Hb9 Interneurons: Reply to Ziskind-Conhaim and Hinckley

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REPLY: We thank Drs. Ziskind-Conhaim and Hinckley for their comments and are appreciative that they find our paper of interest. We, too, find it interesting that the conclusions of our two groups differ.

We initially provided indirect evidence of electrotonic coupling involving Hb9 interneurons in a previous paper (Wilson et al. 2005) and subsequently proceeded to characterize this coupling. In our 2007 Journal of Neurophysiology paper (Wilson et al. 2007a), we demonstrate that there is heterogeneous electrotonic coupling between Hb9 interneurons and non-Hb9 interneurons. We were unable to find evidence of electrotonic coupling between Hb9 interneurons (Wilson et al. 2007a), which most frequently are found in small clusters or pairs in medial lamina VIII or ventral lamina X (Wilson et al. 2005). On the contrary, Hinckley and Ziskind-Conhaim (2006) reported electrotonic coupling between GFP+ neurons in this region in this same strain of mouse. There are several possible explanations for the seemingly contradictory results between the two groups, many of which we outlined in our paper (Wilson et al. 2007a).

We began working with fluorescent protein (XFP)-expressing animals about 6 yr ago and, as physiologists, were surprised to learn that in the various transgenic animals, XFP expression does not necessarily correspond to protein expression. In the Hb9:eGFP line, for example, there are many GFP+ neurons in which Hb9 does not appear to be expressed (Wilson et al. 2005). For this reason, and because we found immunohistochemistry against Hb9 unreliable in juvenile mice, we crossed Hb9lacZ (knock-in) mice into the Hb9::eGFP (transgenic) line to unambiguously identify Hb9 interneurons (through β-gal expression) in anatomical preparations. Further, these crosses enabled us to establish that the GFP+ interneurons we were recording were definitively Hb9+. These mice facilitated the characterization of the electrophysiological properties of Hb9 interneurons and the comparison of their properties to nearby GFP+, Hb9− neurons. We termed these GFP+ Hb9− neurons, which had electrophysiological and morphological properties different from those of GFP+ Hb9+ interneurons, “Type 2” interneurons for lack of more specific details on which to base a name (e.g., transcription factors expressed, synaptology). Our studies have focused primarily on Hb9 neurons, because they can be genetically defined, rather than on Type 2 interneurons.

We concluded that the discrepancy between results from the two groups is most likely a consequence of the recording of different neuronal populations in these mice. We have outlined in detail the anatomy and physiology of the Hb9 interneurons in medial lamina VIII or ventral lamina X (Wilson et al. 2005), and it is neurons that satisfy these criteria that we continued to study in Wilson et al. (2007a). In the juvenile mouse, we do see few solitary Hb9 interneurons that do not meet these anatomical criteria (e.g., there is one on the right-hand side of the spinal cord in Fig. 1D in Wilson et al. 2007a). However, we have not characterized interneurons that we cannot definitively identify and thus know nothing of their physiology. It is possible that Hinckley and Ziskind-Conhaim (2006) have targeted these solitary, ventral interneurons that express Hb9. However, as can be seen in the figures from both groups, there are many more neighboring GFP+, Hb9− interneurons in these mice. It therefore seems to be a reasonable explanation that, in the hemisected early postnatal mouse, these GFP+ non-Hb9 interneurons were targeted for study. Further, it is possible (as discussed in our paper) that in the younger mice, Hb9 (and thus GFP) is transiently expressed in these neurons. We must emphasize that the interneurons that we study and call “Hb9 interneurons” continue to express Hb9 into adulthood and have known, recognizable anatomical and physiological properties.

It can be seen clearly both in our earlier work (Wilson et al. 2005) and in that of Hinckley et al. (2005), and whether using lacZ knock-in animals (Fig. 1 in Wilson et al. 2007a) or anti-Hb9 immunohistochemistry (Fig. 2, C and D in Hinckley et al., 2005), that there are GFP+, Hb9− neurons in the region of Hb9 interneurons. This is particularly evident in the early postnatal period (see Fig. 1 in Wilson et al. 2007a). To ensure that we were recording from Hb9+ GFP+ neurons, we therefore developed and presented electrophysiological criteria for the identification of these Hb9 interneurons (Wilson et al. 2005). Hinckley and Ziskind-Conhaim (2006) adopted this electrophysiological characterization of Hb9 interneurons.

In point 2 of their letter, Ziskind-Conhaim and Hinckley emphasize that they record in the longitudinally hemisected spinal cord, in which they have some problems with light penetration. Because their supporting images are all in transverse planes, the degree to which they can identify GFP, morphology, and laminar location in their longitudinal preparation is not clear. This may also explain why they sometimes record from GFP− neurons. We have not recorded from GFP− neurons in this region, as have Hinckley and Ziskind-Conhaim, and therefore cannot comment on their properties. Perhaps these GFP-interneurons are coupled to Hb9 interneurons.

We used a slice preparation for the dual-cell patch-clamp experiments and acknowledged in our paper (Wilson et al. 2007a) that the different dissections could lead to differences in the extent of electrical coupling between pairs of recorded neurons. However, this does not explain the fact that even in the slice there was electrotonic coupling, and this coupling does not arise from Hb9 interneurons.

Similar to Hinckley and Ziskind-Conhaim, we also have difficulty definitively identifying Hb9 interneurons in the
hemisected spinal cord at early postnatal ages. Therefore in our studies using two-photon excitation microscopy (which would be technically easier in the hemisected cord; see Wilson et al. 2007b), we have used the whole isolated spinal cord and imaged in the coronal plane. As shown in our recent paper (Wilson et al. 2007a), in this plane of imaging, Hb9 interneurons can readily be identified.

In our paper, we demonstrated that Hb9 interneurons are electrotonically coupled to other neurons but we could not demonstrate direct electrotonic coupling between recorded pairs of Hb9 interneurons. Although we initially expected Hb9 interneurons to be coupled to each other, our calcium imaging experiments revealed a number of noncoincident bursts (“failures”) in Hb9 interneurons, both in the slice and in the whole isolated spinal cord. This demonstrated the necessity of proceeding with dual-cell patch-clamp experiments, which revealed a lack of DC transfer between pairs of Hb9 interneurons. It is unfortunate that in their letter, Ziskind-Conhaim and Hinckley do not provide an alternative explanation for the “failures” of coincident calcium transients that we recorded (see also Fig. 6B in Hinckley and Ziskind-Conhaim 2006). Would it not be unlikely to record such failures if there is extensive electrotonic coupling? To investigate potential sources of this heterogeneous coupling, we recorded from non-Hb9 “Type 2” GFP positive neurons and demonstrated these to be one source of this coupling. Whether there are also other neurons coupled to Hb9 interneurons has yet to be investigated.

We have found that in most Hb9 interneurons recorded at room temperature in the slice, the input resistance is around 1 GΩ (Wilson et al. 2005), a value also reported by Ziskind-Conhaim and Hinckley. We did our dual patch-clamp experiments at 30–32°C. Because it has been well documented that input resistance decreases with increasing temperature (for example, Guatteo et al. 2005; Heitler and Edwards 1998; Lee et al. 2005), we were not surprised that our recorded input resistances were somewhat lower than we and Hinckley and Ziskind-Conhaim had previously shown. Further, with respect to the reported input resistances in Hinckley and Ziskind-Conhaim (2006), we were surprised that with a coupling incidence of about 80% and a coupling coefficient of about 12%, these investigators found an associated input resistance of about 1 GΩ. With this sort of coupled network, we would expect the input resistance to be much lower (for example, see McMahon et al. 1989; Rorig et al. 1995; Takeda et al. 2005). It would seem that our findings of a lower coupling incidence, lower coupling coefficients, and moderate input resistances present a coherent picture.

We are grateful for the permission received to use a panel from Fig. 6 of Hinckley et al. (2005) in a recent review (Brownstone and Wilson 2008). This was used to demonstrate that GFP+ neurons in this region are rhythmically active during rhythmic ventral root output. We chose, as indicated in the text of our review, to use this figure to illustrate the rhythmicity recorded in GFP+ neurons during chemically induced rhythmic motor output. Because we have demonstrated that Hb9 interneurons are electrotonically coupled to nearby GFP+ Hb9− (Type 2) neurons, we would expect both of these populations to have synchronous membrane potential oscillations during chemically induced rhythmic activity. Therefore whether the illustrated neuron is Hb9+ or Hb9− (e.g., Type 2) is not significant to our interpretation. We appreciate the authors’ permission to use this figure.

In our 2007 paper, we offer several explanations as to the differences in the results between the studies of the two groups. Given that we find evidence of electrotonic coupling of Hb9 interneurons with other neurons—but, despite extensive sampling, cannot find evidence that Hb9 interneurons are coupled to each other in the more mature mouse—we concluded that the two groups must be recording from different populations of interneurons. An additional explanation to be considered is that, as stated in our paper (Wilson et al. 2007a), “Type 2” interneurons express Hb9 only at early developmental stages and continue to express GFP despite the lack of Hb9 expression at later developmental stages. It is known that the ion channels of some spinal neurons change during the first two postnatal weeks (e.g., Jiang et al. 1999; Song et al. 2006; Wilson et al. 2004) and thus the possibility exists that these “Type 2” interneurons develop a sag potential during early postnatal development. With the acknowledged difficulty of light penetration in the older hemisected spinal cords used by Hinckley and Ziskind-Conhaim and the difficulty in obtaining whole cell recordings in that preparation (Hinckley and Ziskind-Conhaim 2006), it would be useful to know whether the neurons they recorded from P10–P11 mice met the same electrophysiological criteria as in the younger mice and whether they were labeled immunohistochemically with anti-Hb9 antibody.

This is not the first time in the history of science that two research groups have seemingly contradictory results, nor will it be the last time. The data from the two groups have now been published and possible explanations for the differences have been provided. Because these particular interneurons are now being studied by a number of additional groups, it is likely that definitive explanations for these differences will be provided in time. Indeed, given the fact that Hb9 interneurons and their electrotonically coupled neighbors may be of fundamental importance to mammalian locomotor rhythm generation, we hope that Drs. Hinckley and Ziskind-Conhaim agree with us that definitively resolving the reasons why the underlying conclusions differ may provide clues not only as to how adult spinal locomotor networks are assembled during development but also as to how they function.

**REFERENCES**


