

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

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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 part 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 lumbosacral 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.

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 garnered the most attention. These are the LIM and ETS families (whose names

derive from an acronym of the original three proteins that were identified in each family), which are differentially expressed in motoneurons projecting to different muscles. These correlations have formed the basis for hypotheses about the roles of transcription factors in determining the phenotype of motoneuron classes (Pfaff et al. 1996; Sharma et al. 1998; Tanabe et al. 1998). Extension of this approach to spinal interneurons has been slow because subpopulations of interneurons are much harder to identify than motoneurons. Whereas motoneuron subsets can be labeled through individual muscle nerves, the same type of identification is not possible for interneurons. Rather, to determine the synaptic connectivity of interneurons, intracellular recordings are required.

Several studies have demonstrated differential expression of transcription factors in subsets of spinal interneurons during development (Burrill et al. 1997; Liem et al. 1997; Matisse and Joyner 1997; Pierani et al. 1999). In addition to providing important insights into the specification and development of interneurons, the ability to identify unique markers for particular types of interneurons would also be extremely useful for studies of interneuronal function and anatomy. At present, in the absence of such markers, interneuronal studies rely largely on single-cell electrophysiology.

For these reasons, we have examined some of the synaptic connections of interneurons expressing the Engrailed-1 transcription factor (En1) in the developing chick spinal cord. We focused on En1 for several reasons. First, En1 expression has been extensively characterized in the chick spinal cord (Burrill et al. 1997; Davidson et al. 1988; Gardner et al. 1988). Second, expression of En1 in ventral spinal interneurons is extremely well conserved across vertebrate species, being found in the mouse, chick, zebrafish, and *Xenopus* (Davidson et al. 1988; Davis et al. 1991; Gardner et al. 1988; Hatta et al. 1991), suggesting an important developmental role. Third, while studies in knockout mice have suggested that En1 is not required for the survival, migration, or expression of several other transcription factors normally coexpressed (Matisse and Joyner 1997), the protein does appear to be involved in the regulation of axonal pathfinding (Saureissig et al. 1999). Furthermore, En1-expressing (En1+) cells are likely to be GABAergic, and many send their axons into the ventrolateral white matter and

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into the motor column (Saureissig et al. 1999). These and other studies have led to the hypothesis that En1+ cells make monosynaptic connections with motoneurons (Saureissig 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 (adductor = 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 segment 7 (T7) and lumbosacral segment 5 (LS5). All whole cell recordings were obtained using an Axoclamp 2B amplifier and custom written data acquisition software (Labview 4.0). Extracellular suction electrode recordings from ventral roots were amplified ×10,000 and filtered at DC–0.1 kHz. Cells were only accepted for further study if their membrane potential was less than or equal to –40 mV. Single-pulse stimuli of 30–50 μA (0.5 ms) were delivered to dorsal or ventral roots. Data on afferent stimulation were only accepted in cases where an evoked potential was concurrently observed in the ventral root. Such a response was taken as evidence that the root was intact and stimulated effectively.

Whole cell recordings were obtained with 0.5% biocytin added to the patch solution to label the recorded cell. No more than two cells per hemisegment were labeled, and their locations were recorded. The low number of labeled cells in each cord allowed unambiguous matching of the biocytin-labeled cell with its physiologically determined synaptic connections.

Spike triggered averaging

To determine whether a particular interneuron projected monosynaptically to motoneurons, we used the interneuron's spike as a trigger to average synaptic potentials from the ventral roots. For this purpose, we depolarized the membrane potential of the interneuron by ~5–10 mV to produce action potentials at a steady rate of approximately 1–2 Hz. The spikes were used to trigger an averaging program running on

a Macintosh computer that accumulated from 200 to 400 traces of the ventral root recording (Wenner and O'Donovan 1999). Averages were acquired shortly after (~2–5 min) an episode was evoked by electrode penetration of the ventral cord surface, and so the interneuronal network was most depressed at this time minimizing polysynaptic transmission (Fedirchuk et al. 1999). Such a procedure made it unlikely that a potential observed in the averaged ventral root recording would be mediated polysynaptically. When a spike-triggered potential was *not* observed, the data were only included if the ventral root recording revealed the presence of an evoked potential in response to VLF stimulation.

Immunohistochemistry and cell counts

For antibody staining, *embryonic day 10 (E10)* chick embryos were fixed for 2 h in 4% paraformaldehyde/PBS on ice, washed in PBS, sunk in 30% sucrose/PBS, embedded in Tissue Tek and cut serially in a cryostat. Anti-Engrailed antibody (αEnhb-1) (Davis et al. 1991) was used as described previously (Matisse and Joyner 1997). This antibody detects both En1 and En2 proteins, but since En2 is not expressed in the lumbosacral region of the embryonic chick spinal cord at any stage before *E10* (Millet and Alvarado-Mallart 1995 and data not shown) this antibody reveals only En1 protein in this tissue.

Fluorescently coupled secondary antibodies were obtained from Jackson Immunoresearch and used as follows. For single-labeling studies, cy3-conjugated goat anti-rabbit IgG was used at 1:250. For double-labeling in experimental *E10–11* embryos, cy3-goat anti-rabbit IgG was used at 1:500 to detect En1, and fluorescein-conjugated streptavidin was used at 1:100 to detect biocytin.

En1+ interneurons were counted in every fifth 10 μM section through the anterior spinal cord segments (thoracic 7 to lumbosacral 3). Spinal segments were identified as described previously (Matisse and Lance-Jones 1996). 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 (Matisse 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; Matisse 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–LS3 (*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.

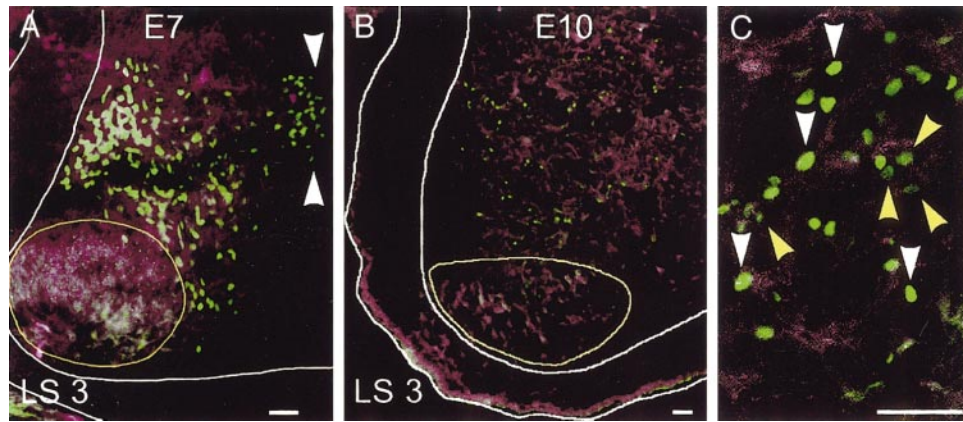


FIG. 1. En1 expression in the lumbar spinal cord (LS) at *embryonic day 7 (E7)* and *E10*. *A* and *B*: transverse sections through LS 3 spinal segments at *E7* (*A*) and *E10* (*B*) stained with antibodies to En proteins (green). Images show ventral hemi-cord. Many En1+ cells migrate ventrally after 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- μ M 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 μ M.

1*C*. 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 lumbar 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. 4*A*). No obvious correlation was observed between the location of En1+/bio+ double-labeled cells and their morphology or synaptic connections (see next section).

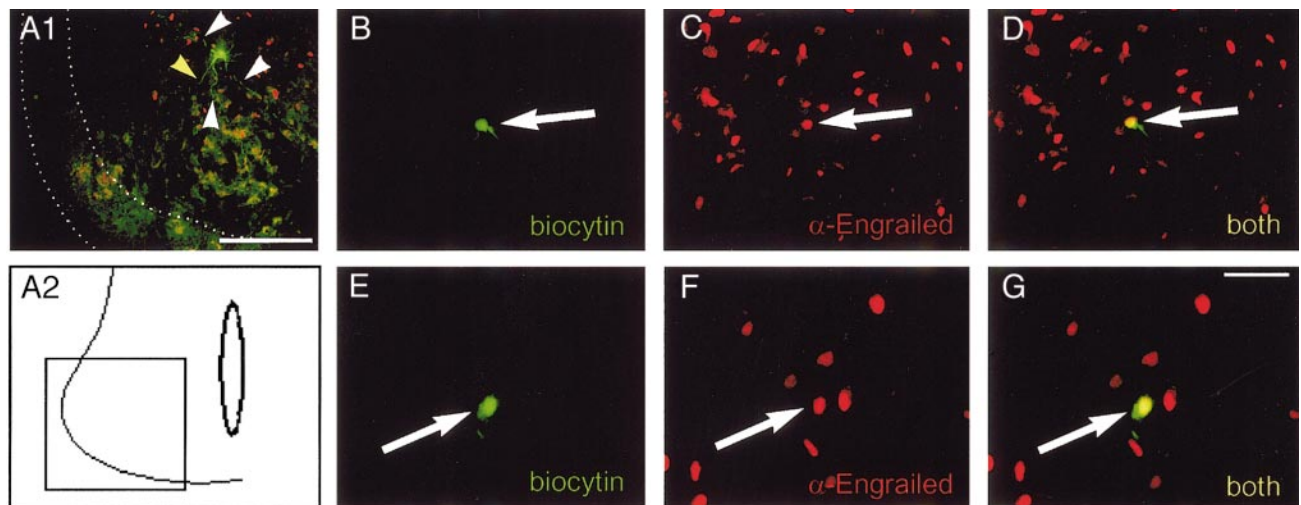


FIG. 2. En1 protein expression in biocytin-labeled, recorded interneurons. *A1*: transverse section double-labeled to reveal En1 protein (red) and biocytin (green). Biocytin labeling can be seen in the cytoplasm of the cell, filling both putative dendrites (white arrowheads) and axon (yellow arrowhead), which is projecting in the direction of the ventral funiculus. *A2*: region of spinal cord enlarged. *B–D*: 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 μ M for *A* and 17 μ M 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 GABA_A 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

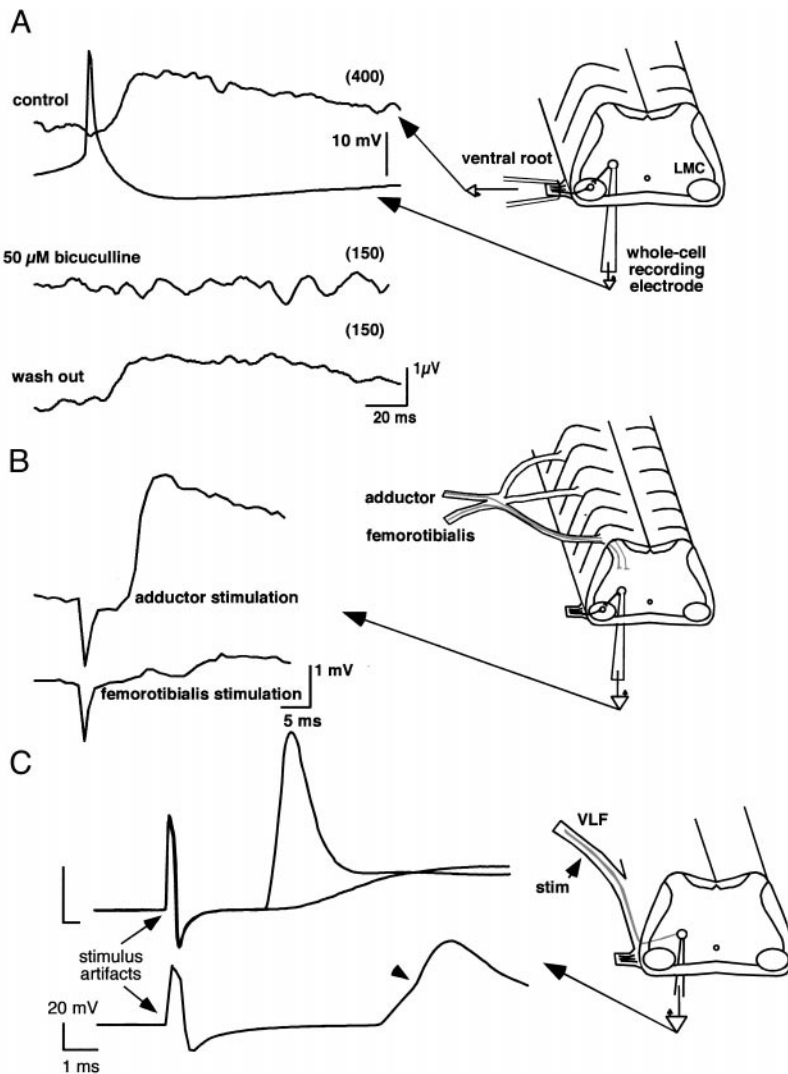


FIG. 3. Physiological assessment of En1+ interneurons. Whole cell and ventral root recordings are shown on the left; schematic representation of the recording configuration is shown on the right. *A*: interneuron spike (bottom trace, control) was used to trigger the acquisition of ventral root recordings 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 GABA_A 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.

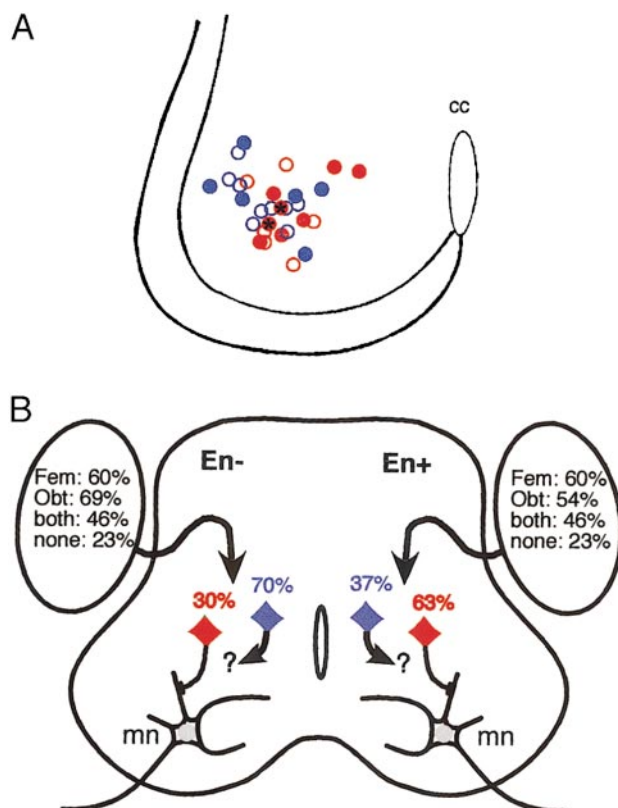


FIG. 4. Distribution and connectivity of En1+ and En1- interneurons. *A*: location of sampled interneurons in the transverse axis. Ventral hemicord is depicted. Filled circles, En1+; open circles, En1-; red, last order; blue, not last order. Interneurons located on opposite sides of the spinal cord were projected onto one side for comparison. See text and METHODS for further details. cc, central canal. *B*: schematic summary of afferent and efferent connectivity of En1+ and En1- interneurons sampled. Connectivity is represented as a percentage of the total number sampled for each category. Thus 30% of the En1- cells made last-order connections (*left side of figure*), representing 7 cells (see text), while 70% did not make last-order connections (16 cells). Afferent input is given in ovals at *top of figure*. mn, motoneurons. See text for further details.

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 (Saureissig 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_A 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+ are monosynaptically connected with 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+ interneurons and motoneurons.

Finally, it is possible that some cells we 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 than 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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