Differential involvement of projection neurons during emergence of spontaneous activity in the developing avian hindbrain

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Running title: Spontaneous activity in projection neurons
Abstract

To better characterize the emergence of spontaneous neuronal activity in the developing hindbrain, spontaneous activity was recorded optically from defined projection neuron populations in isolated preparations of the brain stem of the chicken embryo. Ipsilaterally projecting reticulospinal (RS) neurons and several groups of vestibulo-ocular (VO) neurons were labeled retrogradely with Calcium Green-1 dextran amine, and spontaneous calcium transients were recorded using a CCD camera mounted on a fluorescence microscope. Simultaneous extracellular recordings were made from one of the trigeminal motor nerves (nV) to register the occurrence of spontaneous synchronous bursts of activity. Two types of spontaneous activity were observed: synchronous events (SE), which occurred in register with spontaneous bursts in nV once every few minutes and were TTX-dependent, and asynchronous events (AE) which occurred in the intervals between SE and were TTX-resistant. AE occurred developmentally before SE and were in general smaller and more variable in amplitude than SE. SE appeared at the same stage as nV bursts early on embryonic day 4, first in RS neurons and then in VO neurons. All RS neurons participated equally in SE from the outset, whereas different subpopulations of VO neurons participated differentially, both in terms of the proportion of neurons that exhibited SE, the fidelity with which the SE in individual neurons followed the nV bursts, and the developmental stage at which SE appeared and matured. The results show that spontaneous activity is expressed heterogeneously among hindbrain projection neuron populations, suggesting its differential involvement in the formation of different functional neuronal circuits.
Introduction

Neuronal activity is essential for the proper development and maturation of synaptic connections. Evidently to ensure a background of activity as neural circuits are constructed, the central nervous system exhibits a remarkable propensity for self-generated spontaneous activity that is independent of *de facto* sensory inputs. For example, spontaneous activity is present in developing sensory systems, most notably in the retina, where it is believed to provide a substrate for synaptic interactions necessary for proper synaptic patterning at higher levels of the visual system (Firth et al. 2005; Wong 1999). Underscoring its general relevance for brain development, it is also a characteristic of the developing cortex where it appears to serve the same role (Peinado 2000; Turrigiano 2004; Turrigiano and Nelson 2004). Since spontaneous activity is so widespread in the central nervous system, increasing efforts are being made to understand its spatiotemporal patterning and the mechanisms by which it is produced.

Early embryonic activity is particularly interesting to study in the hindbrain and spinal cord because it causes sporadic movements of the embryo termed embryonic motility (Bekoff 2001; Fortin et al. 1994, 1995; Hamburger et al. 1966; Landmesser and O'Donovan 1984; O'Donovan and Landmesser 1987; Provine 1971; 1972; Ripley and Provine 1972). Embryonic motility appears to be instrumental in ensuring the proper development of bones, joints and muscles (Osborne et al. 2006, Hammond et al. 2007, Gomez et al. 2007). In the chicken embryo, motility has been analyzed in numerous studies (reviewed in Bekoff 2001, Bradley 2001), and the first movements, arising during the fourth day of embryonic development, have durations and intervals quite similar to those of bursts of activity recorded from cranial and spinal nerves in isolated brainstem and spinal cord preparations (Hamburger and Balaban 1963; O’Donovan and Landmesser 1984; Fortin et al. 1994). As development proceeds, patterns of motility become more complex and include limb movements. In
parallel, bursts of nerve activity in isolated brainstem and spinal cord preparations decrease in frequency and begin to exhibit a more episodic pattern by about the sixth day of development (O’Donovan and Landmesser 1984; Fortin et al. 1994, 1995). In the hindbrain of the chicken embryo, the episodic pattern results from maturation of GABAergic inhibition and the appearance of rhythmic interruption of the motor burst (Fortin et al. 1995, 1999; Champagnat and Fortin, 1997), and is dependent on interactions among neurons in different hindbrain segments (Coutinho et al. 2004).

In a comprehensive set of studies using extracellular recording from cranial motor nerves and optical recording of calcium transients in retrogradely labeled motoneurons, we and Bosma and colleagues have demonstrated that spontaneous activity in cranial motoneurons has two distinguishable components. The first, which has only been recorded optically in the mouse embryo, consists of sporadic, long-duration, low-frequency calcium transients that are tetrodotoxin-insensitive (Gust et al. 2003) and may therefore result from intrinsic membrane or synaptic properties of neurons. The other is remarkable in that it consists of tetrodotoxin- and carbenoxolone-sensitive bursts of activity that are synchronized in most if not all cranial nerves (Gust et al. 2003; Thoby-Brisson et al. 2005). The synchrony of these bursts indicates a rapidly spreading activation of cranial motor neurons located at different segmental levels of the hindbrain. Although this activation is clearly generated centrally, since it manifests as motor output from acutely isolated brain stems in vitro (Fortin et al. 1994), relatively little is known about how it is initiated and propagated. In our earlier studies in the chicken embryo, electrophysiological exploration and neuronal staining suggested that many neurons, including bulbospinal neurons with long axons, are co-active with the motor output (Fortin et al. 1995; Champagnat and Fortin 1997; Fortin et al. 1999). More recently, Bosma and colleagues used optical recording of calcium transients to show that synchronous spontaneous activity in the mouse embryo brain stem also occurs in
medially located serotonergic neurons (Hunt et al. 2005). At the pontine level, a subpopulation of these are evidently the primary origin of the spontaneous bursts, which propagate laterally to engage the motoneuron nuclei (Hunt et al. 2006a). A similar sequence of events, with an initiating center either in the medial pons or the rostrolateral medulla, has been seen using voltage-sensitive dye recording in the rat embryo (Momose-Sato et al. 2007). At the embryonic stages studied in the mouse, the medial serotonergic neuron population extends highly ramifying ascending and descending axons to the midbrain and caudal hindbrain, providing a potential substrate for rapid spread of activity along the longitudinal and mediolateral axes of the brainstem (Hunt et al. 2005; Hunt et al. 2006b). Initiation need not be exclusively from the pons, since at the medullary level, after isolation from the pons, an embryonic activity persists and spreads from unidentified dorsal midline structures (Thoby-Brisson et al. 2005).

Although synchronous spontaneous activity clearly spreads from serotonergic neurons near the rostral midline to encompass all the cranial motor nuclei in the mouse embryo, it is not known in any species the extent to which other specific hindbrain neuron populations engage in either the sporadic, asynchronous form or the synchronous form of spontaneous activity. The voltage-sensitive dye recordings of Momose-Sato et al (2007) in the rat embryo suggest a highly distributed network encompassing most regions of the hindbrain, but these recordings do not permit the identification of specific neuron types. Nor is it clear whether all neurons that exhibit spontaneous activity do so to the same degree or in the same temporal pattern. This is an important question because it addresses the issue of whether spontaneous activity arises independently in diverse subpopulations of neurons, as opposed to being driven by a distinct rhythm-generating center. We have therefore undertaken a study of the emergence of spontaneous activity in defined subpopulations of projection neurons in the hindbrain of the chicken embryo. We have focused on two different projection neuron
populations, the reticulospinal (RS) neurons, with long descending axons, and groups of operationally defined vestibulo-ocular (VO) neurons, that is, neurons with long ascending axons that terminate in the regions of the oculomotor and trochlear motor nuclei (Glover and Petursdottir 1991). Large numbers of RS axons have reached the spinal cord by embryonic day (d)4 (Glover and Petursdottir 1991), and large numbers of VO axons have reached the oculomotor nucleus in the midbrain by d5 (Glover 2003). Thus, both of these projection neuron populations can be labeled retrogradely with calcium-sensitive tracers during the early period when spontaneous activity arises, and subpopulations within them can be readily recognized on the basis of position and axon trajectory. This has allowed us to compare patterns of spontaneous activity in discrete and identifiable groups of projection neurons. We specifically analyzed the proportion of synchronized neurons within a given subpopulation, the fidelity with which they followed the general cranial motoneuron bursts, the stability of their bursts and the developmental stage at which synchronization appeared. We show that specific RS and VO neuron subpopulations exhibit both asynchronous and synchronous spontaneous activity, but do so differentially, both with respect to developmental timing and to the fidelity of synchronization. This indicates that spontaneous activity is a more heterogeneous phenomenon than previously recognized.

**Materials and methods**

*Preparation and labeling*

All the experimental procedures were performed according to guidelines established by the Norwegian Experimental Animal Committee and the local veterinary authorities at the University of Oslo and also adhered to EU and NIH regulations for the use of animals in scientific experiments. Fertilized eggs of White Leghorn chickens (Lohman race) were obtained from a local supplier (Samvirkekylling AS, Norway and Morizeau, France) and incubated at 38°C for 3-7 days in a forced-draft incubator, after which time the embryos were
removed, immediately anaesthetized by immersion in ice-cold artificial cerebrospinal fluid (ACSF, containing 120 mM NaCl, 5 mM KCl, 1.5 mM CaCl₂, 1.0 mM MgCl₂, 21 mM NaHCO₃, 0.58 mM NaH₂PO₄ and 15 mM glucose, continuously bubbled with a mixture of 95% O₂ and 5% CO₂ to maintain pH 7.4), staged according to the Hamburger and Hamilton (HH) system (Hamburger and Hamilton 1992), and decapitated at the lower neck. The entire brain was then removed and pinned to a silicone polymer substrate in a dissection dish. To label reticulospinal (RS) or vestibulo-ocular (VO) projection neurons with the dextran conjugated calcium indicator, Calcium Green-1 dextran amine (CGDA, 3kD, Molecular Probes / Invitrogen, USA; O’Donovan et al. 1993), small crystals of the indicator were made on the tips of fine needles and applied to a cut freshly made in the tract in which the respective axons course, the medial longitudinal funiculus (MLF), either in the caudal medulla (RS neurons) or at the level of the trochlear nuclei (VO neurons; Figure 1; see Glover 1995 for details of the technique). The damage to the axons at the application site facilitates uptake of the CGDA which is then transported retrogradely to the neuron cell bodies (Glover et al 1986). To further investigate supopulations of output neurons, the VO neurons were distinguished into subpopulations based on position and axon trajectory according to the scheme described in Díaz et al (2003). The specific subpopulations studied were the ipsilateral Rostral (iR), ipsilateral Caudal (iC) and contralateral Caudal (cC) groups. Because late maturation of spontaneous activity patterns varies in different hindbrain segments, or rhombomeres (Fortin et al., 1999, Coutinho et al., 2004), the RS population was subdivided according to rhombomeric position. To do this, in most preparations in which RS neurons were labeled, trigeminal and facial motoneurons were also labeled retrogradely with tetramethylrhodamine dextran amine (RDA, 3kD, Molecular Probes / Invitrogen, USA) to distinguish the rhombomeric domains. This was done by applying crystals of RDA to the freshly cut proximal stumps of the corresponding motor nerves. In other preparations, RS and
VO neurons were retrogradely labeled differentially by applying RDA to the RS axons and 10kD Alexa Fluor 488 dextran amine (ADA, Molecular Probes / Invitrogen, USA) to the VO axons. All preparations were incubated for 3-5 hours in ACSF at room-temperature (22-25°C) after dextran application until the neurons were sufficiently labeled as monitored under a fluorescence dissection microscope.

**Optical recording**

After being trimmed at the levels of the midbrain and the upper cervical spinal cord, preparations were pinned in a recording chamber and placed on an upright fluorescence microscope (Axioskop, Carl Zeiss, Germany) equipped with a 40x water immersion objective (LUMPlanFl, 0.8 NA, Olympus, Japan), fluorescein and rhodamine filter sets, a 0.4x C-mount adapter lens, a CCD camera (Photometrics Cascade, Roper Scientific, USA) and, as a light source for epi-illumination, a 100W halogen lamp driven by a regulated DC power supply (PAN35-20A, Kikusui Electronics Corporation, Japan). Fluorescence changes caused by spontaneous calcium transients in RS or VO somata were filmed respectively from the ventral or dorsal surfaces of the hindbrain using the CCD camera and an imaging program on a PC (MetaMorph 5, Universal Imaging Corporation / Molecular Devices, USA) at 2-10 frames / second (Figure 1). Separate recordings of 8-16 minutes duration were made in each rhombomere (r) 1 to 6 in the case of RS neurons and in each of the iR-VO, iC-VO and cC-VO subpopulations in the case of VO neurons. By virtue of high amplifier gain at the CCD camera and the low frame rate we could minimize epi-illumination so that dye bleaching and phototoxicity were negligible despite the long recording durations. The preparations were continuously perfused with 30°C ACSF at 2 ml / minute during the entire experiment.

**Nerve recording**
Bursts of action potentials in one of the trigeminal motor nerves were recorded using a glass suction electrode (Figure 1). Electrical signals were amplified by a differential amplifier with a gain of 10,000 and a band width of 0.3-3 kHz, integrated by an envelope integrator with a time constant of 50 msec (DPA-2FS and INT-01-T, NPI Electronic GmbH, Germany), and recorded with a digitizer and a recording program on a PC (Digidata 1322A and Clampex 9, Axon Instruments / Molecular Devices, USA).

**Offline data analysis**

Each optical recording typically included tens of CGDA-labeled neurons. In each optical recording, 16 neurons were selected randomly and analyzed. Using the MetaMorph program, an aperture was placed on each neuron soma, and intensities averaged over the pixels within each aperture were calculated frame-by-frame and saved as text files. The intensities were normalized to the average intensity for each cell measured in background frames acquired during non-active periods, rearranged on a handmade program developed on Delphi (Borland Japan, Japan), and then imported to Clampfit 9 (Axon Instruments / Molecular Devices, USA) to be expressed as waveforms. From these waveforms, the peak amplitude of each recorded synchronous calcium transient was expressed as the percent increase over background light intensity. This was done by measuring from the height of the peak to a straight baseline connecting the middle values of the background noise just prior and just after the transient. Because average noise levels were around 1% in the optical recordings, for subsequent analysis, only synchronous transients representing an increase in fluorescence over background larger than 1% were assessed. It is important to emphasize that the amplitudes of the calcium transients represent changes in light intensity; the relationship of these to membrane potential changes or other events that might be responsible
for the underlying changes in calcium concentration has not yet been characterized in this system.

Within each neuron population, we identified individual neurons that were (1) co-active with all nV bursts, (2) co-active with only some bursts, and (3) not co-active with nV bursts at all. To quantify the degree of participation in synchronous spontaneous activity of a given neuron population, we defined a likelihood index (LI) that accounted for the proportion of spontaneous calcium transients exhibited by individual neurons that were synchronized with the spontaneous bursts in nV and the degree of fidelity of the synchronization across neurons (Figure 2).

Since optical and nerve recordings recorded simultaneously from the same preparation were stored in separate files, alignment of the two types of recording was done visually using Illustrator 10 (Adobe, USA) by importing waveforms from Clampfit 9. Analysis of the frequency of and intervals between calcium transients was made using Clampfit 9. These data (as well as amplitudes) were exported to an Excel spreadsheet for statistical analysis. Variability of synchronized bursts within a given neuron population was estimated from the coefficient of variation of calcium transient amplitudes exhibited by all the neurons of that population in each recording and trends in development were studied by comparing preparations at five coarse developmental stages, denoted by days (d) of development: early d4 (HH stages 23-24), late d4/early d5 (HH stages 25-26), late d5 (HH stage 27), d6 (HH stages 28-29) and d7 (HH stage 30-32).

Results

Synchronous spontaneous activity begins in RS and VO projection neurons after they have started to extend axons
In previous studies, synchronous spontaneous bursts of action potentials recorded extracellularly from cranial motor nerves have been observed starting early on d4 in the chicken embryo hindbrain (Fortin 1994, 1995). We found that spontaneous bursts recorded from the trigeminal nerve (nV) started at stage 24, which is early on d4, in complete agreement with these earlier studies (Table 1). We combined trigeminal nerve recording with calcium recording to assess spontaneous activity in individual neurons within the reticulospinal (RS) or vestibulo-ocular (VO) projection neuron populations starting on d3 (stage 20, RS) and late on d4 (stage 25, VO). At these respective stages the RS and VO projection neuron populations have started to extend axons and can therefore be retrogradely labeled with CGDA and identified by this labeling. We observed spontaneous calcium transients synchronized with the nV bursts as early as stage 24 in ipsilaterally projecting RS neurons (contralaterally projecting RS neurons were not assessed), and as early as stage 26, but not before, in VO neurons. At late stages (d6), more complex synchronized patterns were seen (Figure 3, cC-VO). We call these calcium transients that are synchronized with nV bursts “synchronous events”, or SE. Two conclusions can be drawn from these experiments on the developmental stage at which synchronous activity starts in neuron populations. First, in both RS and VO, SE start after the extension of long axons, since during initial stages of their axon outgrowth (late d4/early d5) SE were not observed. Second, SE starts at different times in RS and VO neurons, contemporaneously with (RS) or about 12-48 hours after (VO) the appearance of synchronous activity in cranial motoneurons.

Asynchronous spontaneous activity

In every preparation that exhibited nV bursts, SE occurred once every few minutes, with a general developmental decrease in frequency (Table 1). In addition to SE, we also observed asynchronous events (AE), which were calcium transients that occurred in the
intervals between SE (Figure 3). Because AE were much more variable in time and amplitude than SE, and therefore more difficult to analyze, we have restricted further quantitative analysis in this report to the SE. Nevertheless, we summarize several consistent features of AE in the following paragraph (we plan to present a more detailed analysis of AE elsewhere).

AE occurred at developmental stages earlier than the onset of SE. Both types of projection neuron exhibited AE from the earliest stages they were labeled, but AE were generally less frequent in VO neurons than in RS neurons. Thus, individual projection neurons generated spontaneous calcium transients independently of each other from an early stage of their development, and then later engaged in the more widespread synchronous activity. AE continued to occur after SE appeared, indicating that neurons could still generate independent calcium transients after engaging in the synchronous network (Figure 3, d6). In contrast to SE, AE varied substantially in amplitude, duration, and frequency in any given neuron and between neurons. Once SE were well established in a given population (within a day after first appearance), AE were always smaller than SE in any given neuron. For example, in RS neurons, AE amplitudes were typically 10-50% of SE amplitudes (Figure 3, r2-RS late d5 – d6). As was the case for SE, AE frequency decreased as development proceeded, most markedly after d6 (data not shown). AE occurred with highly variable timing within the intervals between SE (Figure 3), suggesting that there were no strong temporal interactions or protracted refractory effects between SE and AE. Given their variable timing and smaller amplitudes it is even possible that AE might have occurred simultaneously with, and been occluded by, SE.

Both AE and SE could also be recorded in axons and axon tracts (see Videos 1 and 2, Data Supplements).
Neuron subpopulations differ in their expression of synchronous activity during development

As shown above, RS and VO neurons clearly differed in the stage at which they started to express SE and by the frequency of AE. To investigate whether these differences were related to location along the AP axis or to specific neuronal subpopulations, we compared the percentage of co-active cells and the burst amplitudes in RS neurons located in different rhombomeres and in VO neurons of the iR-VO, iC-VO and cC-VO subpopulations. We first asked, in those preparations that exhibited spontaneous nV bursts, what proportion of neurons in the RS and VO populations expressed SE at different stages (Figure 4A). To quantitate this, we measured the degree of participation in SE of neurons within a given neuron population by calculating a likelihood index (LI) as defined in Figure 2. In this respect the RS and VO neurons differed markedly. Essentially all RS neurons that we recorded from expressed SE in register with each nV burst as soon as SE appeared in RS neurons early on d4 (Figure 4A left), whereas the VO neuron population as a whole did not engage in SE fully until d7 (Figure 4A right). In other words, from the appearance of SE in VO neurons early on d5 and up to d7 there were always some VO neurons that either did not participate in SE at all or did not follow each nV burst. Figure 3 (cC-VO late d5, iC-VO d6) shows that in many cases, when synchrony with the nV activity started, individual neurons were either fully co-activated or not co-activated at all.

We then asked whether the different subpopulations of RS and VO neurons participated unequally in spontaneous activity (Figure 4B). Again, all RS neurons that we recorded from, irrespective of their position along the longitudinal axis of the hindbrain, expressed SE in register with each nV burst. The VO neuron subpopulations, however, participated quite unequally in SE. The cC-VO neurons, like the RS neurons, participated with full fidelity as soon as they expressed SE (early d5). Some iR-VO neurons, by contrast, did not participate fully until d6. Most strikingly, only a small minority of iC-VO neurons
participated in SE through late d5, even though these were located at approximately the same AP level as the cC-VO neurons. On d6 the iC-VO neurons showed a sharp increase in their degree of participation in SE, reaching full fidelity on d7. There was therefore nearly a 2-day lag in the expression of SE by iC-VO neurons relative to the other VO neuron subpopulations. In contrast to late stages of episodic pattern maturation (Fortin et al. 1999, Coutinho et al. 2004), none of these differences in the early pattern of spontaneous activity could be ascribed to a specific rhombomeric origin of the neuron populations.

**Neuronal subpopulations differ in the amplitude of synchronous activity during development**

Within the RS and each of the VO neuron subpopulations, SE amplitude appeared similar among individual neurons in any given preparation. SE amplitude was least variable among RS neurons, and substantially more variable among VO neurons in any given preparation (average coefficients of variation: RS, 7.0; cC-VO, 14.1; iR-VO, 16.2; iC-VO, 19.3). This between-neuron variability was greater than the variability of SE amplitudes exhibited by any given neuron (see Figure 3 for examples). On comparison across preparations, variability was greater in all neuron populations (Figure 5), possibly related to disparity in developmental maturity within the embryo staging categories used.

We noted also that RS neurons generally had higher SE peak amplitudes than VO neurons, especially at early stages (Figure 5A). We therefore assessed SE peak amplitude more carefully in the different RS and VO subpopulations (Figure 5B). In both the RS and VO neurons, mean SE peak amplitude increased with development. No substantial differences in SE peak amplitude were seen among RS subpopulations in different rhombomeric domains. There were clear differences in SE peak amplitude among VO subpopulations, however. The cC-VO neuron group always had a substantially larger mean SE peak amplitude than the other VO groups, which increased developmentally to approach
the amplitudes seen in RS neurons by d6-7. The iR-VO neurons had SE peak amplitudes about a third the size of the cC-VO neurons, with a similar proportional increase over developmental time. The iC-VO group stood out by hardly exhibiting SE at all until d6, and had substantially smaller SE peak amplitudes until d7, when these reached about the same size as in the iR-VO neurons (Figure 5B).

We considered that one possible explanation for the substantial difference in SE amplitudes between RS and VO neurons (and potentially between VO neuron subpopulations) might be the depth within the tissue of the neuron populations. Fluorescent emission from a deeper neuron population might be impeded more than that from a superficial neuron population. We therefore labeled the RS and VO neuron populations differentially with RDA and ADA and examined their locations in the transverse plane (Figure 6). This showed that, although some ipsilateral RS neurons lie more superficially (relative to the ventral surface from which they were recorded optically) than most VO neurons (relative to the dorsal surface from which they were recorded), there is substantial overlap in the relative depths of RS and VO neurons as well as of iC-VO and cC-VO subpopulations. The difference in neuron population depth seems therefore unlikely in itself to explain the difference in SE peak amplitude. Moreover, as noted above, once SE was fully established in these neuron populations, SE peak amplitudes were very similar among individual neurons, despite the fact that these were recorded at quite different depths.

**TTX affects synchronous and asynchronous spontaneous activity differently**

Since the participation in spontaneous activity of projection neurons with long ascending or descending axons suggests a mechanism for rapidly conducting this activity to other regions of the CNS by action potential propagation, we tested the effects of TTX on spontaneous activity. TTX at 2µM completely eliminated SE (and nV bursts) but spared AE
(Figure 7), although AE frequency did decrease (from $0.43 \pm 0.22/\text{min}$ to $0.12 \pm 0.12/\text{min}$, averages obtained from 64 RS neurons in r2 or r3 in 3 preparations at stages 26-27). This indicates that TTX-sensitive sodium currents provide a critical contribution to the synchronization of activity in the chicken embryo hindbrain, as in the mouse embryo hindbrain (Gust et al. 2003). Conduction of action potentials is therefore likely to be involved in local synchronization within the hindbrain, and, by extension, also to the spread of activity to more distant regions.

**Discussion**

This is the first study to assess the relative participation of different populations of projection neurons in the spontaneous activity exhibited by the embryonic hindbrain. Our results show that synchronous spontaneous activity does not develop homogeneously among hindbrain projection neurons. This suggests that spontaneous activity may influence projection neuron populations differentially, and that different projection neuron populations may transmit spontaneous activity differentially to different target regions in the developing CNS.

*Independence of asynchronous and synchronous events*

We have shown that reticulospinal and vestibulo-ocular projection neurons in the hindbrain of the avian embryo each express two types of spontaneous activity, consisting respectively of higher frequency, lower amplitude asynchronous events (AE) and lower frequency, higher amplitude synchronous events (SE). Both types are expressed in the form of calcium transients that can be recorded optically. AE develop first and are variable in amplitude and frequency, both within and between neurons. SE develop at least a half-day (in
VO neurons) or a full day (in RS neurons) later. It is not clear how much earlier AE are expressed relative to SE, because we did not attempt to record earlier than stage 20 (about 3 days of development). AE are already evident, at least in RS neurons, at that stage (our unpublished observations).

AE and SE appear to be independent phenomena produced by different mechanisms. AE are resistant to concentrations of TTX that completely eliminate SE. Thus, neither TTX-sensitive synaptic inputs nor SE (once they appear) are necessary for the elicitation of AE. AE continue to occur after the appearance of SE, but the two bear no obvious temporal relationship to each other once both are present. There is no indication that SE evolve from AE, since SE arise abruptly from an ongoing background of AE in any given neuron. We have never observed any form of temporal amalgamation of AE into SE. AE can occur at variable times within the intervals between SE, including a few seconds seconds prior to or after SE, suggesting that neither triggers the other nor exerts any lengthy refractory effects on the other. However, because there is as yet no experimental situation in which SE have been recorded in the absence of AE, it is difficult to formally exclude that AE may contribute, either developmentally or at a given developmental stage, to the generation of SE.

**Heterogeneity in the developmental expression of SE**

SE are synchronized with spontaneous bursts of action potentials recorded extracellularly from nV. Since most if not all cranial motor nerves generate such bursts simultaneously in the chicken embryo hindbrain at these stages (Fortin et al., 1995), the SE that we have recorded optically in projection neurons clearly represent the coactivation of a widespread network that includes at least motoneurons and RS and VO projection neurons. Nevertheless, quantitative analysis shows that SE are not expressed equivalently among projection neurons. RS neurons express SE abruptly and robustly from the same stages as
motoneurons, whereas VO neurons are recruited into the synchronized network later and more gradually, in a subpopulation-specific manner. VO neuron subpopulations that can be distinguished according to axon trajectory and target innervation exhibit markedly different timetables of SE expression and fidelity of SE participation (likelihood of following nV bursts). Thus, whereas essentially all RS neurons exhibit large amplitude SE in register with each and every nV burst as soon as nV bursts appear, only one of the VO subpopulations (cC-VO) expresses SE as abruptly (albeit later than the RS neurons) and one VO subpopulation (iC-VO) expresses very few SE until substantially later in development. Moreover, SE peak amplitudes are generally smaller in VO neurons than in RS neurons, and a marked variation in peak amplitude is evident among the different VO subpopulations.

One interesting question that remains to be resolved is whether heterogeneity in the development of SE is due to heterogeneity in the development of cellular properties or heterogeneity in the development of network connections. Over developmental time, RS and VO neurons both show a gradual increase in SE peak amplitude, and VO neurons show an increase in SE participation, suggesting a process of increasing differentiation. Heterogeneity might then simply reflect different schedules of general cellular differentiation for different neuron populations, including the appearance and maturation of calcium signaling and metabolism. The RS neurons are among the first neurons in the neuraxis to be born and extend axons, preceding vestibular neurons (McConnell and Sechrist 1980; Glover and Petursdottir 1991). They might already have differentiated cellular properties that permit a full-blown expression of SE as soon as these are triggered, whereas VO neurons (and perhaps other neuron types) might not do so until later and at varying times and degrees specific to each subpopulation.

On the other hand, heterogeneity in the expression of SE might reflect differences in the degree of recruitment into the spontaneously active network of equally differentiated
neuron populations. Early synchronization and stable, robust bursts might indicate that a neuron population is part of or just downstream of the rhythm-generating source of the spontaneous activity, whereas late synchronization and variable burst amplitudes and proportions of synchronized neurons might indicate a more distant relationship to the rhythm generator. In this regard, the RS neuron population might express SE so early because it contains or is tightly linked to the SE rhythm generator. The RS neurons include neurons in the posterior raphe nuclei (Glover and Petursdottir 1991) which are among the serotonergic neurons that appear to be the principal source of synchronous spontaneous activity in the mouse embryo (Hunt et al. 2006a; Hunt et al. 2005). The RS population as a whole is primarily a medial neuron population, and the cC-VO neuron group contains the most medial of the VO neurons. For this reason non-raphe RS neurons and cC-VO neurons might also be readily recruited by synchronous activity originating from the raphe neurons. This might explain why virtually all RS and cC-VO neurons express SE robustly as soon as SE appear. If the same situation holds in the two species, further studies should reveal that serotonergic neurons give rise to the spontaneous activity in the chicken embryo, that medial neurons in general are tightly linked to the serotonergic neurons in both species, and that more lateral populations of projection neurons, such as many of the VO neurons, are less tightly linked or delayed in attaining an equivalent level of connectivity to the spontaneously active network in both species.

Irrespective of whether the differences observed here are due to differential maturation of cellular properties or of network participation, the fact that cC-VO neurons eventually attain SE peak amplitudes similar to those in RS neurons is consistent with different development schedules in different projection neuron populations. In this regard it would be interesting to see whether all VO neurons eventually express SE as robustly as RS neurons (this is difficult to assess beyond d7 because the increasing thickness of the
hindbrain hampers optical recording). It also raises the interesting question of why different VO neuron subpopulations would have such different developmental schedules.

*potential propagation of SE from the hindbrain to other regions?*

The fact that the RS and VO projection neurons already possess long axons at the time they start participating in synchronous spontaneous activity provides a substrate for spreading this activity rapidly to other regions of the nervous system. The first RS axons to reach the cervical spinal cord do so early on d3, and large numbers of RS axons have reached the spinal cord by d4 (Glover and Petursdottir 1991), when synchronous bursts of similar frequency to the SE can first be recorded from lumbar spinal motoneurons *in vitro* (Milner and Landmesser 1999). Bursting in lumbar motoneurons can also be triggered by stimulating the rostral spinal cord (Milner and Landmesser 1999), which would be expected to activate descending RS axons and propriospinal axons. VO axons reach the oculomotor nucleus in the midbrain in large numbers by d5 (Glover 2003). SE clearly arose both in the somata and axons of RS and VO neurons (see Video 2, Data Supplements).

TTX eliminated nV bursts and SE, indicating that TTX-sensitive sodium channels play a critical role in either the generation or the local propagation of SE within the hindbrain, or both. It therefore seems likely that sodium channels also mediate impulse propagation in the projection neuron axons by these stages. An important line of future inquiry will be to assess when and where spontaneous activity originates along the neuraxis and whether impulse propagation in projection neurons is instrumental in activating disparate local origins in stereotyped patterns. In the rat embryo, for example, although spontaneous activity can be initiated either in the spinal cord or the hindbrain, the spinal cord seems to dominate in the intact CNS *in vitro*, particularly at earlier stages and in the special case of oscillatory bursts, which appear to originate primarily in the lumbar spinal cord (Momose-
Sato et al 2007). Spontaneous bursts that originate in the rat embryo spinal cord can propagate to the hindbrain (and beyond), and vice versa (Ren and Greer 2003; Momose-Sato et al 2007), and separation of the hindbrain from the spinal cord leads to more frequent bursts in the hindbrain (Momose-Sato et al 2007). Interactions clearly occur over much of the neuraxis, and the neuronal substrates for these deserve to be characterized.

*Why is synchronous activity expressed heterogeneously among projection neurons?*

An intriguing possibility for variable expression of SE by projection neuron subpopulations is that this might be related to the development of synaptic connections that are specific for mature functional roles. The iC-VO, iR-VO and cC-VO subpopulations have different patterns of connectivity to motoneuron targets, and subserve different roles in the context of vestibulo-ocular reflexes (Glover 2003). The functional connectivity of the VOR has been established with specific connections onto oculomotor motoneuron pools by d7, in the face of synchronous activity in both the VO neurons and the oculomotor motoneurons (Glover 2003; Glover et al. 2008). The differences in SE participation and amplitude exhibited by the VO neuron subpopulations up to d7 might therefore exert differential influences on these neurons as they are growing to and innervating their targets, perhaps contributing to differences in target selection or innervation. We note that the differences we see do not appear to correlate simply with the eventual functional sign of connections, as both the iR-VO and iC-VO groups are believed to be inhibitory and the cC-VO group excitatory (Glover 2003). This does not explain why the iC-VO group in particular lags behind the others. On this backdrop, it will be of great interest in future studies to characterize the degree of participation in SE of other neuron populations to see if this is correlated with mature patterns of connectivity or other function-specific features.
Relation between spontaneous activity and embryonic motility

Much speculation has been made, and some evidence obtained, regarding the purpose(s) of synchronous activity in the developing brain and spinal cord. Potential roles include the maintenance and refinement of synaptic connections (Torborg and Feller 2005), the regulation of the expression of axon guidance molecules (Hanson and Landmesser 2004), and a mechanism for ensuring homeostatic regulation of excitatory and inhibitory balance in developing circuits (Borodinsky et al. 2004; Borodinsky and Spitzer 2007; Spitzer et al. 2004). It is also believed that the connections that underlie spontaneous activity provide a repertoire from which more mature behavioral patterns can be constructed or selected (reviewed in Bekoff 2001). In the context of brainstem function, although many cranial motoneurons are involved in cyclically rhythmic movements (breathing, chewing, swallowing, vocalization, etc), it is unlikely that the full complement of projection neurons studied here exhibit cyclically rhythmic activity in maturity. Regarding breathing, it has been shown recently in the mouse that the rhythm generator responsible for spontaneous activity in the embryo is anatomically, pharmacologically and developmentally distinct from the respiratory rhythm generator (Thoby-Brisson et al., 2005). Some RS neurons are involved in locomotory control and exhibit cyclically rhythmic activity in that connection, but the majority of RS neurons have entirely different functions. Moreover, the normal function of the VO neurons is in episodic reflex movements triggered by ongoing, typically non-cyclical sensory input. Thus, in the vast majority of these projection neurons the SE observed here probably do not represent a primordial version of any mature rhythmic activity pattern. A more general purpose, such as in synaptic development and/or homeostatic regulation, seems to be more likely.

An obvious potential role for early spontaneous activity in RS neurons is in the control of embryonic motility. The chicken embryo starts periodic spontaneous movements
on d4. The first movements are single and multiple neck bends and then a stereotypic S-wave starting from the neck and extending into the trunk. These movements have durations and intervals quite similar to those of the SE in the hindbrain (Hamburger and Balaban 1963, though these authors provided no description of eye or face movements which would suggest a general cranial nerve discharge). The mouse embryo starts the same kinds of movements once every few minutes on E12.5 (Suzue 1996), and this corresponds well to synchronous activity recorded in mouse brainstem and spinal cord preparations in vitro (Abadie et al. 2000; Gust et al. 2003, Yvert et al 2004). Thus, it is quite likely that the synchronous activity we have observed in vitro is a physiological phenomenon that causes the earliest embryonic movements. We have attempted to record the SE in the hindbrain in ovo using fiber optic technology, but have not yet succeeded. This, as well as electromyogram or cranial motor nerve recordings in ovo, should be an important goal for the future, as should be recordings of the same type in the mouse embryo in utero.

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References

Abadie V, Champagnat J, Fortin G. Branchiomotor activities in mouse embryo. 


Table 1. Proportions of preparations that exhibited nV bursts and average burst intervals. The number of bursts recorded in each preparation ranged from 1 to 35. Average intervals shown here are grand averages of the average intervals recorded in all preparations that exhibited at least 6 bursts. Note that between-preparation variability in burst intervals was typically (but not always) greater than within-prep variability (compare with Figure 3).

<table>
<thead>
<tr>
<th>HH stages</th>
<th>proportion (n)</th>
<th>average interval ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>d3</td>
<td>0% (3)</td>
<td>87 ± 42 sec</td>
</tr>
<tr>
<td>early d4</td>
<td>14% (7)</td>
<td>143 ± 69 sec</td>
</tr>
<tr>
<td>late d4 / early d5</td>
<td>50% (18)</td>
<td>234 ± 60 sec</td>
</tr>
<tr>
<td>late d5</td>
<td>82% (11)</td>
<td>238 ± 68 sec</td>
</tr>
<tr>
<td>d6</td>
<td>100% (12)</td>
<td>426 ± 107 sec</td>
</tr>
<tr>
<td>d7</td>
<td>100% (4)</td>
<td></td>
</tr>
</tbody>
</table>
Figure Legends

Figure 1. Experimental plan. The schematic drawing on the left shows locations and projecting pathways of RS (in black) and VO (iR, iC and cC; in gray) projection neurons in the hindbrain (scale bar = approximately 200 microns, for a d6 preparation). The wedges in the corresponding colors indicate application sites of CGDA in the MLF. Rhombomere-derived domains are indicated by dashed lines and numbered, specific cranial nerves and/or ganglia are denoted with roman numerals and a glass suction electrode for extracellular nerve recording is shown at the site of the mandibular (motor) branch of the right trigeminal nerve. Note that contralateral RS neurons, which were labeled but not recorded from optically, are omitted in the drawing. To the right of the drawing is shown the general principle of the recording procedure. Briefly, calcium transients in individual projection neurons (photographic inset, scale bar = 35 microns) were filmed with a CCD camera and presented as waveforms, one for each neuron (“calcium traces”), and action potential bursts from one of the trigeminal nerves were recorded simultaneously (“nerve recording”). See text for details and abbreviations.

Figure 2. Calculation of the likelihood index (LI). Here we consider a schematic example of an optical recording consisting of calcium traces from 5 neurons (neurons 1-5) and the extracellular nerve recording from the trigeminal nerve (nV). In this example asynchronous calcium transients (asynchronous events, or AE) are omitted. During the recording period nV exhibited 3 bursts. The first step is to calculate the proportion of calcium transients that are synchronized with the nV bursts (synchronous events, or SE) in individual neurons. Neuron 1 followed none of the 3 nV bursts and its proportion is 0 / 3 x 100 = 0%, neuron 2 followed one nV burst and its proportion is 1 / 3 x 100 = 33%, neurons 3 and 4 each followed two nV bursts and their proportions are 2 / 3 x 100 = 67%, whereas neuron 5 followed all nV bursts
and its proportion is $3/3 \times 100 = 100\%$. The average of these 5 proportions, 53\%, represents the LI for this population of recorded neurons (LI). Since each recording was taken from a projection neuron subpopulation, each LI represents neurons from a subset of projection neurons in a given preparation. In actual calculations, each LI was based on calcium traces from 16 neurons randomly selected from the population of recorded neurons.

Figure 3. Representative examples of AE and SE in selected projection neuron subpopulations. Sample waveforms arranged in columns, with each column derived from a separate preparation representative of the indicated stage. Recordings from one RS (r2) and two VO (iC-VO and cC-VO) projection neuron subpopulations are shown for each of the three stages. Calcium traces from nine neurons and a trigeminal nerve recording (nV) are shown for each recorded subpopulation. AE are evident in all subpopulations at all stages (most clearly in the r2-RS neurons), whereas SE are evident in the late d5 recordings of r2-RS and cC-VO neurons, and in the d6 recordings of iC-VO neurons. The boxed area of the late d5 recording of r2-RS neurons is magnified at lower left to facilitate comparison of AE (dotted), the nV burst recorded electrically (arrow) and the SE (large traces synchronized with the nV burst).

Figure 4. Developmental changes in degree of participation in SE by RS and VO projection neurons. (A) Average likelihood indices of RS (left) and VO (right) neurons and (B) average likelihood indices of subpopulations of RS (left) and VO (right) projection neurons (color coded as indicated) as a function of developmental time. Note that the data in these graphs are derived only from preparations that exhibited nV bursts. Error bars: standard deviations. The number of preparations is indicated at the base of each column in A. Stages/neuron
populations that were not recorded are indicated by “no recording made” or “n.r.m.” All other instances in which data do not appear indicate lack of SE in recorded neurons.

Figure 5. Developmental changes in SE peak amplitudes in RS and VO projection neurons. (A) Average SE peak amplitudes in RS (left) and VO (right) projection neurons and (B) average SE peak amplitudes of subpopulations of RS (left) and VO (right) projection neurons (color coded as indicated) as a function of developmental time. Note that the data in these graphs are, as in figure 4, based only on preparations that exhibited nV bursts. Error bars: standard deviations. The number of preparations is indicated at the base of each column in A. Stages/neuron populations that were not recorded are indicated by “no recording made” or “n.r.m.” All other instances in which data do not appear indicate lack of SE in recorded neurons.

Figure 6. Relative locations of RS and VO projection neurons in the transverse plane. The projection neurons were labeled retrogradely with RDA (RS) or ADA (VO) in a late d5 preparation. Fluorescent microphotographs at two rostrocaudal levels (indicated by transverse lines in the drawings) are shown adjacent to drawings of the preparation showing the locations of the relevant projection neuron populations (left, RS; right, VO; see Figure 1 for approximate scale of drawings). Dorsal is up in all photographs. White arrows indicate the direction of view of the CCD camera when activity of these neurons was recorded optically. Note that contralateral RS neurons, which were not recorded from in this study, are also seen in the upper left photograph. In addition, the MLF, containing the labeled axons of these neurons, is also visible near the midline. See text for abbreviations. Scale bar = 100 microns.
Figure 7. Differential effect of TTX on AE and SE. Optical recordings of r2-RS neurons from a late d5 preparation are shown. SE were eliminated completely by adding 2 µM of TTX to the perfusate. AE were spared, although their frequency decreased. The time elapsed between recordings was 30 minutes.
Data Supplements

Video 1. A one-minute sequence recorded at the University of Oslo showing AE in the somata and axons of RS neurons. CGDA fluorescence intensity is color-coded using a rainbow LUT, with progression from blue to red to white indicating increase in intensity. Black-and-white versions of this type of imaging served as the raw data for measuring fractional changes in calcium-induced fluorescent light intensity (changes relative to background light intensity in each neuron). Rostral is oriented towards the top and the midline is to the right.

Video 2. A four-minute sequence recorded at C.N.R.S. showing AE in somata and axons of RS neurons, and a single SE at the end of the sequence that involves all the labeled RS neurons. The magnification and spatial resolution are lower than in Video 1. Camera settings are also such that the background CGDA fluorescence in each neuron (used for determining fractional changes as indicated in Video 1) has been set nearly to black. This provides a cleaner image of individual neuronal somata and axons during each calcium transient. Rostral is oriented towards the right and the midline is at the bottom.
nerve recording

amplifier

CCD camera

calcium traces
4A

RS

average likelihood index

0% 10% 20% 30% 40% 50% 60% 70% 80% 90% 100%
early d4 late d4 / early d5 late d5 d6 d7

1 7 3 6 no recording made

4B

RS

average likelihood index

0% 10% 20% 30% 40% 50% 60% 70% 80% 90% 100%
early d4 late d4 / early d5 late d5 d6 d7

n. r. m.

0% 10% 20% 30% 40% 50% 60% 70% 80% 90% 100%

RS

early d4 late d4 / early d5 late d5 d6 d7

1736 2644 no recording made

4A

VO

average likelihood index

0% 10% 20% 30% 40% 50% 60% 70% 80% 90% 100%
early d4 late d4 / early d5 late d5 d6 d7

2 6 4 4 no recording made

4B

VO

average likelihood index

0% 10% 20% 30% 40% 50% 60% 70% 80% 90% 100%
early d4 late d4 / early d5 late d5 d6 d7

R I C C

r1/r2 r3/r4 r5/r6

no recording made
Reticulospinal Vestibulo-ocular
Control

TTX

40%

2 min