Topographical and Physiological Characterization of Interneurons That Express Engrailed-1 in the Embryonic Chick Spinal Cord

PETER WENNER, MICHAEL J. O’DONOVAN, AND MICHAEL P. MATISE

INTRODUCTION

The last few years have witnessed a major advance in our understanding of the molecular mechanisms controlling the specification of spinal motoneurons during development. This progress has occurred because it has been possible to identify the expression of certain transcription factors in different populations of motoneurons (Lin et al. 1998; Tsuchida et al. 1994). Two families of transcription factors have been implicated in controlling the differentiation of various types of neurons including spinal motoneurons. Some of these proteins are also expressed in spinal interneurons, but their function is unknown. Progress in understanding the role of transcription factors in interneuronal development has been slow because the synaptic connections of interneurons, which in turn define their identity, are difficult to establish. Using whole cell recording in the isolated spinal cord of chick embryos, we assessed the synaptic connections of lumbar and sacral interneurons expressing the Engrailed-1 (En1) transcription factor. Specifically we established whether En1-expressing interneurons made direct connections with motoneurons and whether they constitute a single interneuron class. Cells were labeled with biocytin and subsequently processed for En1 immunoreactivity. Our findings indicate that the connections of En1-expressing cells with motoneurons and with sensory afferents were diverse, suggesting that the population was heterogeneous. In addition, the synaptic connections we tested were similar in interneurons that expressed the En1 protein and in many that did not. The majority of sampled En1 cells did, however, exhibit a direct synaptic connection to motoneurons that is likely to be GABAergic. Because our physiological methods underestimate the number of direct connections with motoneurons, it is possible that the great majority, perhaps all, En1-expressing cells make direct synaptic connections with motoneurons. Our results raise the possibility that En1 could be involved in interneuron-motoneuron connectivity but that its expression is not restricted to a distinct functional subclass of ventral interneuron. These findings constrain hypotheses about the role of En-1 in interneuron development and function.

Wenner, Peter, Michael J. O’Donovan, and Michael P. Matise. Topographical and physiological characterization of interneurons that express Engrailed-1 in the embryonic chick spinal cord. J Neurophysiol 84: 2651–2657, 2000. A number of homeodomain transcription factors have been implicated in controlling the differentiation of various types of neurons including spinal motoneurons. Some of these proteins are also expressed in spinal interneurons, but their function is unknown. Progress in understanding the role of transcription factors in interneuronal development has been slow because the synaptic connections of interneurons, which in turn define their identity, are difficult to establish. Using whole cell recording in the isolated spinal cord of chick embryos, we assessed the synaptic connections of lumbar and sacral interneurons expressing the Engrailed-1 (En1) transcription factor. Specifically we established whether En1-expressing interneurons made direct connections with motoneurons and whether they constitute a single interneuron class. Cells were labeled with biocytin and subsequently processed for En1 immunoreactivity. Our findings indicate that the connections of En1-expressing cells with motoneurons and with sensory afferents were diverse, suggesting that the population was heterogeneous. In addition, the synaptic connections we tested were similar in interneurons that expressed the En1 protein and in many that did not. The majority of sampled En1 cells did, however, exhibit a direct synaptic connection to motoneurons that is likely to be GABAergic. Because our physiological methods underestimate the number of direct connections with motoneurons, it is possible that the great majority, perhaps all, En1-expressing cells make direct synaptic connections with motoneurons. Our results raise the possibility that En1 could be involved in interneuron-motoneuron connectivity but that its expression is not restricted to a distinct functional subclass of ventral interneuron. These findings constrain hypotheses about the role of En-1 in interneuron development and function.

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into the motor column (Sauressig et al. 1999). These and other studies have led to the hypothesis that En1+ cells make monosynaptic connections with motoneurons (Sauressig et al. 1999).

In this paper we have directly tested this hypothesis by examining the synaptic connections between En1+ cells and motoneurons. We have also addressed the question of whether En1+ cells constitute a single functional class (defined by their synaptic connections with motoneurons and dorsal root afferents) or whether En1 expression occurs in different interneuronal types. Some of this work has been published in abstract and in meeting proceedings (Wenner et al. 1998a,b).

**METHODS**

**Physiology and biocytin labeling**

Chick embryos were removed from the egg at embryonic days 10 to 11 (E10–E11; stage 36–37) and staged according to the criteria of Hamburger (Hamburger and Hamilton 1951). Embryos were decapitated and the spinal cords isolated as described previously (O’Donovan 1989; O’Donovan and Landmesser 1987) in recirculating cold (12–15°C) Tyrode’s solution (concentration in mM: 139 NaCl, 2.9 KCl, 17 NaHCO3, 12 glucose, 3 CaCl2, and 1 MgCl2). The spinal cord was isolated together with certain muscle nerves (adductors and obturator; femorotibialis = external and medial head). The perfusion solution was slowly brought to room temperature (~21°C), and the cord was transferred to a recording chamber for at least 2 h, before raising the temperature to ~27°C for the remainder of the experiment. For overnight experiments, where recordings were obtained throughout the next day, the temperature was brought to 17°C and left for up to 12 h before beginning the experiment. Afferent muscle nerves (where motoneuron axons have been cut at the ventral root), ventral roots, and a strip of the ventrolateral funiculus (VLF) were drawn into suction electrodes for stimulation and/or recording. Whole cell electrodes (4–8 MΩ, with a K gluconate solution concentration in mM: 10 NaCl, 130 K gluconate, 10 HEPES, 1.1 EGTA, 0.1 CaCl2, 1 MgCl2, and 1 Na2ATP) were driven through the ventral white matter into the ventral horn (after removal of the pia) as described previously (Sernagor and O’Donovan 1991; Wenner and O’Donovan 1999). Recordings were made along the anterior lumbosacral spinal cord between thoracic 7 and lumbosacral 3. Spinal segments were identified as described previously (Matise and Joyner 1999). Three separate embryos were counted at each stage, and the counts were averaged.

The schematic in Fig. 4 was created by making camera lucida tracings of biocytin-labeled cells in each preparation. To combine the data onto a single image, traces were aligned using the ventrolateral margin of the gray matter, and the outlines (white matter and cells) were scaled using the central canal as a second reference point. All data are expressed as means ± SD.

**Image and data processing**

Images were captured on a Princeton Instruments cooled charge-coupled device (CCD) camera and processed in Metamorph software (Universal Imaging) as previously described (Matise and Joyner 1997).

**RESULTS**

**Distribution of En1+ cells in the ventral cord between E7 and E10**

Previous studies in mouse and chick spinal cords have shown that En1 interneurons migrate ventrally and laterally toward the ventral horn after they are born in the ventricular zone at an intermediate dorsoventral position (Gardner et al. 1988; Matise and Joyner 1997). In the present work, we found that the labeled cells lie dorsal to the lateral motor column with few, if any, within the motor column at E7. A few days later at E10, En1 expression was detected in fewer cells per 10 µM section than at E7 measured between segments T7-L3 (E10: 99 ± 20 cells/section, mean ± SD, n = 25 sections; E7: 168 ± 26, n = 18 sections; Fig. 1, A and B). In addition, at E10 the intensity of En1 labeling varied between cells as illustrated in the micrographs of Fig.
These observations suggest that En1 expression is extinguishing in some cells by E10. Alternatively, or in addition, En1 expression might differ between cells and be extinguished later in development.

Distribution and morphology of biocytin labeled En1 expressing interneurons

To compare the connectivity of both En1+ and En1– spinal interneurons, we made whole cell recordings from randomly sampled ventral/intermediate zone neurons. Recording electrodes were filled with a solution containing 0.5% biocytin to label cells for later histological identification. After the recording, the lumbosacral spinal cord was fixed, serially sectioned, and stained for En1 protein and biocytin (see METHODS).

Using this protocol, we identified 62 biocytin-labeled cells in 9 different embryos. Forty-two of these cells had acceptable physiological recordings (for criteria, see METHODS) and were examined further. Nineteen of these 42 cells were labeled with biocytin and also expressed En1 (En1+/bio+). Examples of two double-labeled cells are shown in Fig. 2, B–D (cell 1) and E–G (cell 2). En1+ and En1– biocytin-labeled cells were in similar positions and were widely distributed in the ventral horn, both dorsal and medial to motoneurons (Fig. 4A). No obvious correlation was observed between the location of En1+/bio+ double-labeled cells and their morphology or synaptic connections (see next section).

![Figure 1](image1.png)

**FIG. 1.** En1 expression in the lumbosacral segment (LS) spinal cord at embryonic day 7 (E7) and E10. A and B: transverse sections through LS 3 spinal segments at E7 (A) and E10 (B) stained with antibodies to En proteins (green). Images show ventral hemi-cord. Many En1+ cells migrate ventrally after they are generated from the region of the ventricular zone (VZ) between white arrowheads. B: transverse section through LS3 at E10 showing the position of En1+ cells dorsal to the lateral motor column (LMC) (outlined in yellow) at mid-gestation stages. There are fewer En1+ cells per 10-μM section at E10 than at E7. C: high power view showing that En1 protein is detected at different levels in cells at E10. Cells expressing higher levels of En1 protein (white arrowheads) are intermixed with those expressing lower levels (yellow arrowheads). Dorsal is to the top in all figures. The margins of the gray and white matter are outlined in white, and the LMC in yellow in A–C. Scale bar, 20 μM.

![Figure 2](image2.png)

**FIG. 2.** En1 protein expression in biocytin-labeled, recorded interneurons. A1: transverse section double-labeled to reveal En1 protein (red) and biocytin (green). Biocytin labeling can be seen in the cytoplasm of the cell, filling both putative dendrites (white arrowheads) and axon (yellow arrowhead), which is projecting in the direction of the ventral funiculus. A2: region of spinal cord enlarged. B–G: 2 examples of co-localization of En1 antibody (α-Engrailed) and biocytin in ventral interneurons. B–D and E–G show 2 separate double-labeled cells. B and E: biocytin labeling. E and F: same section showing En1 expression. D and G: merged images showing overlap of staining for biocytin and En1 in a single En1+ cell in the field (arrow). Scale bar, 120 μM for A and 17 μM for B–G.
Physiological examination of the synaptic connections of En1 expressing interneurons

The 19 En1+/bio+ cells could be divided into two groups using the spike-triggered average ventral root potential as a marker for monosynaptic connections with motoneurons. According to this criterion, 11 cells made monosynaptic connections with motoneurons (Fig. 3A). Another cell was identified by its direct recurrent input from motoneurons and was therefore an R-interneuron (as defined by Wenner and O'Donovan 1999). These cells are known to make depolarizing GABAergic connections to motoneurons (Wenner and O'Donovan 1999). Therefore 63% (12/19) of the sample made monosynaptic connections with motoneurons confirming the hypothesis, first proposed in the mouse, that some En1 cells project directly to motoneurons (Sauressig et al. 1999).

We tested 4/12 of these last order interneurons pharmacologically and determined that all of them were GABAergic because the spike-triggered ventral root potential disappeared following bath application of the GABAA antagonist bicuculline (Fig. 3A). Two additional neurons were assumed to be GABAergic because they were identified as R-interneurons, for a total of six GABAergic En+ cells. This functional evidence is consistent with immunocytochemical data from the mouse suggesting that En1+ cells are GABAergic (Sauressig et al. 1999).

The second group of En1+/bio+ interneurons comprised seven cells (37%; 7/19) in which we were not able to resolve a ventral root potential by spike-triggered averaging. The anatomical distribution of this group was no different from that of the first group (Fig. 4A).

Twenty-three of the 42 biocytin-labeled cells that we recorded from did not express the En1 protein. Of these, seven cells (30%; 7/23) projected monosynaptically to motoneurons as determined by spike-triggered averaging. Two of seven of these cells appeared to be GABAergic based on the abolition of the averaged ventral root potential by bicuculline. Collectively, these results suggest that En1+ interneurons had a greater tendency to project to motoneurons than did randomly sampled En1− ventral horn interneurons, although the difference was not statistically significant (P = 0.07; Z-test). We found that En1−/bio+ and En1+/bio+ cells were distributed similarly in the intermediate region of the cord, dorsal to the lateral motor column (LMC) (Fig. 4A).

We also measured the muscle sensory input to spinal interneurons to determine whether En1+ cells could be distinguished by their inputs from different muscle nerves (Figs. 3B

![FIG. 3. Physiological assessment of En1+ interneurons.](http://jn.physiology.org/)
Whole cell and ventral root recordings are shown on the left; schematic representation of the recording configuration is shown on the right. A: interneuron spike (bottom trace, control) was used to trigger the acquisition of ventral root recordings that were then averaged; the number of averaged recordings is shown in parentheses. The ventral root potential, indicative of a synaptic connection to motoneurons observed in control conditions, was abolished in the presence of the GABAA antagonist bicuculline, suggesting the cell was GABAergic. B: whole cell recordings of an En1+ interneuron show that this cell receives strong short latency input from adductor muscle afferents, but only weak input from the femorotibialis muscle afferents. C: all-or-none action potential (42 vs. 41 µA stimulation, respectively) was observed in a whole cell recording of an En1+ interneuron following ventrolateral funiculus (VLF) stimulation, suggesting that the cell projected its axon into the VLF. Because VLF stimulation generates a synaptic potential in interneurons, proof that this spike is antidromic is difficult. Additional evidence supporting antidromic activation is shown in the bottom trace. In an En1+ interneuron a clear A/B break (arrowhead) can be seen in the antidromic action potential.)
and 4B). For this purpose, we stimulated the femorotibialis or adductor muscle afferents, which are known to make connections with different classes of motoneurons (Lee and O’Donovan 1991; Mendelson and Frank 1991; Wenner and Frank 1995). Such stimuli are likely to activate many different functionally distinct classes of afferent within a muscle nerve including those making monosynaptic connections with motoneurons (Lee et al. 1988). We found that the En1+ and En1− interneurons received very similar patterns of afferent input (Fig. 4B): Short-latency (−10 ms, probably monosynaptic) input from femorotibialis muscle afferents was observed in 9/15 En1+ neurons and 9/15 En− cells. Similarly 7/13 En1+ versus 9/13 En1− cells received short latency adductor input; 6/13 En1+ versus 6/13 En1− cells received inputs from both muscle afferents, and 3/13 En1+ versus 3/13 En1− cells received from neither. We also found similar patterns of afferent input in last order interneurons and interneurons that did not make a detectable connection to motoneurons. Thus the expression of En1 does not distinguish between last order interneurons based on the muscle afferent inputs we examined. A summary of the above physiological data is provided schematically in Fig. 4B.

In a few cells, evidence for an axonal projection into the VLF was obtained by stimulating a strip of VLF drawn into a suction electrode and evoking a short-latency, all or none, action potential (Fig. 3C). In two of the En1+ and six of the En1− interneurons we were able to record such a potential. Although these spikes were evoked in an all-or-none manner, it is difficult to be certain that they originated antidromically because VLF stimulation subthreshold for the spike, invariably produced a synaptic potential and often triggered an episode (Fig. 3C, top panel). However, in five cells (1 En+, 4 En−), the VLF stimulus evoked action potential exhibited a clear A/B break consistent with antidromic activation (arrowhead in Fig. 3C, bottom trace).

**DISCUSSION**

In this study we have combined physiological and immunohistochemical approaches to examine the characteristics of ventral interneurons that express the En1 homeodomain-containing transcription factor. We have addressed two questions about this population. First, do some, or all, of the cells make monosynaptic connections with motoneurons? Second, does En1 mark a unique interneuronal population? Our results show that many En1+ cells project to motoneurons and that the population is not uniform but instead exhibits a diverse pattern of synaptic connectivity.

**En1 expression marks a diverse population of ventral interneurons**

Several lines of evidence suggest that En1 expression does not identify a unique interneuronal class. First, the synaptic connections of En1+ and En1− interneurons were similar. Second, the connections of En1+ cells were extremely variable. En1+ cells exhibited a wide range of muscle afferent input; some received input from muscle afferents projecting to one muscle (Fig. 3B), some from a functionally distinct muscle, some from both, and some from neither. While these findings suggest that specific patterns of muscle afferent input are not associated with En1 expression, we cannot exclude the possibility that connections from an untested muscle nerve or different classes of afferent within a single muscle nerve could be associated with the expression of En1. Third, there is evidence suggesting that while some R-interneurons express En1, not all do. We found that two of the En1+ cells in our sample were R-interneurons (Wenner and O’Donovan 1999). R-interneurons are labeled following injections into the rostral or caudal VLF (Wenner and O’Donovan 1999) indicating that their axons project rostrally and caudally. However, in the early mouse cord (up to E12) En1+ cells project an axon rostrally but not caudally in the VLF (Sauressig et al. 1999).

Finally, we found that En1+ cells were widely distributed within the ventral horn and were not concentrated in distinct pools, in contrast to the discrete organization of many functional subclasses of interneurons. For instance, Ia inhibitory interneurons (Hultborn et al. 1971), Clarke’s column (Rethelyi 1968), Hoffman’s nucleus (Eide and Glover 1996), and R-interneurons (Wenner and O’Donovan 1999) are all organized in spatially discrete nuclei or columns. It is unlikely, therefore that En1 expression defines any of these functionally and anatomically distinct interneuronal subpopulations.
Does En1 expression identify interneurons that form monosynaptic connections with motoneurons?

It had been proposed previously that the En1 population may consist of interneurons that make direct synaptic connections onto motoneurons (Sauressig et al. 1999). In this report we have shown that spike-triggered averaging from 63% of the En1 population produced a potential in the ventral roots, indicating that many of these cells did project synaptically to motoneurons. While it is possible that a polysynaptic connection might be responsible for some of the potentials recorded by spike-triggered averaging, we believe this to be unlikely for the following reasons. First, the spike-triggered ventral root potentials acquired in this study were obtained shortly after an episode when polysynaptic transmission is depressed (Fedirchuk et al. 1999). Second, the potentials were resolvable after only 200 or fewer sweeps, indicating the strength and reliability of the response. Finally because developing synapses are immature, polysynaptic connections are particularly susceptible to low-frequency synaptic fatigue (Lee and O’Donovan 1991).

We found that many of the direct connections between motoneurons and En1 cells were blocked by the GABA antagonist bicuculline, consistent with a previous report in the mouse that the En1 cells are GABAergic (Sauressig et al. 1999). Of the 12 En1 cells that exhibited a spike-triggered potential, four of four tested were GABAergic (Fig. 3A). It is quite possible that the other eight last order interneurons were also GABAergic because we have found that most interneuron spike trigger averaged ventral root potentials are blocked in the presence of bicuculline (10/12, 83%, from this study and unpublished observations). It is not yet clear why we have observed such a predominance of monosynaptic GABAergic connections, but one possibility is that such connections are somatic, which would favor their detection in ventral root recordings. Notwithstanding this bias, our results are consistent with the idea that a significant proportion of last order En1 interneurons are GABAergic at E10/11.

One question that is difficult to answer from our work is whether all En1 cells are monosynaptically connected to motoneurons. We found that En1 cells exhibit a higher preponderance of spike trigger averaged ventral root potentials than cells that did not express the protein (63 vs. 30%, P = 0.07). Although we have argued that such potentials are strong evidence of a direct connection to motoneurons, a negative result is more difficult to interpret. False negatives could arise for several reasons. For example, it is possible that the interneuron’s axon was damaged, the synaptic connection was too weak to be resolved by spike-triggered averaging of the ventral root potential, or the interneuron projected to motoneurons in a different segment to that of the ventral root recording. A definitive answer to this question will probably require combined dual intracellular recording from En1 neurons and motoneurons.

Finally, it is possible that some cells identified as En1 at E10 expressed En1 earlier in development but down-regulated expression by E10. We provided evidence that En1 expression appears to be in the process of being down-regulated in chick spinal cord interneurons at the time that the physiological experiments were performed, and this must be considered when interpreting our findings. Preliminary experiments in mice using a lineage tracing technique reveal that En1 is expressed transiently in a larger population of cells than express the protein at later, mid-gestation stages in mouse embryos, and is beginning to be down-regulated by E15.5 (M. P. Matise, personal observations). It is possible, therefore that all cells that express the En1 protein at some point in their history make monosynaptic connections to motoneurons. This hypothesis will have to be tested by making more extensive measurements of the connections of individual interneurons that was possible in the present study, and by combining genetic techniques that allow permanent marking of all cells that express En1 at any time in their developmental history.

Conclusions

The results of this paper illustrate the feasibility of combining electrophysiology and immunocytochemistry to identify the synaptic connections of spinal interneurons that express particular transcription factors. Such experiments are essential to our understanding of the role that transcription factors play in the specification and differentiation of spinal interneurons during development. We have demonstrated that a substantial proportion of En1+ cells make monosynaptic connections with motoneurons but that the protein is unlikely to mark a unique, anatomically defined interneuron sub-class. To determine whether the En1 protein is involved in specifying the projections to motoneurons, physiological studies of ventral interneuron connectivity in animals in which En1 function has been eliminated will be necessary.

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