Developmental reorganization of the output of a GABAergic interneuronal circuit

Running Head: Inhibitory interneuronal circuit reorganization

Huaying Xu, Arthur Clement, Terrence Michael Wright, and Peter Wenner*

Department of Physiology,
Room 601, Whitehead Bldg.,
Emory University, School of Medicine,
Atlanta, GA, 30322

* Corresponding Author:
Peter Wenner
Department of Physiology,
Room 601, Whitehead Bldg.,
Emory University, School of Medicine,
Atlanta, GA, 30340
Phone (404) 727-1517
Fax (404) 727-2648
Email pwenner@emory.edu

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**ABSTRACT**

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 report we demonstrate that the GABAergic R-interneuron circuit undergoes a reorganization in the chick embryo spinal cord between embryonic day 8 and 15 (E8 & E15). R-interneurons receive synaptic input from and project back to motoneurons. By stimulating motoneurons projecting in one ventral root and recording the disynaptic response from motoneurons in adjacent segments, we show that the output of the R-interneuron circuit is reorganized during development. Following stimulation of the LS2 ventral root, disynaptic responses observed in whole cell recordings, became more common and stronger for LS3 motoneurons and less common for the more distant LS4 motoneurons from E8 to E10. Optical studies demonstrated that R-interneurons activated by LS2 stimulation were restricted to the LS2 segment and had a small glutamatergic component at both E8 and E10, but that more R-interneurons were activated within the segment by E10. The recruitment of more LS2 R-interneurons at E10 is likely to contribute to stronger projections to LS3 motoneurons, but the fact that fewer LS4 motoneurons receive this input is more consistent with a functional refinement of the more distant projection of the GABAergic R-interneuron. Interestingly, this pattern of reorganization was not observed throughout the rostrocaudal extent of the cord, introducing the possibility that refinement could serve to remove connections between functionally unrelated interneurons and motoneurons.
INTRODUCTION

Locally projecting inhibitory interneurons prevent hyperexcitable network activity and establish precise patterns of activity in their resident circuits (McCormick and Contreras 2001; Porter 1993; Young 1994). Despite their importance in the adult, we know very little about their incorporation into circuits during development. Inhibitory interneurons have been far less studied than excitatory projection neurons because of a lack of access to the locally projecting axons of the many different classes of inhibitory interneurons. As a result, our understanding of excitatory neuronal development is significantly advanced compared to that for inhibitory neurons; for example, several kinds of synaptic plasticity are better understood for excitatory neurons (LTP, LTD, synaptic refinement).

Synaptic refinement is the process of pruning initially exuberant synaptic projections. This kind of plasticity is thought to be important in allowing developing circuits to adjust to their environment. This process has been studied extensively in excitatory projection neurons, including the neuromuscular junction (synapse elimination), projections throughout the visual system, and many other excitatory projection systems (Campbell and Shatz 1992; Kasthuri and Lichtman 2003; O'Brien et al. 1978; Redfern 1970; Reh and Constantine-Paton 1984; Simon and O'Leary 1992; Sretavan and Shatz 1987; Sur et al. 1984; Tello 1917; Thompson et al. 1979). In the developing mammalian spinal cord, synaptic refinement and axon elimination have been demonstrated extensively for corticospinal projections (Bates and Killackey 1984; Cabana and Martin 1985; Li and Martin 2000; Luo and O'Leary 2005; Martin 2005; Stanfield and O'Leary 1985), and refinement has been postulated for dorsal root afferent projections into the cord (Kudo and Yamada 1985; Saito 1979). A synaptic reorganization that is likely due to synaptic refinement has
been described for one excitatory spinal interneuron that projects to parasympathetic preganglionic neurons (Araki and de Groat 1997).

Until recently it was unknown if any inhibitory neurons experienced synaptic refinement. One set of inhibitory projection neurons, which form tonotopic maps in the auditory system, has been shown to undergo synaptic refinement at a stage when their transmitters (GABA and glycine) were depolarizing (Kandler and Gillespie 2005; Kim and Kandler 2003; Sanes and Friauf 2000; Sanes and Siverls 1991). It is unknown whether this is a general feature of inhibitory neuron development, and would therefore also occur in locally projecting inhibitory interneurons. However, it is clear that local inhibitory circuits profoundly influence the development of their networks (Hensch 2005; 2004).

We have recently identified an accessible inhibitory interneuron (R-interneuron) in the chick embryo spinal cord, which is the avian homologue of the mammalian Renshaw cell (Wenner and O’Donovan 1999) (Figure 1 schematic). At embryonic day 10 (E10) R-interneurons receive monosynaptic input from motoneurons, mediated predominantly by nicotinic receptors. These interneurons make direct projections back onto motoneurons in the same and adjacent segments and are GABAergic (possible small glycinergic contribution). R-interneuron axons can project multiple segments through the ventrolateral funiculus (VLF), and are located in a nucleus dorsomedial to the lateral motor column at both E7 and E10 (Xu et al. 2005). This disynaptic circuit first forms by E7, and the pharmacology of the R-interneuron circuitry is largely unchanged from this point to E15; however, the caudal spread of the output of the R-interneuron circuit appears to change (Xu et al. 2005). Because GABA is depolarizing at these stages the R-interneuron is excitatory and as a result plays a distinct functional role to that in the adult. During development the cell and its circuit are involved in the normal initiation
of the spontaneous network activity observed in the cord at embryonic ages (Wenner and O'Donovan 2001).

In this report we show a developmental reorganization of the R-interneuron circuit, as defined by a change in the strength of certain connections and/or in the number of specific connections within the circuit (not necessarily an anatomical change). This reorganization includes a functional refinement of the more distant GABAergic R-interneuron projection. However, strengthening the connections in the proximity of the stimulated motoneurons at the expense of the more distant connections was not observed uniformly throughout the rostrocaudal extent of the cord. The results are consistent with the possibility that the circuit reorganization could serve to sharpen connections between functionally related R-interneurons and motoneurons.

MATERIALS AND METHODS

Electrophysiology

Experiments were performed on White Leghorn chick embryos aged E8-E15. Embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton 1951). This report will refer to developmental age in days of incubation (i.e. embryonic day 8, E8) corresponding to the Hamburger Hamilton staging criteria, whether this was the actual incubation period or not. Three developmental stages were used in the study: early (E8, stage 34, shortly after the onset of circuit formation at a period when the response was reliably detectable), middle (E10/11, stage 36/37 when previous studies were carried out, referred to as E10), and late (E14/15, stage 40/41, the latest stage when the response can still be reliably observed, referred to as E15).
Embryos were decapitated and eviscerated under continuous superfusion with oxygenated Tyrode’s solution (concentration in mM: NaCl 139, KCl 2.9, NaHCO₃ 17, glucose 12.2, CaCl₂ 3, MgCl₂ 1) 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 and the isolated cord was left overnight to recover at 17°C. The next morning the central portion of the dorsal roots were cut so that we could selectively activate motoneurons following stimulation of the spinal nerve (referred to throughout this report as ventral root stimulation). The preparation 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 as described previously (Xu and Wenner, 2005; Figure 1 schematic; (O’Donovan 1987; 1989)). These ventral root recordings represent the electrotonically degraded motoneuron population potentials as shown originally in the 1940s (Brooks et al. 1948; Eccles 1946) and later using glass suction electrodes as sucrose gap recordings (Brink et al. 1981; Luscher et al. 1979; Roberts and Wallis 1978), and more recently in the in vitro preparation (Butt and Kiehn 2003; O’Donovan 1987; Wenner and O’Donovan 1999). The signals were amplified (1000), filtered (DC to .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.

Amplitudes of the responses in ventral root recordings following ventral root stimulation were normalized to the amplitude of the root potential produced during an episode of spontaneous network activity, where motoneurons throughout the rostrocaudal extent of the lumbosacral cord experience potentials of 20-30 mV in amplitude (O’Donovan 1999). This served to normalize each root recording, thereby accommodating for variability in the fit of the root in the electrode or to accommodate for a partially damaged root. Normalized values were
still variable as they were subject to changes in the excitability of the network or variability in the stimulated root. To reduce this variance the normalized amplitudes were expressed as a percentage of the normalized response in the root adjacent to the stimulated root, which had the largest response (normalization process described in greater detail in Xu and Wenner, 2005).

Whole-cell recordings were obtained from antidromically identified motoneurons (Xu and Wenner, 2005). Meninges were removed from the ventral surface overlying LS1-4 to allow for electrode penetration. Whole-cell electrodes (K-gluconate solution concentration in mM: NaCl 10, K-gluconate 94, KCl 36, HEPES 10, EGTA 1.1, CaCl2 0.1, MgCl2 1, Na2ATP 1) containing 5μM QX-314 to block action potentials (usually after 10 min dialysis) were then targeted to the lateral half of one side of the cord. Recordings were obtained from motoneurons using whole-cell electrodes (5-15 MΩ). To reduce variability in potential responses following ventral root stimulation, resting membrane potential was maintained at –70mV ±2mV. It is unlikely that variability was introduced based on variable dialysis of the cell, as the predominantly dendritic chloride-mediated conductances are not believed to be significantly influenced by the patch solution (Chub et al. 2006; Chub and O'Donovan 2001). Only cells with resting potentials more hyperpolarized than –40 mV, strong network driven potentials, and expression of antidromic action potentials following ventral root stimulation were accepted into the database.

Optical recordings.

To visualize the interneurons activated following stimulation of either ventral roots (R-interneurons) or the VLF (most interneurons) we isolated the spinal cord from thoracic segment 5 to lumbosacral segment 8, and retrogradely loaded many interneurons with
calcium-sensitive dye (Ca$^{2+}$ Green-1 dextran 10,000 MW; Molecular Probes). Interneurons were retrogradely labeled through their axonal projections into the VLF, which was drawn into a suction electrode containing ~ 20% w/v 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 dye back to interneuronal cell bodies. We removed the dorsal half of the cord to better visualize the dye-loaded cells in a ventral half preparation, where the R-interneuron circuit is located. This was accomplished by removing the dorsal and lateral pia mater (along with dorsal roots) and the cord was then pinned to a Sylgard block, ventral side up. A vibratome blade (Lieca VT 1000) was then positioned at the ventral surface of the cord. To create consistent ventral half preparations the blade was moved laterally away from the cord and then descended 230$\mu$m (at E8) or 300$\mu$m (at E10) toward the dorsal part of the cord and a horizontal cut was performed along the cord’s rostrocaudal axis. This generated approximately equal dorsal and ventral half pieces. The ventral-half preparation (Figure 5 schematic), along with intact ventral roots, was then transferred to the recording chamber and held in position with an anchor. Ventral roots were then drawn into suction electrodes for motoneuron stimulation and recording.

Images were continuously acquired of the dorsal aspect of the ventral-half preparation (Figure 5) through an inverted microscope (Olympus IX 70) to videotape using an intensified ccd video camera (Stanford Photonics) as the ventral root was stimulated (50Hz, 200 ms). 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. Images were downloaded onto a Macintosh computer and analyzed on ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/,
To determine if the interneurons fired action potentials following ventral root stimulation or during network activity we constructed difference images ($\Delta F$). These were generated by subtracting a 30 frame average obtained prior to the activity (background image) from an average of 8 consecutive frames during the peak of R-interneuron activity or a network driven activity. During episodes of network activity virtually all labeled neurons showed changes in fluorescence or became optically active (O'Donovan et al. 1994). A median filter (1 pixel radius) was used to better resolve optical activity. To better identify the active cells these difference images were stretched across their dynamic range (i.e. contrast enhanced – if pixel intensity values of optically active cells ranged from 0-20, these values were then stretched to occupy the entire 256 value range of the lookup table). These processed images were then used to draw regions of interest (ROIs) corresponding to optically active interneurons. By increasing or decreasing the stretch across the dynamic range we could resolve additional ROIs in the image. This provided an initial set of ROIs (R-interneurons or interneurons) that were adjusted (ROIs were added or removed) by monitoring the ROIs during playback of the video, which provided the most compelling evidence (see supplemental videos of spontaneous network activity at E8 and E10). ROIs were accepted as R-interneurons (following ventral root stimulation) or optically active interneurons (during network activity) if these ROIs, of 70 contiguous pixels or more (to distinguish individual cells from a part of the neuron – dendrite, axon), displayed an increased intensity of at least 2 SD over the mean background (O'Donovan et al. 1994). For acceptance into the database, preparations had to demonstrate multiple optically active R-interneurons that provided single cell resolution following stimulus trains (10x, 50Hz) to the ventral roots (Figure 5). In this way we could be relatively confident that the dorsal-ventral cut was close enough to the R-interneuron population, and that the stimulated ventral root was intact.
**Statistical analysis**

Student’s t-tests, or one way ANOVAs followed by Tukey posthoc tests were performed on the data to detect significant differences (JMP software), unless mentioned otherwise. For sequential antagonist applications in optical imaging a paired t-test was used. Means and corresponding standard errors are presented.

**RESULTS**

**Functional reorganization of the R-interneuron circuitry.**

*ventral root recording*

Previously, we had reported preliminary evidence suggesting that caudal projections of the R-interneuron circuitry may become more locally focused between E8 and E15, although this was not quantified. Here we extend these studies by stimulating a ventral root and quantifying the responses in adjacent ventral roots, either rostral or caudal to the stimulated root. These recordings allow us to sample the inputs of the entire population of motoneurons within a segment and provide a synchronous comparison of the output of the activated R-interneurons to motoneurons in different rostral or caudal segments.

Single suprathreshold stimuli (30μA, 0.5 ms) were delivered to the LS6 ventral root while recording LS5, 4, 3, and 2 ventral roots through tight fitting glass suction electrodes and averaging the responses (Figure 1A, n=3-20 recordings/average). The amplitudes of these responses were normalized twice (see methods for normalization procedure). In this way we were assessing the strength of the circuit’s rostral projection to different ventral roots. The LS3 ventral root response following LS6 stimulation became significantly weaker from E8 to E15.
(Figure 1; 39.8 ± 2.1% at E8; 17.6 ± 6.6% at E15). At E8 the responses at LS5 and LS4 were similar, but by E15 the LS4 response appeared to be reduced compared to LS5, however this did not reach significance (p=0.09 one tailed t-test). In contrast, the already weak projection to LS2 did not appear to change. Therefore a relative weakening of the rostral projection of the R-interneuron circuitry was observed from E8 to E15, but not in all roots.

We also quantified changes in the caudal projection of the R-interneuron circuit by stimulating LS2 and recording LS3-6 ventral roots (Figure 2). In addition, to better define the period of circuit reorganization, we tested the circuit at E8, E10, and E15. Following LS2 stimulation, the normalized LS4 root response was 111.5 ± 13.4% of the value for LS3 at E8, suggesting that the circuit projected to LS4 motoneurons with the same, or greater strength as it did to LS3 motoneurons, despite the greater distance. The projection to LS5 and LS6 was sequentially weaker. However, later in development the LS4 root response was significantly weaker than the LS3 response (50.3 ± 7.4% at E10; 61.1 ± 6.1% at E15). Because of the normalization process it was possible that the LS4 responses were reduced relative to LS3, not because of changes in the R-interneuron circuit but because the episode potential amplitude increased for LS4 relative to LS3 at the later stages. This did not appear to be the case as the amplitude of the episode potentials were not significantly different for the different roots at any of the ages, and if anything decreased for LS4 and LS5 at the later stages (LS3:LS4:LS5; E8 – 1033±312 : 954±244 : 920±288μV, E10 - 864±191 : 562±80 : 532±148μV, E15 - 329±62 : 243±34 : 285±68μV). As in the rostral projection of the circuit, the relative strength of the projection 4 segments away (caudally) did not change with development. These data show that developmental changes in the rostrocaudal projection of the R-interneuron circuitry occurred in a specific manner. The findings demonstrate that there was a functional
reorganization of the circuitry underlying the ventral root response. To better understand the mechanisms underlying the reorganization we recorded intracellularly from motoneurons.

**Whole cell recording of motoneurons.**

The functional reorganization of the R-interneuron circuit could occur through changes in the number of motoneurons receiving the disynaptic input, and/or in the strength of the inputs to each motoneuron. To examine how the circuit achieved the functional reorganization we obtained whole-cell recordings from motoneurons, which provided information about the strength of R-interneuron inputs, as well as the frequency of motoneurons receiving the disynaptic input. The clearest example of reorganization described above was observed following LS2 stimulation in the potentials produced in LS3 and LS4 ventral roots between E8 to E10. To test how functional reorganization occurred in this part of the circuit, we recorded from antidromically identified LS3 and LS4 motoneurons at E8 and E10, while stimulating the LS2 ventral root. It is likely that the potentials produced were mediated predominantly by a disynaptic circuit (motoneuron to R-interneuron and R-interneuron back to motoneuron) (Wenner and O'Donovan 1999; Xu et al. 2005). In neither whole cell nor ventral root recordings did we ever see potentials whose onset was less than 10 ms following the stimulation of adjacent ventral roots, suggesting that there were no direct monosynaptic connections between these motoneurons that could have contributed to the observed potential. The amplitudes of the potentials were measured at a latency corresponding to the peak of the potential recorded in the ventral root (~100 ms). This was typically also the peak of the potential in the intracellular record.

We observed that following LS2 stimulation the average potential in LS3 motoneurons receiving input was significantly increased from E8 to E10 (Figure 3A & B, for number of
motoneurons recorded see Figure legend or Table 1). This change cannot be explained by changes in the input resistance of the motoneurons, which decreased from E8 to E10 (661±85MΩ to 457±61MΩ; n=18, n=6). The amplitude of the LS2-evoked potential in LS4 motoneurons did not change from E8 to E10 (Figure 3B). Therefore, following LS2 stimulation, there was increase in the potentials of LS3 compared to LS4 motoneurons.

We also examined the percentage of LS3 or LS4 motoneurons receiving input from ventral root stimulation at E8 and E10 (Figure 3C). The percentage of LS3 motoneurons receiving input following LS2 stimulation increased from E8 to E10. However, the percentage of LS4 motoneurons receiving input decreased during the same period, and thus the percentage of LS3 and LS4 motoneurons receiving LS2-evoked input was the same at E8, but significantly different by E10 (Figure 3C). The results of relative increases in LS3 versus LS4 motoneurons for both amplitude and percentage receiving R-interneuron input were consistent with the functional reorganization observed in the ventral root recordings described above.

We sought to determine how common this pattern of relative strengthening of the more local circuitry was. We therefore stimulated the LS3 and LS4 ventral roots while recording from LS3 or LS4 motoneurons. The strongest inputs were, at both ages, from the most local stimulus (i.e. LS3 motoneurons received the strongest input from LS3 stimulation, Figure 4A). However, in neither segment was there a significant change from E8 to E10 following stimulation of either ventral root. If anything the LS4-evoked response got weaker in LS4 motoneurons at E10 (Figure 4B). Next we looked at the frequency of LS3 or LS4 motoneurons receiving input following stimulation of LS3 or LS4 ventral roots (Table 1). We observed a dramatic increase in the frequency of LS3 motoneurons receiving LS4 evoked input by E10. On the other hand, the frequency of other inputs to these motoneurons did not change from E8 to E10. Collectively, these data demonstrate that disynaptic projections following LS2
stimulation are strengthened for adjacent segments compared to more distant segments (LS3 versus LS4) by E10. However, this local strengthening was not observed uniformly throughout the rostrocaudal extent of the cord.

**Optical recordings show distribution and percentage of activated R-interneurons at E8 and E10.**

The ventral root and motoneuron recordings from the LS3 and LS4 segment demonstrated that the disynaptic circuit undergoes a functional rearrangement. Following LS2 stimulation, by E10 the circuit had weakened its projection to LS4 compared to LS3 motoneurons. Because we were assessing a disynaptic circuit, changes could have occurred in the recruitment of R-interneurons, and/or in the R-interneuron projection to motoneurons. To better understand where in the circuit changes occurred, we tested the recurrent connection to R-interneurons. This was accomplished using calcium imaging of interneuron cell bodies by retrogradely-labeling many interneurons from their axonal projections into the VLF (see methods). 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. 1994; O'Donovan et al. 1993). While our technique labeled many different species of interneuron, we could selectively activate R-interneurons in a particular segment by delivering stimulus trains to the associated ventral root. A column of cells dorsomedial to motoneurons became optically active following ventral root stimulus trains (Wenner and O'Donovan 1999, 2001). We monitored optically active interneurons in the LS2 or LS3 segment. Experiments were carried out in the presence of bicuculline to block the depolarizing output of the R-interneuron, and prevent the possible recruitment of other neurons. This technique allows us to selectively image R-interneurons (Wenner and O'Donovan, 2001).
At both E8 (Figure 5A-D, supplemental video) and E10 (Figure 5E-H, supplemental video) optical recordings showed that R-interneurons in LS2 or LS3 could only be activated by stimulating the LS2 or LS3 ventral root, respectively (8 of 8 ventral root stimulations, 5 preparations at E8; 8 of 8 ventral root stimulations, 5 preparations at E10). Even when R-interneurons were very close to each other at the LS2/3 border (within ~100μm, Figure 5C & G), only one root was capable of driving an R-interneuron to become optically active, never both, suggesting that the motoneurons only activated R-interneurons in the same segment. Moreover, we never observed R-interneurons following stimulation of both roots that had not been observed by one of the roots individually (spatial facilitation), and the optical signals produced were no larger than stimulation of the individual effective root. This suggests that stimulation of the ineffective root did not provide significant subthreshold input. Together the results imply that motoneuron recurrent collaterals are limited in their rostrocaudal projection to their own segment at both E8 and E10 and argue against a functional rostrocaudal refinement of the recurrent collateral at these stages.

While the functional rostral and caudal spread of the motoneuron recurrent collateral did not appear to change during this developmental period, it was still possible that within a segment the number of activated R-interneurons following stimulation of that segments ventral root was changed between E8 and E10. To address this possibility we counted the number of optically active R-interneurons in a segment following stimulation of that segment’s ventral root, and marked a region of interest (ROI) for each R-interneuron (Figure 5B & F, arrowheads). To account for variability in the labeling procedure we normalized the number of optically identified R-interneurons (ventral root stimulation) to the number of optically active interneurons during an episode of network activity when virtually all the labeled neurons become active and exhibit changes in fluorescence (O'Donovan et al., 1994, Figure 5D & H,
see methods for a detailed explanation of technique). The number of optically active neurons during episodes of network activity was higher at E8, possibly due to the fact that cells were smaller at this stage and the tissue was more translucent, so more interneurons are sampled in our focal plane (Figure 5D & H). Across all E8 preparations, 41 of the 217 optically active interneurons in the focal plane were R-interneurons (5 segments, 4 preparations) while at E10, 54 of the 147 optically active interneurons were R-interneurons (6 segments, 4 preparations). When comparing the average percentage of R-interneurons from the E8 (19.8±2.9%) and E10 (35.5±5.8%) preparations we saw that a significantly higher proportion of optically active cells were R-interneurons at E10 (p<0.05, two tailed t-test). This increased R-interneuron population could result from an increase in the strength of existing motoneuron recurrent collateral connections and/or newly formed connections at the later stage. In summary, by E10 more R-interneurons were recruited, and their projection to LS3 motoneurons were stronger, yet fewer LS4 motoneurons received this R-interneuron input.

**Optical recordings show glutamatergic component to the recurrent activation of R-interneurons at both E8 and E10.**

A recent report suggested that the ventral root-evoked ventral root response was mediated by glutamatergic, in addition to GABAergic and nicotinic transmission. In order to determine if motoneuron-evoked activation of R-interneurons was partly dependent on glutamatergic transmission, we made optical recordings of R-interneurons as described above at E8 and E10. Further, this allowed us to test if a developmental change in the glutamatergic component of R-interneuron activation could play a role in the circuit reorganization. We monitored the average pixel value of ROIs before, 30 minutes after the addition of, and 45 minutes after the washout of the AMPA/Kainate receptor (CNQX, 50µM) and NMDA receptor
(AP5, 50μM) antagonists (Figure 6; in the presence of bicuculline throughout). We found that after addition of the glutamate receptor antagonists to the bath all the ROIs continued to exhibit detectable signals, but the intensity of the optical signal was reduced at both ages to a similar extent (see Figure 6 legend). Upon washout, the signals were stronger than before glutamatergic antagonists were added (see Figure 6 legend). This finding suggests that reduced optical signals were not due to bleaching, and are consistent with the compensatory nature of the cord excitability following transmitter antagonist application (Chub and O'Donovan 1998). At E10, optical signals were reduced in either CNQX or AP5, each to a smaller extent than when both antagonists were present (CNQX – 90.8±1.7%, n=23, p<0.0001; AP5 - 87.9±2.7%, n=17, p<0.005). The findings suggest that the recurrent activation of R-interneurons has a glutamatergic component. Further, the extent of this glutamatergic contribution to the recurrent activation of R-interneurons did not change between E8 and E10, and thus was not likely to underlie the reorganization of the circuit.

**DISCUSSION**

Despite their importance in network function, very little is known about the development of inhibitory interneurons. We have determined that the disynaptic GABAergic R-interneuron circuitry undergoes a functional reorganization between E8 and E10. We first assessed the R-interneuron circuitry (Figure 1) by stimulating one ventral root and recording the evoked potential in the adjacent ventral roots. By E10 parts of the circuit had become more locally focused. Whole cell recordings from motoneurons demonstrated that the disynaptic potentials produced by LS2 ventral root stimulation became stronger and more frequent in LS3 motoneurons and less frequent in LS4 motoneurons from E8 to E10. Finally, optical imaging experiments were used to demonstrate that R-interneurons are spatially restricted to the
segment of the stimulated motoneurons at both stages, but that more R-interneurons were likely activated within a segment at E10. We identified a glutamatergic component to the recurrent activation of R-interneurons that was similar at E8 and E10. Based on these results we propose that the circuit rearrangement is mediated through at least 2 processes. First, LS2-activated R-interneuron projections to some LS4 motoneurons were functionally silenced, suggesting that local projecting inhibitory interneurons undergo refinement. Second, the increase in activated R-interneurons within a segment at E10 likely contributes to the stronger projection to LS3 motoneurons. Because this pattern of favoring of the more local projections of the circuit did not occur uniformly across different rostrocaudal segments, the functional significance of the reorganization may be to sharpen connections between functionally related motor and R-interneurons, as discussed below.

**Mechanisms underlying inhibitory circuit reorganization.**

Ventral root evoked ventral root responses demonstrated that caudal and rostral projections of the R-interneuron circuit became more concentrated in nearby segments later in development. The observation that the circuit modifications occurred in both directions makes it unlikely that the reorganization was dependent on different maturational states along the rostrocaudal axis of the cord. Intracellular motoneuron recordings obtained following LS2 ventral root stimulation demonstrated a change in the relative strength of the response in LS3 versus LS4 motoneurons by E10. We determined that fewer LS4 motoneurons and more LS3 motoneurons received the disynaptic response at E10. This suggests that one of the ways the circuit had been modified was through changes in the types of motoneurons (LS3 versus LS4) to which the activated R-interneurons projected. In addition, from E8 to E10 the amplitude of the disynaptic potential became larger in LS3 motoneurons but not LS4 motoneurons.
Because motoneuron input resistance was smaller at E10 the disynaptic currents were likely even more increased for LS3 motoneurons, and slightly increased for LS4 motoneurons.

Circuit reorganization could also come about as a result of changes in the recruitment of R-interneurons following motoneuron stimulation. Previous reports in the neonatal cat have demonstrated that there is a developmental elimination of synaptic boutons of the motoneuronal recurrent collateral, but this refinement process did not reduce the rostrocaudal extent of recurrent boutons (Cullheim and Ulfhake 1982; Remahl et al. 1985). Using calcium imaging techniques to assess the number and location of R-interneurons, we demonstrated that motoneuron recurrent collaterals were only capable of recruiting R-interneurons in the same segment as the activated motoneurons at both E8 and E10, consistent with a previous anatomical study showing that the recurrent collateral did not extend rostrocaudally (Velumian and Poliakova 1992).

Although there was no change in the rostrocaudal recruitment of R-interneurons, we did find that within a segment, more R-interneurons were recruited (higher percentage of optically active interneurons) following ventral root stimulation at E10. This is consistent with the idea that the recurrent motoneuronal connections to R-interneurons continue to form after E8, one day after motoneurons are first able to activate R-interneurons (Xu and Wenner, 2005). An alternative possibility is that a similar number of R-interneurons are recruited at both ages, but that our imaging technique was unable to pick up some R-interneurons that spike at E8, possibly because they do not fire enough action potentials to produce a detectable calcium transient. Such a possibility would suggest that the R-interneuron population is close to our threshold for optically detecting their activity. We do not favor this possibility because blockade of an excitatory (glutamatergic) component of the synaptic drive to R-interneurons resulted in a reduction in the intensity of the optical signal, yet all of the optically active cells were still
detectable. This suggests that the R-interneurons were not close to the threshold for detection. Several observations argue against the possibility that more R-interneurons project into the VLF by E10, and are therefore retrogradely labeled with the calcium dye: there are fewer total optically active interneurons during network activity at E10, many interneurons are known to project several segments by E4.5 (Oppenheim et al. 1988), and whole-cell recordings of R-interneurons were more commonly obtained at E10 than at E8 (Xu and Wenner, 2005).

Although several transmitters are known to mediate the disynaptic R-interneuron circuit (nicotinic, glutamatergic, GABAergic), it is unlikely that the circuit reorganization could be explained by developmental changes in the relative contributions of these different transmitters. No clear changes in transmitters were observed in the ventral root evoked ventral root potential from E8 to E15 in a previous study (Xu et al., 2005). The study did find a small glutamatergic contribution to the ventral root response, consistent with previous work suggesting that the motoneuron input to Renshaw cells in the mouse is partly glutamatergic (Mentis et al. 2005; Nishimaru et al. 2005). It remained possible that the motoneuronal recurrent activation of R-interneurons in the chick embryo also had a glutamatergic component and that its relative contribution could change during development. In the current study we found that indeed there is a small glutamatergic contribution to the activation of most R-interneurons, but that it does not change from E8 to E10, and therefore is unlikely to contribute to the reorganization.

Taken together, these results are consistent with a reorganization of the R-interneuron circuit between E8 and E10, where more R-interneurons are recruited following ventral root stimulation at E10. This increased population then projects more strongly to each LS3 motoneuron (larger amplitude potentials) and projects to more LS3 motoneurons. These R-interneurons, during the same period project to fewer LS4 motoneurons (see below).
Potential synaptic refinement of the GABAergic R-interneuron.

Although recruiting more R-interneurons at E10 would be expected to lead to stronger projections to LS3 motoneurons, the observation that fewer LS4 motoneurons received input from these activated R-interneurons at E10 was surprising. This finding suggests that there was an elimination of functional R-interneuron synaptic projections to LS4 motoneurons, rather than the loss of a subpopulation of R-interneurons that make the longer range projection. The results make it likely that R-interneuron projections to some LS4 motoneurons were functionally silenced between E8 and E10. This finding suggests that inhibitory interneurons within a network can undergo a functional synaptic refinement. Additional studies will be necessary to determine the period and anatomical extent of this refinement.

Much like the studies of excitatory projection neurons relaying a topographic sensory map, recent studies suggest that inhibitory projection neurons that relay a tonotopic map in the auditory system undergo a functional refinement, followed by a structural pruning later in development (Kandler and Gillespie 2005; Kim and Kandler 2003; Sanes and Friauf 2000; Sanes and Siverls 1991). Functional refinement in the auditory system, as appears to be the case for the R-interneuron, occurs at a developmental stage when GABA and glycine are depolarizing due to high intracellular chloride concentration (Kandler and Gillespie 2005; Kim and Kandler 2003). Therefore, inhibitory refinement may be a general phenomenon that shares certain underlying mechanisms with excitatory refinement (depolarization leading to calcium entry).

Functions of circuit reorganization.

R-interneuron circuit reorganization would likely affect the expression of spontaneous network activity that is experienced by the embryonic cord. R-interneurons are known to play
an important role in the initiation of this spontaneous network activity (Wenner and O'Donovan 2001), which is important in the maturation of synaptic strength, axon pathfinding, and limb development (Gonzalez-Islas and Wenner 2006; Hall and Herring 1990; Hanson and Landmesser 2004; Jarvis et al. 1996; Persson 1983; Roufa and Martonosi 1981; Ruano-Gil et al. 1978; Toutant et al. 1979).

Another consequence of the R-interneuron circuit reorganization is that the circuit is more locally focused later in development. The pattern of rostrocaudal projections of the homologous Renshaw cell circuit in the adult cat is such that the strongest projections are those closer to the activated motoneurons, and this has been termed the proximity principal (Eccles et al. 1961; Eccles et al. 1954; McCurdy and Hamm 1994; Renshaw 1941; Thomas and Wilson 1967). The Renshaw circuit achieves its rostrocaudal spread via the Renshaw axonal projection through the VLF (Hultborn et al. 1971; Jankowska and Smith 1973; McCurdy and Hamm 1994; Ryall et al. 1971). Similarly, R-interneurons project rostrocaudally to motoneurons in adjacent segments through the VLF (Wenner & O'Donovan, 1999; Xu & Wenner, 2005). The R-interneuron circuit reorganization found in this study could contribute to a more mature proximally projecting circuit, like that of the adult cat Renshaw circuit.

While R-interneuron circuit reorganization does appear to concentrate the circuit’s projections closer to the activated motoneurons when stimulating at LS2 or LS6, the totality of our results suggest a more complex phenomenon. For instance, from E8 to E10 there was no change in the response (frequency receiving input or amplitude of potential) in LS3 motoneurons to LS3 ventral root stimulation (same segment, Figure 4). During the same period there was a dramatic increase in the proportion of LS3 motoneurons that received LS4 input (more distant, Table). Therefore, a general rule that assumes all R-interneurons are the same, in that they all weaken their more distant projections and strengthen their closer
connections, is overly simplistic. Therefore a more complex set of rules must be at play. It is possible that the reorganization (strengthening or weakening) could serve to sharpen the connections between functionally related R-interneurons and motoneurons from E8 to E10, and could act to remove functional mismatches in interneuronal connectivity as has been proposed previously (Glover 2000; Mears and Frank 1997; Seebach and Ziskind-Conhaim 1994). For example, it may be that LS2 stimulation activates a population of R-interneurons that are most related to adductor motoneurons, and at E8 these project to adductor (thigh) and femorotibialis (leg extensor) motoneurons in the LS3 segment, and in the LS4 segment to iliofibularis (leg flexor) motoneurons, among others. By E10 the connections to adductor and femorotibialis motoneurons might be strengthened, while the connections to LS4 iliofibularis motoneurons are weakened or silenced. Because we did not identify motoneurons by the muscle they innervated in this study, but rather by the segment they occupied, we were not in a position to assess connectivity across functional subclasses. Alternatively, it is possible that rostrocaudal focusing does not occur across different motoneuron species, but occurs within a particular motor pool and would thus be dependent on the unique rostrocaudal location of that motor pool. Regardless, we were able to determine that circuit reorganization does occur from E8 to E10, and it will be important in the future experiments to test if certain motoneuron species lose or strengthen their R-interneuron inputs. Any of the above mentioned changes would affect interneuronal circuits and therefore impact locomotor activity in the more mature animal.

Another aspect of the reorganization that is inconsistent with a simple proximity-based sharpening is the observation that long-range (4 segment) R-interneuron projections did not appear to weaken in the ventral root recordings. Renshaw cells are thought to project only 1 or 2 segments at most in the cat and mouse. Recent reports, in the developing mouse and rat,
have suggested the possibility that there are different classes of interneurons that receive
direct input from motoneurons, and that some project multiple segments, and may use a
different transmitter than Renshaw cells (Hanson and Landmesser 2003; Machacek and
Hochman 2006; Mentis et al. 2005). It is possible that the interneurons mediating multisegment
ventral root responses in the chick embryo are similar to the cells described in the developing
rat and/or mouse. These longer projecting neurons would have distinct functions from the
classical Renshaw cells, and have been proposed to provide a feedback excitation and would
maintain ongoing activity in the more mature system (Machacek and Hochman 2006). In the
chick embryo, these long projecting cells may not undergo refinement because it may be
important to coordinate the activity of some cells that are separated by multiple segments in
the more mature cord. Alternatively, if these multisegmental projections are glutamatergic
(Machacek and Hochman 2006; Mentis et al. 2005) then they may undergo refinement, but at
a later stage in development when other excitatory projections in the spinal cord refine.
Figure Legends

Figure 1  Reorganization of the rostral spread of the R-interneuron circuitry. A) Schematic shows LS6 R-interneuron projecting to motoneurons in various segments (LS3-6). The cut face of the spinal cord is expanded to show motoneuron (MN) recurrent collateral connecting to the R-interneuron (R-int). LS6-evoked response can be recorded in ventral roots using suction electrodes. Ventral root recordings (LS3-LS5) are first normalized to episode potentials in the same root and then to the normalized LS5 response at E8 and E15. Ventral root recordings were averaged and shown in traces on the right at different stages of development. Thus the noise associated with each trace can vary significantly (i.e. because the episodic potentials are small for the LS3 recordings, the noise becomes large following the normalization procedure). B) Rostral spread of the R-interneuron circuitry changes during development. Graph shows that normalized LS3 ventral root response to LS6 stimulation was significantly weakened by E10 compared to E8 (student t-test). Data points represent n=5 preparations for E8, and n=3 preparations at E10 (exceptions shown in parentheses). Error bars denote standard error.

Figure 2  Caudal projection of the R-interneuron circuitry changes during development. Graph shows that the normalized LS4 ventral root response to LS2 stimulation is significantly weakened at E11 and E15 compared to E8 (student t-test). Data points represent n=4 preparations (exceptions shown in parentheses, n=7 preparations for E15 LS3 root potential to which other E15 potentials were normalized). Error bars denote standard error.

Figure 3  Whole cell motoneuron recordings show LS2-evoked potentials in LS3 and LS4 motoneurons at E8 and E10. (A) Schematic shows recording configuration while in an LS3
motoneuron, which can be identified by its antidromic action potential (antidromic shown for E8 LS3 motoneuron in upper trace). The amplitude of the LS2-evoked potential in LS3 motoneurons increases from E8 to E10, while the potential is similar in LS4 motoneurons at both stages. Baseline before stimulus delivered was set to −70 mV. Multiple peaks are common in these recordings and are likely due to R-interneurons that often fire multiple times (Wenner and O'Donovan, 1999). (B) The amplitude of the LS2-evoked potential in LS3 motoneurons (n=17 at E8, n=16 at E10) significantly increased from E8 to E10, while there was no change for LS4 motoneurons (n=16 at E8, n=13 at E10). Error bars denote standard error. (C) The percent of motoneurons receiving input following LS2 stimulation was similar for LS3 (17 of 23) and LS4 (16 of 20) motoneurons at E8 but decreased for LS4 (13 of 21) and increased for LS3 (16 of 17) motoneurons by E10.

Figure 4  LS3 and LS4-evoked potentials do not change significantly from E8 to E10. Average amplitudes for LS3-evoked (A) or LS4-evoked (B) potentials observed in whole cell recordings from LS3 and LS4 motoneurons are shown. LS3 motoneurons receive the largest amplitude potentials from LS3 stimulation, while LS4 motoneurons receive the largest amplitude potentials from LS4 stimulation. However, the amplitude of these disynaptic inputs do not change from E8 to E10. Number of motoneurons recorded are shown in parentheses. Error bars denote standard error.

Figure 5  Calcium imaging experiments demonstrate that R-interneurons are only activated by motoneurons within the same segment at both E8 and E10. The dorsal aspect of the ventral half preparation is imaged as shown in the schematic. Background subtracted, pseudocolored images are shown during the peak of optical activity in E8 preparations following LS3 ventral
root (VR) stimulation (A), LS2 VR stimulation (B), or VLF stimulation evoking an episode of network activity where virtually all the labeled cells become active (D). Arrowheads show the positions of R-interneurons in the total population of optically active cells. Panel C shows panels A and B pseudocolored red and green, respectively and overlapped to show the proximity of R-interneurons activated by different roots. E-H are the same as A-D but at E10.

**Figure 6** Calcium imaging demonstrates that the motoneuron-evoked activation of R-interneurons has a small glutamatergic component at both E8 and E10. LS2 ventral root stimuli activate R-interneurons in the presence of bicuculline, to block the output of the R-interneuron, at both E8 (A) and E10 (B). Top images show pseudocolored averaged images during the peak of the ventral root response that demonstrate a change in the fluorescence (ongoing image – background image before stimulation). The white outline in images represents the ROI, whose average intensity is measured over time and plotted in the traces shown below the images. After adding glutamatergic antagonists to the bath, the optical response is slightly reduced at both stages (E8 - Figure 6A, 75.2±4.4%, n=6; E10 - Figure 6B, 73.0±4.9%, n=26; not significantly different between E8 and E10 p=0.42, but significantly different from 100% at both E8 and E10 p<0.00001). Washout of the glutamatergic antagonists, still in the presence of bicuculline, demonstrates a recovery of the optical response that is stronger than the original response in bicuculline (E8 – 151±3.9%, n=6; E10 - 168.1±8.0%, n=46; not significantly different between E8 and E10 p=0.45, but significantly different from 100% at both E8 p<0.0001, and E10 p<0.00001). AU is arbitrary units and represents changes in fluorescence intensity in the ROI.
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Figure 2

Stimulate LS2 Ventral Root

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<td><strong>E10</strong></td>
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