

Hb9 Versus Type 2 Interneurons

Lea Ziskind-Conhaim and Christopher A. Hinckley

Department of Physiology and Center for Neuroscience, University of Wisconsin Medical School, Madison, Wisconsin

TO THE EDITOR: In a recent issue of the *Journal of Neurophysiology*, Wilson, Cowan, and Brownstone (2007) investigated the incidence of electrical coupling between different spinal interneurons. Both Wilson et al. and our group use the Hb9: GFP transgenic mouse line that was generated in the laboratory of Thomas Jessell. In a recently published report, Wilson et al. (2005) proposed that these mice have two populations of green fluorescent protein (GFP)-positive interneurons in medial lamina VIII. One population expresses the HB9 protein (Hb9 interneurons [Hb9 INs]) and the second group does not. They called the second group of interneurons “type 2 interneurons” (type 2 INs). Based on that report, Hb9 and type 2 INs differ in their morphological and electrophysiological properties.

We have recently demonstrated abundant electrical coupling (>80%) and synchronous activity between Hb9 INs in both newborn and juvenile mice (Hinckley and Ziskind-Conhaim 2006). Wilson et al. (2007) could not confirm our findings, and therefore concluded that the electrical connections we demonstrated were probably between type 2 INs.

In their paper, Wilson et al. (2007) used calcium imaging and whole cell paired recordings to demonstrate synchronous calcium oscillations between Hb9 INs. However, current transfer by intracellular current injection (see Fig. 2) was not apparent. When recording from type 2 INs, they reported that only one of five pairs was electrically coupled. In addition, they found electrical coupling in only one of nine pairs of Hb9 and type 2 INs. Based on their data of synchronous calcium waves and a total of two electrically coupled pairs, they proposed that the observed synchrony of calcium oscillations resulted from electrical coupling between Hb9 INs and adjacent neurons, but not coupling among Hb9 INs.

We do not dispute the authors' conclusion that Hb9 INs might be electrically coupled to other GFP-positive neurons. We did not investigate this interesting possibility. However, their conclusion that we recorded from type 2 INs does not take into account the strict criteria, in addition to antibody staining, that we use to identify Hb9 INs. These criteria are: high-input resistance, the absence of hyperpolarization-dependent depolarization sag, small cell size, and their distribution along the midline in lamina VIII.

We believe that Wilson et al. reached the wrong conclusion for the following reasons.

1) Wilson et al. characterized type 2 INs by their hyperpolarization-dependent depolarization sag (see Fig. 1). As we emphasized in two earlier papers (Hinckley and Ziskind-Conhaim 2006; Hinckley et al. 2005), one of the criteria that we used to identify Hb9 INs was the linear current–voltage (I – V) relation at membrane potentials between -60 and -180 mV.

Traces from Figs. 1 and 2 of our 2006 paper are shown here to demonstrate this point. Hyperpolarization-dependent depolarization sags were not recorded in GFP-positive/Hb9-positive neurons, but were obvious in GFP-negative Hb9-negative neurons adjacent to Hb9 INs. We did not use data from neurons in which membrane hyperpolarization evoked depolarization sag.

We do occasionally record from GFP-negative neurons, as we realize after processing the tissue for neurobiotin labeling. We use the labeling to identify the neurons from which we recorded. However, we do not use data from cells if negative current injections generate depolarization sags.

2) Wilson et al. suggested that we recorded from neurons larger than theirs as additional support that we used data from type 2 INs. Their conclusion is based on images in our publication of neurobiotin-filled neurons (Hinckley et al. 2005; Figs. 8 and 9). Unfortunately, Wilson et al. did not provide corresponding images of their own, which would have allowed readers to judge for themselves whether their conclusions were justified. There is only one image of the cell body of type 2 IN (Wilson et al. 2005; Fig. 6). The morphological properties of type 2 INs remain a mystery because they have never appeared in the literature. It should be noted that the cell bodies shown in Wilson et al.'s earlier report (Wilson et al. 2005; Fig. 5F) closely resemble the cell bodies of our Hb9-positive interneurons (Hinckley et al. 2005; Figs. 2D and 4; see Figs. 3 and 4). If there is a difference in size, it is very small, in the range of 2–3 μ m.

It should be emphasized that we record from Hb9 INs in longitudinally hemisectioned spinal cords. Because the hemicord is placed with the medial side up, we cannot visualize GFP-expressing interneurons for whole cell recordings (using differential interference contrast optics) if they are distributed laterally to Hb9 INs.

Moreover, based on input resistance measurements, it is unlikely that our Hb9 INs are larger in size than the neurons from which Wilson et al. recorded. The input resistance of our neurons is high, frequently >1 G Ω (Hinckley and Ziskind-Conhaim 2006). Similar to our findings, Wilson et al. (2005) stated that the average input resistance of Hb9 INs is 900 M Ω . The input resistance of type 2 neurons is significantly lower (600 M Ω).

3) We use immunohistochemical techniques different from those used by Wilson et al. to identify Hb9 expression in medial lamina VIII interneurons. We reported that 3–9 interneurons/100- μ m sections are Hb9-positive in postnatal mice (Hinckley et al. 2005; see Fig. 3). Wilson et al. (2005) counted an average of 4 ± 2 Hb9-positive neurons in 30- μ m sections in spinal cords of juvenile mice. In more recent studies we counted 3.3 ± 1.6 (SD, $n = 3$ cords) Hb9 INs/70- μ m section in spinal cords of P9 mice (unpublished data) with a maximum of 7 Hb9 IN/section.

4) We attempted to demonstrate neurobiotin transfer between coupled Hb9 INs, but dye-coupling was not evident in our experiments, and thus we did not discuss it in our 2006

Address for reprint requests and other correspondence: L. Ziskind-Conhaim, Department of Physiology and Center for Neuroscience, University of Wisconsin Medical School, Madison, WI 53706 (E-mail: lconhaim@physiology.wisc.edu).

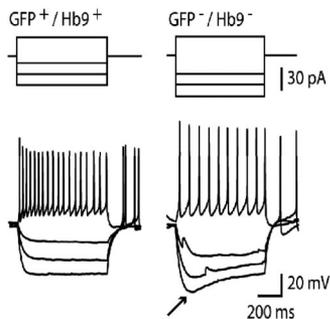


FIG. 1. Voltage responses to positive and negative currents in GFP⁺/Hb9⁺ neuron and GFP⁻/Hb9⁻ neuron. Lower input resistance and transient sags (arrow) characterize the GFP⁻/Hb9⁻ neuron.

paper. Apparently Wilson et al. detected extensive dye-coupling between type 2 INs (stated as data not shown in Wilson et al. 2007).

5) Wilson et al. concluded that we probably recorded from their type 2 INs, but this does not explain why we demonstrated that five of six pairs were coupled in juvenile mouse, whereas they recorded current transfer from only one of five pairs of type 2 INs. We conducted experiments only if membrane potentials were more negative than -50 mV and input resistances were ≥ 700 M Ω .

Based on their Fig. 5C, the input resistance of the Hb9 IN was 300 M Ω , threefold lower than the 900 M Ω reported in their 2005 paper. The input resistance of the type 2 IN was 200 M Ω , threefold lower than the input resistance reported in that paper. Moreover, we estimated that the coupling coefficient in that pair ranged from 0.5 to 3% (Wilson et al. 2007; Fig. 5B). This is significantly lower than the values reported by most other investigators. We reported that the average coupling coefficient between Hb9 INs was 12%.

6) Dr. Brownstone recently asked permission to use one of our published recordings that illustrated membrane oscillations and firing in Hb9 INs in phase with L2 motor output. We were delighted to grant this permission for a review that he was preparing. In Fig. 1 of that review (Brownstone and Wilson 2008) he placed our figure next to an image of GFP-positive cells labeled with Fos protein following locomotor activity. His figure legend implies that Fos protein-labeled neurons are the same Hb9 INs illustrated by our figure. If he believes we were recording from type 2 INs, then it is not clear why he would choose our figure to illustrate membrane oscillations in Hb9 INs.

In summary, we applied several rigorous criteria for the identification of Hb9 INs that we found to be electrically

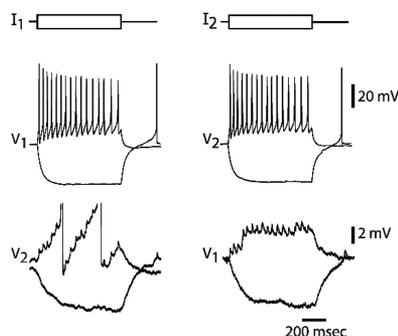


FIG. 2. Whole cell paired recording in Hb9 interneurons (INs). Flat hyperpolarizations with no transient sags were recorded in electrically coupled Hb9 INs.

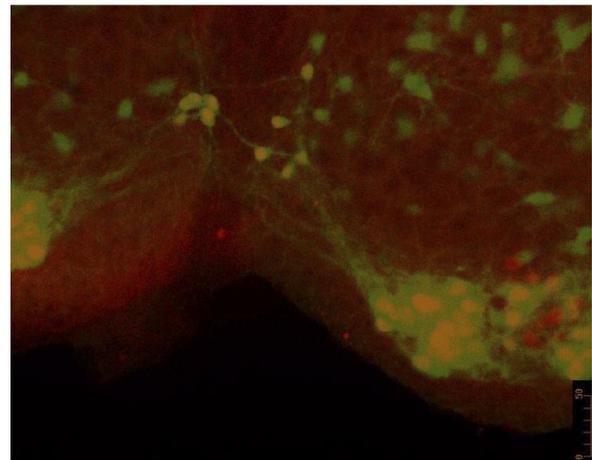


FIG. 3. Where are the type 2 INs? Transverse slice of lumbar segment L2 of a P2 mouse stained with Hb9 antibody (red). The coexpression of the Hb9 protein and green fluorescent neuron (GFP, yellow) is apparent in medial lamina VIII interneurons (Hb9 INs) and motoneurons.

coupled. To explain the absence of electrical coupling in the recordings of Wilson et al. other explanations must be considered. These include: low-input resistance of the neurons recorded from, loss of electrical coupling in the process of slice preparation, different extracellular and pipette solutions, and higher temperature.

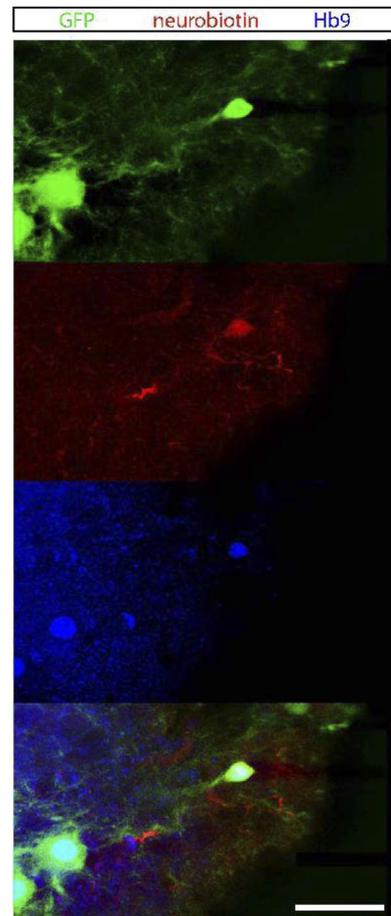


FIG. 4. Expression of the Hb9 protein and GFP in medial lamina VIII interneuron that was labeled with neurobiotin during whole cell recording. The diameter of this Hb9 IN is 11 μ m (scale bar: 50 μ m).

Thank you for your attention.

REFERENCES

Brownstone RM, Wilson JM. Strategies for delineating spinal locomotor rhythm-generating networks and the possible role of Hb9 interneurons in rhythmogenesis. *Brain Res Rev* 57: 64–76, 2008.

Hinckley CA, Hartley R, Wu L, Todd A, Ziskind-Conhaim L. Locomotor-like rhythms in a genetically distinct cluster of interneurons in the mammalian spinal cord. *J Neurophysiol* 93: 1439–1449, 2005.

Hinckley CA, Ziskind-Conhaim L. Electrical coupling between locomotor-related excitatory interneurons in the mammalian spinal cord. *J Neurosci* 26: 8477–8483, 2006.

Wilson JM, Cowan AI, Brownstone RM. Heterogeneous electrotonic coupling and synchronization of rhythmic bursting activity in mouse Hb9 interneurons. *J Neurophysiol* 98: 2370–2381, 2007.

Wilson JM, Hartley R, Maxwell J, Todd AJ, Lieberam I, Kaltschmidt JA, Yoshida J, Jessell TM, Brownstone RM. Conditional rhythmicity of ventral spinal interneurons defined by expression of the Hb9 homeodomain protein. *J Neurosci* 25: 5710–5719, 2005.