Early postnatal development of GABAergic presynaptic inhibition of Ia proprioceptive afferent connections in mouse spinal cord

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Sonner PM, Ladle DR. Early postnatal development of GABAergic presynaptic inhibition of Ia proprioceptive afferent connections in mouse spinal cord. J Neurophysiol 109: 2118–2128, 2013. First published January 23, 2013; doi:10.1152/jn.00783.2012.—Sensory feedback is critical for normal locomotion and adaptation to external perturbations during movement. Feedback provided by group Ia afferents influences motor output both directly through monosynaptic connections and indirectly through spinal interneuronal circuits. For example, the circuit responsible for reciprocal inhibition, which acts to prevent co-contraction of antagonist flexor and extensor muscles, is driven by Ia afferent feedback. Additionally, circuits mediating presynaptic inhibition can limit Ia afferent synaptic transmission onto central neuronal targets in a task-specific manner. These circuits can also be activated by stimulation of proprioceptive afferents. Rodent locomotion rapidly matures during postnatal development; therefore, we assayed the functional status of reciprocal and presynaptic inhibitory circuits of mice at birth and compared responses with observations made after 1 wk of postnatal development. Using extracellular physiological techniques from isolated and hemisected spinal cord preparations, we demonstrate that Ia afferent-evoked reciprocal inhibition is as effective at blocking antagonist motor neuron activation at birth as at 1 wk postnatally. In contrast, at birth conditioning stimulation of muscle nerve afferents failed to evoke presynaptic inhibition sufficient to block functional transmission at synapses between Ia afferents and motor neurons, even though dorsal root potentials could be evoked by stimulating the neighboring dorsal root. Presynaptic inhibition at this synapse was readily observed, however, at the end of the first postnatal week. These results indicate Ia afferent feedback from the periphery to central spinal circuits is only weakly gated at birth, which may provide enhanced sensitivity to peripheral feedback during early postnatal experiences.

presynaptic inhibition; development; proprioceptive; spinal cord; GABAergic feedback

PROPRIOCEPTIVE FEEDBACK is critical for normal locomotion. Primary proprioceptive neurons provide information to motor circuits in the spinal cord by virtue of their different sensory endings in the periphery. The effects of feedback provided by group Ia proprioceptive afferents have been the subject of study for many years (Baldissera et al. 1981; Eccles et al. 1957; Prochazka 1996; Proske 2006; Zehr and Stein 1999). Stretch-activated channels associated with the annulospiral terminations of these afferents on intrafusal muscle fibers provide the molecular substrate for high sensitivity to changes in muscle length (Banks 2005; Simon et al. 2010). These sensory signals are transmitted directly to motor neurons (MNs) and interneurons related to motor control in the spinal cord. Readily observable deficits in coordinated movements of various genetic mutants lacking Ia afferent feedback illustrate the critical importance of this sensory modality in voluntary locomotion (Arber et al. 2000; Levanon et al. 2002; Tourtellotte and Milbrandt 1998).

Group Ia afferent proprioceptive feedback affects MN output and consequent locomotion, via both direct and indirect excitation and indirect inhibition of target MNs (Eccles et al. 1957; Eccles and Lundberg 1958; McCrea et al. 1995). Direct communication with MNs is accomplished through the monosynaptic stretch reflex, in which Ia afferents that convey information from a particular muscle in the periphery establish glutamatergic excitatory connections with MNs projecting back to the same muscle or with MNs innervating close synergists, but do not synapse on MNs that project to antagonist muscles (Eccles et al. 1957; Mears and Frank 1997). The indirect inhibitory pathway of Ia afferent feedback that has been studied most extensively is that of reciprocal inhibition, which acts to prevent co-contraction of antagonist muscles (Jankowska 1992). This effect is mediated by a class of glycine-releasing interneurons termed Ia inhibitory interneurons (IaINs) located near MN pools in the ventral horn of the spinal cord (Curtis et al. 1968; Jankowska and Lindstrom 1972). Distinct subsets of these interneurons receive monosynaptic input from axon collaterals of Ia afferents carrying sensory information from either flexor or extensor muscles and act to inhibit MNs innervating antagonist muscles (Eccles and Lundberg 1958). For example, extensor Ia afferents, such as those innervating the knee extensor quadriceps muscle group, activate subsets of IaINs that inhibit MNs projecting to flexor muscles of the knee.

Both direct and indirect Ia afferent feedback can be limited by presynaptic inhibition, which acts to reduce synaptic transmission between Ia afferents and their target neurons. Activation of axo-axonic contacts arising from GABAergic interneurons onto Ia afferent terminals results in depolarization of the synaptic terminal and limits sensory neurotransmitter release (Alvarez 1998; Hughes et al. 2005; Rudomin and Schmidt 1999). Presynaptic inhibitory control of synaptic transmission can be highly selective, affecting only subsets of terminals arising from a single Ia afferent collateral (Lomeli et al. 1998; Quevedo et al. 1997). Furthermore, studies of adult locomotion indicate the degree of presynaptic inhibition can change rapidly, in concert with ordered muscle contraction during locomotion (Capaday et al. 1995; Gossard et al. 1989, 1990; Gossard and Rossignol 1990; Rossignol et al. 2006; Stein 1995).

The development of direct and indirect Ia feedback pathways, as well as the gating of Ia afferent feedback by presynaptic inhibition, is of fundamental interest in understanding the acquisition of normal motor skills. Are these circuits effective in altering MN output at birth, as neonates begin to develop purposeful motor control? Studies attempting to answer this question have focused primarily on analysis of developing rodents. The answer is clear for the direct monosynaptic con-
connections of the stretch reflex circuit. This pathway is first observed at E19.5 in rats (Kudo and Yamada 1985) and at E17.5 in mice (Mears and Frank 1997). While the strength of the reflex pathway increases over the first postnatal week in rodents, stimulation of Ia afferents, even in embryonic stages, can lead to action potentials in MNs (Mears and Frank 1997). Reciprocal inhibition can also be detected at birth, but initial reports suggest the strength of inhibition may increase over the first postnatal week (Wang et al. 2008). Presynaptic inhibition has been measured primarily through detection of primary afferent depolarization (PAD) in the form of a dorsal root potential (DRP) that originates on central terminals of afferents in the spinal cord and then is conducted antidromically into the periphery along sensory axons. DRPs are readily detected from early neonatal rodent preparations (Hayes et al. 2012; Vinay and Clarac 1999). Nevertheless, dorsal roots contain a variety of proprioceptive and cutaneous afferents, and it is unclear whether presynaptic inhibition of Ia afferents, or of proprioceptive afferents in general, is functional in early neonatal mice.

In this study, we investigated the status of reciprocal inhibition and of presynaptic inhibition of group Ia afferents at birth and at the end of the first postnatal week in mice. Using an isolated spinal cord preparation and extracellular physiological assays focusing on antagonistic knee flexor and extensor motor groups, we found reciprocal inhibition to be as robust, stable, and effective at birth as at 1 wk of age. These results suggest that la afferent feedback control of flexor and extensor MNs through reciprocal inhibition is likely to be fully functional at birth. In contrast, proprioceptive evoked gating of Ia afferent input by presynaptic inhibitory circuits is not sufficiently strong at birth to alter direct activation of MNs by Ia afferents. By the end of the first postnatal week, however, robust presynaptic inhibition of Ia afferent synaptic transmission is consistently observed.

MATERIALS AND METHODS

Spinal cord preparation. All procedures for animal experiments were approved by the Wright State University Animal Care and Use Committee. Neonatal mice (C57BL/6) from two different age groups were used in this study. Mice in the first group were studied on the day of birth or the first postnatal (P) day (P0/P1). A second group of mice were studied after 1 wk of postnatal development at either P7 or P8. Isolated spinal cords dissected in continuity with selected peripheral nerves were prepared as described previously (Mears and Frank 1997). Briefly, mice were anesthetized by hypothermia induction in an ice water bath and then perfused transcardially with 5 ml of ice-cold, oxygenated (95% O2-5% CO2) artificial cerebrospinal fluid [ACSF; containing in mM: 127 NaCl, 1.9 KCl, 1.2 KH2PO4, 1 MgSO4·7H2O, 26 NaHCO3, 16.9 D(+)-glucose monohydrate, and 2 CaCl2]. The spinal column and attached lower limbs were dissected free and immersed in a recirculating bath of cold (16–18°C), oxygenated ACSF. The spinal cord was exposed by dorsal laminectomy of the spinal column and careful removal of the dura. All spinal cords, regardless of the age of the preparation, were hemisected to maximize oxygen penetration into the cord. Previous studies have shown increased function of monosynaptic sensory-motor connections following hemisection (Jiang et al. 1999). The hemisected cord was then removed from the vertebral column together with several peripheral nerves projecting to muscles of interest in the periphery. These included the nerve bundle projecting to the knee flexor muscles posterior biceps and semitendinosus (PBST) and the nerve supplying the knee extensor quadriceps (Quad). The saphenous (Saph), a purely cutaneous nerve, was also dissected free in some preparations. The final preparation was transferred to the recording chamber and allowed to recover for 1 h while the recirculating oxygenated ACSF equilibrated to room temperature (22°C). All recordings were made at room temperature.

Peripheral nerve recordings. Extracellular recordings of either sensory or motor axon responses were obtained using suction electrodes (see Fig. 1A for diagram). The nerve to be recorded from, usually PBST, was placed in a suction electrode (A-M Systems, Sequim, WA). Responses were recorded with an EX4-400 Quad Channel Differential Amplifier (1,000× gain, 2 Hz low cut, 500 Hz high cut; Dagan, Minneapolis, MN) and digitized at 20 kHz with the use of WinLTP software (WinLTP, Bristol, UK). Compound action potentials (CAPs) in the PBST were evoked by stimulation of afferent fibers in dorsal root L5 (DRL5). DRL5 was cut just proximal to the dorsal root ganglion and carefully pinned to the bottom of a Sylgard-covered recording chamber to allow for stimulation. A matrix electrode [catalog no. MX21AEW(RT1); FHC, Bowdoin, ME] was positioned on the cut DRL5 and triggered using a constant current stimulus isolator (A365; World Precision Instruments, Sarasota, FL). Positive unipolar current pulses (0.1-ms duration) required to elicit the maximal size of the PBST CAP varied between preparations but ranged from 1 to 5 mA. Sweeps where only DRL5 was stimulated were referred to as test pulses (T).

Conditioning pulses of muscle nerves were delivered through suction electrodes. Most commonly, afferents in Quad nerve were stimulated using a second constant current stimulus isolator (200 μA, 0.1-ms duration). The ventral roots of lumbar segments 2–4 were cut to eliminate effects mediated by antidromic stimulation of Quad motor axons (Wang et al. 2008). Sweeps with a test pulse (T) to DRL5 were alternated every 10 s with sweeps in which a conditioning pulse of the Quad preceded a test pulse to DRL5 (C+T) by a predetermined interval (Fig. 1B). Intervals ranged from 0 ms (synchronous stimulation of Quad and DRL5) to 32 ms (Quad stimulation 32 ms before DRL5) in 2-ms increments. Longer conditioning intervals of 40 and 50 ms were also tested. For each interval, alternating T and C+T pulses were presented six times each and the responses were averaged offline for later analysis before a new conditioning interval was tested. In some experiments, the entire series of conditioning intervals were presented multiple times to test the stability of responses over time. In these cases, the order in which intervals were tested was randomized from series to series to control for the possibility of order-dependent effects. Average traces were analyzed using custom routines in MATLAB (The MathWorks, Natick, MA) to rectify and integrate the area of the waveforms of the average CAPs from the initiation of the rise phase to the maximum peak of the hyperpolarization period. Ratios of the resultant areas of the T and C+T CAPs were calculated, referred to as response ratios, and plotted against the conditioning stimulus interval. Latency-to-peak values were measured in WinLTP.

The intensity used for the conditioning stimulation pulse was determined by stimulating the peripheral Quad nerve and recording the response from DRL3 (from which a proportion of quadriceps afferents enter) to determine the threshold for quadriceps afferents. We then ran a full series of T/C+T pulses with Quad stimulation of 200 μA (ranging from 7.75–10T; n = 3). We determined the conditioning intervals associated with maximal short- and long-interval inhibition and re-ran the response ratio paradigm at these conditioning intervals at various multiples of threshold (1.5T, 3T, and 5T). We determined that there was not a significant difference among the degrees of inhibition at the various thresholds studied (1.5T, 3T, 5T, and 7.75–10T) at either the short-interval inhibition (P = 0.95, 1-way ANOVA) or long-interval inhibition (P = 0.97, 1-way ANOVA). Of note, both short- and long-interval inhibitions were observed at 1.5T.

In preparations designed to measure primary afferent depolarization responses, all dorsal roots of the lumbar spinal cord (L1–L6) were kept intact, whereas all of the corresponding ventral roots (L1–L6) were cut to eliminate any contribution from motor axon activation. No test pulses were given. Sensory afferent responses in the PBST nerve were measured.

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using a suction electrode following stimulation of Quad or other nerves. Stimulation pulses (200 μA) were presented at 0.1 Hz.

Statistical comparisons between small samples were performed using the nonparametric Wilcoxon rank sum test. Comparisons between larger groups were performed using Student’s t-test. Data are reported as means ± SE, except in Figs. 1 and 5, in which data points plotted in the graphs represent the mean ± SD. Results were considered to be significant if P ≤ 0.05.

Pharmacology. To determine the relative contributions of glycnergic and GABAergic pathways in inhibiting MN responses, oxygenated ACSF containing either 5 μM bicuculline or 0.4 μM strychnine was allowed to recirculate in the bath for 5 min before recordings began to allow time for the drugs to take effect. Concentrations were chosen to maximize specificity for their respective receptors, as described previously (Wang et al. 2008). All chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

RESULTS

We adapted an extracellular recording assay utilizing the isolated spinal cord preparation to measure changes in motor output evoked by proprioceptive afferents via interneuron-mediated pathways (Wang et al. 2008). Motor neurons in the L5 lumbar spinal segment were activated through monosynaptic inputs from Ia afferents by electrical stimulation of the L5 dorsal root (see Fig. 1A for diagram). Stimulation of the entire dorsal root activates Ia afferents projecting to various muscles and results in broad activation of MNs projecting to many muscles in the hindlimb. To observe effects on selected MN pools, recordings were made from identified muscle nerves in the periphery where the area of the CAP is a function of the number of MNs driven to threshold by activation of DRL5 Ia afferents that project to that particular
muscle. We chose to record the CAP response of MNs innervating two major knee flexor muscles, the posterior biceps and semitendinosus (PBST), which together are supplied by a distinct muscle nerve bundle that branches from the common sciatic nerve.

To test the effectiveness of interneuron-mediated proprioceptive pathways in modulating the PBST CAP, conditioning pulses to afferents supplying knee extensor muscles, Quad, were applied at varying intervals before the DRL5 test pulse. Stimulation of Quad nerve alone evoked no CAP response in the PBST nerve, confirming previous reports that Quad afferents are not monosynaptically connected to PBST MNs (data not shown) (Baldissera et al. 1981; Eccles and Lundberg 1958; Wang et al. 2008). Trials utilizing a Quad conditioning pulse followed by the DRL5 test pulse (C+T trials) were interleaved with test pulse-only trials (T trials) at a frequency of 0.1 Hz (Fig. 1A). An increase or reduction in CAP area during C+T trials indicates greater or fewer PBST MNs reach threshold compared with T-only trials, respectively. PBST CAP area thus serves as a quantitative measure of proprioceptive modulation of MN output via interneuron pathways.

Efficacy of inhibitory circuits at 1 wk of age. In P7/P8 mice, varying the interval between the conditioning and test pulse (conditioning pulse given 0–50 ms before the test pulse; see MATERIALS AND METHODS for details on stimulation protocols) produced two distinct phases of inhibition of PBST motor output (Fig. 1, C and D). One reached a maximum degree of inhibition (70.6 ± 8.8%; n = 10) at short intervals (6.5 ± 0.8 ms), and the second reached a maximum degree of inhibition (51.6 ± 12.3%) at longer intervals (22.8 ± 0.5 ms). Conditioning pulse intervals between these two regions of greatest inhibition still evoked inhibition of the test pulse, but to a lesser extent, suggesting separate circuits may mediate the two phases of inhibition. Previously published results indicate that the short-interval inhibition observed here was consistent with classic disynaptic reciprocal inhibition elicited by activation of antagonist Ia afferents (Wang et al. 2008). We found that application of the glycine receptor antagonist strychnine (0.4 μM) abolished short-interval inhibition of the PBST nerve, consistent with the fact that reciprocal inhibition is mediated by glycinergic IaINs (Fig. 1E; n = 5) (Fyffe 1991). Application of strychnine also appeared to result in a general facilitation, likely due to disinhibition of MNs (control: 49.9 ± 5.5% inhibition; 0.4 μM strychnine: 27.9 ± 13.4% facilitation; P = 0.01, Wilcoxon rank sum test; n = 5). The longer interval component of inhibition was unaffected by strychnine (control: 44.5 ± 16.4% inhibition; 0.4 μM strychnine: 45.7 ± 5.5% inhibition; P = 0.81, Wilcoxon rank sum test; n = 5), however, indicating this proprioceptive pathway is mediated by nonglycinergic interneurons (Fig. 1E).

In separate preparations, we found that long-interval inhibition was blocked by addition of the GABA A receptor antagonist bicuculline (control: 87.6 ± 3.0% inhibition; 5 μM bicuculline: 16.6 ± 5.7% inhibition; P < 0.05, Wilcoxon rank sum test; n = 3), whereas short-interval inhibition was unaffected (Fig. 1F; control: 92.3 ± 2.4% inhibition; 5 μM bicuculline: 73.6 ± 9.4% inhibition; P = 0.12, Wilcoxon rank sum test; n = 3). These experiments provided evidence for two pharmacologically distinct interneuronal pathways through which proprioceptive information can modulate motor output.

To assess the stability of the two phases of inhibition, in several experiments conditioning stimulation intervals were randomized and the whole range of values were tested repeatedly for a total of five series, encompassing more than 3 h of total recording time. We consistently observed that the short-interval maximal glycine inhibition was robust and highly stable throughout the entire recording period (Fig. 2, A–C; 1st series: 70.6 ± 8.8%; 5th series: 70.8 ± 6.3%; n = 10; P = 0.95). Interestingly, we observed that the long-interval GABAergic inhibition was more variably effective in its ability to inhibit the PBST. In more than half of the preparations (n = 6/10), long-interval maximal GABAergic inhibition had an initially low degree of inhibition (1st series: 29.8 ± 14.8%) that would increase upon repetitive proprioceptive stimulation (Fig. 2, A and C; 2nd series: 43.0 ± 13.4%; 3rd series: 53.7 ± 12.6%; 4th series: 63.8 ± 12.1%; 5th series: 62.7 ± 12.1%; P < 0.005, 1st vs. 5th series). In the remainder of preparations (n = 4/10), however, the long-interval maximal GABAergic inhibition initially had a higher degree of inhibition (1st series: 84.2 ± 1.4%) that was reduced upon repetitive Quad stimulation (Fig. 2, B and C; 2nd series: 75.2 ± 2.0%; 3rd series: 71.1 ± 2.6%; 4th series: 71.6 ± 4.8%; 5th series: 69.8 ± 4.6%; P < 0.05, 1st vs. 5th series). Overall, these data point to the stability of the short-interval glycine inhibition compared with the variability of the long-interval GABAergic inhibition, in response to repetitive proprioceptive activity from the Quad, observed at the first postnatal week of development.

We next sought to determine the number of synaptic linkages involved in mediating these pharmacologically distinct windows of inhibition by making a series of measurements of the times required to generate various aspects of the responses that were recorded. Response latencies in our experiments are the combination of both peripheral and central conduction times of the various elements in the proprioceptive pathways. We began by measuring the latency of known monosynaptic connections between Ia afferents and MNs at two relevant locations in the lumbar spinal cord.

First, we measured the time required to elicit a monosynaptic response at ventral root L5 (VRL5) following stimulation of DRL5. Strong, synchronous activation of DRL5 afferents provided by electrical stimulation of the whole dorsal root elicits excitatory postsynaptic potentials (EPSPs) in MNs that lead to action potentials, and both of these responses can be recorded on the ventral root (Kudo and Yamada 1987; Shneider et al. 2009; Wang et al. 2008; Whelan et al. 2000). Although measuring the onset of the EPSP in MNs provides insight into the time required to initiate a monosynaptic response in MNs, the time required to generate an action potential response in the postsynaptic neuron is of greater importance in our current experiments because information will only be passed to the next neuron in a pathway after an action potential is generated. The latency to the peak of the action potential response at VRL5 recorded at room temperature was determined to be 5.6 ± 0.4 ms (Fig. 3A; n = 4). Because the latency-to-peak response is influenced by the fluctuating state of excitability of MNs in L5, some trial-to-trial variability might be expected in these measurements. We found, however, the standard deviation of the latency-to-peak recorded at VRL5 (0.055 ms) to be in agreement with that reported for monosynaptic connections in other studies measuring only the onset latency variability (Vrieseling and Arber 2006; Wang et al. 2008). Similarly, we found the monosynaptic latency-to-peak measured at VRL3 following DRL4 stimulation to be 4.9 ± 0.2 ms (Fig. 3A; n = 4). Again, variability between trials was low (SD = 0.10 ms) and in
line with reports for monosynaptic onset latencies derived from intracellular recordings (Wang et al. 2008). On the basis of these measurements, we estimated the average latency-to-peak required for the monosynaptic reflex in the lumbar spinal cord at room temperature to be $\approx 5.3$ ms (average of 5.6 and 4.9 ms).

Last, we measured the time required to evoke a monosynaptic response at VRL3 following stimulation of the Quad nerve from the periphery (Fig. 3A; $6.7 \pm 0.1$ ms; $n = 4$). This pathway includes a greater peripheral conduction component, in this case Ia sensory axons carrying the signal to the spinal cord via the Quad nerve. This latency was measured at DRL4 following stimulation of the Quad nerve as $2.0 \pm 0.2$ ms ($n = 6$). The distance from Quad to the recording site at DRL4 was also measured to estimate peripheral conduction velocity of group I sensory afferents at this age ($2.52 \pm 0.36$ m/s; $n = 4$). Subtracting the peripheral conduction time would leave an average central monosynaptic latency of Quad to VRL3 of $4.7$ ms ($6.7$ ms $- 2.0$ ms $= 4.7$ ms), a value in general agreement with measurements made directly from DRL4 to VRL3.

We then inferred the sum of two central monosynaptic latencies would result in a reasonable value for a central disynaptic latency, or the two-step relay between the Ia afferent and an interneuron and then to the motor neuron target (Fig. 3B; $5.3$ ms $+ 5.3$ ms $= 10.6$ ms). Does this estimate agree with our observations that the interval between Quad and DRL5 stimulation producing maximum inhibition of the test pulse in PBST is $6.5 \pm 0.8$ ms? If our estimate for disynaptic linkage ($10.6$ ms) is added to the time required for conduction of the signal along Quad sensory axons...

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**Fig. 3.** Predicted durations of individual stages of proposed pathways mediating inhibition of PBST responses based on measurements of monosynaptic connectivity between Ia afferents and MNs at P7/8. A: latency-to-peak values (means $\pm \text{SE}$) of various monosynaptic connections measured in lumbar spinal cord. B: schematic of predicted proprioceptive pathways that mediate Quad afferent effects on PBST MN responses. Average measured durations (black brackets) are shown for various stimulus and recording combinations. Estimated duration of 1 central synaptic relay (purple) was used to predict central latencies of the Ia interneuron (IaIN)-mediated reciprocal inhibitory pathway (blue) and GABAergic presynaptic inhibitory pathway (red). VR, ventral root.
from the periphery (2.0 ms), then we would estimate that maximal inhibition of PBST MNs would likely occur ~12.6 ms following stimulation of the Quad nerve. Monosynaptic excitation of L5 MNs, including PBST MNs, by DRL5 Ia afferents requires only 5.6 ms. Therefore, if Quad were stimulated 6.5 ms before DR5L, then monosynaptic excitation of PBST MNs would occur ~12.1 ms after stimulation of Quad, which falls within the time window in which reciprocal inhibition would be expected when evoked by Quad stimulation. A study of another disynaptic pathway in spinal cord, again recorded at room temperature, reported disynaptic inputs occurred with a latency about 5 ms longer than monosynaptic inputs (Machacek and Hochman 2006). In addition, other reports using similar methodologies have demonstrated disynaptic reciprocal inhibition mediated by IaINs is likely the active pathway mediating the short-interval inhibition observed in our experiments (Talpalar et al. 2011; Wang et al. 2008).

We employed similar logic to estimate the number of synaptic relays in circuits that may mediate the long-interval inhibition observed in our experiments. Inhibition mediated by two central interneurons in a trisynaptic pathway would have a latency of ~15.9 ms (5.3 ms + 5.3 ms + 5.3 ms), based on our measurements of monosynaptic communication in the spinal cord (Fig. 3B). Accounting for Quad peripheral conduction time (2.0 ms), this would suggest that trisynaptic inhibition of PBST MN output would begin at ~18 ms. Analysis of preparations where short-interval inhibition is blocked by addition of 0.4 μM strychnine suggests that the long-interval inhibition begins to be observed at intervals of 16–20 ms (average of 16.8 ± 0.8 ms; n = 5; see Fig. 1E for example). These observations are then consistent with a model where the long-interval inhibition observed in our experiments is mediated by a trisynaptic response elicited by Quad afferent activation.

**Long-interval inhibition is mediated via GABAergic presynaptic interneurons.** Activation of trisynaptic presynaptic inhibitory circuits by stimulation of Quad afferents could potentially reduce the PBST CAP evoked by stimulation of the DR5L by blocking transmission of DRL5 Ia afferents on PBST MNs. We investigated this possibility by modifying a common strategy to measure PAD. PAD is often measured at the dorsal root and evoked by stimulation of an adjacent dorsal root (Bautista et al. 2012; Bos et al. 2011). In our preparation, we instead recorded antidromically conducted action potentials in the PBST nerve in the periphery following stimulation of the Quad nerve (Fig. 4A). All ventral roots were cut in these experiments to eliminate possibilities for contamination from MN axon responses, meaning that all responses in the PBST would be from sensory axons and evoked by stimulation of Quad sensory afferents via projections in the L3 and L4 dorsal roots. In all preparations at P7 (n = 5), a cluster of CAPs was observed in the PBST in each sweep following Quad stimulation at 0.1 Hz (Fig. 4B, top trace). These responses were greatly attenuated at increased stimulation frequencies (1.0–5.0 Hz), excluding the possibility the CAPs resulted from direct activation of PBST sensory afferents (data not shown). We found PBST primary afferent CAPs were blocked by bath application of 5 μM bicuculline (n = 3). In all three tests in which bicuculline was applied, no CAPs were observed under any stimulus condition (Fig. 4B, bottom trace). CAPs were observed again after 1 h of washout with ACSF (data not shown). Overall, these results strongly support the likelihood that long-interval Quad afferent inhibition of sensory-evoked activation of PBST MNs is likely mediated by GABAergic presynaptic inhibition of DRL5 primary afferent terminals via a trisynaptic circuit.

**Efficacy of proprioceptive circuits at birth.** Previous studies have clearly demonstrated that monosynaptic connections between Ia afferents and target neurons in the spinal cord, including MNs and IaINs, exist at birth (Kudo and Yamada 1987; Pinco and Lev-Tov 1993; Wang et al. 2008; Ziskind-Conhaim 1990). Therefore, we used our CAP analysis assay to determine the functional efficacy of reciprocal inhibition and presynaptic inhibition in reducing PBST motor output in early neonates (P0/P1). In contrast to the two phases of short- and long-interval inhibition observed at P7, only one phase of inhibition was observed at P0/P1 with maximum inhibition at an interval of 20.0 ± 0.9 ms (Fig. 5, A and B; n = 6). Quadriceps group I sensory afferent conduction velocities were measured as being only 0.58 ± 0.04 m/s (n = 3), so the longer interval required for maximum inhibition is likely a function of slower action potential conduction velocities. We wondered if...
bicuculline (Fig. 5).

A greater degree of facilitation was observed, in strychnine, at inhibition was very similar at P0/P1 (5.7% reduction at P0/P1 and 70.6% inhibition; 0.4 µM strychnine: 66.0 ± 9.9% facilitation; P < 0.05, Wilcoxon rank sum test). Interestingly, a greater degree of facilitation was observed, in strychnine, at birth than at P7 (P < 0.0005, Wilcoxon rank sum test). In addition, the single phase of inhibition was not blocked by bicuculline (Fig. 5C; n = 3; control: 52.2 ± 7.03%; 5 µM bicuculline: 46.5 ± 11.3%; P = 0.83, Wilcoxon rank sum test). These manipulations suggest only disynaptic reciprocal inhibition may be strong enough to block activation of MNs at birth.

Reduction in the area of the CAP in a peripheral nerve such as the PBST indicates fewer MNs are driven to threshold and fire action potentials in response to a given stimulus, in this case via activation of monosynaptically connected Ia afferents from DRL5. As a consequence, this assay can be utilized to quantify the effectiveness of a particular proprioceptive pathway in modulating MN output. We found that the percent reduction of the PBST CAP through disynaptic reciprocal inhibition was very similar at P0/P1 (n = 6) and at P7 (n = 10) (65.4 ± 5.7% reduction at P0/P1 and 70.6 ± 8.8% at P7; P = 0.63, 2-tailed, unpaired t-test). In a subset of these P0/P1 preparations (n = 3) we tested the stability of reciprocal inhibition over three full series of conditioning latencies and found glycnergic reciprocal inhibition was indeed stable (1st series: 54.5 ± 13.5%; 3rd series: 47.3 ± 9.1%; P = 0.83, Wilcoxon rank sum test). Similar to the observation at P7, this suggests that reciprocal inhibition is a functionally relevant and effective pathway through which MN activation can be modulated even at birth.

GABAergic presynaptic inhibition of proprioceptive afferents is weak at birth. Our finding that conditioning stimulation via Quad afferents evokes only a strychnine-sensitive inhibition of PBST MN activation at birth suggests presynaptic inhibition of DRL5 Ia afferents may be ineffective at this developmental stage. Nevertheless, GABAergic PAD can be elicited by stimulating and recording from adjacent dorsal roots in neonatal rats (Vinay and Clarac 1999), indicating that a large nerve bundle containing a variety of proprioceptive and cutaneous sources is capable of eliciting PAD. To investigate potential differences between these two results, we employed our modified PAD assay to attempt to record antidromic action potentials in sensory afferents of the PBST (n = 9; Fig. 6A). In contrast to what was observed at P7, we found stimulation of Quad afferents in P0/P1 preparations failed to induce any sensory action potentials in the PBST nerve even very high stimulation intensities (>0.5 mA), which likely recruit many classes of sensory afferents, failed to induce action potentials in the PBST (data not shown). Interestingly, activation of afferents in the purely cutaneous saphenous nerve were capable of evoking some sensory afferent action poten-
Nevertheless, robust antidromic sensory action potentials could only be recorded in the PBST nerve following stimulation of the entire DRL4 (Fig. 6D). Our results with broad stimulation of DRL4 are consistent with previous reports demonstrating dorsal root potentials at birth, responses that we could observe also in the preparations (Fig. 6, B–E). Together, these experiments suggest stimulation of proprioceptive afferents at birth fails to elicit presynaptic inhibition of other Ia proprioceptive afferents but that this circuitry becomes effective by the end of the first postnatal week.

**DISCUSSION**

Proprioceptive information provides critical feedback to spinal circuits that can be used to alter motor output in response to perturbations in the periphery. Significant processing of proprioceptive information is conducted via interneuron-based pathways, yet relatively little is known regarding the functional status of interneuronal pathways in early postnatal animals. In this study, we assayed the ability of two interneuronal pathways to block motor neuron output using an acute in vitro isolated spinal cord preparation derived from early postnatal mice. We found that disynaptic reciprocal inhibition circuitry is as effective at birth as at P7 in blocking MN activation. We also found that proprioceptive afferents do not evoke presynaptic inhibition of Ia afferents at birth. In contrast, by P7 proprioceptive afferents are able to elicit presynaptic inhibition as has been reported in adult animals. These results suggest the mechanism of presynaptic inhibition, which acts to gate the flow of proprioceptive information into the circuits of the spinal cord, is only weakly established at birth but increases by the end of the first postnatal week.

Our finding that reciprocal inhibition of PBST via Quad afferents was as effective at blocking PBST activation at birth as at P7 differs from results published by Wang et al. (2008). They reported minimal reciprocal inhibition effectiveness at P0 (reduction of test pulse response of ~12%). By P3, however, reciprocal inhibition strength was similar to that found at P7 (~42% and ~45% reductions, respectively). In contrast, intracellular recordings made from PBST MNs in the same study indicated the amplitude of reciprocal inhibition potentials was essentially unchanged between P0 and P7 (Wang et al. 2008). Differences in extracellular recording strategies may explain these apparently contradictory findings. Wang et al. recorded the compound response of all MNs exiting VRL5, whereas we recorded specifically the response of PBST MNs via measurements of the PBST CAP in the periphery. Their strategy focused on selective afferent stimulation (PBST afferents), with the converse trade-off of nonspecific recording from the whole ventral root. In contrast, our approach focused on selective recording of PBST MN responses activated by stimulation of a larger population of afferents, including synergists of PBST, via whole dorsal root stimulation. At birth, stimulation of only PBST afferents may be insufficient to drive many MNs to threshold, thus reducing the response measured at VRL5. In fact, Wang et al. reported EPSPs were difficult to obtain with this stimulation paradigm at P0/P1 (Wang et al. 2008). On the other hand, robust CAP responses were always obtained in our preparations at P0/P1, likely a consequence of activation of PBST afferents together with other synergistic DR afferents. In our experiments, PBST CAP amplitude was consistently reduced by conditioning stimulation of Quad afferents at appropriate intervals. This effect was sensitive to strychnine, indicating it is mediated by glycinergic transmis-
GABA antagonists as well as differences in GABAA receptor during development. For example, differences in sensitivity to postnatal stage. Afferent responses to GABA may also change with the intermediate zone of the spinal cord. If this were the case, conditioning stimulation of Quad should be able to initiate antidromically propagated action potentials in PBST afferent collaterals terminating at more dorsal positions in the cord. Because no action potentials were recorded, however, it is more likely that presynaptic inhibition is weak at birth and that responses can only be generated if large enough groups of axons are synchronously activated.

It should be noted that the present results can only provide information regarding presynaptic inhibition status on Ia afferent connections with MNs. Recent studies utilizing an isolated spinal cord and hindlimb preparation in neonatal rats (P1–P4) reported robust, phasic DRP evoked in contralateral dorsal root during supported locomotion (Hayes et al. 2012). The authors suggest these responses may be initiated by Ib afferents in the contralateral limb, particularly toe muscle afferents, and that the likely target of PAD, and hence the source of contralateral DRP, are Ib afferents (Devanandan et al. 1965; Hayes et al. 2012). Although our experiments cannot confirm or disprove this hypothesis, the general lack of PAD-evoked antidromic action potentials observed in the periphery following stimulation of Quad afferents suggests PAD on other proprioceptive afferents, including Ib afferents, is weaker at birth than at P7.

At the onset of a voluntary contraction, presynaptic inhibition of Ia afferent terminals on MNs projecting to the contracting muscle is reduced, whereas presynaptic inhibition of Ia afferent terminals on nonparticipating muscles is increased (Hultborn et al. 1987). Selective gating of Ia afferent input allows sensory feedback to influence MN activity to compensate for unpredictable length and/or velocity differences (Meunier and Piersot Deseligny 1989). Multiple descending systems exert control on the interneurons involved in presynaptic inhibition (Rudomin et al. 1983; Rudomin and Schmidt 1999). In the developing system studied presently, both the local circuits that effect presynaptic inhibition as well as descending systems that selectively increase and/or decrease the strength of presynaptic inhibition during voluntary movements are immature. Thus, before both of these systems mature, Ia afferent signals on MNs are not gated in the way they are in adults. This situation may be advantageous for neonatal animals as they learn to interact with the environment. Proprioceptive feedback from either passive or intentional limb movements are unfiltered by presynaptic inhibition and may serve to maximize sensitivity or to calibrate various feedback-sensitive pathways, including the stretch reflex and reciprocal inhibition, to respond to the widest range of signal intensity. This may be particularly important when considering the fact that the response profiles of muscle spindle afferents during early postnatal development are still somewhat immature in coding signals of muscle stretch (Vejsada et al. 1985). Analysis of the acquisition of locomotor skills in genetic experiments in mice with reduced or increased presynaptic inhibition may provide additional insight into its role in postnatal development.

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DISCLOSURES
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AUTHOR CONTRIBUTIONS

P.M.S. and D.R.L. conception and design of research; P.M.S. and D.R.L. performed experiments; P.M.S. and D.R.L. analyzed data; P.M.S. and D.R.L. interpreted results of experiments; P.M.S. and D.R.L. prepared figures; P.M.S. and D.R.L. drafted manuscript; P.M.S. and D.R.L. edited and revised manuscript; P.M.S. and D.R.L. approved final version of manuscript.

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