Mechanisms of Spontaneous Activity in Developing Spinal Networks

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ABSTRACT: Developing networks of the chick spinal cord become spontaneously active early in development and remain so until hatching. Experiments using an isolated preparation of the spinal cord have begun to reveal the mechanisms responsible for this activity. Whole-cell and optical recordings have shown that spinal neurons receive a rhythmic, depolarizing synaptic drive and experience rhythmic elevations of intracellular calcium during spontaneous episodes. Activity is expressed throughout the neuraxis and can be produced by different parts of the cord and by the isolated brain stem, suggesting that it does not depend upon the details of network architecture. Two factors appear to be particularly important for the production of endogenous activity. The first is the predominantly excitatory nature of developing synaptic connections, and the second is the presence of prolonged activity-dependent depression of network excitability. The interaction between high excitability and depression results in an equilibrium in which episodes are expressed periodically by the network. The mechanism of the rhythmic bursting within an episode is not understood, but it may be due to a ''fast'' form of network depression. Spontaneous embryonic activity has been shown to play a role in neuron and muscle development, but is probably not involved in the initial formation of connections between spinal neurons. It may be important in refining the initial connections, but this possibility remains to be explored. © 1998 John Wiley & Sons, Inc.* J Neurobiol 37: 131–145, 1998

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Spontaneous neural activity is an important and incompletely understood feature of developing neurons and networks. The type of activity produced by the developing nervous system depends on the level of differentiation of individual neurons and their networks. Before synaptic networks have formed, individual neurons or clusters of electrically coupled neurons express spontaneous calcium transients (Yuste et al., 1995; Gu and Spitzer, 1997, see also this volume). Later in development, a different type of activity emerges which depends upon the global structure of the emerging networks as well as the properties of their individual neurons.

In this review, we will focus upon network-driven activity in the developing spinal cord of the chick embryo between stages 25 and 40 [corresponding to embryonic day 5–15 (E5–15)]. Most of the experimental work has been performed using an isolated preparation of the spinal cord that generates spontaneous activity for many hours when superfused with an appropriate salt solution. Activity produced by this *in vitro* preparation is composed of recurring episodes of rhythmic bursting similar to activity recorded electromyographically from the embryo *in ovo.* However, it lacks the richness and variety of the *in ovo* behavior (Fig. 1). Despite these differences, the enormous simplification and experimental accessibility that accompany isolation

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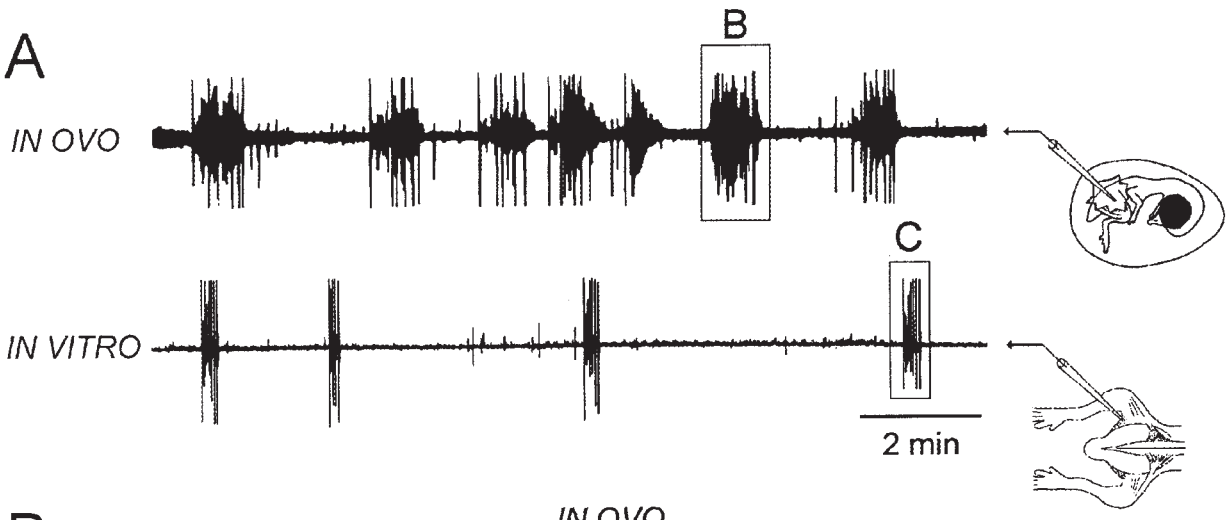
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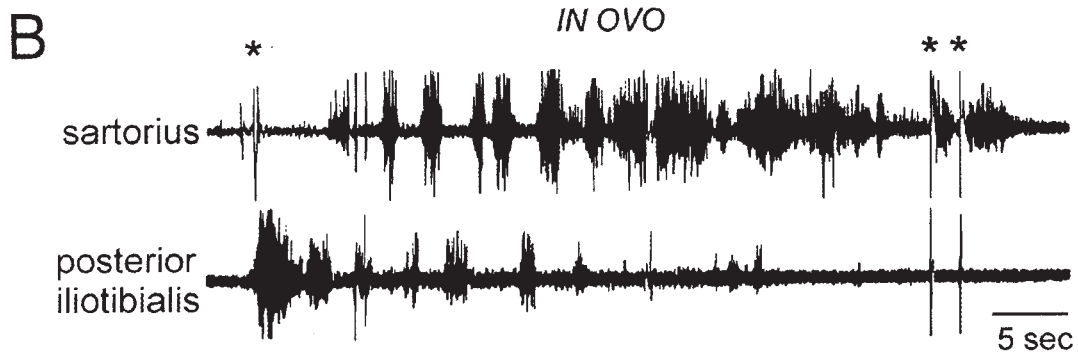
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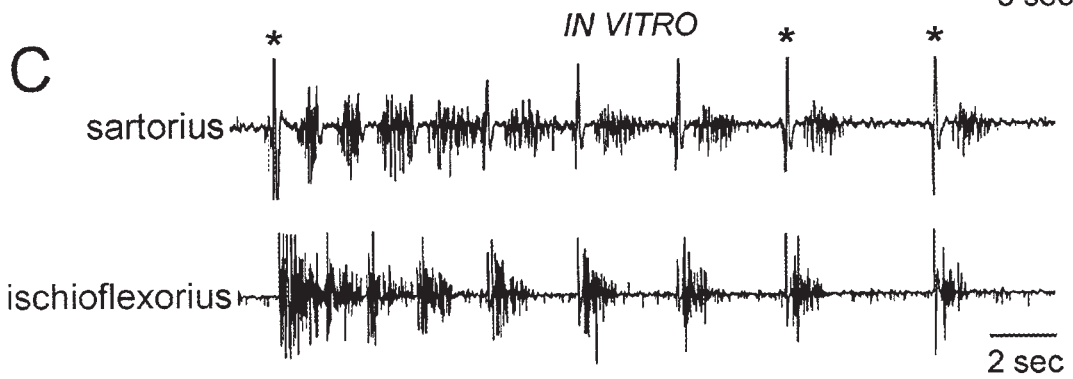
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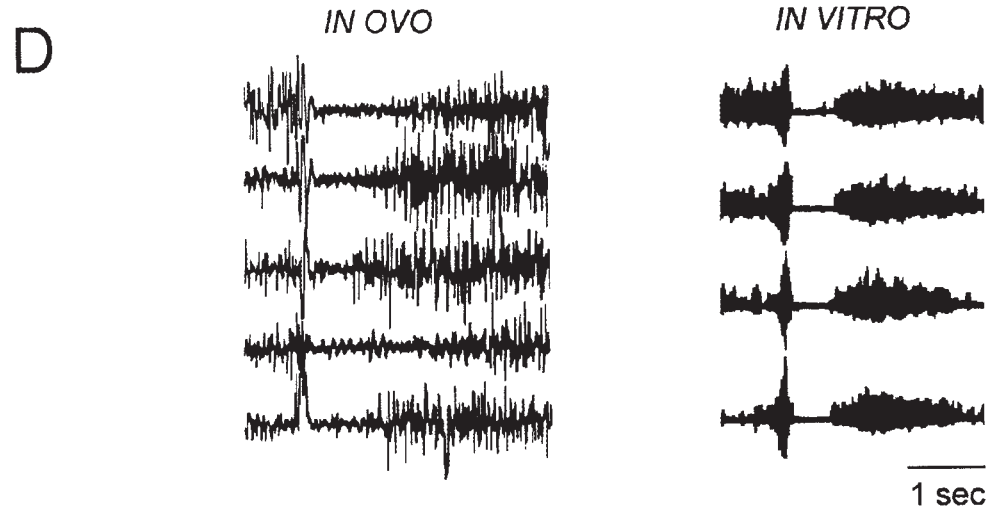
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of the spinal cord justify its use for studying the underlying mechanisms of spontaneous activity.

SPONTANEOUS ACTIVITY GENERATED BY THE ISOLATED SPINAL CORD AND ITS COMPARISON TO EMBRYONIC MOTILITY *IN OVO*

When the spinal cord is removed from the embryo and maintained *in vitro*, it is initially quiescent for one to several hours, depending on the age of the embryo. Once activity begins, it continues for as long as the cord can be maintained viable (typically 1–2 days). At E10, for example, the activity is composed of recurring episodes of rhythmic firing that can be recorded from motoneurons and interneurons. Figure 1 illustrates the activity recorded from hindlimb muscles of an isolated spinal cord and hindlimb preparation, and compares it to similar recordings obtained from the embryo *in ovo* at the same stage of development. The recordings were made from two muscles whose functions are antagonistic in the adult: the sartorius muscle which is a hip flexor, and either the ischioflexorius or the posterior iliotibialis which are knee extensors. Both of the extensor muscles have a similar function and pattern of discharge.

Initially, we will describe the similarities between the *in vitro* and *in ovo* motor output and then consider the differences. In each preparation, activity is periodic, and within episodes, motoneurons and interneurons are rhythmically active. Flexor and extensor motoneurons discharge alternately during the episode [Fig. 1(A–C)]. At the beginning of an episode and also some of the cycles, there is often a brief high-amplitude discharge that is synchronized in flexors and extensors [asterisks Fig. 1(B,C)]. This discharge is followed by a pause in firing that is longest in the sartorius and other flexor motoneurons [Fig. 1(D)] (Landmesser and O'Donovan, 1984). This behavior—a synchronized discharge followed by a pause in firing—is important because we believe it is a ''fingerprint'' of sartorius motoneuron behavior in response to a depolarizing synaptic input (see below and Fig. 4). As such, its presence *in ovo* suggests that the mechanisms generating this behavior are similar *in ovo* and *in vitro.*

There are, however, several differences in the activity generated by the two preparations. First, activity produced by the isolated spinal cord is more regular and occurs less frequently than that *in ovo.* Moreover, in the isolated cord, individual bursts are similar from cycle to cycle, whereas recordings within the egg reveal highly variable bursts during each episode [Fig. 1(B)]. The reasons for these differences are not understood, but are probably related to the reduced nature of the isolated spinal cord preparation and the lower temperature (27–30°C) of the *in vitro* experiments.

SPONTANEOUS EPISODIC ACTIVITY IS A PROPERTY OF DEVELOPING SPINAL NETWORKS AND DOES NOT DEPEND ON THEIR DETAILED SYNAPTIC ARCHITECTURE

Spontaneous activity produced by the isolated spinal cord can be first detected from motor nerves or ventral roots at about E4–5 and exhibits a characteristic sequence of changes with further development. At the earliest stages of network formation, the spontaneous activity is composed of a single, recurring, depolarizing event that can be recorded from the ventral roots (O'Donovan and Landmesser, 1987). As the cord matures, its output becomes progressively more complex, consisting of recurrent multicycle episodes.

Figure 1 Comparison of spontaneous activity generated by hindlimb muscles recorded in the egg (*in ovo*) or in an isolated spinal cord/hindlimb preparation (*in vitro*). The top traces (A) show electromyographic recordings from the sartorius muscle *in ovo* and in an isolated preparation of the spinal cord with the hindlimbs attached (*in vitro*). Several episodes are shown, and the boxes surround two that are displayed in more detail in (B) (*in ovo*) and (C) (*in vitro*). These detailed episodes show electromyographic recordings from the sartorius and one of its functional antagonists (either the posterior iliotibialis or the ischioflexorius) and illustrate the multicycle activity within a single episode. The asterisks identify the synchronized discharge in several cycles. (D) Comparison of the synchronized discharge and the subsequent pause (the ''fingerprint'' referred to in the text) in several cycles of sartorius activity recorded either from the muscle *in ovo* or from the muscle nerve *in vitro.* Data are from stage 36 embryos. [(A–D) were modified from Landmesser and O'Donovan (1984).]

Spontaneous activity is not generated exclusively by motor networks, because it can also be expressed by isolated pieces of the dorsal horn whose networks are concerned with sensory rather than motor functions. However, the developmental changes in spontaneous output occur later in the dorsal horn than in isolated sections of ventral cord, consistent with the delayed development of dorsal networks. For example, at a time when isolated sections of ventral cord produce multicycle episodes, the isolated dorsal cord is only capable of generating single cycles (Wenner and O'Donovan, unpublished observations). Similarly, when dorsal networks have developed multicycle activity, the isolated ventral networks are quiescent (Chub and Baev, 1991). Spontaneous rhythmic activity, which closely resembles that in the spinal cord, can also be recorded from the isolated brain stem (Fortin et al., 1995), whose circuitry is presumably very different from the spinal cord.

These findings suggest that spontaneous rhythmic activity is a feature of network development that depends upon the state of network maturity rather than its detailed or specific connectivity. This idea has been supported by a series of pharmacological experiments designed to alter network architecture (Chub and O'Donovan, 1998a). These experiments have shown that spontaneous rhythmic activity can be generated by networks composed predominantly of either $GABA_A$ and glycinergic connections or excitatory amino acid and cholinergic connections.

SPONTANEOUS, EPISODIC ACTIVITY DEPENDS ON THE HIGH EXCITABILITY OF DEVELOPING NETWORKS COUPLED WITH THE PRESENCE OF ACTIVITY-DEPENDENT DEPRESSION OF NETWORK EXCITABILITY

If spontaneous activity does not depend upon the detailed connections of neurons within the network, then how is it generated? We believe that two general features of developing networks are crucial for the expression of spontaneous activity. The first is the predominantly excitatory nature of developing synaptic connections, and the second is the presence of activity-dependent depression of neuronal and network excitability. It has been proposed that the conjunction of these two characteristics is responsible for the expression of spontaneous activity in the developing cord (O'Donovan and Chub, 1997, 1998a) or in cultured spinal networks (Senn et al., 1996).

THE EXCITATORY NATURE OF NETWORK CONNECTIONS

During development, the classical inhibitory neurotransmitters GABA and glycine can depolarize spinal neurons (Obata et al., 1978; Wu et al., 1992; Sernagor et al., 1995; Nishimaru et al., 1996). It is assumed that this occurs because the chloride concentration of developing spinal neurons is maintained at a higher concentration than in the adult. This has been established experimentally for the Rohon–Beard cells of amphibian embryos (Rohrbough and Spitzer, 1996). Thus, the absence of conventional hyperpolarizing inhibition results in the high excitability of developing spinal networks.

ACTIVITY-DEPENDENT DEPRESSION OF NETWORK EXCITABILITY

Following an episode of spontaneous activity, network excitability is depressed for several minutes. This depression is manifest in several ways as illustrated in Figure 2. First, the membrane potential of many spinal neurons is hyperpolarized by up to 10 mV after an episode and progressively recovers as a depolarizing ramp in the interval between successive episodes (Chub and O'Donovan, 1995). Second, stimulation of several synaptic pathways, whose neurons are believed to be rhythmically active during an episode, produces smaller synaptic potentials in motoneurons and interneurons after an episode. The amplitude of the evoked potentials progressively recovers in the interval between episodes (Fedirchuk and O'Donovan, 1996). In addition, spontaneously occurring synaptic potentials are depressed following an episode and increase in amplitude before the next episode. The origin of these spontaneous potentials is not clear: They could arise either from discharging neurons or, alternatively, from spontaneous release of transmitter (Chub and O'Donovan, 1998b).

MECHANISM OF EPISODE GENERATION BY SPINAL NETWORKS

To understand how activity is generated by developing networks, we will consider the recovery of excitability that follows the occurrence of an epi-

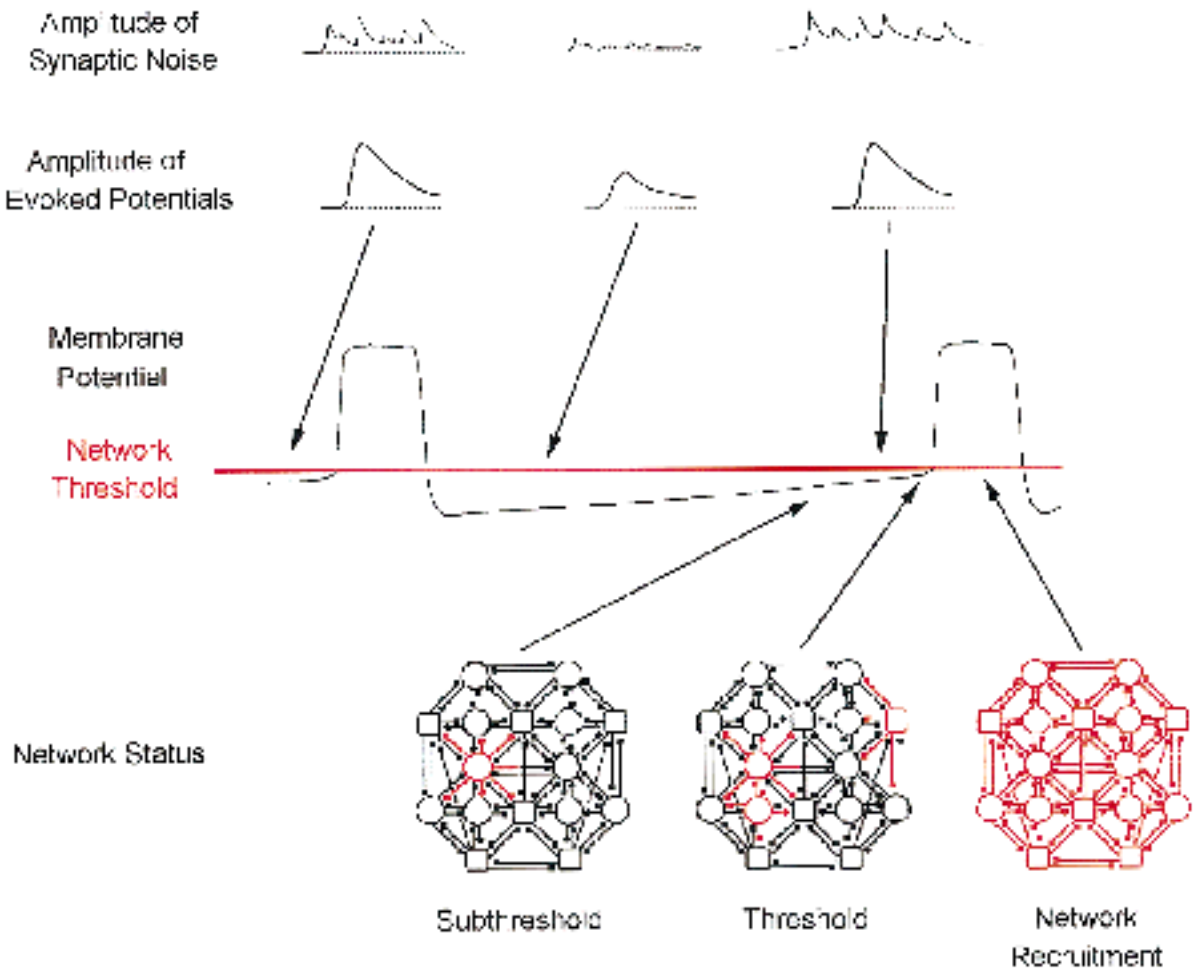

Figure 2 Mechanism hypothesized to be responsible for the occurrence of spontaneous episodes of activity in developing spinal networks. The top trace shows the intracellularly recorded synaptic noise in a spinal neuron before and after an episode. Below is shown the amplitude of evoked synaptic potentials recorded at the same time. The continuous traces show the membrane potential of a spinal neuron during two episodes of spontaneous activity. Below this is the presumed state of the network showing active (red) and inactive (black) neurons.

sode (Chub and O'Donovan, 1997, 1998a) (Fig. 2). As we have discussed, the membrane potential of spinal neurons is hyperpolarized and the amplitude of evoked and spontaneous synaptic potentials falls to a minimum after an episode. During recovery, the membrane potential progressively depolarizes and the amplitude of evoked synaptic potentials increases. These changes can be viewed as a progressive increase in the functional connectivity and excitability of the network. Concomitant with these changes is an increase in the frequency and amplitude of spontaneous synaptic events which will cause some neurons, near threshold, to fire. As network excitability increases further, the discharging neurons may recruit other neurons [Fig. 2(C), subthreshold]. Eventually, a point will be reached when the connectivity is high enough to sustain a rapid propagation of activity throughout the network. Once this occurs, neurons will be explosively recruited and an episode will begin. The network would remain in a state of indefinite excitation if processes were not engaged to depress network excitability. Because of such depression, network excitability eventually falls below the level that can sustain network recruitment and the activity stops. According to these ideas, activity is not initiated in a particular class of cells or in a particular part of the cord. It will be initiated where the connectivity to other parts of the network is high enough to sustain regenerative recruitment of the whole network.

The mechanism we have just described would lead to episodes comprising a continuous discharge throughout the episode. We occasionally observe such episodes, but generally rhythmic bursting occurs within the episode. At present, we do not understand the generation of this rhythmicity, but propose that it could be due to the existence of a short-term form of activity-dependent network depression that limits activity within each cycle—a possibility discussed in more detail below.

MECHANISMS RESPONSIBLE FOR ACTIVITY-DEPENDENT DEPRESSION OF NETWORK EXCITABILITY

The mechanisms responsible for the activity-dependent depression of network excitability are only partially understood. The interepisode depolarizing ramp appears to be regulated by some aspect of GABA and possibly glutamatergic function. $GABA_A$ receptors are implicated because local application of bicuculline to a single neuron can hyperpolarize its membrane potential by several millivolts when the drug is applied just before an episode. When the drug is applied just after an episode, it does not affect membrane potential. $GABA_A$ receptors are further implicated because application of low doses of bicuculline (5 μM) can greatly depress the frequency of occurrence of spontaneous activity (Chub and O'Donovan, 1995).

These results suggest two possibilities that are not necessarily exclusive. Either GABA is progressively released into the extracellular space in the interval between episodes or, alternatively, some aspect of GABA conductance or receptor function is changing in the presence of a constant concentration of extracellular GABA. The importance of GABA in the regulation of early spontaneous activity is striking because a population of GABA-immunoreactive interneurons appears in the ventral part of the spinal cord, only to disappear later in the development of both chick [Fig. 3(A)] (Antal et al., 1994) and rat spinal cord (Ma et al., 1992). The fate of these neurons is not clear, although they may experience a transmitter switch from GABA to glycine. As the number of GABA-immunoreactive neurons declines in the ventral part of cord, the number of glycinergic neurons progressively increases with some neurons exhibiting immunoreactivity for both transmitters (Berki et al., 1995) [Fig. 3(B)].

The postepisode depression of evoked synaptic potentials could be accounted for by several mechanisms. The simplest is a prolonged form of synaptic

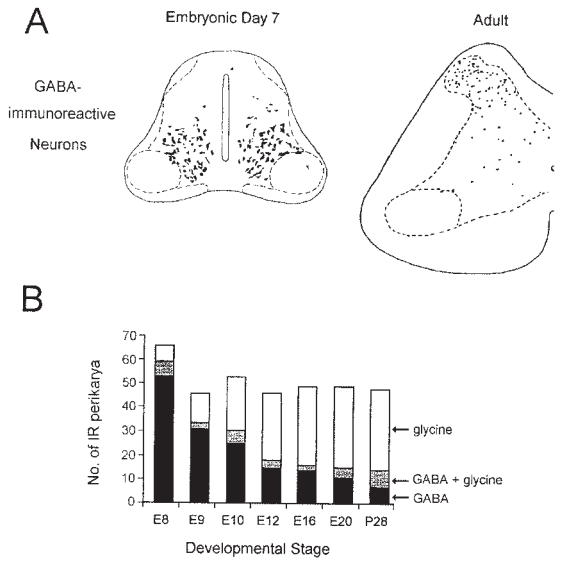

Figure 3 Early in development, GABA-immunoreactive neurons are restricted to the ventral part of the spinal cord, but in the adult they are found predominantly in the dorsal horn. (A) Comparison of transverse sections of the lumbosacral cord embryonic day 7 and the adult (postnatal day 28) showing the distribution of GABA immunoreactive neurons at the upper segments of the lumbosacral cord. (B) Histogram showing developmental changes in the number of neurons immunoreactive for GABA, glycine, and both transmitters at segments LS1–3 of the lumbosacral cord. [Modified from Antal et al. (1994) and Berki et al. (1995).]

depression—a ''slow depression'' that could originate at the level of either the postsynaptic receptor or presynaptic terminals. It is known, for example, that neuronal firing or elevations of intracellular calcium concentration can depress $GABA_A$ currents in some hippocampal and cerebellar neurons (Alger and Pitler, 1995; De Koninck and Mody, 1996). Another possible mechanism was originally proposed to explain the termination of swimming episodes in *Xenopus* tadpoles. Dale and Gilday (1994) argued that ATP released from active neurons is converted into adenosine, which progressively builds up to depress transmitter release presynaptically. It is also possible that depletion of transmitter follows the prolonged discharge within an episode. This mechanism has been implicated in synaptic depression seen at developing muscle afferent–motoneuron synapses in the neonatal rat (Lev-Tov and Pinco, 1992) and in cultured spinal neurons (Streit et al., 1992).

One puzzling observation about postepisode depression is that it does not reach a maximum imme-

diately after firing stops in the episode. Rather, the maximum depression of evoked and spontaneous potentials occurs about 1–1.5 min after the end of the episode. We do not know why this occurs, but it appears that the ''slow'' depression requires some time to develop fully.

WHAT IS THE SOURCE OF THE RHYTHMIC SYNAPTIC DRIVE TO MOTONEURONS?

Muscle nerve recordings reveal that flexor and extensor motoneurons receive a rhythmic depolarizing synaptic drive during each episode of spontaneous activity. The depolarizations recorded from the muscle nerve represent the synaptic drive in a population of motoneurons decremented electrotonically along the nerve (O'Donovan, 1989; Lee and O'Donovan, 1991). The rhythmic depolarizations are synchronized in flexor and extensor motoneurons but are somewhat longer in the flexor motoneurons (O'Donovan, 1989; Sernagor et al., 1995). Whole-cell recording from individual identified motoneurons and local application of transmitter antagonists to motoneuron cell bodies has revealed that motoneurons receive a complex synaptic drive composed of glutamatergic, GABAergic, glycinergic, and cholinergic components (Sernagor and O'Donovan, 1991; Sernagor et al., 1995). The relative proportion of the various classes of input varies between flexor and extensor motoneurons and has not been fully characterized. However, GABAergic inputs appear to be particularly important for the timing of sartorius motoneuron discharge because local injection of $GABA_A$ receptor antagonists into the sartorius motor nucleus can abolish the pause in sartorius discharge (Sernagor et al., 1995). However, GABAergic inputs are not essential for the pause in the firing of sartorius motoneurons. Spontaneous activity can recover in the presence of bath-applied $GABA_A$ antagonists, and under these conditions the glutamatergic drive to motoneurons is increased and the pause in sartorius firing persists (Sernagor et al., 1995). We have interpreted this result to indicate that a powerful enough depolarizing synaptic conductance can prevent action potential generation either by shunting or by voltage clamping the somatic membrane (Sernagor et al., 1995). Intracellular recordings have confirmed that the pause in each cycle of sartorius discharge occurs at the peak depolarization of sartorius motoneurons and is accompanied by a significant increase in membrane conductance (O'Donovan, 1989) (Fig. 4). We believe that this mechanism is responsible for the characteristic ''fingerprint'' of sartorius discharge illustrated in Figure 1(D) which occurs both *in vitro* and *in ovo*.

The mechanism for rhythmic bursting within an episode is not understood. We initially considered the possibility that pacemaker neurons might be responsible for rhythmogenesis, but now this seems unlikely because the overwhelming majority of embryonic spinal neurons do not express voltage-dependent bursting. We can also exclude voltage-dependent *N*-methyl-D-aspartate (NMDA) oscillations, which have been implicated in rhythmogenesis of some species (Grillner and Matsushima, 1991; Hochman et al., 1994; Rioult-Pedotti, 1997). This is because the current–voltage relationship for the rhythmic synaptic drive of spinal neurons is very linear, showing none of the voltage-dependence characteristic of the NMDA channel (Chub and O'Donovan, 1998a). Furthermore, although NMDA oscillations can be induced in spinal neurons in the presence of tetrodotoxin (TTX), such oscillations are rare when other synaptic conductances are active (Chub et al., 1997). Most compelling, however, is the observation that rhythmic activity can occur following blockade of NMDA receptors (Barry and O'Donovan, 1987; Chub and O'Donovan, 1998a). We can also exclude the possibility that the rhythmic activity within an episode is produced by some form of inhibition between reciprocally connected interneuronal centers, because it occurs in the presence of bicuculline and strychnine (Chub and O'Donovan, 1998a; Sernagor and O'Donovan, 1995). In addition, reciprocally connected centers would experience out-of-phase or alternating synaptic drive (Selverston, 1992; Roberts and Tunstall, 1990), and we have never observed this in either motoneurons or interneurons.

The mechanism we favor is that rhythmicity within an episode occurs because of the existence of a second type of activity-dependent network depression which recovers with a time constant of seconds or less (Chub and O'Donovan, 1997; Rinzel and O'Donovan, 1997). This ''fast'' depression limits the activity of the network during each cycle of activity. As this depression recovers rapidly, the network can retrigger many times within an episode. This mechanism is similar to that proposed to account for rhythmic activity in cultured spinal networks (Senn et al., 1996). At present, the existence of such fast depression is hypothetical, and it could be manifest either at the level of synapses or by activity-dependent changes in active membrane conductances (Turrigiano et al., 1994).

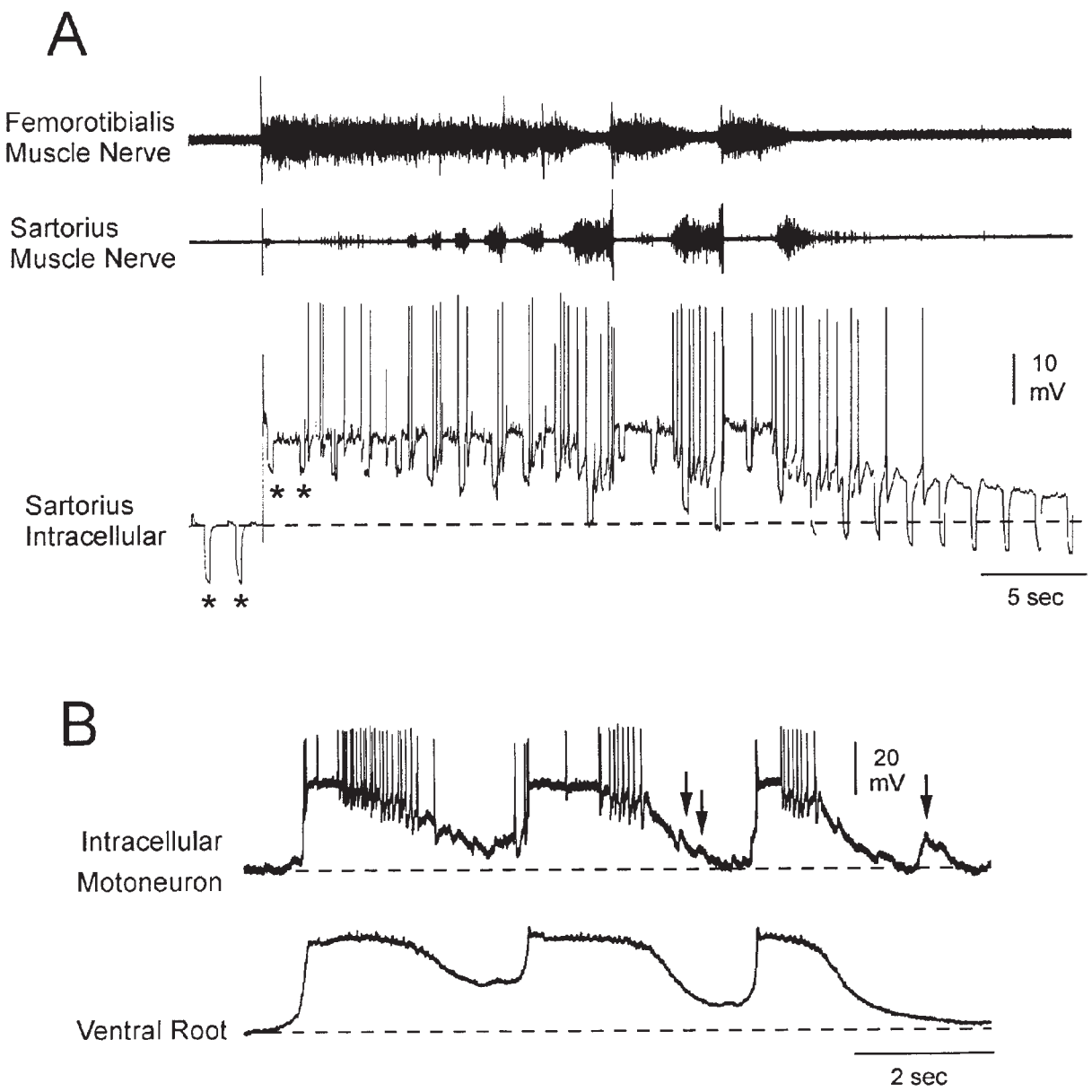

Figure 4 (A) Intracellular recordings from an identified sartorius motoneuron in the developing spinal cord compared with the electrical activity recorded from the sartorius and the femorotibialis muscle nerves. The downward voltage deflections (asterisks) show the membrane responses to an injected current pulse to illustrate the increased conductance (reduced voltage deflection) that coincides with the peak depolarization and the pause in firing. (B) Intracellular recording from a ''sartorius-like'' motoneuron together with a slow potential recording from the ventral roots. Notice the double discharge that occurs in each cycle. The delay after the initial, short burst (''the fingerprint'') is the result of a large depolarizing synaptic input which causes firing to stop at the peak depolarization and then resume as the membrane potential decays to rest. Notice also the synaptic noise (arrows) on the falling phase of the intracellular depolarization that could be involved in retriggering the network.

It should be emphasized that if the activity of individual neurons were perfectly synchronized during the first cycle of an episode, then once their firing had stopped (because of the hypothesized short-term depression), the network would not trigger again. In actual spinal networks, however, neuronal activity is not perfectly synchronized. Intracellular recordings have shown that the firing of individual neurons is not completely synchronized throughout the network. As illustrated in Figures 1 and 4, flexor and extensor motoneurons exhibit different firing patterns, and the same is true for interneurons. Discharge persists significantly longer in some neurons than others, and this delayed firing could retrigger the network. It is also possible that spontaneous transmitter release could retrigger each cycle in a manner similar to that proposed for the initiation of an episode. Indeed, spontaneous potentials can often be observed at the end of a cycle and can be quite large [up to 10 mV, as illustrated in Fig. 4(B)].

WHAT NETWORKS AND NEURONAL TYPES ARE ACTIVE DURING DEVELOPMENT?

We have hypothesized that episodes of rhythmic activity are generated by highly excitable networks coupled with some form of activity-dependent network depression. However, to obtain more direct evidence for these mechanisms, it is necessary to identify the active networks and to characterize the synaptic connections and properties of their constituent cells. Such information is also essential for understanding the function of spontaneous activity in network development.

The cell types experiencing rhythmic synaptic drive have not been completely characterized, but evidence is accumulating that short-range propriospinal neurons whose axons travel in the ventrolateral funiculus (VLF) are an important source of some of the synaptic drive to motoneurons. First, transection of the VLF leads to a substantial reduction in the depolarizing synaptic drive of motoneurons caudal to the cut (Ho and O'Donovan, 1993). Second, stimulation of the VLF produces powerful, short-latency synaptic potentials in motoneurons. Finally, whole-cell and optical recordings have shown that a subset of these propriospinal interneurons are rhythmically active (O'Donovan and Ritter, 1995). However, the VLF population is heterogeneous, containing many different types of interneuron, and it is likely that non-VLF interneurons are also rhythmically active. Therefore, to define the active population further, we have begun to record from individual interneurons to characterize their properties and synaptic connections.

In the adult mammal, surprisingly few interneuronal classes have been identified (reviewed in Baldiserra et al., 1981). These include Renshaw cells, which mediate recurrent inhibition of motoneurons; the 1a inhibitory interneurons mediating reciprocal inhibition of antagonist motoneurons; the 1b interneurons responsible for the inhibition of motoneurons from Golgi tendon organs; and a number of other less well characterized spinal neurons. Among these interneurons, Renshaw cells are probably the easiest to identify because they are the only known interneuronal targets of motoneurons. In the chick cord, ventral root stimulation evokes bicuculline and strychnine-sensitive synaptic potentials that can be recorded in other motoneurons (O'Donovan, 1989; Whelan and O'Donovan, 1997). Recently, we have identified a class of interneurons that appear to mediate these responses (Wenner et al., 1997). These cells receive direct input from motoneurons and also project monosynaptically onto motoneurons, suggesting that they are the avian equivalent of the mammalian Renshaw cell. They are rhythmically active and should therefore provide some of the GABAergic and glycinergic rhythmic drive to motoneurons. For example, they could be responsible for some of the GABAergic drive to sartorius motoneurons that underlies their pause in discharge. If so, they would be expected to fire during, or just before, the sartorius pause—an idea which is readily testable.

To understand the participation of these and other interneuronal classes in rhythmogenesis, it will be necessary to develop techniques that allow them to be manipulated selectively. At present, this is difficult to achieve experimentally, but the burgeoning field of molecular biology may offer the necessary tools to achieve this important goal.

Specific networks are difficult to delineate in any part of the nervous system without characterizing their constituent neurons. Nonetheless, it is possible to infer the operation of certain networks from the pattern of muscle or motoneuron discharge during spontaneous activity. *In ovo* electromyography, which has been performed throughout development, has revealed that the basic synergies of muscle activity that characterize locomotion arise early in development and become progressively refined thereafter (Bekoff, 1976). These findings suggest that the developing networks responsible for locomotion and perhaps other rhythmic hindlimb behaviors are

activated during spontaneous activity. However, the role of such activity in the development of these networks is unclear, as we discuss below.

FUNCTION OF SPINAL NETWORK ACTIVITY

Spontaneous motoneuron activity is known to be important in the development of muscle and joints (Ruano-Gil et al., 1978; Toutant et al., 1979; Persson, 1983; Hall and Herring, 1990; Jarvis et al., 1996), in the neurochemistry and gene expression of spinal neurons (Kalb and Hockfield, 1992; Garner et al., 1994; Mendelson, 1994; Spitzer, 1995), in the intramuscular branching patterns of motoneurons (Dahm and Landmesser, 1988), and in the survival of motoneurons during development (Pittman and Oppenheim, 1978). While these processes will clearly influence network function, it has not yet been possible to demonstrate a clear role for activity in the development of behaviors that depend on spinal circuits or on the initial formation of connections within spinal circuits. In a particularly telling experiment, embryonic tadpoles were raised in anesthetics or α-bungarotoxin to block neural activity during periods of network development. This experiment revealed no permanent changes in the swimming behavior of the tadpoles after an initial 1- to 2-day period of reduced activity (Haverkamp and Oppenheim, 1986). This result suggests that the swimming circuitry developed appropriately in the absence of network activity. In another experiment, Mendelson and Frank (1991) investigated the effects of altering muscle activity on the formation of synaptic connections between muscle afferents and lumbosacral motoneurons in the chick embryo. In normal embryos, muscle afferents from a particular muscle project monosynaptically to motoneurons innervating the parent muscle and its synergists, but not to antagonist motoneurons (Lee and O'Donovan, 1991; Mendelson and Frank, 1991). This highly specific pattern of connections was preserved when the nicotinic, cholinergic antagonist curare was applied *in ovo,* during the time the connections were forming (stages 28–42). Curare applied in this manner not only paralyzes the embryo, thereby preventing the normal activation of the muscle afferents, but also alters the central generation of activity by the spinal cord (Landmesser and Szente, 1986). Although the manipulation did not affect the specificity of connections, the synaptic potentials produced in motoneurons were about twice their usual amplitude.

At face value, these experiments suggest that spontaneous activity is not involved in the specificity of connections between spinal neurons. However, this interpretation is only valid given two assumptions. The first is that activity has been blocked or altered significantly by a particular experimental manipulation. This is generally very difficult to assess because it is usually impossible to monitor the activity of the relevant neurons during the period of blockade. Second, as we have reported, spinal networks can compensate for pharmacological blockade *in vitro* (Chub and O'Donovan, 1998). Whether such compensation can also occur *in ovo* is not known, but its presence would clearly confound the interpretation of activity-blocking experiments.

Despite these concerns, the available evidence, although very limited, suggests that the initial formation of connections between spinal neurons probably does not depend upon activity. In other parts of the nervous system, where activity has been demonstrated to play a role, it is in the rearrangement or pruning of excess connections. This is true both for the innervation of muscle and in the development of spatially ordered maps in the mammalian visual system (see Fields and Nelson, 1992, for review; but see Harris, 1980, for an opposing view in the development of the amphibian retino-tectal system). However, it is not known if the initial connections between spinal neurons or between afferents and spinal neurons are in excess of their final, adult numbers. If such excess connections do exist in spinal circuits, then it seems reasonable to speculate that activity may play a role in their withdrawal or rearrangement. Consistent with this idea is the result of Mendelson and Frank (1991) showing that chronic curarization, which depresses both muscle and spinal activity, leads to larger amplitude EPSPs than in control animals.

In adult mammals, it is known that single muscle spindle afferents project to all of the motoneurons innervating the homonymous or parent muscle and to a significantly smaller fraction of synergist or heteronymous motoneurons (Luscher et al., 1979). It is conceivable that single afferents project to all of the homonymous and heteronymous motoneurons early in development, and that establishment of the adult pattern requires activity. This hypothesis is testable by comparing the projection of single or small groups of muscle afferents onto homonymous and heteronymous motoneurons at different times in development. Using conventional electrophysiological methods, this would be difficult because a large sample of motoneurons would be required. However, recently developed optical methods may offer a tractable alternative (O'Donovan et al., 1993; Wenner et al., 1996).

In the retina, it has been shown that the firing of ganglion cells is correlated among adjacent cells (Meister et al., 1991). This correlation is thought to be important in the establishment of visual circuitry (Katz and Shatz, 1996; Shatz, 1994). In the spinal cord, the majority of spinal neurons within a given segment receive a depolarizing synaptic drive that is synchronized to within 10–20% of the cycle duration. However, little is known about the correlation in the firing of individual spinal neurons, and a documented function for such synchrony for the formation of spinal networks has not been forthcoming.

While synchrony is traditionally considered in terms of the timing of action potential activity among neurons, it should be emphasized that network oscillations are likely to result in spatially coordinated concentrations of extracellular and intracellular ions and other neurochemicals. We know that network-driven oscillations of intracellular calcium are synchronized across large numbers of spinal neurons (Fig. 5). The significance of such temporally and spatially coordinated variations in the chemistry and possibly gene expression of neuronal populations is not clear, but it may be important in determining the several aspects of neuronal development. For example, it has recently been shown that neurotrophins act synergistically with activity in influencing dendritic morphology in the ferret cortex (McAllister et al., 1996). Since it is known that neuronal activity can lead to the release of neurotrophins (Canossa et al., 1997), the synchronous activation of neuronal populations may facilitate this synergistic interaction.

Synchronization will also serve as an amplifier of neuronal activity by increasing both the depolarization of individual neurons and the number of active neurons by temporal and spatial facilitation of their synaptic inputs. Since neural activity is clearly involved in several aspects of neural differentiation and gene expression, synchrony may have evolved to maximize the number of neurons exposed to spontaneous activity.

Finally, it is recognized that the frequency and pattern of neuronal spike trains can have important effects on muscle and neuron development. For example, the frequency of firing of motoneurons is an important factor in the development of muscle fiber types and can regulate the expression of muscle proteins and other aspects of the muscle phenotype (Jarvis et al., 1996; Calvo et al., 1996). Firing pattern has been shown to affect the expression of cell adhesion molecules and ion channels on dorsal root ganglion cells (Fields and Itoh, 1996; Li et al., 1996).

When considering the effects of frequency on spinal network development, it is important to realize that the relevant frequencies vary over a very wide range. For example, episodes occur at a frequency of about 1/min *in ovo* at E10, whereas within an episode, the cycle frequency is rapid (1–2 Hz) initially and progressively declines throughout the episode (Figs. 1 and 4). The firing rate of individual neurons is also complex, reaching 50 Hz on the rising phase of the depolarization but slowing to 5–10 Hz later in the cycle (Fig. 4). The significance of these frequencies and whether any processes are sensitive to them are not known. One possibility is that intra- or extracellular chemical pathways are sensitive to these frequencies, and that such pathways regulate particular aspects of neuronal development. This idea is plausible because it has recently been shown that the kinase activity of calmodulin is sensitive to the frequency of calcium oscillations (De Koninck and Schulman, 1998). Moreover, studies of *Xenopus* spinal neurons have shown that spontaneous calcium spikes and waves, which occur at different frequencies, encode distinct neuronal processes. Spontaneous waves appear to be important in neurite extension of growth cones, whereas the spikes modulate transmitter expression (Gu and Spitzer, 1997).

CONCLUDING REMARKS

We have presented here, in rather broad strokes, the general mechanisms we believe operate to generate spontaneous activity within the spinal cord. However, we have not obtained direct experimental proof for these ideas, and we need to consider precisely what constitutes experimental proof. For example, we have proposed that widespread excitation coupled with activity-dependent network depressors combines to produce spontaneous episodes of activity. How can this be proved? We know that the major transmitters are all depolarizing, but we also know that under certain conditions the conductance change produced by these transmitters can be functionally inhibitory (O'Donovan, 1989; Sernagor et al., 1995). Therefore, it will be essential in future experiments to define precisely the conditions under which inhibition or excitation predominates within the network. Second, we know very little about the specificity, strength, and extent of connections between individual neurons within spinal networks. Such information is necessary to constrain mathematical models of network operation (Rinzel and O'Donovan, 1997). We also have scant knowledge

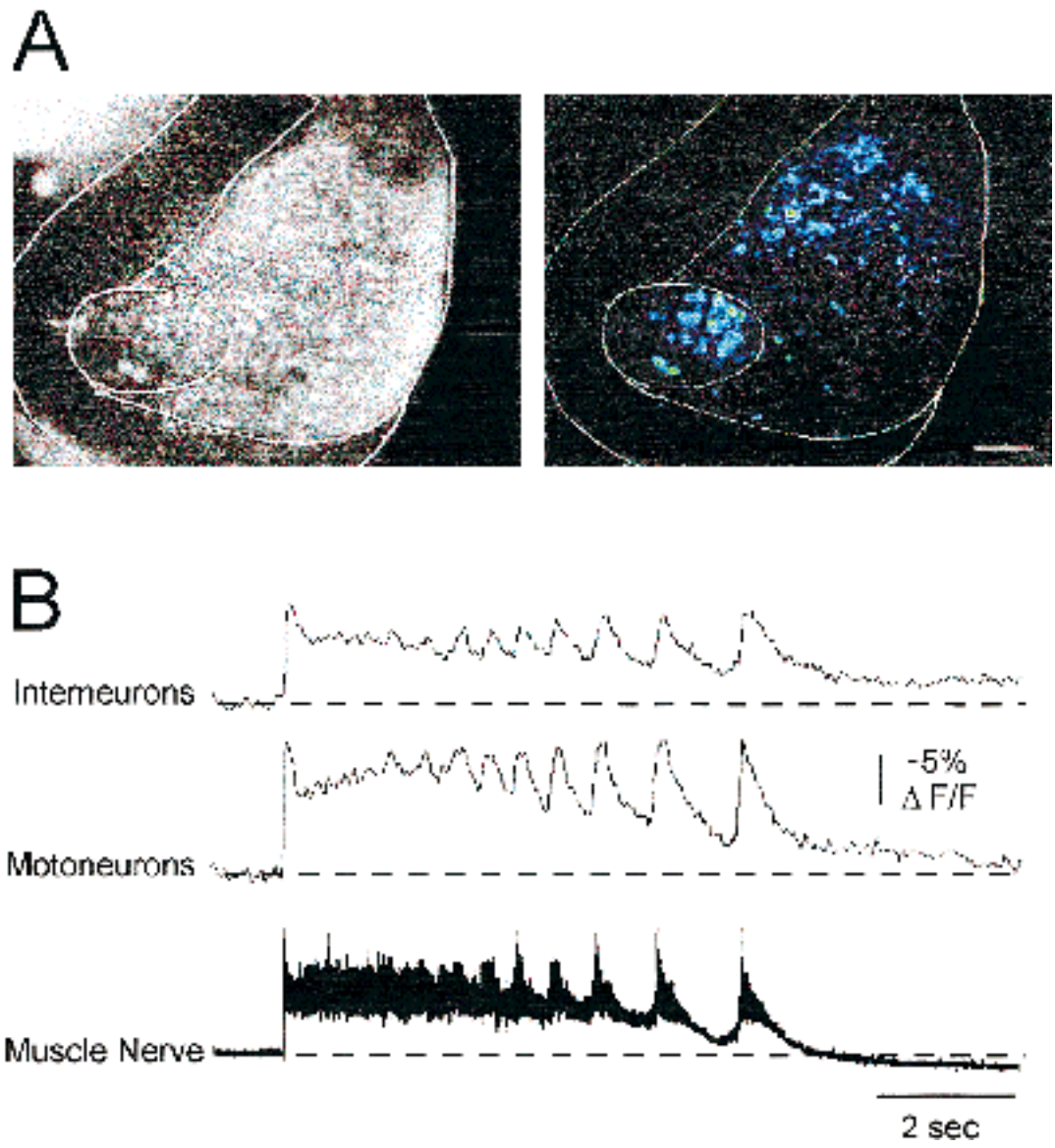

Figure 5 Calcium imaging of an episode of rhythmic activity in the developing spinal cord. The videomicrographs (A) show the cut transverse face of the spinal cord labeled with Fura-2am (left) and a difference image obtained during 1 s of an episode of rhythmic activity. The difference image was obtained by subtracting an image averaged from 1 s of activity during the episode from an image obtained just before the episode began. Calibration bar = 100 μm. (B) Comparison of the time course of the Fura-2 fluorescence change and the electrical activity recorded from a muscle nerve during an episode of rhythmic activity. The fluorescence changes were measured from the intermediate region of the cord (interneurons) or the lateral motor column (LMC). Data in (A,B) were obtained from E11 embryos. [Modified from O'Donovan et al. (1994).]

about the precise mechanisms responsible for the depression of network excitability by neuronal activity and the extent to which it is causally responsible for terminating network activity. One approach would be to manipulate network depression to show that it could either increase or decrease the duration of spontaneously occurring episodes. Unfortunately, there is no simple way of doing this selectively at the network level without interfering with other processes. One possible avenue for future experiments is to use genetic manipulations targeted to the synapse or the postsynaptic receptors with the

goal of manipulating the extent of synaptic depression within the network.

Another area of ignorance is the functional role of neural activity during the development of spinal networks. There several obstacles to demonstrating convincingly what neural activity might do during development. First, there is the problem of manipulating neural activity in an appropriate manner. This is usually done pharmacologically, but such manipulations inevitably lack specificity and operate indiscriminately across multiple networks. Moreover, it is usually very difficult to introduce selective patterns of activity or alter the level of synchrony within the developing networks. Another problem arises because the output of spinal networks is very robust and can compensate for major pharmacological perturbations (Chub and O'Donovan, 1998). Again, targeted genetic manipulations of particular cell classes or of particular networks offer promise for future experiments.

Finally, there are numerous questions that we have not addressed in our experiments. For instance, we know nothing about the potential role of neurotrophins either in the regulation of network excitability or in controlling network plasticity. Similarly, we have only limited knowledge of the role of neuropeptides in network function. Recently, Carr and Wenner (1998) have shown that antagonists of the calcium gene-related peptide (CGRP) can slow spontaneous activity within the spinal cord. It seems reasonable to assume that many other peptides are also being released into the spinal cord, but we have little or no direct information about this possibility. Similarly, biogenic amines are known to modulate network excitability, but if or when they are being released within developing spinal networks is currently unclear.

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