Timing and mechanism of a window of spontaneous activity in embryonic mouse hindbrain development

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Spontaneous activity (SA) in the developing vertebrate brain is required for correct wiring of circuits and networks. In almost every brain region studied to date, SA is recorded during a period of synaptogenesis, and may deploy ionic mechanism(s) that are not expressed in the adult structure. Eventually the conditions in the immature neurons that allow SA are replaced with ion channels found in the mature neuron; this replacement may itself require SA. In the embryonic (E) 11.5 mouse hindbrain, SA is initiated by a subgroup of serotonergic neurons derived from former rhombomeres 2 and 3; SA events propagate rostrally and caudally along the midline, and into the lateral hindbrain. In this review, I describe the properties of mouse hindbrain SA and the developmental window during which it is expressed, summarize the known mechanisms by which SA arises, and describe other brain regions where this SA is similar (chick hindbrain) or influential (mouse midbrain).

Keywords: motor patterns; hindbrain; spontaneous activity; serotonin

Introduction

The mature brain is wired to respond to signals, either from sensory inputs or other neurons, which cause an electrical response; with the exception of a few neuron types, few CNS neurons participate in electrical signaling by large synchronous groups, except under unusual conditions. The situation is quite different in the developing brain, in which large regions of the brain synchronously express spontaneous activity. Such spontaneous activity (SA) has been shown to be required for a variety of developmental processes in network formation, including neuronal proliferation, cell body migration, neurite extension and pathfinding, and synaptic formation and maintenance.\(^1\) SA has been recorded in a number of CNS structures, sometimes during a discrete window that corresponds to a period of specific network formation; such structures include the cortex, hippocampus, retina, cochlea, and spinal cord.

One example of network formation control by SA is found in the connections between the retina and its major target, the lateral geniculate nucleus of the thalamus. The retina undergoes waves of SA which are able to initiate in any region, but which may be elicited by a specific group of retinal neurons, the starburst amacrine cells.\(^2,3\) Alterations in the frequency or spread of SA waves alter the specificity and coarseness of the retinal map on the LGN, demonstrating that the mechanisms that elicit and propagate SA are crucial in correct network formation.\(^4,5\)

Our work concentrates on the development of the hindbrain, which in the adult becomes the pons, medulla and, very rostrally, the cerebellum. These structures receive sensory input and deliver motor output to the face and head, coordinate sensory input in the cerebellum, contain many axon tracts between the spinal cord and higher CNS, and are home to many neurons of the reticular formation, including serotonergic neurons. The hindbrain, in early development, is organized in the rostral-caudal axis by segmentation into rhombomeres, cell-specification segments setting up transversely arranged bands of gene expression. As development proceeds, longitudinal tracts are formed that course between vestibular networks, the spinal cord, and higher CNS structures.
Figure 1. Spontaneous activity propagates from an initiation zone (InZ). Inset (left) shows main features of hindbrain, in open book preparation. On the right are the borders of the former rhombomeres, designated by numbers (r). Red circles show the cell bodies of motoneurons from the trigeminal (V) and facial (VII) nerves, with exit points shown as red asterisks (EP). Blue circles near midline indicate location of serotonin-positive cell bodies (caudal group does not develop until E12.5.); green box is initiation zone (InZ). (A)–(D) Live $[\text{Ca}^{2+}]_m$ imaging of hindbrain, with midline crossing diagonally from upper left (rostral) to lower right (caudal). Single event initiating at center of screen, propagating both rostrally and caudally. High $[\text{Ca}^{2+}]_m$ levels are shown in red, superimposed on bright-field image of hindbrain. Frames taken every 0.4 sec.

The serotonergic neurons originating in the rostral hindbrain later develop into the raphe nuclei of the reticular formation. The raphe innervates many brain structures and is involved in a wide range of complex behaviors, such as sleep, circadian rhythms, and mood and is composed of several groups of 5HT-positive neurons. We have previously demonstrated that the early differentiating neurons of the serotonergic raphe play a role in early hindbrain development, where they act as a driver or initiator zone (InZ) that mediates synchronous SA in the hindbrain, and in the adjoining midbrain as well.

In this review, I will discuss the properties of the initiator region in driving hindbrain SA. When rhombomeric segmentation ends, synchronized SA emerges in the mouse hindbrain, driven by the InZ, which initiates over 85% of the events. This pacemaker persists in initiating events between E11.5 and E13.5, and then becomes inactive. We have observed a similar phenomenon in chick hindbrain, with differences attributable to the more mature properties of chick hindbrain. This conservation suggests that SA is important in network development in the hindbrain. We have also observed that the events initiated in the hindbrain trigger waves of SA in the more rostral midbrain, the first time that an identified initiator can control activity in a different brain structure.

Results

Description of mouse hindbrain spontaneous activity

SA was examined using the open-brain preparation, in which the dorsal midline is opened and the hindbrain placed in a $[\text{Ca}^{2+}]_m$ imaging chamber (Fig. 1, inset). The positions and trajectories of the branchiomeric motoneurons (trigeminal and facial) were delineated by retrograde labeling, and the serotonergic neuronal groups identified using immunocytochemistry. In the E11.5 mouse hindbrain, more than 85% of recorded events initiate from the midline in former rhombomere 2; we thus term this region the initiator zone (InZ). InZ-initiated events propagate rostrally and caudally along the midline, as well as laterally (see later). Figure 1A shows an event initiating in the midline InZ (red color indicates increase in $[\text{Ca}^{2+}]_m$); this event then propagates in both the rostral and caudal directions along the midline (Figs. 1B–D). Interestingly, events moving in the rostral direction propagate at a more rapid velocity ($359 \pm 5 \mu\text{m/s}$) than the same event propagating caudally ($168 \pm 35 \mu\text{m/s}$) ($n = 23$), suggesting that differential mechanisms mediate propagation in the two directions. Other rostral midline regions can initiate events (approximately 15%), but we have never observed initiation of events by lateral regions.
InZ-triggered events also propagate laterally from the midline. Figure 2A shows the placement of multiple $\text{[Ca}^{2+}\text{]}$, imaging sites on the hindbrain, starting from the midline InZ and covering the entire medio-lateral extent of the hindbrain. Changes in fluorescence at each site are plotted from bottom to top in Figures 2B and C, demonstrating that events initiate medially and propagate laterally. When a longitudinal cut is made between the InZ and a lateral position, the activity in the midline is not affected, while the lateral activity is smaller, significantly lower in frequency, and not coordinated with the midline, after the cut.\(^9\)

We used pharmacological manipulation to elucidate interactions that elicit hindbrain SA. Blockers of glutamatergic, GABA\(_A\) and GABA\(_B\), nicotinic, glycinergic, dopaminergic, tachykinin, purinergic, and histaminergic receptors did not abolish activity.\(^11\) The only transmitter system that appears to be required for expression of hindbrain SA is serotonin (5HT), acting through 5HT\(_2A\) and 5HT\(_2C\) receptors. In addition, gap junctional coupling appears to be crucial to SA, as block by the relatively nonspecific agents octanol, carbenoxolone, and mefloquine all abolish SA, while the addition of ammonium transiently augments SA.

Transverse cryosections of the E11.5 hindbrain labeled with an antibody against serotonin showed that serotonin-positive neurons were located with 125 m of the midline of the hindbrain, and send
Figure 3. Immunocytochemistry against serotonin (5HT) demonstrates newly postmitotic neurons at E11.5. (A) Transverse section showing serotonin-positive neurons leaving the ventricular zone, and clustering in the marginal zone very close to the midline (white line). Axons cross the floor plate ventral to the ventricular zone. Scale bar is 50 μm. (B) Parasagittal section 30 μm from midline showing rostral cluster of serotonin-positive neurons stretching from isthmus (at rostral end) to the rostral edge of former r4 (as measured in dextran-identified animals; not shown). Scale bar is 100 μm.

their axons both contralaterally across the floor plate and into the marginal zone (Fig. 3A). In sagittal sections, the serotonin-positive neurons were clustered in a group that extended from the isthmus (hindbrain–midbrain organizer) to the rostral end of former rhombomere 4 (Fig. 3B), encompassing the identified InZ. These neurons send axons across the isthmus into the midbrain, which becomes highly innervated by E13.5, and into more rostral regions of the brain. (A second serotoninergic group, located caudal to former rhombomere 4, develops at E12.5 and innervates the spinal cord.) Counting serial transverse sections showed that within the midline InZ region, 85% of the postmitotic neurons were serotonin-positive, suggesting that the InZ is composed almost exclusively of serotoninergic neurons. This combined evidence of initiation site, proportion of serotoninergic neurons within the InZ, and pharmacological dependence of SA on 5HT receptors, implies that the developing neurons of the rostral raphe are the drivers of SA in the hindbrain.

Developmental window of SA

SA in mouse hindbrain is a transient phenomenon, recorded only in the interval of E9.5–E13.5. Early experiments examining the development of SA utilized recordings from identified trigeminal and facial motor neurons, retrogradely labeled by Texas Red-conjugated dextrans injected into the target branchial arches (Fig. 4A). At E9.5, individual neurons have spontaneous events which are long in duration, and neurons undergo events independently (Fig. 4B). Twenty-four hours later, at E10.5, events are significantly shorter in duration, suggesting that some mechanism for more rapid termination of events has developed; events are still independently expressed in individual neurons (Fig. 4C). After an additional 24 h of development, at E11.5, events between individual motor neurons become highly synchronized, and almost no independent events are observed (Fig. 4D).

The synchronized SA, initially observed in identified motoneurons lateral to the midline, was determined to be driven by the midline InZ, as shown by the medio-lateral mapping experiments described above. Thus, at E11.5, the InZ becomes active, and synchronizes SA across the entire hindbrain (green area, Fig. 5A), driving neurons that are already close to threshold in propagating waves of activity. The relative frequency of midline activity is slightly higher near the pontine flexure (PF) (right histogram, Fig. 5A), which includes the InZ. The InZ remains active until E13.5, but the area of the hindbrain that is able to be synchronized by InZ input diminishes over time. Thus, the midline remains...
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Figure 4. Developmental sequence of SA in dextran-identified motoneurons. (A) Living motoneurons retrogradely filled with dextran (red) by injection into target tissue, and loaded with fluo4-AM (green). Single identified neurons were recorded for fluctuations in [Ca^{2+}]_i. Scale bar is 15 μm. In B–D, each color trace represents recording from an individual neuron. (B) At E9.5, events in individual neurons are long duration, and independent. (C) At E10.5, events are significantly shorter in duration, and events in individual neurons are still independent. (D) At E11.5, events are short-duration, and highly synchronized between neurons.

driven by the InZ at E12.5, although the most rostral and caudal extremes have a reduced frequency of activity (green area and histogram, Fig. 5B); lateral regions of the hindbrain no longer respond to the InZ signal. By E13.5, the rostral and caudal regions of the midline respond to InZ activity at only very low frequency (green area and histogram, Fig. 5C), and by E14.5, no SA is recorded in the hindbrain. The area that responds to the InZ at E13.5 is only 14% of that at E11.5. The absolute frequency of InZ-initiated events decreases by approximately twofold between E11.5 and E12.5, remains constant to E13.5 (inset, Fig. 5), and is disappears at E14.5.

These experiments show that SA in the hindbrain is expressed over the interval of E9.5–E13.5, but each day of that period has a unique and characteristic form of SA. Independent spontaneous events at E9.5 have long durations, and shorten significantly at E10.5; events in all neurons are then synchronized by InZ input at E11.5. After this 24-h period of whole-hindbrain response to the InZ driver, the lateral areas begin to become refractory at E12.5, followed by the midline outside of the InZ at E13.5. Thus, the unique aspects of SA on each developmental day overlie other developmental events in the hindbrain.

Mechanisms of SA initiation

The ability to observe the relationship between several cells or groups of cells was integral to the discovery of the propagation of SA in the hindbrain. We then examined the electrophysiological properties of individual neurons in medial versus lateral positions within the hindbrain. Neurobiotin was included in the pipette in order to measure the coupling of the recorded neuron to neighboring neurons. Within the delineated InZ (up to 100 μm from the midline), in current clamp recording, neurons had spontaneous events comprised of two components: the first was a large amplitude, spike-like event; the second was a slower plateau (Fig. 6(A)1). Outside of the midline area comprising the InZ, neurons had spontaneous events that were small in amplitude, and consisted of only the plateau component (Fig. 6(A)2). In several tens of recordings, the components were measured and compared to the position in the hindbrain. These data showed that the spike was recorded only in medial neurons, and the plateau was significantly larger in medial neurons.
Figure 5. Between E11.5 and E13.5, SA retracts from lateral, then medial tissue. Hindbrains from each stage were laid in open-book configuration, and drawings to scale made of each stage. Boxed frames in each figure correspond to camera frames during each recording, which were positioned over each hindbrain in random order; a multiple array of sites within each frame were then recorded. Scale bar applies to A–C. (A) At E11.5, SA is recorded in the entire hindbrain (green area). At the midline, the relative frequency of activity was highest in the most rostral frame (graph on right of figure); in lateral region, frequency was highest in more caudal region, suggesting that propagation is more efficacious at that level of the tissue. (B) At E12.5, SA is largely retracted from the lateral regions, and is found only in midline tissue; frequency of activity is highest just above the pontine flexure (PF). Dashed line indicates most extreme lateral extent of SA. (C) At E13.5, SA is even more restricted within the midline, with frequency highest at the most rostral end of the hindbrain near the isthmus. Events are able to propagate to PF, and then stop well before the end of the hindbrain. Inset shows absolute frequency of SA at each stage, which is significantly higher at E11.5.

We sought to identify the underlying currents that engendered the differential expression of event properties between medial and lateral neurons. When the tissue containing the recorded cells was reacted for the neurobiotin from the pipette, medial neurons had a small cluster size, while lateral neurons were coupled to significantly more neighboring neurons (Figs. 6(B)1 and 2). The neurobiotin-permeable connections are likely to be gap junctions, as octanol and mefloquine block SA, while ammonium transiently augments SA. Thus, midline neurons have small, but significant, cluster sizes that may be important in initiating activity, while lateral neurons have connections to more neighboring neurons, most likely to aid in propagation of events.

We then asked whether the differential expression of neuron coupling was reflected in the resting conductance of the neurons. Using voltage clamp recording, we applied ramp potentials from $-110$ to $+30$ mV. Resultant currents in lateral cells showed a much larger resting conductance compared to those in medial cells, likely a consequence of the increased coupling between neurons (Fig. 6C).

In addition, the resultant currents in medial neurons showed a small inflection near $-40$ mV, raising the possibility that an inward current was expressed in medial cells, but not evident in lateral cells. We used Cs$^+$-based pipette solutions to ask whether hindbrain neurons expressed inward currents. We found that medial, but not lateral neurons, expressed an inward current that was sensitive to Ni$^{2+}$, peaked at about $-45$ mV, and had kinetics similar to T-type Ca$^{2+}$ channels (inset, Fig. 6C). Thus, medial neurons have inward currents that are available near the resting potential of the neuron, and have relatively little resting conductance, both properties that would allow them to act as initiators of activity. Lateral neurons, in contrast, have no inward currents and have high resting conductance, consigning them to a follower behavior in the hindbrain.
Figure 6. Physiological properties of medial vs. lateral neurons. (A1 and 2) Representative traces in current clamp mode from a medial neuron (A1) and a lateral neuron (A2), showing the two-component spontaneous events (spike plus plateau) in medial neurons, and events with only plateaus in lateral neurons. Dotted line in A1 indicates 0 mV for both figures. (B1 and 2) Representative neurobiotin-reacted fills from medial (left) and lateral (right) neurons, showing that lateral neurons are coupled to significantly more neurons via gap junctions. (C) Main graph shows resultant currents in voltage clamp from a voltage ramp from $-110$ to $+30$ mV, showing that lateral neurons (gray trace) have high resting leak conductance, while medial neurons (black trace) have significantly less. Inset shows inward current responses in a medial neuron to depolarizations from a holding potential of $-80$ mV in voltage clamp; 5 mV steps to $-40$ to 0 mV elicit inward currents resembling T-type Ca$^{2+}$ currents. Only medial neurons express measurable inward currents.

SA in chick hindbrain
To characterize the relevance of hindbrain SA across species, we examined the expression of SA in developing chick hindbrain. In chick hindbrain, SA was recorded at E5, but only under conditions of increased $[K^+]_o$ (5 mM, in contrast to our standard 2.5 mM). During the developmental period between E6 and E9, intervals between events were very regular, and increased from approximately 4 min at E6 to 18 min at E9 (Fig. 6). In contrast to mouse hindbrain SA, which has a single initiation point near the former r2, chick SA was initiated from two sites, one near the former r2, and the other more caudally located, at the former r4 (Fig. 7). Another striking difference between chick and mouse was the involvement of spinal cord in chick SA; when the preparation included the spinal cord, lateral waves of spinal cord activity could be seen that appeared to be synchronized with hindbrain SA; however, the presence of the spinal cord was not required for independent hindbrain SA. Spinal cord involvement in mouse hindbrain SA was never observed, regardless of the length of spinal cord that was present.

Pharmacologically, SA in chick was not exclusively dependent on 5HT receptors, as blockers of nAChR, GABA$_A$, and glycine receptors all blocked activity. As chick develops more rapidly than mouse, it may not be surprising that other neurotransmitter systems are involved in chick SA, as many more axon tracts and networks are in place at these developmental stages in chick.

We examined the serotonergic system in E6 chick hindbrain, and found that both the rostral and caudal serotonergic groups were in place, with extensive axon extension and cell body migration to more mature positions. Interestingly, the gap in serotonin-positive cells between the rostral and caudal group was situated more rostrally in chick than in mouse, at approximately former rhombomere 2; thus, both initiation points are located in the caudal serotonergic group. The anatomical differences between chick and mouse are summarized in Figure 7, which shows the initiation points (asterisks) and serotonergic midline groups (both rostral and caudal in chick, and the rostral and future caudal in mouse). These comparative experiments show that hindbrain SA is strongly conserved within vertebrate species suggesting a role for hindbrain SA in network formation.

Hindbrain SA drives activity in the midbrain
Events from the hindbrain InZ propagate rostrally and caudally along the midline; those that
propagate caudally dissipate well before they encounter the spinal cord. Events that propagate in the rostral direction can cross the isthmus (also known as the hindbrain–midbrain organizer), and sweep across the tegmentum of the midbrain.\(^\text{16}\) Events initiated in the hindbrain InZ travel along the midline towards the isthmus, where they pause before propagating into the midbrain (Fig. 8). The ability to cross the isthmus is dependent on developmental stage, as hindbrain events at E12.5 have a higher probability of crossing into the midbrain than those at either E11.5, even though the frequency of activity in the hindbrain decreases slightly during that interval. This is likely due to the increased length of serotonin-positive projections towards the midbrain and crossing the isthmus over this developmental period; since the hindbrain events travel rostrally along these projections, their increasing length may facilitate crossing the isthmus. Events cross into the midbrain along the midline through the isthmus; they then propagate rostrally and laterally to fan across the entire tegmentum of the midbrain. Groups of nicotinic and GABAergic neurons, located laterally to the midline, are likely crucial in the lateral propagation,
as it is abolished by blockers of those receptors. Strikingly, although each wave of midbrain activity crosses the developing dopaminergic neurons, those neurons are not themselves required for SA, as blockers of either D1 or D2 receptors do not affect midbrain SA. However, the dopaminergic neurons may themselves contribute to, or be influenced by, the waves of midbrain activity.

In the chick midbrain, it has been shown that concentric torii of gene expression are located within the tegmentum; each torus opens facing caudally, and each underlies a specific mature neuron population. The most caudal and medial group is the anlage of the dopaminergic neurons of the substantia nigra, while more lateral torii underlie GABAergic and cholinergic neurons. The propagation of midbrain SA events occurs in a fan which moves orthogonally to the established torii of gene expression. We have observed hindbrain-to-midbrain propagation in chick (unpublished) as well as in mouse, implying that along with conservation of hindbrain SA between species, hindbrain-driven midbrain events are also conserved.

**Discussion**

SA in mouse hindbrain is first recorded as individual neurons engaged in spontaneous events that are not coordinated. Synchronized waves of InZ-driven SA are expressed exactly at E11.5, when the transient borders between rhomobomeres disappear. Segmented gene expression is the conserved mechanism for establishment of anterior–posterior identity in vertebrates and invertebrates; this segmentation determines insect segmental identity as well as hindbrain neuronal fates (for example, the differential determination of trigeminal versus facial motoneurons). We postulate that as the role of the anterior–posterior fate determination by rhombomeric segregation decreases, waves of SA that repeatedly emanate from one site become the mechanism by which neurons localize their spatial relationship. A single point source of propagating excitation allows developing neurons in a longitudinal or horizontal group to discriminate neighbor relationships; those that are close neighbors may then reinforce their developing synapses in a Hebbian manner.

This hindbrain InZ is the first example where an identified driver causes SA in a neighboring brain structure, the midbrain. The frequency of midbrain events is determined by the ability of serotonergic axons to carry the event through the isthmus into the midbrain; as these axons are longer at E12.5 than E11.5, midbrain activity is higher in frequency at that stage. The frequency decreases at E13.5, as the midline of the hindbrain becomes refractory to the InZ signal. The phenomenon of the hindbrain driving the midbrain may be a mechanism by which connections between the two structures are guided: for example, axons of the medial and lateral longitudinal funiculi derive from neurons in the rostral hindbrain and midbrain, and may require SA in order to pathfind appropriately. In addition, the rostrally directed axons of the serotonergic neurons are themselves elongating extensively, and the robust midline SA may be an important guidance mechanism by which they find and traverse the isthmus.

Interestingly, the strongest and longest-lasting axis of SA propagation is rostro-caudal, which places SA propagation orthogonal to the former transverse bands of gene expression which were initially established by rhombomere segmentation. As these waves of activity enter the midbrain, they fan rostro-laterally from the isthmus across the tegmentum, again moving orthogonally across bands of gene expression. It is possible that in each of these cases, spontaneous electrical activity is replacing gene patterning mechanisms in neuron specification or differentiation.

At E11.5, both the frequency of events and the total area that is able to be driven by each event is significantly higher than later stages. We have yet to determine the mechanism by which first the lateral (at E12.5), then the midline neurons (at E13.5), lose their ability to be driven by the InZ. Possibilities include the down-regulation of connexin expression such that the signal cannot propagate between cells or the up-regulation of K⁺ channels that oppose depolarizing influences.

When the InZ is cut into successively smaller pieces the probability of SA decreases as the pieces get smaller. This phenomenon suggests that the InZ is composed of a network of serotonergic neurons interacting to initiate events which then propagate into other hindbrain regions. Although individual cells undergo spontaneous electrical events without obvious evidence of synaptic input from other neurons, and ionotropic receptors are not involved in hindbrain SA, the events may be a consequence of...
electrical coupling and G-protein-coupled receptor input.

A further area of study is the ionic mechanism(s) which turn off the expression of SA within the InZ. One mechanism may be the developmental down-regulation of T-type Ca\textsuperscript{2+} channels, which have a window current near the resting potential which might allow SA; without such a depolarizing drive, cells may not undergo spontaneous events.

The changing pattern of SA in the hindbrain, from independent long-duration events (at E9.5), to all-over synchronized waves (at E11.5), to retraction back to the InZ (at E13.5), is unique to the hindbrain. The mechanisms that cause each step—independent SA, synchronization, and retraction—are not yet understood, nor is the mechanism of differential ion channel expression between medial and lateral neurons. However, this robust phenomenon is maintained between relatively distant vertebrates (mouse and chicken), and thus has been conserved strongly. It is possible that alterations resulting from brain insult, fever, or fetal seizures may disrupt the balance of this pattern over the developmental window of SA, resulting in abnormal network properties not just in hindbrain, but midbrain as well. An additional point to consider is that maternal use of serotonergic modulators may influence the synchronization, frequency or propagation of hindbrain SA, possibly altering developing circuits.

**Conflicts of interest**
The author declares no conflicts of interest.

**References**


