus, transients propagate at least partially via gap junctions. Unfortunately, in our attempt to localize Cx36 in the chick hindbrain using immunohistochemistry, the available antibodies did not appear to recognize the chick protein.

The T-Type calcium channel is blocked by both mibefradil and nickel chloride. Applying 50 μM mibefradil reduces the frequency of activity by 88.81 ± 6.85% ($p = 0.004$, $n = 3$). At 10 μM, it reduces the frequency by 82.11 ± 5.33% ($p = 0.0007$, $n = 4$) NiCl is especially useful as it can differentiate between subtypes of the Ca_v3 subfamily of Ca^{2+} channels. At 100 μM, it blocked the activity entirely ($p = 0.003$, $n = 3$), while at 10 μM, it reduced the frequency of activity by 65.04 ± 12.69% ($p = 0.03$, $n = 4$). As $Ca_v3.2$ (α1H) is sensitive to NiCl in the low micromolar range, it is likely that this subunit is expressed in chick hindbrain (Yunker, 2003). Immunocytochemistry using an antibody against $Ca_v3.3$ showed that this channel is also highly expressed in many cell bodies of the hindbrain (data not shown).

Barium, which blocks M- and inwardly rectifying-potassium currents, approximately doubles transient frequency, increasing it to 205 ± 8.04% of control when applied at 500 μM ($p = 0.01$, $n = 3$). It also increases the amplitude of events. This suggests that one of these currents (or both) depresses the excitability of the system. On the other hand, E4031, a HERG-channel blocker, had no effect. Tetraethylammonium, another potassium channel blocker had small, nonsignificant effects on the activity at E6.

Tetrodotoxin-sensitive sodium channels also mediate SA. Applying 2 μM TTX completely stops activity ($p = 0.0003$, $n = 2$), thus voltage-gated sodium channels are necessary.

Serotonergic Cells are Present in and along the Midline and are Divided into a Rostral and a Caudal Cluster in the E6 Hindbrain

Two main groups of presumptive 5HT-positive cells are found in the E6 chick hindbrain (see Fig. 6). The rostral group is present more dorsally and extends rostrally to very near the midbrain border [Fig. 6(A,D,E)]. The second group is present more caudally, tending also toward the ventral surface [Fig. 6(B,C,E)]. A gap containing no 5HT-positive cells is located in the former r2 (the level of the nV exit point) and separates the two groups. The caudal group is present more extensively along the mediolateral axis than the rostral group [Fig. 6(D,E)], as it can be observed in the more lateral parasagittal section.

The presence of serotonergic cells primarily along the midline suggests that 5HT may be especially important in mediating the midline activity and that (an)other transmitter system(s) may be more involved in mediating lateral activity.

Nicotinic Acetylcholine Receptors are Present Throughout the E6 Hindbrain

Signal for the presumptive nAChR showed that the receptor is diffusely present throughout the majority of the E6 hindbrain (see Fig. 7). In transverse sections in lateral regions, the receptor is highly expressed within the trigeminal ganglion and in the motor nucleus of the trigeminal [Fig. 7(A)]. In midline areas, a region of low expression characterizes the edge of the midline, while the axon-rich core of the

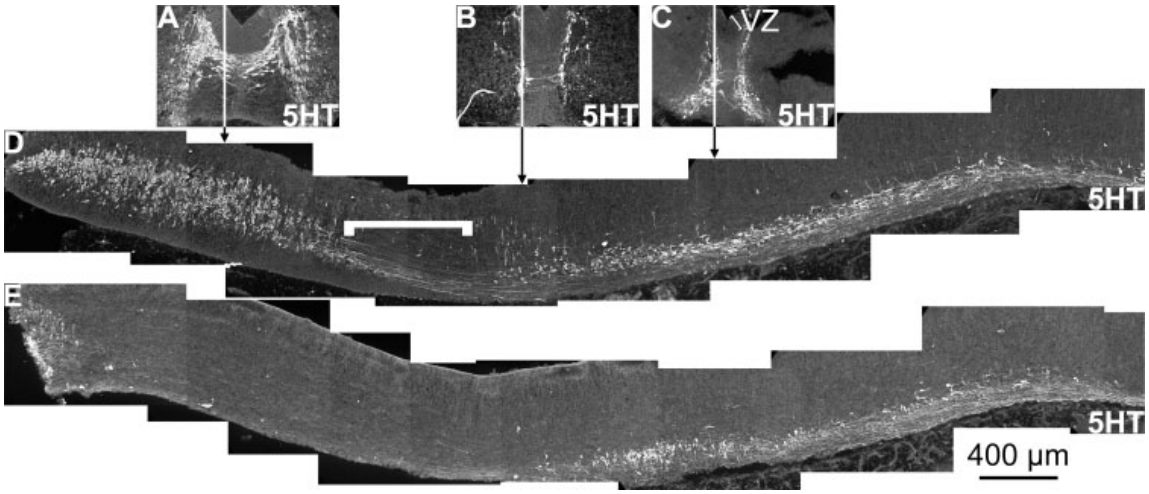

Figure 6 Serotonergic cells and fibers are abundant in the E6 hindbrain. (A–C) Transverse cryosections stained for serotonin. The rostral-caudal positions of the sections are indicated by the arrows pointing to D. (D–E) Sagittal and parasagittal cryosections stained for serotonin. Section E is approximately 270 μm from the midline. Serotonergic cells are present in two groups: A rostral-dorsal group (A,D) and a ventral-caudal group (C,D,E). A region of low serotonergic cell density separates the two groups (bracket; B,D,E). The caudal cell group extends more widely along the mediolateral axis, as it can be observed in the parasagittal section. For all sections, dorsal is up. For slices D and E, rostral is to the left. Section C has a tear in the lateral region. VZ = ventricular zone.

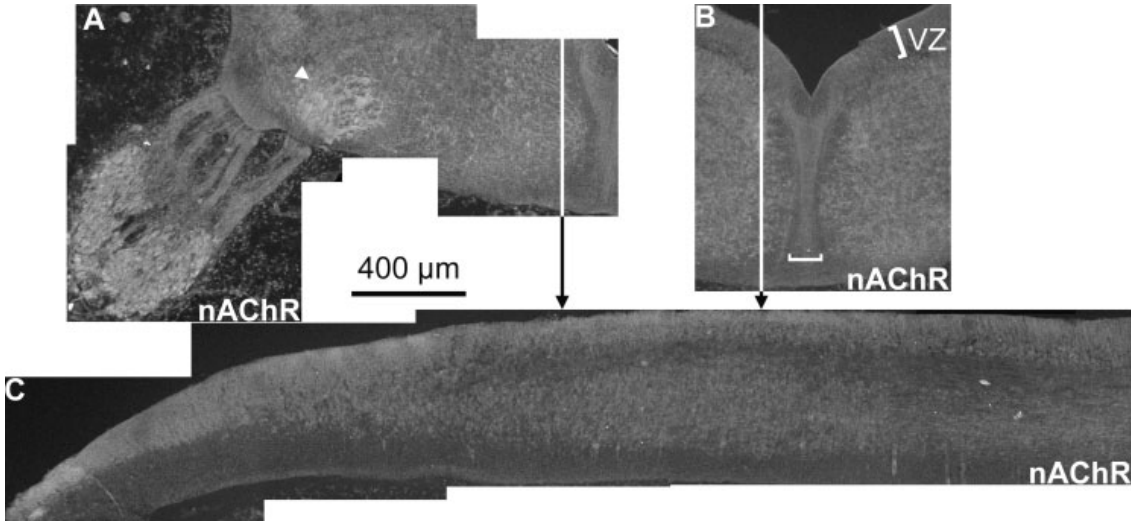

Figure 7 The nicotinic acetylcholine receptor is widely distributed in the E6 hindbrains. (A–B) Transverse cryosections stained for nAChR. The rostral-caudal positions of the sections are indicated by the arrows pointing to C. (A) Bright nAChR staining in the trigeminal nerve and motor nucleus (arrowhead) in lateral regions. (B) A more caudal section shows nAChR expression is present diffusely throughout the mediolateral and dorsoventral axes. The axon-rich region (bracket) of the commissural region expresses nAChR. (C) A parasagittal cryosection just off of the midline shows regions of nAChR expression. For all sections, dorsal is up. For slice C, rostral is to the left. VZ = ventricular zone.

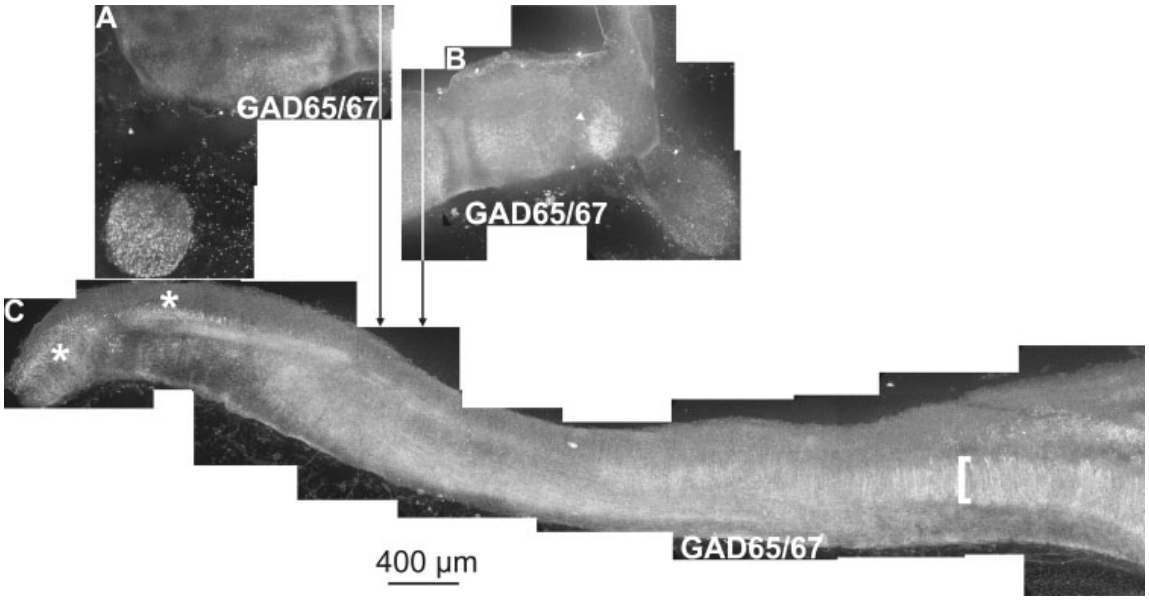

Figure 8 Glutamic acid decarboxylase 65/67 is present throughout the E6 hindbrain. The rostral-caudal positions of the sections are indicated by the arrows pointing to C. (A) A transverse cryosection stained with an antibody directed at an oligopeptide common to GAD65 and GAD67 shows bright staining in the trigeminal nerve root (bottom left) and near the midline. (B) A transverse section 240 μm caudal to A shows expression of GAD65/67 throughout the hindbrain and near the motor nucleus (arrowhead) of the trigeminal nerve. (C) A parasagittal section just off of the midline shows GAD65/67 expression throughout the length of the hindbrain and a few cell bodies near the isthmus (asterisks). In the caudal regions, GAD65/67 staining appears in a band of fibers (bracket). For all sections, dorsal is up. For section C, rostral is to the left.

midline is stained [bracket, Fig. 7(B)]. Expression of the receptor is especially pronounced along the dorsal surface, particularly near the rostral extreme of the hindbrain, as observed in parasagittal sections [Fig. 7(C)]. As expected, no expression of the receptor appeared in cell bodies. Because we were unable to successfully stain for the synthetic enzyme of acetylcholine (choline acetyltransferase), we were unable to determine the location of the cholinergic neurons. However, given that only the nicotinic (i.e. not the muscarinic) acetylcholine receptor is necessary for the SA, specifically staining for this receptor reveals more precisely the postsynaptic cells involved in SA.

Glutamic Acid Decarboxylase 65/67, the Synthetic Enzyme of GABA, is Present Throughout the E6 Hindbrain

We assessed the presence of GABA by staining with an antibody directed at an oligopeptide common to GAD65 and GAD67. We found that presumptive GAD65/67 is highly expressed in cell bodies in the trigeminal ganglion and motor nucleus [Fig. 8(A,B)]. A few cell bodies within other regions of the hindbrain itself show expression, but the staining is primarily diffuse, indicating expression within fibers. Aside from a group of cell bodies found dorsally near the rostral border of the hindbrain (asterisk), no cells appear to be organized into nuclei [Fig. 8(C)], although there are sparse discrete cell bodies found in the marginal zone near the midline. Along the midline, there is little staining, but as with nAChR staining, the axon-rich region in the core of the midline shows greater GAD65/67 expression [Fig. 8(B)].

DISCUSSION

In this article, we characterize the phenomenon of spontaneous $[Ca^{2+}]_i$ activity in the chick hindbrain. SA decreases in frequency during a discrete developmental window (E6-E9) before ceasing at E10, and is regulated by a number of transmitter systems (5HT, ACh, GABA, glycine). Along the midline, transients originate from at least two discrete points, one immediately caudal to the nV exit point and one just caudal to the nVII exit point; waves from the two origins propagate within the midline and collide between the

origins. In some experiments, additional caudal initiation points are seen. Waves in the lateral tissue appear to arise from the spinal cord when it is attached; the caudal hindbrain can self-initiate when it is not.

Earlier work examining the presence of activity in chick using cranial nerve recordings suggested that such spontaneous activity would be present, and that it would have more than one point of origin (Fortin et al., 1994, 1995). Fortin et al., 1995 show a periodicity of approximately 6 min at HH stages 24–36 (E4–6); this is in close agreement with our measurement of an ITI of approximately 4 min at E6. The discrepancy between the onset of activity in the earlier reports (e.g. E4) might be due to the higher external K^+ (8 m*M*) used in those experiments compared to the current work (2.5 m*M* K^+); the relative depolarization caused by a higher potassium concentration could cause the network to become active at an earlier stage. Similarly, the use of 5 m*M* external K^+ may allow spontaneous activity, though restricted in the area that it encompasses, in stage 24 (E4) chick hindbrain (Momose-Sato et al., 2009); lengthening of the interburst interval over developmental time was also shown in this work.

Chick SA is similar in many ways to that recorded in embryonic mouse hindbrain (Hunt et al., 2005, 2006a,b). For example, the onset of the activity in both animals occurs soon after the period of segmentation is complete, and the rostral point of origin in the chick is anatomically very close to that in mouse, medial to the exit point of nV. A striking feature of SA in the chick is the precision of event timing at each developmental stage. This could be a result of summation of excitatory synaptic inputs or expression of a slow voltage-gated current such as I_H, leading to a constant rate of depolarization and tightly timed events. This is substantially different from mouse, where transient frequency does not change as dramatically over the 3-day window of development, and the interval between events is not uniform. The mechanism(s) generating such timing could likely be elucidated using patch clamp recording of synaptic or depolarizing inputs.

Mouse SA is pharmacologically dependent only on serotonergic receptor activation, while the chick activity has multiple pharmacological participants. However, of the transmitter systems that are required for the activity in chick, only serotonergic neurons are situated near where the midline activity arises; cell bodies expressing GAD and nAChR are found rostrally (in midbrain) or laterally. This, and the different propagation patterns in the midline and lateral tissue, introduces the possibility that activity in different locations is mediated by different transmitter systems. However, we were unable to pharmacologically dissect midline from lateral activity, suggesting that they ultimately depend on one another. Preliminary experiments using spinal cord-hindbrain preparations suggest that this dependence remains in this more intact preparation (Easton and Bosma, unpublished results). Changes in pharmacological dependence over development of the chick hindbrain has recently been shown by Mochida et al., 2009, where the influence of ACh decreases as GABAergic input becomes more important. Our pharmacology was conducted at E6, where both inputs were shown to be important.

The two invariant points of initiation are closely synchronized, with the rostral point slightly preceding the caudal group in temporal initiation in 90% of cases. The synchronization is so close that the two waves propagating in opposite directions collide in the midline, although the collision site fluctuates because of the variability in propagation rates in both directions. Separation of the two points of origin silences the rostral, but not the caudal, site. This suggests either that the caudal initiation point, just below the nVII exit point, is intrinsically stronger, or that it continues to initiate because the lateral activity persists in these conditions. Unlike mouse, where the rostral (pontine) serotonin group initiates virtually all activity, in the chick hindbrain, the caudal (medullary) serotonergic group is the major initiator of spontaneous midline activity, albeit dividing the function between the two invariant initiation sites.

Mouse transients propagate rostrally, caudally, and laterally from a single midline site of origin while chick transients propagate rostrally in the lateral tissue and have multiple sites of initiation along the midline. Indeed, activity in medial sites of initiation in the chick appears to possibly arise following activation by lateral activity, which precedes it. As seen in Supporting Figure 1d, when the spinal cord is attached, the activity spreads from the spinal cord into the hindbrain. Thus, the spinal cord appears to drive hindbrain activity. However, when the spinal cord is not attached (as was true in the majority of experiments described in this article), the activity continues unabated. In those cases, the activity in lateral regions appears to derive from the caudal hindbrain, perhaps arising from hindbrain neurons that project to the reticulospinal tract. While the spinal cord seems to drive activity in the hindbrain, the hindbrain is nonetheless capable of self-initiation. Though a number of specific details of the SA differ between species, conservation of the main phenomenon suggests that some event(s) during the development of hindbrain networks depend(s) on the

expression of SA. In comparison, SA in embryonic rat demonstrated that at E15, SA was mostly expressed in spinal cord, while a medullary contribution was observed at E16. At that later stage, the medullary component became stronger upon transection from the spinal cord (Momose-Sato et al., 2007).

Chick and mouse brains mature at quite different rates; the E6 chick hindbrain is much more differentiated than the E11.5 mouse hindbrain. At this stage in the chick, several major projection tracts and the reticular formation are quite differentiated (Glover and Petursdottir, 1991) while those in the mouse are just beginning to develop. In mouse, rostral hindbrain projections have not yet reached the spinal cord at E11.5, but by E12, projections to the spinal cord from the more rostral hindbrain (pontine; former r2) resemble those of the mature animal. This is past the point in time at which SA in the mouse hindbrain commences (E11.5), demonstrating that other axonal tracts from the spinal cord that participate in chick hindbrain SA are not yet available for participation in mouse at E11.5 (Auclair et al., 1999), and other neurotransmitter systems are not involved (Hunt et al., 2006a). In chick, however, medullary brain stem projections are present at E3 and by E4–5, multiple pontine projections are differentiated (Glover, 1993). Thus, chick reticulospinal projections are mature as compared to mouse, and thus can participate in SA in chick, but not mouse, hindbrain.

The expression pattern of 5HT-expressing neurons differs significantly between chick and mouse. At E6 in the chick, the serotonergic raphe is quite differentiated. The rostral pontine group (which becomes the dorsal raphe nuclei) appears at E4, while the caudal medullary groups forming discrete and identifiable B1–B3 nuclei appear at late E4 or early E5 (Sako et al., 1986; Okado et al., 1992). The rostral group extends only as far caudal as the level of the trigeminal nerve (up to the former r2). A region devoid of serotonergic cells appears at the level of the trigeminal nerve (within the former r2). The caudal group of cell bodies begins just caudal to the trigeminal nerve, in the former r3. In mouse at E11.5, the stage at which SA begins, the serotonergic raphe has just begun expression in the more rostral pontine nuclei, and the neurons are clustered near the midline of the former r1–r3 (Hunt et al., 2005). The caudal medullary group in mice begins expression of 5HT at E12.5 in the former r5–r7 region, and it is not until E13.5 that the murine raphe resembles the maturity of the E6 chick (Hunt et al., 2006b). The two groups border the former r4, which contains no serotonergic neurons (Jensen et al., 2008). Thus, in chick, the gap between the two serotonergic populations is more rostral than that in mouse (see Figure 9) (Sako et al., 1986; Okado et al., 1992). This implies that slightly different axial patterning may cause the rostral shift in the chick serotonergic neurons.

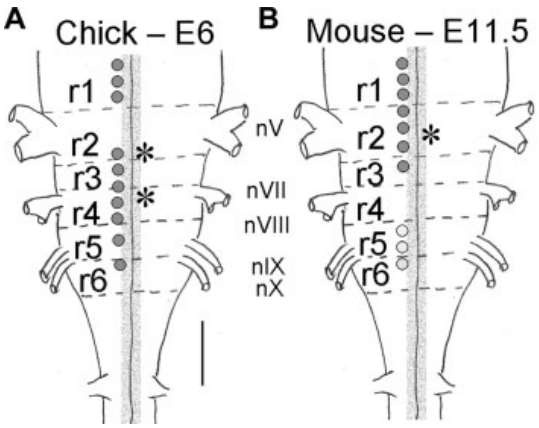

Figure 9 Comparative summary of spontaneous activity in chick and mouse. Schematic drawings of chick (A) and mouse (B) hindbrains at the stage of onset of SA observed using $[Ca^{2+}]_i$ imaging (chick E6, mouse E11.5). Dashed lines indicate former rhombomere (r) boundaries and are shown for reference as the boundaries have disappeared in both species by the stages shown. In chick, serotonergic neurons (gray circles) are found along the commissural region (stippled bar), with a gap of expression in the former r2; in mouse, the 5HT-positive neurons are found in the former r1-r3, but the caudal group has not yet appeared (white circles). In mouse, the former r4 has a gap of expression of serotonergic neurons. Asterisks indicate points of origin for SSA: Two are recorded in chick, both within the caudal serotonergic group, while one is recorded in mouse, in the rostral serotonergic group. Scale bars are 1 mm for chick, 0.5 mm for mouse.

Our data show that the rostrocaudal position of the serotonergic clusters in chick places the beginning of the caudal cell group in the same position as the rostral-most initiation point of midline activity; both are just caudal to the nV exit point, within the former r3. As the rostral and caudal points of origin are roughly 0.75 mm apart, the caudal point of origin is also within the caudal serotonergic cell cluster. The caudal serotonergic cluster first begins to appear consistently at early E5, increasing in size by E6 (Sako et al., 1986). SA can be induced at E5 by using low concentrations of TEA, although no transients are recorded without this added depolarization; by E6 transients are observed spontaneously. This implies that the circuits underlying the transients are developing concomitantly with the differentiation of the caudal cell raphe group. Based on the proximity of the caudal 5HT cell cluster to the points of origin, the similar

time course of the cell cluster's development and the appearance of activity, and the necessity of serotonin receptors in generating the activity, we suggest that the caudal serotonergic cell cluster underlies the medial points of origin. This is in contrast to the mouse embryonic hindbrain, in which activity originates in the former r2 and is driven by a portion of the rostral (pontine) medullary raphe group (Figure 9, asterisks).

The expression of SA in chick requires serotonergic receptor activation, but it is clear that other transmitters are involved in the activity as well. Localization of both nAChR-bearing and GAD65/67-expressing processes using immunohistochemistry shows that few organized cell bodies are found in the area where midline activity initiates. The expression pattern of GAD65/57 that we observe is similar to that seen in embryonic rat hindbrain (Tran et al., 2004), in which GABA65-positive sensory commissural neurons originating in the spinal cord project rostrally in the ventral axon tracts, turning to more lateral areas in the hindbrain before ascending and terminating in the midbrain. It is possible that the fibers in the more lateral areas of the hindbrain mediate the lateral activity that is synchronized with the midline activity, and that sensory input from caudal hindbrain or spinal cord influences SA in the hindbrain. GAD expression is observed in early postnatal trigeminal motor neurons (Turman et al., 2001) and trigeminal ganglion cells (Hayasaki et al., 2006). The presence of both GABAergic and serotonergic axons within the hindbrain midline commissural region suggests that either or both may mediate excitation of the hindbrain initiation.

The involvement of both GABA and glycine in chick hindbrain SA suggests that the Cl^- reversal potential is sufficiently positive to threshold to generate an excitatory response to transmitter input. This is a facet of synchronized waves of excitation in many regions of the developing CNS (Rivera et al., 2005). Up-regulation of the chloride-extruding KCC2 K^+/Cl^- cotransporter ends this excitation by bringing the Cl^- reversal potential to more negative values. Application of bumetanide, a blocker of KCC2, had no effect on chick SA, suggesting that KCC2 expression is low at E6. Whether the dependence on GABA and glycine continues over the entire window of SA in chick hindbrain is unknown. In retina and spinal cord, SA dependence switches between different transmitters (see Moody and Bosma, 2005 for review). In mouse hindbrain, a slight dependence on norepinephrine develops by E13.5 (Hunt et al., 2006a), and the change in ITI observed in chick hindbrain may indicate a change in transmitter dependence as well; this change may include a reduced role of GABA and/or glycine, or a switch to inhibition by these transmitters as KCC2 expression is up-regulated.

SA in chick hindbrain also relies on mechanisms that are utilized in other systems where SA is recorded. Gap junctional coupling is important in propagating waves of excitation in retina, mouse hindbrain, hippocampus and other brain regions (for review, see Moody and Bosma, 2005). Mouse hindbrain SA is not sensitive to TTX until synchronization is also observed (Gust et al., 2003), and many systems acquire synchronicity and TTX-sensitivity simultaneously. The expression of T-type Ca^{2+} channels is suggestive of an ability of neurons to undergo spontaneous depolarizations, as this channel passes a relatively large amount of "window" current at negative potentials, allowing spontaneous depolarization of the membrane.

SA has been shown in many parts of the developing nervous system to be required for correct network formation. Work in the retina in several vertebrate species has shown that SA in neighboring retinal ganglion neurons strengthen their synapses closely together and maintain the retinotopic array onto CNS structures (Shatz, 1996). The phenomenon of SA in the chick and mouse hindbrain is highly conserved from birds to mammals; this suggests that there are developmental events in this brain structure that require SA for correct differentiation. Since, at the time activity arises in chick, the reticular formation is largely in place, it is likely that patterning of that structure does not require the presence of SA, although the maturation of function within that system may require activity. Interestingly, the projection of rostral connections to the spinal cord is just being formed at the time when activity begins in the mouse (E11.5–E12; Auclair et al., 1999), and the transients recorded in mouse at E11.5 may be important in patterning projection neurons during that time period. If true, this would suggest that SA, despite having many similarities, may have multiple or different functions in mouse and chick hindbrain.

The SA expressed in mouse and chick hindbrain might also be important in patterning the axons of the 5HT-expressing raphe neurons themselves. The serotonergic cell bodies are born near the ventral floorplate, migrate dorsally, and send their axons rostrally (B4–9), caudally (B1–3), and contralaterally (all groups). Although the 5HT neuronal expression is more anatomically distributed in the chick at E6, when activity begins, it is possible that transmitter release, cell body migration or synaptic patterning may be regulated by SA. In both species, the neurons

that comprise the respiratory nuclei in the caudal hindbrain may be influenced by the earlier-expressed SA. The similarities between anatomical position, neuronal type underlying SA, and timing of appearance of SA suggest that this phenomenon is important enough to persist through evolution, even though differences between species are apparent.

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