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Central Pattern Generators Deciphered by Molecular Genetics

Minireview

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Central pattern generators (CPGs) are localized neuronal networks that have the ability to produce rhythmic movements even in the absence of movement-related sensory feedback. They are found in all animals, including man, and serve as informative model systems for understanding how neuronal networks produce behavior. Traditionally, CPGs have been investigated with electrophysiological techniques. Here we review recent molecular and genetic approaches for dissecting the organization and development of CPGs.

To understand normal brain function and diseases of the brain it is of great importance to understand how an assembly of nerve cells can generate the processing power required for different brain functions. Traditionally, neuronal networks have been studied with electrophysiological, anatomical, and imaging techniques, and such techniques have provided vast insights into their general organization. In this minireview we will discuss new molecular genetic approaches, which recently have been applied to the studies of central pattern generators (CPGs), the neuronal networks that control rhythmic movements, like breathing, feeding, swimming, and walking. CPGs provide a continuous, measurable and reproducible physical output, making it possible to relate the network activity with an actual behavior, and although complex, their structure is relatively simple compared to networks controlling higher brain function. Further, since their assembly likely follows similar principles to those guiding the assembly of most, if not all, neural circuits, CPGs represent a useful model system for studying the organization of neuronal networks generally.

Central Pattern Generators

A comprehensive understanding of any network requires identification of the participating neurons and deciphering of the wiring diagram. In the case of CPGs, so far, a complete wiring diagram has only been obtained for a small number of rhythmic motor systems, such as the Crustacean gustatory CPG and the swimming CPGs in the tadpole and the lamprey (diagrammed in Figure 1). While the layout of the mammalian CPG will no doubt be quite different from these more primitive systems, they nonetheless provide a useful model for demonstrating the components that make up a functional CPG. In the lamprey CPG (illustrated in Figure 1), each one of the about 100 spinal segments contains a CPG unit, with CPG neurons on both the left and right side of the cord (Grillner, 2003). The basic unit is composed of two types of interneurons: excitatory glutamatergic neurons (Es) and inhibitory glycinergic commissural interneurons (Is). Es provide monosynaptic excitation of motor neurons (Ms) while commissural interneurons with axons crossing in the midline ensure segmental alternation: when one side is active, all neurons on the other side are silenced. Several organizing principles have emerged from studies of these CPGs. First, the behavioral output generated by the CPG depends on the patterned activity of its component neurons, and in turn CPG depends on the neurons' intrinsic membrane properties (Harris-Warrick, 2002). Thus, in order to understand the behavior of the network overall, it is critical to identify and characterize the membrane properties of each type of participating neuron in order to understand the entire network. The assembly of ion channels and their distribution in the membrane of CPG neurons will dictate how they react to and interpret synaptic inputs from other neurons in the network. In addition, neuromodulators such as glutamate acting on metabotropic glutamate receptors will affect the intrinsic membrane properties and may reconfigure the motor output.

Although the mammalian spinal cord has been studied for a century, we still have only a very basic knowledge about the networks controlling rhythmic movements in mammals (see Kiehn and Butt, 2003, and references therein). The enormous complexity of the mammalian nervous system makes identification of CPG neurons and establishment of the connectivity pattern very cumbersome. For example, finding rhythmically active interneurons in the mammalian spinal cord can be compared to a fishing expedition, since interneurons of different types are intermingled in the rhythm-generating areas. Paired recordings to establish connectivity are extremely time consuming and very difficult. The large number of cells further complicates efforts to establish the role that a specific type of neuron plays in the network. Since it is very difficult to manipulate cellular properties of a specific population of neurons using standard pharmacological approaches, there is a critical need to find new methods that in combination with classical electrophysiological and anatomical techniques can be used to tackle these issues. Below, we shall discuss advantages and disadvantages when using genetic approaches for deciphering CPG networks.

Functional CPG Analysis by Elimination or Activation of Network Neurons

The function of neurons in the CPG network can be efficiently determined by their selective killing or inactivation. This approach was pioneered in the Crustacean gustatory CPG, located in the stomatogastric ganglion

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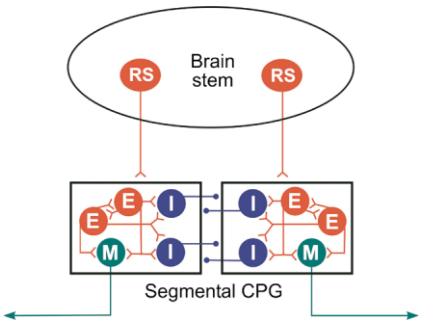

Figure 1. Schematic Model of the Basic Connectivity Pattern in the Lamprey CPG

Each segment along the spinal cord is thought to contain a CPG network with neurons on the left and right side of the cord. The segmental CPG networks are connected with fibers running along the cord (not shown in the figure). The CPG activity is turned on by activity in glutamatergic fibers originating from neurons in the reticular formation. These descending fibers are acting on NMDA and non-NMDA receptors. Excitatory ipsilaterally projecting neurons (E) provide the rhythmic excitation of motor neurons (M) and other Es. The commissural interneurons (Is) that have axons crossing the midline inhibit all CPG neurons and MNs on the other side of the cord, ensuring that when one side is active the other is shot off. Adapted from Grillner, 2003.

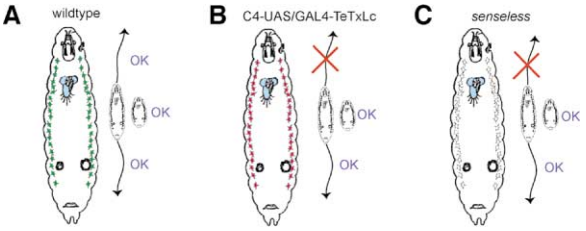

Figure 2. *Drosophila* Larvae Locomotion Studies Suggest that a Functional *Drosophila* CPG Can Develop in the Absence of Sensory Feedback

(A) Schematic model illustrating that wild-type larvae display peristaltic movements as well as backward and forward movements. Green cells schematically represent normal sensory cells, and blue marks the CNS. (B) In larvae where the tetanus toxin light chain (TeTxLc) is expressed in sensory neurons to block their transmission, forward movements are impaired, while backward and peristaltic movements are unaffected. Red cells represent sensory cells with transmission blocked. (C) In *senseless* mutant larvae that lacked sensory neurons, peristaltic movements and backward movements were normal, while forward movements lacked the normal periodic bursts of locomotor activity. Unfilled cells mark the sensory cells no longer present in the mutant.

(STG). The STG contains a small CPG with less than 30 neurons that control the rhythmic movements of the foregut in crustacean. Miller and Selverston (1979) injected Lucifer Yellow into individual neurons in this small network and photoinactivated individual or classes of neurons, thereby directly revealing their function in the network. Unfortunately, single-cell ablation is not expected to give any measurable effects on the behavior in the complex vertebrate CPGs where presumably large groups of cells control specific network functions. Therefore, ablation and loss-of-function approaches that target large groups of cells are needed. An example of a successful application of this approach is a recent study that was able to selectively kill neurons in the pre-Bötzinger complex (preBötC) that express the substance-P receptor neurokinin-1 (NK1R) (Gray et al., 2001). Previous experiments have suggested that neurons positive for NK1R are essential for rhythm generation from the preBötC, a region in the brainstem proposed to contain the neuronal circuits generating respiration (see references in Gray et al., 2001). A substance-P-saporin conjugate that is internalized by the NK1 receptor was used to kill the NK1R-expressing neurons, resulting in a highly irregular breathing pattern. Although not without some caveats (for instance, the substance-P-saporin conjugate can also be taken up by cells expressing other substance-P receptors than the NK1R; it is possible that other neuronal populations not expressing NKR1 contribute to the observed phenotype), these experiments provide a good example of how selective cell ablation can be used to elucidate the contribution of particular cells and cell populations in a network.

A considerable limitation of the previously described technique is that it can only be used in cases where populations of neurons can be defined by the expression of specific receptors.

On the other hand, genetic manipulation provides a more versatile approach for selective cell killing. As one example, targeted expression of the tetanus toxin light chain (TeTxLc), a toxin which blocks spike-evoked neurotransmitter release by inhibiting synaptobrevin, can be used to block the activity of specific groups of neurons. This approach has been used successfully in *Drosophila* to identify a set of neurons that are important for the normal, forward crawl of larvae (Suster and Bate, 2002; Suster et al., 2003). The authors used 150 different Gal4 strains to target expression of TeTxLc to various populations of neurons in the CNS of *Drosophila* larvae. Five of these lines had specific locomotor deficits, one of which displayed an unusual circling behavior. By crossing this Gal4 strain (4S-Gal4) to a number of reporter strains, including UAS-GFP-nuclear LacZ, the expression pattern for the eliminated neurons could be analyzed. These data showed that about 200 neurons were affected in the circling animals, including major groups of serotonergic, dopaminergic, and peptidergic interneurons. Selective expression of TeTxLc in dopaminergic and serotonergic but not peptidergic neurons replicated most aspects of the unusual circling behavior, suggesting that monoaminergic neurons play an essential role for normal locomotion in *Drosophila* larvae.

It is well established that a CPG can generate rhythmic output in the absence of phasic sensory inputs, but it has been unclear how important sensory feedback is for proper development of the CPG circuit. This problem is difficult to address with mechanical deafferentation since it requires delicate surgical manipulations of early embryos. This question has recently been addressed in *Drosophila* larvae using the UAS-Gal4 system. Suster and Bate (2002) expressed TeTxLc exclusively in the peripheral nervous system to block neurotransmitter release from sensory neurons, thereby removing sensory input to the CPG during development. Although the movements of the genetically modified larvae were somewhat impaired, the larvae were still able to hatch and move over a substrate, demonstrating that the CPG is functional in the transgenic animals (Figure 2). To

eliminate the possibility that the silent sensory axon terminals in the nerve cord could serve to support development of the CPG, the authors took advantage of a null mutation in the *senseless* gene. In *senseless* mutant larvae, almost all sensory neurons are missing throughout development. In spite of this, peristaltic and backward movements were still functional, demonstrating that development of basic CPG function does not require sensory input. In contrast, bursts of forward movement were virtually absent, suggesting an important role for sensory feedback for generating forward movements in *Drosophila* larvae.

In addition to inactivation and elimination of specific neurons, CPG networks can also be studied by activating neurons through depolarization. This can be achieved by expression of a mutated ionotrophic glutamate receptor that causes chronic depolarization. This novel technique has been used to determine how locomotion is controlled in *Caenorhabditis elegans* (Zheng et al., 1999). Other genetic techniques to eliminate neurons from neuronal circuits include overexpression of potassium channels that leads to electrical silencing of specific groups of neurons (Nitabach et al., 2002) and selective killing of neurons by the expression of diphtheria toxin subunit A (DTA) (Lee et al., 2000).

The experiments just described demonstrate that selective elimination or activation of neurons within a CPG network are powerful methods for identifying neurons important for CPG function. However, it remains to be established whether such genetic techniques will be readily applicable to mammalian systems. A prerequisite for extending these approaches to mammalian CPGs is that specific genetic markers for groups of spinal interneurons are needed. To this end, several labs have identified a number of transcription factors that specify both motor neuron and interneuron populations in the developing spinal cord (Jessell and Sanes, 2000; Sharma and Peng, 2001; Goulding et al., 2002). The promoters of such transcription factors can be used to selectively drive expression of neuronal reporters (Sapir et al., 2003; Wenner et al., 2000) or expression of genes that modify or kill neurons, like potassium channels or DTA. Since the transcription factors have dynamic expression profiles, they may be absent at the time when the CPG matures. Moreover, a particular transcription factor may define a heterogeneous group of cells, encompassing more than one assembly of interneurons (e.g., Renshaw cells, Ia interneurons, and Ib interneurons; see Sapir et al., 2003). To date, we are not aware of a single molecular marker that defines a homogeneous group of spinal cord interneurons. Therefore, in order to progress by genetic manipulations, more suitable promoters will be needed. Gene expression studies may point to candidate promoters, but expression of genes driven by the chosen promoters may fail to reproduce the desired pattern of expression in mice either because of unfavorable integration site in the genome or because the full promoter sequence is difficult to identify. A recent study has suggested an exciting alternative approach using bacterial artificial chromosomes (BACs) that contain very large pieces of DNA, thereby considerably improving the chance of using the full promoter sequence. The BACs, in which green fluorescent protein (GFP) was placed under the control of suitable promoters, were then used to create transgenic mice. This large-scale screen aims to create an atlas of CNS gene expression at the cellular level in vivo (Gong et al., 2003), and so far, over 150 verified transgenic mouse GFP lines can be found in the publicly available database (www.gensat.org). This study represents a major effort and an impressive new tool to identify specific neuronal populations, and one can't help but dream of a similar library with transgenic mice that express Cre protein, which would allow conditional knockout of genes in limited neuronal populations.

One potential drawback to genetic ablation is that chronic manipulations may lead to compensatory mechanisms that might obscure the functional defects that we are looking for. For example, expression of DTA driven in germ cells may lead to premature death of the developing embryos. One way around this problem is to use inducible promoter systems, such as promoters driven by the reverse-tetracycline-controlled transactivator.

Connectivity Tracing Using Genetics

Even in smaller CPGs, it is a tremendous task to study the connectivity patterns of the neurons using classical, dual microelectrode recordings. For example, Getting (1988) calculated that in the 12 neuron CPG controlling escape swimming in the sea snail *Tritonia*, there are 132 possible connections between the neurons. These numbers scale up as the complexity of the nervous system increases, making it impractical to deduce the pattern of connectivity between the single neurons. Guidance is therefore needed from broader scale anatomical tracings of circuit connections, for instance, using microinjection of tracer compounds and dyes. Such tracers can be taken up by dendrites and axon terminals and transported either anterogradely or retrogradely in the labeled neurons. Although these tracing techniques have given us tremendous insights into how the brain is wired on a broad scale, they have given limited information about the precise network wiring, since the tracers cannot be injected selectively into a specific phenotype of neurons that are embedded in the network.

Recent studies have addressed this issue by expressing either anterograde or retrograde (Maskos et al., 2002; Yoshihara et al., 1999) tracers under the control of promoters specific for a particular neuronal cell type. In these transgenic mice, the tracer is not only expressed in the cell of origin but is also transported transsynaptically, allowing mapping of the afferent (retrograde transsynaptic tracing) and/or efferent (anterograde transynaptic tracing) connections. Using this type of technology, it has been possible to demonstrate multisynaptic transfer in cerebellar and olfactory pathways (Yoshihara, 2002) and in the spinal cord of mice (Sapir et al., 2003). In another study, by coexpressing a reporter gene that can jump synapses with a reporter gene that remains in the cell of origin, Maskos et al. (2002) were able to discriminate neurons that produced the tracers from those that were transsynaptically labeled. Transgenic mice carrying these types of reporter constructs have an enormous potential for advancing studies of connectivity in the developing and mature mammalian CPG. There are, however, several problems that need to be considered. One is the strength of the promoter. Weak promoters may lead to a limited production of the desired marker

in the cell of origin, resulting in insufficient labeling. This can be solved by introducing a conditional allele where a strong promoter is followed by a loxP-flanked stop signal and the marker of interest (Yoshihara, 2002). The weak promoter is instead used to drive expression of the Cre protein, which will delete the stop codon and turn on the marker. Very strong promoters, on the other hand, may cause unwanted side effects, such as degeneration of the neuronal population under study. Another problem is that at least some tracers, such as plant lectins, are transported both anterogradely and retrogradely (Yoshihara, 2002), and it thus becomes difficult to discriminate whether a neuron is post- or presynaptic to the cell of origin. Moreover, if the tracer passes more than one synapse, there is a risk that the labeling will encompass the entire network, leaving little information about the actual network connectivity. Finally, tracing studies can only present a static picture of the network connections and don't represent the dynamic interactions between the individual neurons.

Pathfinding Defects Used as a Tool to Understand CPG Organization

Another means to gain insight into the make up and functional role of a network is through the study of mutants in which the development of the circuit is perturbed. During development, a large number of axon guidance molecules operate to set up precisely the wiring of neuronal networks, and by studying specific axon guidance defects it might be possible to gain new insights into the organization of CPGs. As an example, the zebrafish mutant *space cadet* was identified in the Tuebingen screen as one of several genes involved in proper development of the network wiring in zebrafish larvae (Granato et al., 1996). *Space cadet* mutants exhibit motor defects both in response to escape stimuli and during ordinary swimming, and anatomical studies revealed that a subset of hindbrain commissural fibers, called spiral fibers, in rhombomere three were strongly reduced in size in *space cadet* (Lorent et al., 2001). Formation of the hindbrain commissural spiral fibers coincides with the onset of the mutant phenotype, and tracing experiments revealed that commissural spiral fibers failed to make normal connections to Mauthner neurons in mutant larvae, suggesting a functional link between the commissural defect and the swimming phenotype. Brain lesions induced by cutting the hindbrain commissural spiral fibers at the midline of fish larvae reproduce the mutant phenotype, supporting a role for the commissural spiral fibers in controlling normal motor behavior. In summary, by carefully studying the axon guidance defect in *space cadet*, it was possible to reveal the role of spiral fibers for normal swimming performance in zebrafish.

Similar studies are now being applied to the mammalian locomotor CPG. For instance, recent genetic studies have revealed a role for Eph Receptor/Ephrin signaling in the development of the mammalin locomotor circuit. Mice with a targeted deletion of the axon guidance molecule EphA4 move with a rabbit-like gait and in addition have axon guidance defects in the anterior commissure and the corticospinal tract (Dottori et al., 1998). The peculiar gait and the defect in the corticospinal tract were identical in mouse mutants that carry a deletion in the ephrinB3 locus produced at Regeneron and by

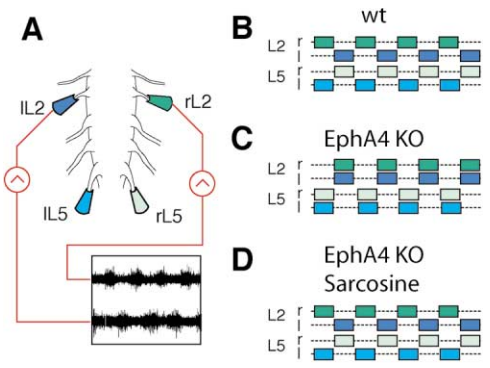

Figure 3. Mice Carrying an Inactivated Allele of the EphA4 Axon Guidance Molecule Have a Reorganized CPG

(A) Experimental setup to measure fictive locomotion. Normal rhythmic activity can be recorded in the vitro spinal cord via electrodes attached to ventral roots at lumbar (L) level 2 and 5 on the left (l) and right (r) side of the cord. Locomotor activity induced by a combination of 5HT and NMDA. (B) In wild-type animals, the activity pattern corresponds to normal walking, with segmental left-right alternation at lumbar level 2 and 5. The recordings also monitor the alternating rhythm between the ipsilateral ventral roots L2 and L5 that approximately corresponds to the alteration of activity between extensor and flexor muscles. (C) In EphA4 mutant animals, the alternating activity between the right and left ventral roots at the segmental level is switched to synchrony, corresponding to the rabbit-like gait seen in intact mutants. (D) The synchronous locomotor pattern in EphA4 mutants is reversed back to normal alteration by addition of the glycine reuptake inhibitor sarcosine (for details, see Kullander et al., 2003).

the Henkemeyer lab (Kullander et al., 2001; Yokoyama et al., 2001). EphrinB3 is a ligand for the EphA4 receptor, and cells that express ephrinB3 repel axons carrying EphA4. In vitro measurements from ventral motor roots in isolated spinal cord preparations replicated the right-left synchronous activation pattern observed in both mutants (Kullander et al., 2003) (Figure 3). The peculiar synchronous gait could therefore be sufficiently explained by a local defect in the lumbar spinal cord circuitry. Strengthening of inhibitory action by addition of sarcosine, which blocks reuptake of the neurotransmitter glycine, reverted the synchronous activation pattern back to normal alteration, thus indicating that the balance between excitation and inhibition over the midline was shifted toward excitation in the mutants (Figure 3D). In both EphA4 and ephrinB3 mutant mice, fibers aberrantly cross the midline in the spinal cord, and these fibers were shown to originate from EphA4-positive neurons in the spinal cord that normally do not project fibers across the midline. This finding indicated that the defective rhythmic pattern originated from excitatory EphA4 neurons that normally project ipsilateral, a hypothesis that was further supported by the fact that more than 50% of the glutamatergic neurons in the spinal cord were also EphA4 positive. Together, these data implicate Ephrin signaling in the establishment of the lumbar spinal cord circuitry and thus both provide new insight into how the network controlling rhythmic walking is organized, as well as identified a useful marker for neurons that are part of the locomotor CPG.

Manipulation of Cellular Properties

Our understanding of how specific ionic membrane properties contribute to CPG function is still rudimen-

tary, even in systems where the CPG connectivity is well understood (see Harris-Warrick, 2002, and references therein). Although membrane properties can be readily investigated in individual neurons, it is difficult with traditional pharmacological tools to assess the role of these properties in shaping the network activity. Because pharmacological perturbations act globally on network neurons, membrane properties change in many different neuron types at once. Genetic overexpression or targeted deletion of ionic channels in a cell type-specific manner could overcome these shortcomings. A more direct approach was used by Zhang et al. (2003), who overexpressed hyperpolarization-activated and cyclic nucleotide-gated channels (HCN) in single neurons in the crustacean stomatogastric ganglion (STG). When mRNA coding for HCN channels was injected, it enhanced the I_h current in the injected neurons. The firing behaviors of the injected cells were strongly affected (cells were depolarized and firing faster and for longer times during the rhythmic cycles), and the function of the CPG was changed. These experiments demonstrate the feasibility of cell-specific manipulation of ionic conductances in small CPGs. The importance of such experiments is tremendous, but at the moment, overexpression of ion channels in specific populations of CPG interneurons have not been attempted in mammals. Again, a major limitation to achieving this goal has been the development of promoters for targeting specific neuronal populations.

Closing Thoughts

Genetic approaches are emerging as important tools to dissect and understand CPG circuits not only at the molecular and cellular levels but also at the network and physiological level. At this point, a critical step toward the use of molecular techniques is the genetic identification of interneuronal subpopulations. We are optimistic that future studies will bring more genetic markers for interneuronal subpopulations. We are also convinced that a multidisciplinary approach combining molecular genetic strategies with electrophysiological and anatomical techniques eventually will allow researchers to decipher the functional organization of CPGs, including those controlling rhythmic movements in mammals.

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