

A New Class of Spinal Interneurons: The Origin and Function of C Boutons Is Solved

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C boutons, a major synaptic input to motor neurons, were first described years ago, but their origin and functional significance were unknown. In this issue of *Neuron*, a group of cholinergic interneurons located near the central canal is identified as the source of C boutons. The rhythmic activity in these neurons during locomotion increases motor neuronal excitability, thereby potentiating the strength of muscle contraction.

An understanding of how the spinal cord controls motor behavior requires a full knowledge of the circuits that integrate descending input from the brain with local sensory information to generate the appropriate patterns of activity in motor neurons. A detailed functional and anatomical description is becoming available for motor neurons and their connections with muscles. A similar analysis of the interneurons that drive motor neurons has been far more difficult, however. Until recently, the dominant method used was to make blind recordings from interneurons in adult cats. This method, however, is not amenable to differentiating among different classes of interneurons, studying their intrinsic electrical properties and the descending and sensory inputs they receive, or determining how they influence motor behavior.

New molecular techniques available in the mouse are now breaking this impasse. During the last several years it has become possible to fluorescently label restricted subclasses of interneurons based on their selective expression of transcription factors. The cells can then be targeted for electrophysiological recordings in neonatal mice, thereby facilitating the study of their intrinsic electrical properties, synaptic inputs, synaptic projections to other neurons, and transmitter phenotypes. Moreover, the unique transcription factors make it possible to functionally silence a single subclass of cells and assess its individual contribution to motor behavior. Using these techniques, Zagoraïou and coworkers have now performed such an in-depth characterization of a new subclass of interneurons (Zagoraïou et al., 2009) and in the

process have solved a long-standing mystery regarding the source and function of cholinergic input to spinal motoneurons.

C boutons, a source of cholinergic input to motor neurons, were first identified on the cell bodies and proximal dendrites of spinal motor neurons 40 years ago (Conradi and Skoglund, 1969), but the neuronal source of this input and its functional significance were unknown. Recently, C boutons were found to originate from a heterogeneous population of intermediate Dbx1⁺ interneurons located lateral to the central canal (Miles et al., 2007). In a screen for genes expressed only in intermediate and ventral spinal interneurons, Zagoraïou and coworkers found that one of these genes, *Pitx2*, is also confined to a group of interneurons located in a longitudinal cluster near the central canal at lumbar, thoracic, and cervical levels of the spinal cord. By generating mice in which Pitx2⁺ neurons expressed a fluorescent protein (FP), Zagoraïou et al. determined that virtually all of the C boutons originate from Pitx2⁺ neurons. These Pitx2⁺ neurons therefore provide a major source of cholinergic input to spinal motor neurons.

Pitx2 is a paired-like homeodomain transcription factor, and Pitx2⁺ interneurons form a small subset of V0 interneurons, which are identified by their expression of Dbx1 (Pierani et al., 2001). Many Pitx2⁺ neurons are cholinergic (referred to here as V0_C neurons), as revealed by expression of the vesicular acetylcholine transporter vAChT and the ACh synthetic enzyme choline acetyltransferase (ChAT). V0_C neurons therefore provide a major source of cholinergic input to spinal motor

neurons. The majority of projections from V0_C neurons are to ipsilateral motor neurons, as revealed by *trans*-synaptic labeling via motor neurons following unilateral injection of pseudo-rabies virus into hindlimb musculature. Another class of spinal interneurons, Renshaw cells, also receives strong cholinergic input, but this input does not originate from V0_C neurons. A schematic representation of the location and connectivity of V0_C interneurons based on these findings is shown in Figure 1.

Although the majority of Pitx2⁺ neurons are cholinergic at rostral lumbar levels, at more caudal lumbar levels the proportion of these neurons that express vGlut2 (V0_G neurons) increases. No Pitx2⁺ neurons coexpress both cholinergic and glutamatergic markers. The synaptic targets and functions of V0_G neurons are unknown. Zagoraïou and coworkers suggest that the differentiation of Pitx2⁺ neurons into V0_C versus V0_G might be controlled by Notch signaling, analogous to the differentiation of V2 interneurons into glutamatergic V2a and GABAergic V2b subsets (Peng et al., 2007). An alternative hypothesis is that different levels of neural activity lead to different transmitter phenotypes. In the embryonic *Xenopus* spinal cord, individual neurons first express multiple transmitter phenotypes: glutamatergic, cholinergic and GABAergic. Reduction of neuronal Ca²⁺ activity results in the selection of the glutamatergic phenotype, whereas with increased activity levels, GABAergic phenotypes predominate (Borodinsky et al., 2004). In a similar manner, differing levels of synaptic input to developing Pitx2⁺ neurons might result in the

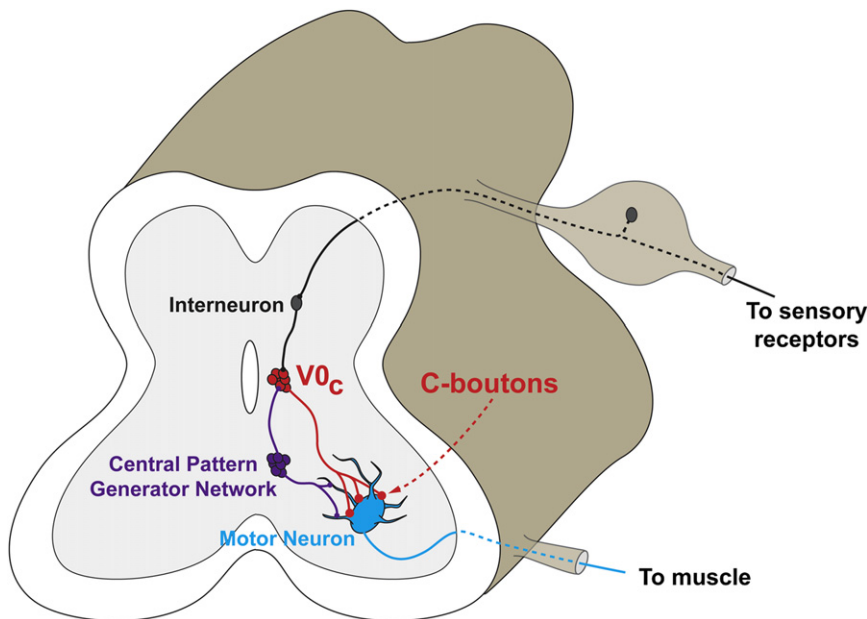


Figure 1. Schematic Diagram of V0c Connectivity

emergence of separate populations of $V0_G$ and $V0_C$ interneurons. This hypothesis could be tested by determining if both transmitter phenotypes are expressed in individual $Pitx2^+$ neurons at early stages and if genetic manipulations to lower activity levels in these neurons result in a change in the proportion of neurons expressing one or the other transmitter phenotype.

Clusters of muscarinic m2 receptors (Hellström et al., 2003) and voltage-dependent delayed rectifier K^+ channels ($K_{V2.1}$) (Muennich and Fyffe, 2004) are closely aligned with C boutons in the postsynaptic motor neurons. Activation of m2 receptors modulates the gating characteristics of these K^+ channels and leads to a reduction in the duration of the action potential. This reduces the amplitude of the afterhyperpolarization following each action potential and thereby increases motor neuronal excitability. Pharmacological blockade of m2 receptors in the isolated neonatal mouse spinal cord reduces the amplitude of the rhythmic bursting of motor neurons during fictive locomotor activity, presumably because the afterhyperpolarization is increased, thereby reducing motoneuronal excitability (Miles et al., 2007). These results suggest that m2 muscarinic inputs to motor neurons are active during the generation of locomotor behavior, serving

to increase the response of motor neurons to inputs from the central pattern generator network.

With the identification of all C boutons as the synaptic terminals of $V0_C$ interneurons, Zagoraïou and coworkers proceeded to study how this synaptic input modulates the activity of motor neurons. First, they assessed the electrical properties of the interneurons themselves in hemisectioned lumbar spinal cords from neonatal mice. Most $Pitx2^+$ neurons are tonically active, with large afterhyperpolarizations, and they respond to maintained current injection with steady trains of action potentials. They receive only indirect sensory input, because stimulation of dorsal roots elicits excitatory input with relatively long and variable latencies. Stimulation of the segmentally aligned ventral root does not elicit synaptic input, arguing against any direct input from recurrent motor neuronal collateral axons. These results corroborate the anatomical presence of $vGlut2^+$ but not $vGlut1^+$ or cholinergic synaptic terminals on $Pitx2^+$ neurons. In spinal cord slices, where the neurons are likely to be isolated from central pattern generator networks, FP-labeled cells increase their firing rate in response to drug cocktails that induce fictive locomotor activity, but this activity is not rhythmic, suggesting that these neurons do not contribute directly to the

generation of rhythmic bursting of motor neurons during locomotion.

In contrast to the absence of rhythmicity in spinal cord slices, Zagoraïou et al. found that the firing patterns of FP-labeled neurons in isolated hemisectioned cords are rhythmically modulated when fictive locomotor behavior is induced with a drug cocktail. Activity is increased in synchrony with the rhythmic activity of ipsilateral motor neurons recorded from the ventral root of the same spinal segment. The bursts of activity in FP-labeled cells begin slightly after the corresponding motor neuronal bursts, suggesting that $Pitx2^+$ neurons do not contribute directly to the initiation of the motoneuronal burst. Instead, a common input, presumably from the central pattern generator network, appears to drive both neuronal types. For example, bursts of excitatory postsynaptic currents in FP-labeled cells occur in synchrony with the motoneuronal bursts, and occasional action potentials in FP-labeled cells occurring during interburst intervals are temporally correlated with short interburst periods of motoneuronal activity in the segmentally aligned ventral root.

As described above, earlier work demonstrated that acute blockade of m2 muscarinic receptors leads to a decrease of firing frequency of motor neurons during fictive locomotion in isolated cords (Miles et al., 2007). Having identified $Pitx2$ as a specific transcription factor for the interneurons that provide the major m2-mediated input to motor neurons, Zagoraïou and coworkers could then assess the contribution of this cholinergic input to motor neuronal activity in adult, behaving mice. To silence the $Pitx2^+$ -motoneuron pathway, the authors first tried a Cre-mediated deletion of ChAT in $Pitx2^+$ neurons. This deletion eliminated ChAT expression in only half of the C boutons, however, an insufficient fraction to conduct a meaningful test of the effect of this input on behavior. The authors then deleted ChAT expression in all $Dbx1^+$ neurons. This manipulation affects more than the $Pitx2^+$ neurons, but these neurons are the only affected ones that synapse directly onto motor neurons. After the deletion, the number and location of C boutons on motor neurons did not change, but without ChAT, they were synaptically silent.

Despite the absence of functional C boutons, these mice developed normally, reached adulthood, and had grossly normal motor behavior. Nor did the deletion disrupt the normal activation patterns of hindlimb motor neurons during walking. In a quantitative assessment of motor activity, the normal alternation of activity in ipsilateral extensor (gastrocnemius, Gs) and flexor (tibialis anterior, TA) muscles is preserved, as is the normal alternation of activity in ipsilateral and contralateral extensor (TA) muscles. The loss of input from C boutons, however, became apparent during swimming. The peak activity in EMG recordings during swimming (6-fold higher than during walking in wild-type mice) is reduced by about 40% in the deletion mutant. This reduction is comparable to the 20%–40% reduction in burst amplitude observed with muscarinic m2 receptor

blockade during fictive motor activity in the isolated cord (Miles et al., 2007). The observation that only a portion of the increased activity in Gs motor neurons during swimming is lost in these mice makes clear that multiple mechanisms contribute to the modulation of motor activity during locomotion. Nevertheless, Zagoraïou and coworkers have demonstrated that this combination of molecular genetic techniques, electrophysiological recordings and quantitative assessments of behavior is able to unravel the role of a specific restricted class of interneurons in controlling and modulating behavior. The 40 year mystery of the function of C boutons is solved.

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Rab3 GTPase Lands Bruchpilot

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Active zones are the sites of neurotransmitter release, but their assembly mechanisms are poorly understood. In this issue of *Neuron*, Graf et al. perform a genetic screen in *Drosophila* and uncover a novel role for the Rab3 GTPase in organizing the active zone at the neuromuscular junction.

Neurons communicate signals to postsynaptic cells through asymmetric, intercellular junctions called synapses. At chemical synapses, such as the fly larval neuromuscular junction (NMJ), the presynaptic terminal is at the end of the nerve axon, containing neurotransmitter-laden synaptic vesicles that cluster around specialized regions, named active zones (AZs) (Figure 1A). The AZ is also the site where voltage-gated calcium (Ca²⁺) channels cluster (Figure 1A). Opening of the Ca²⁺ channels upon arrival of an action potential at the terminal allows

entry of Ca²⁺, which activates the exocytic machinery and promotes the fusion of synaptic vesicles that are docked and primed for release. The release of neurotransmitters in the synaptic cleft activates postsynaptic receptors that are juxtaposed to the AZ. Hence, the subcellular organization of AZ and postsynaptic receptors ensures an efficient coupling of transmission. The assembly site and composition of AZ are therefore important aspects of synapse development and function (Jin and Garner, 2008; Kittel et al., 2006).

At the ultrastructural level, AZs are readily distinguished by electron-dense proteinaceous material (the cytoplasmic matrix at the AZ, or CAZ), which acquires characteristic shapes at different synapses, as for example the T bar-shaped structure observed at the fly NMJ (Figure 1B) (Jin and Garner, 2008). The poor solubility of CAZ components has hindered their biochemical purification and identification. However, by combining genetic, molecular, electrophysiological, and imaging approaches, key properties of the CAZ proteins have recently been