Development of an Inhibitory Interneuronal Circuit in the Embryonic Spinal Cord

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Xu, Huaying, Patrick J. Whelan, and Peter Wenner. Development of an inhibitory interneuronal circuit in the embryonic spinal cord. J Neurophysiol 93: 2922–2933, 2005. First published December 1, 2004; doi:10.1152/jn.01091.2004. Locally projecting inhibitory interneurons play a crucial role in the patterning and timing of network activity. However, because of their relative inaccessibility, little is known about their development or incorporation into circuits. In this study, we characterized the functional onset, neurotransmitters, rostrocaudal spread, and funicular distribution of one such spinal interneuronal circuit during development. The R-interneuron is the avian homologue of the mammalian Renshaw cell. Both cell types receive input from motoneuron recurrent collaterals and make direct connections back onto motoneurons. By stimulating motoneurons projecting in a given ventral root and recording the response in adjacent ventral roots, we demonstrate that the R-interneuron circuit becomes functional between embryonic day 6 (E6) and E7. This ventral root response is observed at E11 and at E14 until it can no longer be detected at E16. Using bath-applied neurotransmitter receptor antagonists, we were able to demonstrate that the circuit is predominately nicotinic and GABAergic from E7.5 to E15. We also found a glutamatergic component to the pathway throughout this developmental period. The R-interneuron projects three or more segments both rostrally and caudally through the ventrolateral funiculus. The distribution of this circuit may become more locally focused between E7.5 and E15.

INTRODUCTION

Understanding how neural circuits are formed has long been a central issue in the study of developmental neurobiology. The great majority of work has investigated the most accessible neurons within a network, these neurons are typically excitatory projection neurons. For example, many different developmental characteristics have been identified in spinal motoneurons, i.e., processes of synapse elimination, axonal pathfinding, and cellular specification by homeobox transcription factors (Goda and Davis 2003; Jacob et al. 2001; Shirasaki and Pfaff 2002). Much less is known about the development of inhibitory interneurons that play a crucial role in patterning activity and setting the excitatory state of the circuitry (McBain and Fisahn 2001). One reason for this paucity of knowledge is that inhibitory interneurons are less accessible, and as a result, few classes of locally projecting inhibitory interneurons have been characterized, even in the adult. Possibly the best-studied inhibitory spinal interneuron is the Renshaw cell, first identified in the adult cat (Fig. 1 schematic) (Eccles et al. 1954; Renshaw 1946). These cells receive direct nicotinic input from motoneurons and make predominantly glycinergic projections back to motoneurons. Although little is known about the development of Renshaw cells and their connections, we do know they arise from a population of cells in the embryonic spinal cord that expresses the engrailed-1 homeobox transcription factor (Goulding et al. 2002; Sapir et al. 2004; Wenner et al. 2000).

In this report, we describe the circuit development of an accessible inhibitory interneuron in the chick embryo spinal cord, the R-interneuron, which is likely to be the avian equivalent of the mammalian Renshaw cell (Wenner and O’Donovan 1999, 2001). Previous reports have partially characterized the R-interneuron circuit at embryonic day 10 (Ritter et al. 1999; Wenner and O’Donovan 1999, 2001). Both Renshaw cells and R-interneurons are easily identified because they receive monosynaptic nicotinic input from motoneuron recurrent collaterals as determined by ventral root stimulation. Further, both are likely to originate from engrailed-1-positive cells (Sapir et al. 2004; Wenner et al. 2000). R-interneurons are located in a position dorsomedial to the lateral motor column (LMC), and they project their axons through the ventrolateral funiculus (VLF) (Wenner and O’Donovan 1999, 2001). R-interneurons make direct projections back onto motoneurons, but their neurotransmitter appears to be GABA rather than glycine (Wenner and O’Donovan 1999). Interestingly, glutamate may play some role in the R-interneuron circuit, as glutamate receptor antagonists are capable of reducing transmission through the circuit at E10 (Tabak et al. 2001). We examine the disynaptic R-interneuron circuit by stimulating one ventral root and recording the response in the adjacent ventral root (henceforth referred to as ventral root response).

R-interneurons provide an opportunity to study interneuronal development from molecules to circuitry and could therefore begin to provide a context for understanding the role of various transcription factors in the differentiation of interneurons. We have therefore characterized some of the developmental features of this circuit from early to late embryogenesis and have asked the following questions. 1) Which neurotransmitter receptors are involved in the circuit during development? 2) When does the circuit first develop and how does it change? 3) What is the rostrocaudal spread or distribution of the circuit and how does it change during development?

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METHODS

Electrophysiology

Experiments were performed on White Leghorn chick embryos aged embryonic days 5–18 (E5–E18). Embryos were staged according to Hamburger and Hamilton (1951). This report will refer to developmental age in days of incubation (i.e., E7) corresponding to the Hamburger Hamilton staging criteria, whether this was the actual incubation period or not. Three developmental stages were chosen to study the distribution of this circuit and its pharmacology: early (referred to as E8 throughout the text but includes E7.5–E8, stages 32–34; shortly after the onset of circuit formation at a period when the response was reliably detectable), middle (referred to as E11 throughout the text but includes E10 and E11, stages 36/37; when previous studies were carried out), and late (referred to as E14 throughout the text but includes E14 and E15, stage 40/41; the latest stage when the response can still be reliably observed).

Embryos were decapitated and eviscerated under continuous superfusion with oxygenated Tyrode's solution (concentration in mM: 139 NaCl, 2.9 KCl, 17 NaHCO₃, 12.2 glucose, 3 CaCl₂, and 1 MgCl₂) cooled to ~15°C. A ventral laminectomy was performed, and the spinal cord, including thoracic to sacral segments, was freed from the underlying dorsal lamina. The central portion of the dorsal roots were cut so that we could selectively activate motoneurons after stimulation of the spinal nerve (referred to throughout this report as ventral root stimulation). The isolated spinal cord was warmed to room temperature and transferred to a recording chamber. Tyrode's solution was superfused at a constant temperature of 26–28°C. Tight-fitting glass suction electrodes were used to record and stimulate the ventral roots. In E18 preparations, the spinal cord was midsagitally hemisected to allow for better O₂ diffusion and viability. Up to five electrodes were placed on different ventral roots and spontaneous or stimulated episodes of activity provided a control response that allowed the amplitude of the recordings to be normalized for each root. We used this rate of stimulation because at faster rates a decline in the amplitude of the recurrently evoked potentials could be detected. The recurrent pathway was monitored by stimulating one ventral root while recording potentials produced in adjacent ventral roots. We stimulated the LS2 root and recorded motoneuron population potentials from the LS3-6 ventral roots. In

FIG. 1. The ventral root response at E8 and E14 is more sensitive to GABA<sub>A</sub> receptor antagonist than glycinergic receptor antagonists. A and B: a significant reduction in the ventral root response is observed after the addition of a GABA<sub>A</sub> receptor antagonist (bicuculline). Ventral root recordings show a reduction of the response at early and late stages (A). The average normalized reduction is shown in the bar chart of B. C: a reduction in the ventrolateral funiculus (VLF)-evoked response [in the presence of bicuculline, 2-amino-5-phosphonopentanoic acid (AP5), 6-cyano-7-nitroquinoxalene-2,3-dione (CNQX), mecamylamine] before and after adding the glycine receptor antagonist strychnine at 1 and 5 μM at early and late stages shows 1 μM is sufficient to block glycinergic transmission. D and E: a partial reduction in the ventral root response is observed after the addition of a glycine receptor antagonist (1 μM strychnine). Ventral root recordings show reduction of the response at early and late stages (D). The average normalized reduction is shown in the bar chart of E. Schematic shows recording configuration and R-interneuron circuitry. *, stimulus artifacts. Error bars represent SE.

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few cases, we stimulated LS2 and recorded LS3, then LS4, then LS5, and then LS6 separately, always stimulating 5 min after the previous episode. Results were no different and were combined. In one set of experiments, we lesioned the VLF with a fine pin. To prevent damage to the ventral roots, the overlying pia was cut with microscissors. The signals were amplified (1,000 times), filtered (DC to 0.3–5 kHz), and digitally recorded using Axograph acquisition software (Axon Instruments) onto a Macintosh computer. Further analyses of the data were performed off-line.

Whole cell recordings were obtained from motoneurons and interneurons (located in the R-interneuron region) at E6 using the dissecting procedures described previously for E10 embryos (Wenner and O’Donovan 1999, 2001). Briefly, meninges were removed from the ventral surface overlying LS1-4 to allow for electrode penetration. At E6, whole cord preparations were pinned ventral side up. Whole cell electrodes [K-glucorurate solution (in mM): 10 NaCl, 130 K-glucorurate, 10 HEPES, 1.1 EGTA, 0.1 CaCl2, 1 MgCl2, 1 Na2ATP] were then targeted to the lateral half of one side of the cord. At depths of ≤120 μm, the great majority of cells displayed an antidromic action potential after ventral root stimulation and were considered motoneurons. At depths >120 μm, most cells did not display an antidromic action potential, and these cells were considered to be interneurons if they had action potentials in response to a depolarizing current. For interneuron recordings, electrodes were targeted to the R-interneuron region (35–55% of the distance from the lateral border to the midline; 120–200 μm below the ventral surface). R-interneurons were targeted differently at E7-7.5. Here the dorsal half of the cord was removed. The dorsal and lateral pia was left on (along with dorsal roots), and the cord was pinned ventral side down and cut horizontally along its rostrocaudal axis into approximately equal dorsal and ventral halves. The vibratome blade (Lieca) was positioned at the dorsal surface of the cord and set to cut after a 400-μm descent. The ventral half preparation was then transferred to the recording chamber and pinned ventral side down; adductor and femototibialis muscle nerves were then drawn into suction electrodes for motoneuron stimulation and recording. R-interneuron recordings were targeted, 35–55% of the distance from the lateral border to the midline, ~50 μm dorsal to the adductor motor nucleus (see Wenner and O’Donovan 2001). Recordings were easily obtained from spinal neurons using whole cell electrodes that were of a slightly higher resistance (10–20 MΩ) than those used in previous reports at E10. Only cells with resting potentials more hyperpolarized than ~40 mV, strong network driven potentials, and expression of action potentials were accepted into the database. Two E7 R-interneurons were identified by a synaptic potential at latencies between 4 and 5 ms (Wenner and O’Donovan 1999).

Pharmacological studies

The pharmacology of the recurrently evoked potential was investigated by bath applying GABA, glycine, acetylcholine, or glutamate receptor antagonists. To block the GABA A receptors, we used bicuculline methiodide (20–30 μM; Sigma Chemicals). Glycinergic transmission was tested using 1 μM strychnine HCl (Sigma). Glutamatergic N-methyl-D-aspartate (NMDA) and AMPA receptors were blocked by using 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), sodium salt (5 μM; Research Biochemicals), and 2-amino-5-phosphono pentanoic acid (AP5; 50 μM, Sigma), respectively. Nicotinic cholinergic receptors were blocked using mecamylamine HCl (25–50 μM, Research Biochemicals). Ventral root responses were compared in the absence and presence of bath-applied antagonists at equivalent periods after an episode. This was necessary because the ventral root response is depressed following an episode and then gradually recovers in the following minutes (Fedirchuk et al. 1999).

Optical recordings

To visualize the interneurons activated after stimulation of ventral roots we retrogradely loaded interneurons with calcium dye (Ca2+ Green-1 dextran 10,000 MW; Molecular Probes). Interneurons were labeled through VLF drawn into a suction electrode containing ~20% wt/vol of the dye dissolved in distilled water containing 0.2% Triton X-100 detergent (O’Donovan et al. 1993). This configuration was left overnight to allow retrograde transport of the calcium-sensitive dye back to interneuronal cell bodies. For imaging R-interneuron activity at early stages (E6-7), the cord was cut transversely (Wenner and O’Donovan 1999). Images were continuously acquired to videotape using an intensified video camera (Stanford Photonics) as the ventral root was stimulated (20Hz, 100–200 μs). The tissue was illuminated using a 75-W Xenon Arc lamp with an excitation filter of 450–490 nm, dichroic of 510 nm, and a barrier filter of 520 nm. Various ND filters were used to reduce photodynamic damage. During the experiment, video data (30 fps) were stored on S-VHS tape (Sony SVO-9500MD). Images were digitized off-line, frame by frame, and processed on Metamorph software (Universal Image Systems). To determine if the R-interneurons were activated following ventral root stimulation at different ages, we constructed difference images normalized to the background (ΔF/IF). These were generated by subtracting a 30-frame average obtained prior to the stimulus (background image) from an average of 8 consecutive frames during the peak of R-interneuron activity. Resulting images were then divided by the background image. Cellular optical activity was defined as that (ΔF/IF) that showed an increase of >2 SD over the background.

Statistical analysis

Student’s t-test, or one-way ANOVAs followed by Tukey post hoc tests were performed on the data to detect significant differences (JMP software). To determine if a significant reduction was observed after application of antagonists a one-tailed, paired t-test was used. Means and corresponding SEs are presented.

RESULTS

In this investigation we explore the properties and development of the Renshaw-like circuit in the embryonic chick from E5 (stage 27) to E18 (stage 44). Our main approach was to activate the R-interneuron pathway by stimulating one ventral root and recording the evoked potential in adjacent ventral roots (motoneuron to R-interneuron and R-interneuron back to motoneuron, Fig. 1 schematic). This evoked potential will be referred to throughout the paper as a ventral root response. Ventral root recordings are thought to be a faithful representation of the potentials experienced by the motoneuron population (O’Donovan 1987, 1989).

Neurotransmitters of the circuit underlying the ventral root response during development

One of the aims of this study was to examine the development of contributing neurotransmitters in the R-interneuron pathway. We tested the pharmacology of the circuitry by assessing the effects of different antagonists on the ventral root responses between E7.5 and E15.

GABA

The amplitude of the ventral root response was examined before and after bath application of the GABA A receptor antagonist bicuculline (20–30 μM). We stimulated the LS2
ventral root and recorded responses in adjacent roots 5 min after an episode of network activity. We then bath applied bicuculline for ≥30 min and again measured the response 5 min after an evoked episode. Bicuculline significantly reduced the ventral root response at both E8 (12.2% of control response, \( P < 0.001, n = 9 \); Fig. 1, A and B) and E14 (19.3% of control response, \( P < 0.05, n = 7 \); Fig. 1, A and B). In two of nine roots at E8 (4 preparations) and three of seven roots at E14 (6 preparations) bicuculline abolished the response, demonstrating that the ventral root response was not mediated through a direct motoneuron to motoneuron connection.

**Glycine**

We then tested for a glycineergic component to this pathway. Because an earlier report suggested that strychnine could significantly block GABA\(_\text{A}\) receptor transmission at higher concentrations (Jonas et al. 1998), we did not want to use more than 1 \( \mu \text{M} \) strychnine. To be confident that this concentration was sufficient to block existing glycineergic transmission, we bath-applied several neurotransmitter antagonists to better isolate glycineergic transmission (glutamate-CNQX and AP5; nicotinic-mecamylamine; GABA-bicuculline). We then stimulated the VLF four to six segments rostral to the recorded ventral roots. The residual response was reduced by 1 \( \mu \text{M} \) strychnine but was not further reduced by increasing the strychnine concentration to 5 \( \mu \text{M} \) (at E8: reduced to 72.0 and 73.3% by 1 and 5 \( \mu \text{M} \), strychnine, respectively, \( n = 6 \); at E14: reduced to 47.1 and 44.2% by 1 and 5 \( \mu \text{M} \) strychnine, \( n = 11 \); Fig. 1C). We therefore reasoned that 1 \( \mu \text{M} \) strychnine was sufficient to block glycineergic transmission in our preparation and tested its effectiveness on the ventral root response at both early and late stages (Fig. 1, D and E). Although strychnine still reduced the response in a significant manner (70.5%/56.9%, \( P < 0.005, n = 8\)/\(n = 4 \); E8/E14) it never abolished the signal and was far less effective than bicuculline. These results suggest that GABA is the predominate transmitter of the R-interneuron even at the latest stages observed. The effectiveness of strychnine, however, did increase while that of bicuculline decreased slightly at E14.

**Nicotinic cholinergic**

Renshaw cells and R-interneurons at E10 are known to receive nicotinic cholinergic input from motoneurons. We tested transmission through nicotinic receptors at early and late stages. Bath application of the nicotinic cholinergic antagonist, mecamylamine (25–50 \( \mu \text{M} \)), dramatically reduced the ventral root response both at early (to 7.8%, \( n = 10, P < 0.01 \)) and late stages (to 21.8%, \( n = 7, P < 0.005 \)) but was slightly less effective at later stages (Fig. 2). In 10 of 10 roots (3 preparations) at E8 and 3 of 7 roots (6 preparations) at E14, the response was reduced below detection.

**Glutamatergic**

To test the importance of glutamate in this pathway, we bath applied the AMPA receptor antagonist CNQX (5 \( \mu \text{M} \)) and the NMDA receptor antagonist AP5 (50 \( \mu \text{M} \)) at all three stages. Because chloride mediated conductances become stronger after addition of these antagonists (Chub and O’Donovan 1998, 2001), we carried out the experiment in the following manner.

**Developmental progression of ventral root response**

In an initial set of experiments (\( n = 7 \)) at stages younger than E7 (stage 31), we could not detect a ventral root response after a single stimulus or trains of stimuli delivered to the
adjacent ventral root (E5.5–6.5, stage 28–30, see Fig. 4), despite the observation of robust episodic potentials (>200 μV) from both the stimulated and recorded ventral roots. Episodes of network driven activity are experienced in virtually all motoneurons as they are depolarized 20–30 mV for durations of ≤1 min (referred to as episodes). The observation of these episodic potentials in the root neurograms demonstrates that both the stimulated and recorded root were intact, that the root was fit tightly in the electrode, and that the cord was in good health (Fig. 4).

Starting at E7 we could detect a small depolarizing potential in a ventral root recording after a train of stimuli delivered to the adjacent ventral root (n = 3/3; Fig. 4). By E7.5 a ventral root response could often be detected after a single ventral root stimulus, and this occurred more reliably at E8. From E8 to E15, a robust depolarizing response could be observed after single shocks or trains of stimuli (Fig. 4). The properties of the ventral root-evoked responses to a single stimulus changed during development and this can be seen in Figs. 1–3, where potentials (normalized to each other) allow a comparison of the form of these signals at different ages. The onset latency of the depolarization decreased from 22.6 ± 1.9 ms at E8 embryos to 15.4 ± 1.5 ms at E11, and to 12 ± 0.5 ms at E14. The half-width of the evoked response also became shorter at later stages of development (E8: 319 ± 40.1, E11: 252 ± 27.7, and E14: 108.8 ± 7.7 ms, n = 3–5). Similarly, the 10–90% rise time was 72.1 ± 9.9, 49.5 ± 2.8, and 20.3 ± 1.2 at E8, E11, and E14, respectively (n = 3–5). The amplitude of ventral root response (normalized to the episode potential for that root) was reduced from early to late stages (not shown). In eight ventral roots (LS3-6 after LS2 stimulation and LS5-2 after LS6 stim-

ulation), the normalized potentials were, in each case, reduced at E14 compared with E8. From E16-18, it was not possible to detect any ventral root response, although we were able to record depolarizing potentials during episodes.

**Ventral root response, E5/6**

To better define the onset of the functional circuit, we carried out further experiments at early time points. In initial experiments at E5.5 to E6.5, no ventral root response was observed. We later observed that the intervals between episodes were shorter at these early stages (E6: 140 ± 30 s, typically single-cycle episodes as shown in Fig. 5, A and 5B; left; E8: 477 ± 60 s, typically 2 cycles/episode—Fig. 5B, right; E10: 761 ± 90 s, many cycles—Fig. 5D, right; or E14: 495 ± 70 s, many cycles). Our inability to detect a ventral root response at these earlier stages might have resulted from a post episode depression that has been described for the E10 preparation (Fedirchuk et al. 1999). In the initial set of experiments at E5.5–6.5, we were not paying attention to the timing of the previous episode, and because episodes at these stages were more frequent, it is possible that we did not observe a ventral root response because it had not recovered after the previous episode. To test this possibility, the interepisode interval was determined in each preparation; the ventral root response (stimulating LS1-4) was then recorded just before the next episode would be expected to occur. We reasoned that the spinal circuitry would be relatively recovered from depression if a spontaneous episode could occur. In two E5 (stage 27) preparations, no response was observed when five different ventral roots were given stimulus trains (Fig. 5A) delivered late in the interepisode interval. Only 2 of 15 ventral root stimulations at E6 (stage 29) resulted in a ventral root response, one of which could evoke an episode. In these two cases, the suction electrode was clearly away from the cord, and we were still able to evoke the response after reducing the stimulus intensity.
from 30 to 20 μA, making it very unlikely that we were directly activating interneurons in the cord. We thus find it likely that the circuit was just becoming functional in these two E6 preparations. In 13 of 15 root response, no ventral root response was observed (n = 7 embryos; Fig. 5B). By contrast in E8 and E11 preparations, it was routinely possible to evoke episodes in the interepisode interval (E8: 4 of 4 preparations, Fig. 5C; E11: 2 of 4 preparations, Fig. 5D). Ventral root stimulus trains did not evoke episodes of activity at E14 (Fig. 5E, 0 of 4 preparations).

One of the advantages of the extracellular ventral root recordings is that they provide information about the entire population of motoneurons projecting in that root. However, because they are electrotonically degraded signals, it is possible that a response could be detected in a whole cell recording from the motoneuron cell body. The ventral root recordings described in the preceding text directed us to the E6 embryo, at or before the onset of the functional circuit. Therefore whole cell recordings were obtained from antidromically identified LS2-4 motoneurons at E6. Antidromically identified motoneurons were observed in the lateral half of the cord at electrode penetration depths of 30–120 μm below the ventral surface of the cord. In the lateral half of the LS2-3 cord at these depths, neurons routinely displayed antidromic action potentials (1.6 ± 0.1-ms delay, Fig. 5F) and were therefore motoneurons. When the ventral root simulation was reduced in intensity, the all-or-none nature of the antidromic could be seen (average failure at 6.9 μA); in many of the motoneurons a very small (<5mV) residual response could be seen (Fig. 5F). These responses in motoneurons were similar to antidromic action potentials in their time course and onset latency. These below-threshold responses could reflect action potentials in other motoneurons transmitted through gap junctions (Fulton et al. 1980) or represent our stimulus carried electrotonically back to the cell body, but because of their short latency and their maintenance during stimulus trains, they are not synaptic events. In none of the 23 motoneurons did we observe a synaptic response to stimulations of the adjacent ventral root (30 μA) or homonomous ventral root at an intensity below that for antidromic activation (single or trains: 7 × 20 Hz, >5 min after the previous episode, Fig. 5F). Although whole cell recordings from motoneurons did not show ventral root evoked synaptic inputs, other synaptic inputs were observed (spontaneous miniature post synaptic currents-mPSCs and strong episodic currents). These data suggest that our stimuli are effective in antidromically activating E6 motoneurons and that the circuit underlying the ventral root response first becomes functional between E6 and E7.

Development of motoneuron input to R-interneurons

If the circuit is not functional in a significant way by E6, then R-interneurons may not yet be driven to fire after antidromic motoneuron stimulation, and/or interneurons may not yet make functional connections back to motoneurons. We therefore asked whether the recurrent activation of interneurons, which clearly exists at E10 (Wenner and O’Donovan 1999), is functional at E6-6.5. This was accomplished using two different techniques (optical and intracellular interneuron recordings).

First, we assessed R-interneuron spiking activity indirectly through calcium imaging. Many different classes of ventrally located interneurons can be labeled retrogradely from their axons that project through the VLF. Sections of the VLF are drawn into suction electrodes containing a calcium-sensitive dye. The dye is allowed to diffuse retrogradely overnight so that labeled interneuron cell bodies can be optically imaged the next morning. An increase in the fluorescence of calcium dye-labeled neurons is a strong indication that a neuron is experiencing spiking activity (O’Donovan et al. 1993, 1994). While our technique labels many different species of interneuron, we can selectively activate R-interneurons by delivering stimulus trains to that segment’s ventral root. At E10, a column of cells dorsomedial to motoneurons becomes optically active after ventral root stimulus trains (Wenner and O’Donovan 1999, 2001). However, when this was done at E6.5 (n = 3/3, not shown), no optical activity was observed, suggesting there...
were no calcium-green-labeled interneurons that could be driven to spike by motoneuron stimulations. During episodic activity, however, strong optical activity was recorded (not shown). While these optical studies are beneficial because they provide information about large numbers of interneurons, they do not resolve subthreshold potentials and only provide information about labeled neurons.

To address these limitations, we made whole cell recordings from E6 interneurons. Electrodes were targeted to a region 120–200 μm deep and 35–55% of the mediolateral distance from the lateral edge to the midline (the R-interneuron region at E10) (Wenner and O'Donovan 2001). Here, most neurons were considered to be interneurons because they did not experience an antidromic action potential following ventral root stimulation. Of 68 interneurons recorded in this way, none showed any response to a ventral root stimulation (single or train: 7 × 20 Hz, 5 min after previous episode, Fig. 6A). The data are consistent with the hypothesis that the recurrent collateral has not yet formed functional connections to R-interneurons at this stage.

The first optical signal from interneurons could be evoked at E7 after a train of stimuli delivered to the ventral root (Fig. 6C; n = 3/3). The dye-labeled cells that became optically active after ventral root stimulations were located dorsomedial to the motor column as previously reported at E10 (Wenner and O’Donovan 1999), suggesting that recurrently activated interneurons are spatially restricted shortly after the connection first becomes functional. To assess the recurrently activated population more directly, we made whole cell recordings from 92 interneurons targeted in the R-interneuron region at E7-7.5. Here, we removed the dorsal half of the cord and targeted the R-interneuron region from the dorsal aspect of the ventral half preparation. In only two (2.2%) of these cells were monosynaptic responses observed (based on latency, 4–5 ms) after motoneuron stimulation, suggesting these were R-interneurons (Fig. 6D, top). We were less likely to record from an R-interneuron targeting the R-interneuron region at E7-7.5 than randomly recording from the ventral half of the cord at E10 (5.1%) (Wenner and O’Donovan 1999). None of the other 90 interneurons from which we recorded received any input from

**Fig. 6.** Interneuron activity in the region of R-interneurons at E6 and E7. **A:** whole cell recordings of E6 spinal interneurons in R-interneuron region. **Top:** episodic potential but no response to corresponding ventral root stimulation. **B:** transverse cut face of the lumbar spinal cord at E7 showing many different calcium green-labeled interneurons. **C:** optical activity evoked after stimulus trains applied to the ventral root showing functionally activated R-interneurons at E7. Image is a 10-frame average of the background-subtracted difference image (ΔF). Low to high activity calibration shown (blue to white). **D:** whole cell recordings of E7 spinal interneurons in R-interneuron region. **Top:** whole cell recording from 1 of 2 R-interneurons recorded at E7 after femorotibialis muscle nerve stimulation (dorsal roots cut). This trace is generated by subtracting a trace from a different cell in same region, after femorotibialis stimulation, to reduce the stimulus artifact. Potential of 5-ms delay shows monosynapticity. Trace below that of the R-interneuron shows an interneuron in the approximate R-interneuron region (near adductor motoneurons) (Wenner and O’Donovan 2001) that does not receive input after stimulation of muscle nerve. Asterisk marks stimulus artifact.
motoneuron stimulation unless an episode was evoked even though we were targeting the R-interneuron region. Collectively, these findings suggest that the connection from motoneurons to R-interneurons first forms between E6 and E7.

**Rostrocaudal distribution of the R-interneuron circuit**

We examined the development of the rostrocaudal distribution of the R-interneuron circuit by delivering single supra-threshold stimuli (30 µA, 0.5 ms) to the LS2 ventral root while recording LS3-5 ventral roots through tight-fitting glass suction electrodes and averaging these responses (Fig. 7, n = 3–20 recordings/average). It is likely that the responses to single stimuli are disynaptic (motoneuron to R-interneuron and R-interneuron back to motoneuron) as considered in the discussion. Furthermore, the onset of the response in LS4 was only 1.9 ± 1.0, 3.8 ± 1.1, and 2.8 ± 1.1 ms after that in LS3 at E8, E11, and E14, respectively. This is less than would be expected if an additional synapse were involved (Wenner and O’Donovan 1999). Therefore we could activate a population of R-interneurons through ventral root stimulation and look at the distribution of the output of these activated R-interneurons. In some experiments, like that shown in Fig. 7, the amplitudes of these responses were normalized twice. First to the episode potential in that root, and then to the normalized potential seen in LS3 (see Fig. 7 legend). In many cases, it appeared that the ventral root responses were more locally distributed later in development. An example of this is shown in Fig. 7, where the LS3 and LS4 responses to LS2 stimulation are similar at E8, but at later stages, LS3 responses are stronger than LS4 responses. Such a finding could arise through a refinement process in the R-interneuron circuit.

To better understand the development of the caudal extent, or reach, of the circuit we observed ventral root responses in roots up to five segments caudal to the stimulated root. A connection was considered absent if a potential was not observed, and the noise of a given root recording (normalized to the episode potential) was <10% of the signal recorded in the LS3 ventral root (1 of the largest signals recorded). If a ventral root response was not observed and the noise was >10% of the LS3 signal, then this was not included in the data pool. While LS2 ventral root stimulation always produced an observable potential in the adjacent three roots (LS3-5), potentials in LS6 and 7 were not always observed at earlier stages (Table 1). We also examined the rostral projection of the R-interneuron circuit. Here, the LS6 ventral root was stimulated while recordings were acquired from the LS5–LS2 ventral roots. The rostral projection of the circuit was observed in every case for the adjacent three segments (LS5-3), and only once at the earlier stages was the projection to four segments away (LS2) not observed (Table 2). The findings demonstrate the ventral root evoked circuitry extends at least three to four segments both rostrally and caudally.

To determine which white matter tracts propagate the ventral root response, a separate set of experiments were performed. At E11, the LS2 ventral root was given a single stimulus, and adjacent ventral roots up to four segments caudal were recorded before and after transversely lesioning the VLF between LS2 and LS5. The normalized amplitude of ventral root responses were significantly reduced to 29.0 ± 6.3% (n = 12 roots, 4 preparations, P < 0.001) of their original values after lesions to the lateral half of the ventral funiculus and the ventral half of the lateral funiculus. This is consistent with the idea that the R-interneuron axons.
known to project through the VLF (Wenner and O’Donovan 1999), carry the rostrocaudal spread of the ventral root response.

**DISCUSSION**

The main findings of this paper show that the R-interneuronal pathway becomes functionally active between E6 and E7 and can no longer be detected after E15. Our data demonstrate that the pathway is dependent on nicotinic cholinergic, GABAergic, and glutamatergic transmission at the onset of circuit function and maintains this pharmacology throughout the period of embryogenesis studied. The distribution of the ventral root response extends multiple segments rostrally and caudally through the VLF but may become more locally focused during development.

The ventral root response provides a means of studying the R-interneuron circuit. Motoneuron stimulation from the ventral root monosynaptically activates a small population of interneurons (R-interneurons) (Wenner and O’Donovan 1999). R-interneurons often fire multiple action potentials in response to a single ventral root stimulus and make direct connections back onto motoneurons. This suggests the disynaptic R-interneuron circuit makes a significant contribution to the ventral root response at E10. Interneurons that did not receive monosynaptic inputs after ventral root stimulation, rarely received any action potentials unless an episode was evoked (E7-10 this study; Wenner and O’Donovan 1999). Because ventral root responses that evoked episodes were not included in this study, it is unlikely that circuits, other than the R-interneuron circuit, contributed significantly to the ventral root response.

**Developmental onset and maturation of the R-interneuron circuit**

The R-interneuron circuit becomes functional between E6 and E7, as ventral root stimulus trains rarely produced a ventral root response before E7. At later stages stimulus trains produce a ventral root response and typically evoke episodes (this study; Wenner and O’Donovan 2001). Further, no response was observed in whole cell recordings from E6 motoneurons in the same and adjacent segment to that of the stimulated root. It seems likely that the disynaptic circuit becomes functional once the recurrent connection from motoneurons to R-interneurons forms between E6 and E7. Imaging studies demonstrated that labeled interneurons were optically active after ventral root stimulus trains at E7 but not at E6. In addition, none of the whole cell recordings from E6 interneurons in a location where R-interneurons exist at E7 and E10 showed any motoneuron input (this study; Wenner and O’Donovan 1999, 2001). On the other hand, it seems possible that the second leg of the circuit, from R-interneurons to motoneurons, may be formed by E6. It is known that interneurons can project from the lumbar to brachial region by E4.5 (Oppenheim et al. 1988) and that connections, presumably from local interneurons, are formed onto motoneurons starting at E4 (Oppenheim et al. 1975). Moreover, whole cell recordings demonstrated synaptic inputs to E6 motoneurons (during episodes and as spontaneous miniature postsynaptic currents). These findings are consistent with the idea that the R-interneuron circuit becomes functional between E6 and E7 when the recurrent connection first forms. Because these are negative results it is not possible to absolutely determine that the connection does not exist in E6 embryos, but if so, it must be weak. One possibility is that the recurrent connection does not form until the motor axons innervate the target muscle around E5 (Landmesser and Morris 1975).

By E7 a ventral root response can be detected after a stimulus train. By E7.5, a single stimulus can produce a weak ventral root response, and from E7.5 to E11, the response becomes easier to detect. At E14, the response normalized to the episode potential was reduced compared with earlier stages, suggesting a weakening of the circuit. The latency of the ventral root response is reduced from early to late stages and has a progressively shorter duration. Several possible mechanisms could account for this observation: R-interneurons could be recruited and/or fire action potentials more quickly, axon conduction velocity could increase (axons are likely becoming larger and myelination begins around E13—Chu-Wang and Oppenheim 1978), changes in the kinetics of receptor conductances, and the driving force could also contribute to the development of smaller and faster ventral root responses.

At later stages (E16-18) no ventral root response could be detected. While these older preparations may be less viable than younger embryos (Wilson et al. 2003), we were able to record depolarizing potentials either by stimulating the cord or recording spontaneous episodes of activity. Alternatively, the ventral root response may be difficult to elicit at older stages because the reversal potential of chloride mediated conductances (E_Cl) in motoneurons may be approaching the resting membrane potential. Consistent with this hypothesis is the observation that ventral root stimulation was less excitatory at E14, and the normalized ventral root responses were smaller in late versus early stages. In late embryonic development of the rodent spinal cord, chloride-mediated conductances become less excitatory because of the expression of the mature form of a chloride pump (Hubner et al. 2001; Nakayama et al. 2002; Ren and Greer 2003; Rivera et al. 1999; Whelan 2003). Alternatively, as cells increase in size, they are likely to have a smaller input resistance. This could result in smaller potentials if the currents remain unchanged and thereby could reduce the excitability of the circuit. Further studies will be necessary to definitively resolve these possibilities.

**Pharmacology of the ventral root response**

At E10, GABAergic R-interneurons receive nicotinic input from motoneurons. The pharmacology of the ventral root response was tested from E7.5 to E15. GABA, acetylcholine, and glutamate appear to play important roles in the circuit throughout this period, whereas glycine may play only a small role. Our findings suggest that the pharmacology does not

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**TABLE 2. Caudorostral spread of R-interneuronal pathway during development**

<table>
<thead>
<tr>
<th>Stimulate LS6</th>
<th>LS5</th>
<th>LS4</th>
<th>LS3</th>
<th>LS2</th>
</tr>
</thead>
<tbody>
<tr>
<td>E8</td>
<td>5/5</td>
<td>5/5</td>
<td>5/5</td>
<td>3/4</td>
</tr>
<tr>
<td>E14</td>
<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
</tr>
</tbody>
</table>

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change during embryonic development, consistent with the idea that constituent elements of the circuit remain unchanged.

R-interneuron circuitry

GABA, GLYCINE. Early in development (E8) many of the interneuron cell bodies in the ventral part of the cord are immunopositive for GABA but by E15-16 very few are and instead are immunopositive for glycine (Antal et al. 1994). Although the Antal study does not directly assess the neurotransmitter of these cells, it has led to the suggestion that there may be a conversion from a GABAergic to a glycinergic neuron. A similar conversion has been described previously in the auditory nucleus (Nabekura et al. 2004) and evidence consistent with this neurotransmitter conversion has been shown in the spinal cord (Gao et al. 2001). Additionally, in the adult cat, glycine appears to be the predominate transmitter for Renshaw cells (Cullheim and Kellerth 1981; Eccles et al. 1954). We therefore tested the possibility that GABAergic R-interneurons switch their transmitter to glycine between E7.5 and E15.

We found that at both early and late stages, GABA was the predominate transmitter and that glycine had only a small contribution, if any. In 5 of 16 ventral root responses, the potential was abolished by the GABAergic antagonist, suggesting there was no detectable glycinergic component to these responses. Because strychnine has been reported to reduce GABA A transmission (Jonas et al. 1998), it is possible that there is no glycinergic component to the pathway at any of the stages studied. Recurrently activated interneurons in the adult chicken may therefore be GABAergic. There is some evidence supporting a GABAergic component to the mammalian Renshaw cell circuitry (Cullheim and Kellerth 1981; Marchetti et al. 2002; Schneider and Fyffe 1992). Alternatively, the switch in transmitter may occur later in development. All of the receptor antagonists were slightly less effective at E14, except strychnine. This might be expected if the penetrance of the drugs decreases as the tissue gets larger with age. Despite this, strychnine was slightly more effective at reducing the ventral root response at late stages, suggesting that a transmitter switch to glycine might be at the beginning of such a transition.

ACETYLCHOLINE. Previous studies in the cat and rat have demonstrated that ventral root stimulation evokes a cholinergic activation of Renshaw cells (Curtis and Ryall 1966a–c; Durado and Sargent 2002; Eccles et al. 1954; Headley et al. 1975). Based on whole cell recordings in the E10 chick embryo, R-interneurons receive nicotinic cholinergic input from motoneurons (Wenner and O’Donovan 1999). The nicotinic component of the ventral root response has not been carefully quantified and has never been assessed at different developmental stages. We determined that blocking nicotinic cholinergic transmission dramatically reduced the ventral root response at all ages tested. A small residual response was observed in a few cases and this could be due to incomplete nicotinic receptor blockade or the existence of separate transmitter (see following text).

At E5-6 and E10, acetylcholine has been shown to be important in the generation of spontaneous episodes of activity (Milner and Landmesser 1999; Wenner and O’Donovan 2001). In this study, we have shown that ventral root stimulation does not evoke episodes at E5-6. Therefore if motoneurons are the source of the acetylcholine, important in episode generation at this stage, then it is likely that it is released in an activity independent manner.

GLUTAMATE. In a recent report Tabak et al. (2001) showed that glutamatergic antagonists caused a significant reduction in the ventral root response at E10. Our work confirms this finding and shows that glutamate antagonists, throughout the developmental period studied, reduce the ventral root response by ~50% just 5 min after application of the antagonist. Interestingly 30 min after adding the drugs we saw an increased response (a compensation) at E11 but not at E8 or E14.

Where in the circuit does glutamate play a role? In the cat, anatomical evidence exists for ventral root afferents (DRG cells) that project into the cord through the ventral root (Maynard et al. 1977). In the rat, ventral root stimulation can activate a glutamatergic input to motoneurons that is thought to be mediated by glutamatergic ventral root afferents making direct connections to motoneurons (Jiang et al. 1991). We cannot rule out the possibility that ventral root afferents play a role in the ventral root response; however, two observations make this less likely. First, bicuculline, alone, can completely abolish the ventral root response, and second the great majority, and possibly all of the axons projecting in the ventral roots in the chick embryo are motoneuronal (Chu-Wang and Oppenheim 1978). A separate possibility is that R-interneurons project to and recruit glutamatergic interneurons that then project to motoneurons in adjacent segments. However, the glutamate antagonist and the GABA A receptor antagonist were each capable of nearly abolishing the response at E8 (30 min AP5/CNQX application). Because we know that R-interneurons project directly to motoneurons at E10, it seems unlikely that an intermediate glutamatergic interneuron can explain these results. Our findings are more easily explained through a direct glutamatergic effect on R-interneuron excitability. Tonic glutamatergic input to R-interneurons could increase the excitability of R-interneurons, or some motoneuron recurrent collaterals could release glutamate. Consistent with this latter idea, a recent report demonstrated that some motoneuron recurrent collaterals ending on Renshaw cells are glutamatergic rather than cholinergic (Herzog et al. 2004). Other reports suggest that motoneurons release glutamate and acetylcholine at the neuromuscular junction (Grozdanovic and Baumgarten 1999), raising the possibility that motoneuron recurrent collaterals may activate R-interneurons by cholinergic and glutamatergic transmission.

Developmental spread of the ventral root response

Based on previous studies in the cat and chick embryo, Renshaw cells and R-interneurons are probably activated by only the closest motoneurons well within a segment (Eccles et al. 1961; Ryall et al. 1971; Velumian and Poliakova 1992). Several electrophysiological and anatomical studies have, in combination, demonstrated that the majority of the rostrocaudal spread of the adult Renshaw circuit can be attributed to the Renshaw axonal projection in the ventral white matter (Jankowska and Lindstrom 1971; Jankowska et al. 1978; van Keulen 1979). This projection is thought to be no more than two segments at the most (Jankowska and Smith 1973; McCurdy and Hamm 1994; Ryall et al. 1971). Similarly, R-
interneurons project to motoneurons in adjacent segments and project through the VLF (Wenner and O’Donovan 1999; this study). However, the embryonic R-interneuron appears to project for at least, and typically more than, three segments rostrally and caudally. Therefore there may be a refinement or pruning of these long range ventral root responses, or rather, they may persist in the adult chicken. The process of synaptic refinement has been described in many developing systems where the projections of excitatory neurons are initially more exuberant and are then pruned back (Godard and Davis 2003). Interestingly, between E8 and E14 we have preliminary observations that are consistent with a synaptic refinement of the R-interneuron circuitry, where the ventral root response appears to become more focused to local segments. Further experiments will be necessary to determine if there is refinement in this circuit, but if true, it raises the intriguing possibility that a locally projecting GABAergic interneuron undergoes synaptic refinement like that of many excitatory projection neurons.

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