Early Postnatal Development of Reciprocal Ia Inhibition in the Murine Spinal Cord

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Abstract

The pathway mediating reciprocal inhibition from muscle spindle afferents (Ia axons) to motoneurons (MNs) supplying antagonist muscles has been well studied in adult cats, but little is known about how this disynaptic pathway develops. As a basis for studying its development, we characterized this pathway in mice during the first postnatal week, focusing on the projection of quadriceps (Q) Ia axons to posterior biceps-semimembranosus (PBSt) MNs via Ia inhibitory interneurons. Synaptic potentials in PBSt MNs evoked by Q nerve stimulation are mediated disynaptically and are blocked by strychnine, implying that glycine is the major inhibitory transmitter, as in adult cats. The specificity of neuronal connections in this reflex pathway is already high at birth; Q afferents evoke inhibitory synaptic potentials in PBSt MNs, but afferents supplying the adductor muscle do not. Similar to this disynaptic pathway in cats, Renshaw cells inhibit the interposed Ia interneurons, as they reduce the disynaptic input from Q axons but do not inhibit PBSt MNs directly. Reciprocal inhibition functionally inhibits the monosynaptic excitatory reflex in PBSt MNs by P3 but this functional inhibition is weak at P1. Finally, deletion of the transcription factor Pax6, which is required for the development of V1-derived Renshaw cells, does not block development of this pathway. This suggests either that Pax6 is not required for the phenotypic development of all V1-derived spinal interneurons or that these inhibitory interneurons are not derived from V1 precursors.

Key Words: IPSP; EPSP; Ia interneuron; Ia sensory neuron
Introduction

The electrophysiological properties of mature spinal interneurons (INs) have been studied for many years, beginning with the pioneering studies of Renshaw and Lloyd (Lloyd 1941; Renshaw 1941), and there is a wealth of information concerning their synaptic inputs and outputs. Two types of inhibitory INs that have received extensive study are the Renshaw cells (RCs) that mediate recurrent inhibition and the Ia inhibitory INs mediating reciprocal inhibition to MNs (Eccles et al. 1954; Hultborn et al. 1971b; Hultborn and Udo 1972). Renshaw cells receive cholinergic excitatory input from a restricted group of MNs and, in turn, synaptically inhibit those same MNs. Ia INs are excited by Ia sensory axons innervating muscle spindles and specifically inhibit MNs supplying antagonistic muscles. Renshaw cells also receive powerful inhibition from other spinal INs (Alvarez et al. 1997), and Ia INs are inhibited by Renshaw cells (Hultborn et al. 1971b).

Despite our detailed knowledge of these pathways in adult animals, relatively little is known about how these pathways develop. The classical electrophysiological techniques used to characterize these INs and their synaptic connections in adult cats are difficult to apply to animals suitable for developmental studies like the embryonic chicken or fetal or neonatal mouse. In the past decade, however, a combination of genetic techniques and immunohistochemical labeling of specific proteins have revealed many aspects of the origin of different classes of spinal interneurons. The V1 class is derived from a specific group of neuronal precursors that express Pax6, and then transiently express Engrailed-1 (En1) postmitotically (Alvarez et al. 2005; Sapir et al. 2004). Based on their transmitter
phenotype, all V1 INs are inhibitory, and both Renshaw cells and Ia inhibitory INs are members of this class.

Electrophysiological studies have revealed that RCs are present in chick embryos by embryonic day (E) 7 (Wenner and O'Donovan 1999), and they express En1 (Wenner et al. 2000). In mice, RCs have an absolute requirement for expression of Pax6 (Sapir et al. 2004). The basic synaptic inputs to and outputs from RCs are already established by these stages, as these pathways must be functional for them to be identified physiologically. There is also evidence for some rearrangements of their inputs and outputs. Mature RCs in cats do not receive functional inputs from primary sensory afferents, but during the first two postnatal weeks in mice, these inputs are prominent, only to disappear in the subsequent weeks (Mentis et al. 2006). In E8 chicken embryos, RCs at the lumbrosacral (LS) 2 level project to MNs at LS3 and LS4, but by E10 the projections to LS 3 have increased while those at LS 4 have decreased (Xu et al. 2007; Xu et al. 2005).

Reciprocal connections between contralateral motoneurons and between ipsilateral flexor and extensor motoneurons develop prenatally in rats, although these connections are initially excitatory (Delpy et al. 2008; Nakayama et al. 2002). A few days before birth, these connections, which are mediated by GABA_A and glycine receptors, become inhibitory, consistent with the change in the reversal potential for GABA- and glycine-mediated synaptic transmission (Delpy et al. 2008). It is unknown, however, whether these reciprocal connections are mediated by Ia INs or even if they depend on sensory input from muscle. In the experiments described here, we demonstrate using intracellular recordings that reciprocal inhibition mediated by Ia INs occurs via a disynaptic
glycinergic pathway and is functional in neonatal mice. Ia input from antagonist muscles inhibits the monosynaptic reflex discharge of MNs. Evoked activity in RCs also inhibits the Ia-evoked disynaptic inhibitory potentials in MNs. The specificity of synaptic inputs and outputs is already established by P0; inputs from functionally antagonistic muscles evoke inhibition but inputs from other muscles do not. Deletion of the \( \textit{Pax6} \) gene, which eliminates RCs, does not eliminate reciprocal inhibition, suggesting that Pax6 is not required for the development of Ia INs.
Material and Methods

Animals. Neonatal mice of the C56B1/6 strain were used within the first postnatal week for all experiments with wild type mice. P0 was defined as the first 24 hours following birth. All research was approved by the Institutional Animal Care and Use Committee at Tufts University School of Medicine and conformed to NIH guidelines. The $Pax_6^{-/-}$ mice used in these experiments have been fully described in an earlier publication (Sapir et al. 2004).

Preparation. An isolated spinal cord preparation was used for all experiments. Details of this preparation have been described previously (Mears and Frank 1997). Briefly, neonatal mice were anesthetized with hypothermia, perfused with 5 ml of cold saline, decapitated, skinned, and eviscerated. Dissection was performed in recirculating cold oxygenated (95%$O_2$, 5%$CO_2$) saline. The solution used for dissection and recording contained (mM): NaCl (127), KCl (1.8), KH$_2$PO$_4$ (1.2), CaCl$_2$ (2.4), MgSO$_4$ (1.3), NaHCO$_3$ (25), dextrose (20.5). Following laminectomy, the quadriiceps (Q), adductor (Add), and posterior biceps semitendinosis (PBSt) nerves in the right hindlimb were dissected in continuity with the spinal cord. In some experiments, the Q muscles were left connected to the preparation. The cord was hemisected along the dorsoventral midline and positioned in the recording chamber with its cut medial surface uppermost. The preparation was maintained in rapidly circulating (~35 ml/min) saline that was gradually warmed to room temperature (22 – 25°C).

The functional presence of the Ia IN pathway in $Pax^{-/-}$ mutant mice was explored in a separate series of experiments that were performed as part of our earlier investigation of Renshaw interneurons (Sapir et al. 2004). In that study, identical procedures were used
for the dissection and recording methods, but the bath temperature was maintained at
30°C. As a result, the synaptic latencies recorded in that series of experiments are shorter
than those at an equivalent stage of postnatal development reported in the other
experiments reported here.

Electrophysiology. For intracellular recordings, MNs were impaled with sharp glass
micropipettes (120-180 MΩ) filled with 2 M potassium methylsulfate and 0.5% Fast
Green. In most experiments, 500 mM QX-314 was added to the pipette solution to block
production of somatic action potentials (AP’s). QX-314, diffusing from the electrode tip,
blocked somatic AP’s after several minutes, facilitating the measurement of synaptic
inputs that would otherwise evoke orthodromic activation. Motoneurons were identified
by antidromic AP’s evoked by stimulation of the appropriate muscle nerve. The MNs
included in this report had resting potentials more negative than -50 mV and synaptic
potential amplitudes that were stable throughout the period that measurements were
made, which lasted from a few minutes up to one hour, when inhibitory synaptic
transmission was blocked with strychnine. In these latter cases, the quality of the
penetration was assessed by measuring the monosynaptic input from PbST sensory
axons, which was not blocked by strychnine.

Muscle nerves were stimulated via individual suction electrodes with square pulses of
0.1 ms duration at supramaximal levels. The stimulus intensity was increased until the
synaptic response was just supramaximal, which was usually between 2 and 3 V. For
responses from the Add nerve, which often evoked little or no visible response, the same
stimulus strength was used as for the Q nerve. In extensive earlier experiments using the
same preparation at similar postnatal stages, the stimulus strength needed to activate Ia
afferents and MNs in the Add and Q nerve was always very similar, so it is very likely that all Group I afferents were activated in the experiments reported here. Ia afferents in Q muscles were activated selectively in some preparations by tapping the distal tendon of the muscle with a piezoelectric probe (Arber et al. 2000; Lichtman and Frank 1984). This activates Ia axons but not Ib axons both in adult cats and in the neonatal mouse preparation used here (Chen et al. 2002). The stimulation frequency was typically 0.2 – 0.3 Hz, which produced little or no synaptic fatigue. The resulting potentials, filtered at 3-10 KHz, were recorded digitally at 20 KHz. Individual traces (5-50 traces, depending on the variability of the responses) were averaged either on- or off-line and stored for subsequent analysis using custom software (LabVIEW, National Instruments).

For extracellular recordings of responses in MNs, compound AP’s were recorded from the cut ventral root (VR) with a tight-fitting glass suction electrode. Only the distal end of the cut root was placed in the suction electrode to minimize the contribution of synaptic potentials to the recordings. Signals were filtered with 0.1 Hz and 3 KHz high and low-pass filters, digitized at 20 KHz and stored for subsequent analysis as above. Eight to twenty individual traces were averaged, and the response was measured as the peak amplitude of the compound AP.

The amplitudes of disynaptic responses were measured using a variation of the monosynaptic model technique used previously to analyze monosynaptic Ia input to MNs (Mendelson and Frank 1991; Wang et al. 2007). This method was used as a way of estimating the amplitude of disynaptic inputs to all MNs, including ones in which these inputs were small or nonexistent. For each MN, the Q nerve was stimulated to evoke a just-threshold response, and this response was stored as a model trace for that MN. The
stimulus strength was then increased until the synaptic response was maximal, about 2X threshold, and the responses to stimulation of the Q and Add nerves were recorded. After the experiment, the model trace was scaled to fit the rising phase of each response, and the amplitude was calculated as the scaled amplitude of the model trace. Occasionally even a just-threshold response to Q input had multiple components, and in those cases a model trace from another MN in the same preparation was used. Fluctuations in synaptic latency for an individual synaptic response (refer to Fig. 2B) were calculated by measuring the individual latencies of more than 30 single traces (described in Wang et al. 2007). Average values in the text are given as ± 1 S. E. M. Error bars in all figures are also ± 1 S.E. M.

The effect of RC inhibition on the disynaptic IPSPs was measured by alternately stimulating the Q nerve alone and the Q nerve together with L3-4 VRs (to activate RCs). The stimulus strength for activating MNs via the VR’s was set at the level we found to be approximately 2X threshold in our earlier studies of Renshaw cell activation (Sapir et al. 2004). In some experiments we recorded from the ventral surface of the cord with a suction electrode while stimulating the VRs to confirm that MNs were being antidromically activated, and the required stimulus strength was found to be the same as in the earlier experiments, 2 – 3 V at 0.1 ms. The two sets of traces were then sorted and analyzed separately (Fig. 5). The same procedure was used to determine the effect of Q nerve stimulation on the strength of the monosynaptic PBSt reflex (Fig. 8). The alternating stimulus technique avoided any progressive change in the amplitude of the responses between the two sets of measurements.
Bicuculline methiodide (5 µM) and strychnine hydrochloride (either 0.4 or 2 µM, both reagents from Sigma-Aldrich) were used to study the pharmacology of the inhibitory potentials. After a stable recording was obtained, the drug(s) were added to the perfusion medium. Blockade was usually seen within a few minutes.
Results

Reciprocal inhibitory responses in P7 mice

As a first step in exploring the neonatal development of reciprocal Ia inhibition in mice, we characterized this pathway in isolated spinal cord preparations from P7 animals. This time point is near the end of the period when isolated preparations survive well for electrophysiological studies, and the adult pattern of monosynaptic connections between Ia afferents and MNs is well established. We focused primarily on the disynaptic pathway between Ia afferents supplying the quadriceps (Q) muscles and motoneurons supplying the posterior biceps–semitendinosis (PBSt) muscles. This pathway has been extensively studied in adult cats (Eccles and Lundberg 1958; Hultborn et al. 1971b; c). Q Ia afferents excite Ia inhibitory interneurons (IaINs) and these interneurons project in turn to PBSt MNs located 2-3 spinal segments more caudally. These muscle groups have largely antagonistic functions, with the Q group causing knee extension and the PBSt group causing knee flexion. Muscle stretch therefore activates homonymous MNs via the direct excitatory projection of Ia afferents to homonymous MNs and reciprocally inhibits MNs supplying antagonist muscles via the IaINs.

Intracellular recordings were made from PBSt MNs following stimulation of the Q muscle nerve. Inhibitory responses were recorded in nearly all PBSt MNs. Immediately after electrode penetration, a hyperpolarizing inhibitory postsynaptic potential (IPSP) was frequently recorded in response to Q stimulation. As the resting potential became more negative over the next few minutes, presumably as K⁺ diffused from the pipette into the MN, this synaptic potential became depolarizing (Fig. 1), and continued to increase in amplitude for 5 to 10 minutes. The reversal occurred at a membrane potential of –65 to –
70 mV, in the range of the reversal potential for inhibitory synaptic potentials in neonatal rodent MNs (Delpy et al. 2008; Stein et al. 2004).

**Disynaptic inhibition of the reciprocal inhibitory pathway**

Several characteristics of these IPSPs suggest that they are mediated via a polysynaptic pathway. First, polysynaptic responses are often depressed to a greater extent with repetitive stimulation than monosynaptic responses in young animals, even at relatively low frequencies of stimulation (Lee and O'Donovan 1991). Stimulation of the Q nerve at 3 Hz caused a reduction of more than 80% in the amplitude of the response compared to the response amplitude at 0.3 Hz (Fig. 2A, upper traces). This is a greater depression than for the monosynaptic input from Ia fibers to MNs, which is about 50% at 3 Hz (Fig. 2A, lower traces, see also Wang et al. 2007). Second, the latency of individual IPSPs was more variable than for monosynaptic Ia responses in MNs. Within a series of individual monosynaptic responses, latencies vary only slightly, because conduction times and synaptic delays are nearly the same from trial to trial. For polysynaptic connections, however, fluctuations in the amplitudes of the synaptic potentials in the INs produce a variability of the time at which an action potential, and hence synaptic release, occurs (Berry and Pentreath 1976). The S.D. of latencies of more than 50 individual traces of Ia IPSPs was 0.39 ± 0.06 ms (n=6, Fig. 2B), which is substantially larger than the S.D. of Q Ia monosynaptic latencies (0.08 ± 0.01 ms) at a similar stage of postnatal development (Wang et al. 2007). These results, taken in conjunction with the fact that all known direct connections of large caliber muscle afferents are excitatory, implies that reciprocal Ia IPSPs are mediated polysynaptically.
A quantitative analysis of the latency of these IPSPs indicates that these connections involve only two synaptic relays. The distribution of synaptic latencies of all Q synaptic potentials in PBSt MNs at P7 was narrow, with an average latency of $7.8 \pm 0.1$ ms and a range of 6 to 9 ms ($n = 43$, Fig. 4A). We estimated the latency for a disynaptic pathway from Q Ia afferents to PBSt MNs by summing the latency of the monosynaptic $Q \rightarrow Q$ EPSP (4.3 ms) and the latency of one additional monosynaptic delay. For this additional latency, we chose the central latency of the $Q \rightarrow Q$ monosynaptic connection, taking the central latency as the total synaptic latency minus the peripheral conduction time of Q afferents ($4.3 \text{ ms} - 1.5 \text{ ms}$), which would include the conduction time of Ia afferents from the dorsal root into the ventral horn and the synaptic delay at the Ia-MN synapse. This estimate is slightly smaller than the measured latency of $Q \rightarrow \text{PBSt}$ connections (7.1 vs. 7.8 ms), but it does not include the time between the onset of the synaptic potential and the onset of the orthodromic AP in the Ia IN. These considerations make it unlikely that there is sufficient time for an additional, third, synaptic relay. The reciprocal Ia inhibitory pathway in mice is therefore mediated disynaptically, as in adult cats.

**Glycinergic transmission at reciprocal inhibitory synapses**

In adult cats, the synaptic connection between Ia INs and MNs is mediated by glycine, in that strychnine abolishes the response (Curtis et al. 1968b; Stuart and Redman 1990). 2 µM strychnine also completely blocks the early component (the first 2 – 3 ms) of this synaptic potential in P7 mice (3/3 cases, data not shown). This concentration, however, also blocks about 50% of the GABA mediated portion of IPSP’s in neonatal rat motoneurons (Jonas et al. 1998). Approximately 90% of the potential was also blocked.
by 0.4 µM strychnine (2/2 cases, Fig. 2C), a concentration that blocks virtually all of the glycine-mediated response but only ~20% of the GABA-mediated response (Jonas et al. 1998). Bicuculline (5 µM), which is specific for GABA-mediated responses, caused high levels of spontaneous activity in the isolated neonatal mouse cord preparation, precluding meaningful tests of its effect on the IPSP. These results suggest that at P7, most of the inhibition mediated by Ia INs is glycinergic, with GABA contributing ~10% of the response, a result that is consistent with studies of spontaneous miniature IPSPs in embryonic and neonatal rat motoneurons (Gao et al. 2001). All synaptic responses were completely blocked by 10 µM CNQX, as expected (data not shown). These results indicate that the disynaptic component of these responses is mediated by both excitatory and inhibitory synapses, consistent with glutamatergic excitation from muscle afferents to Ia INs followed by mixed glycinergic/GABAergic inhibition from these INs to antagonist MNs.

We next verified that these disynaptic IPSPs were elicited by activity in Ia sensory axons. It is difficult to activate Ia fibers selectively by electrical stimulation in neonatal preparations because both Ia fibers, which supply muscle spindles, and Ib fibers, which supply Golgi tendon organs, have similar electrical thresholds. Instead, Ia fibers were activated selectively by tapping the surface of the muscle with a probe (Arber et al. 2000, also see Materials and Methods; Lichtman and Frank 1984), an adequate stimulus for spindle afferents but not for Golgi tendon organs (Chen et al. 2002). In all four cases tested, selective activation of Q Ia afferents elicited a synaptic potential in PBSst MNs, and this potential was blocked by 2 µM strychnine (Fig. 2D). The latency of the synaptic response was longer than that elicited by direct electrical stimulation of the Q nerve, but
this increase is in accord with the increased latency of the excitatory monosynaptic Ia-mediated response in Q MNs (Arber et al. 2000) and is caused by the extra delay imposed by mechanical activation of Ia axons. The average amplitude of the responses elicited by single taps of the Q muscle was $3.9 \pm 1.0$ mV ($n=4$), or over one half of the response to electrical stimulation of the Q nerve (see below). It is unlikely that a single muscle tap activates all the muscle spindles, suggesting that stimulation of all Ia fibers would elicit an even larger response. These results suggest that Ia afferents mediate a significant fraction of all the reciprocal disynaptic inhibition in this preparation and that the inhibition is primarily glycineergic.

Specificity of the reciprocal inhibitory pathway

A characteristic feature of reciprocal Ia inhibition in adult cats is the specificity of these connections. Ia afferents innervating knee extensor muscles evoke inhibition in MNs supplying knee flexors but afferents innervating other muscles do not (Eccles and Lundberg 1958). For example, Q Ia afferents evoke disynaptic inhibition in PBSt MNs but Ia afferents innervating the Add muscle, a hip adductor, do not. In mice, Q afferents evoke strong inhibition in PBSt MNs by P7, as described above, so we explored the specificity of this pathway by testing for input from Add afferents to PBSt MNs.

Synaptic responses elicited in PBSt MNs by Add afferents were generally small compared to those evoked by Q afferents. In contrast to the tightly clustered synaptic latencies of Q inputs, the latencies of Add inputs were highly variable (lower traces in Fig. 3, also compare panels A and C in Fig. 4). One third of the responses (9/26) had latencies as short as the monosynaptic connections of Ia afferents with their homonymous
MNs (5.0 – 5.7 ms), and these responses persisted in 2 µM strychnine (data not shown). These potentials are likely to be monosynaptic inputs, consistent with the fact that Add and PBSt muscles are, to some extent, synergistic; both produce extension and lateral rotation of the hip. One fourth of the responses (7/26) had latencies longer than 20 ms; these are unlikely to represent disynaptic responses. Fewer than half of the responses therefore had latencies consistent with disynaptic input from Ia INs.

We estimated the amplitude of any disynaptic component of these responses by fitting all 26 examples with a scaled response of the disynaptic Q input to PBSt MNs recorded during the same experiment (Fig. 3, see Materials and Methods). Histograms of disynaptic response amplitudes in PBSt MNs evoked by stimulation of the Q and Add nerves are shown in Fig. 4B and D. The amplitudes of Add responses were small. The average amplitude was 0.5 ± 0.1 mV, and 23 of 26 responses were less than 1 mV. In comparison, all Q inputs were larger than 2 mV, and their average amplitude was 6.9 ± 0.5 mV (n= 27). There is therefore little or no disynaptic inhibition from Add afferents in PBSt MNs. To assess this specificity in individual mice, we calculated a Specificity Index (SI, refer to Wang et al. 2007), comparing the inputs from Q and Add nerves. For the 6 mice tested, the SI was 0.87 ± 0.05, which means that on average, Q inputs were ~14 times larger than Add inputs. Together, these results show that reciprocal Ia inhibition is already highly specific for these muscles by P7.

**Reciprocal inhibition from PBSt afferents to Q MNs in P7 mice**

In adult cats, there is reciprocal Ia inhibition between sensory and motor neurons supplying many different pairs of muscles (Eccles and Lundberg 1958), in addition to the
well known pathway from Q to PBSt. We tested one of these other pathways in P7 mice by examining the input from PBSt muscle afferents to Q MNs, which is present in cats. Surprisingly, the synaptic latencies of these inputs were significantly longer than in the Q → PBSt pathway, 10.0 ± 0.2 ms (n = 7) vs. 7.8 ± 0.1 ms (n=43). Some of these responses were initially hyperpolarizing, so they may represent a polysynaptic inhibitory pathway from muscle afferents to MNs. Their increased latencies preclude a rigorous assessment of whether they represent disynaptic Ia reciprocal inhibition, however, so their detailed characteristics were not explored further.

**Renshaw cells project to Ia interneurons**

In addition to the excitatory inputs Ia INs receive from Ia fibers, these INs also receive inhibitory inputs from RCs (Hultborn et al. 1971a). Activation of RCs by stimulation of the L5 and L6 ventral roots in adult cats results in an attenuation of the Ia IN IPSP in PBSt MNs elicited by stimulation of the Q nerve. Stimulation of the ventral roots alone does not evoke an IPSP in PBSt MNs, providing evidence that RCs at L5 and L6 do not inhibit PBSt MNs directly. We explored this characteristic of reciprocal Ia inhibition in P7 mice. To activate the analogous RCs in mice, we stimulated VRs L3 and L4; this was followed by stimulation of the Q muscle nerve, which activated only sensory fibers because the VRs were cut. Ventral root stimulation alone evoked no obvious synaptic response in PBSt MNs. When the VR was stimulated before the Q muscle nerve, however, the reciprocal IPSP was reduced (Fig. 5A). The largest inhibition occurred with an inter-stimulus delay of ~6 ms. The extent of attenuation was variable, ranging from 0 to 44% (average reduction of 18 ± 3%, n=15 cells in 8 preparations, Fig. 5B). Although
the variability of Renshaw inhibition might reflect the immaturity of this pathway at P7, our findings demonstrate that this second characteristic of reciprocal Ia inhibition, previously only described in adult cats, is present in mice and already well developed 1 week after birth.

Neonatal development of reciprocal inhibitory pathways.

The pathway from RCs to MNs is functional at birth (Sapir et al. 2004), but little is known concerning when other interneuronal pathways develop. To explore the early postnatal development of the Ia inhibitory pathway, we measured the synaptic responses elicited in PBSt MNs in response to Q and Add stimulation at P0, P1 and P3. The results obtained with the two P0 mice were very similar to those at P1, and results from these stages are presented together.

Stimulation of the Q nerve elicited IPSPs in PBSt MNs at both P1 and P3. Initially, these responses were frequently hyperpolarizing (13/35 cases at P1) but rapidly became depolarizing as the resting potential improved (Fig 6 A, B). Further evidence that these potentials were IPSPs mediated principally by glycine was provided by the observation that 2 µM strychnine reduced the responses by more than 90% (Fig. 6 C,D). The stabilized resting potentials of PbST MNs at the three different postnatal periods were very similar (P1: 70.9 ± 3.5 mV, P3: 73.4 ± 3.0 mV, P7: 72.8 ± 2.3 mV).

The latencies of these IPSPs were longer than at the earlier developmental times, presumably the result of less complete myelination of sensory axons. At P1, the average latency of $Q \rightarrow \text{PBSt}$ IPSPs was $14.4 \pm 0.3$ ms (n=35), and at P3, the latency was $11.2 \pm 0.3$ ms (n=34) compared to 7.8 ms at P7. These values are nearly identical to the sum of
the Q → Q monosynaptic latency plus the central latency of the Q → Q monosynaptic pathway (Fig. 7A) There is therefore insufficient time for more than two synaptic relays, implying that Q → PBSt IPSPs at these earlier stages are also mediated disynaptically, as at P7 and in adult cats.

The high specificity of Ia IN connections at P7 provided the opportunity to determine if this pathway is also specific at earlier stages. The inhibitory responses evoked by Q in PBSt MNs had amplitudes of 6.6 ± 0.6 mV (n = 17) at P1 and 6.5 ± 0.5 mV (n = 23) at P3, not significantly different from these responses at P7 (ANOVA p > 0.8; Fig. 7B). Inputs from Add had variable latencies, as at P7, so we used a model IPSP elicited by Q stimulation to estimate the amplitudes of disynaptic inputs from Add. Add inputs were small at both P1 and P3 (Fig. 7B), demonstrating that even immediately after birth, the reciprocal inhibitory pathway is highly specific. The average SI was 0.88 ± 0.02 at P1 and 0.80 ± 0.05 at P3 (n=5 mice at each of P1 and P3; Q inputs ~15 and ~9 times larger than Add inputs, respectively), demonstrating that the specificity was high in each individual mouse. The specificity of this disynaptic pathway implies that both the projection of Ia afferents onto Ia INs and the projection of these INs onto their target MNs are specific shortly after these connections are first made and well before hindlimbs are used for weight bearing locomotion.

There is evidence that Ia INs and RCs are both derived from the V1 class of embryonic spinal interneurons (Sapir et al. 2004). They might therefore, have similar patterns of synaptic inputs. Indeed, RCs receive direct excitatory input from Ia axons during the first postnatal week, similar to the Ia input to Ia INs, but this input is subsequently silenced (Mentis et al. 2006). This suggests that RCs and Ia IN might share
other attributes during development. We therefore asked if Ia INs initially receive excitatory cholinergic input from MNs, like RCs, that are subsequently silenced. At P3, stimulation of VRs L3 and L4 did evoke a synaptic potential in PBSt MNs, but the latency of this response was 3-4 ms longer than that mediated via the disynaptic reciprocal inhibitory pathway at this stage. This latency would be consistent with a trisynaptic linkage (L3-4 MNs → RCs → Ia INs → PBSt MNs) but is too long to represent a direct input from L3-L4 RCs to PBSt MNs, which would be disynaptic if evoked by VR stimulation. Moreover, this would require that the RC input to Ia INs be excitatory, which is unlikely by P3. There is thus no evidence that postnatal Ia INs ever receive direct excitatory input from MNs. These physiological data are consistent with morphological evidence showing that of all V1 interneurons, only RCs receive cholinergic inputs (Alvarez et al. 2005), although they do not provide strong evidence for it because excitatory inputs from MNs to Ia INs might be subthreshold.

**Functional consequences of reciprocal inhibition**

Reciprocal inhibition acts to inhibit motoneurons that project to antagonist muscles. Functional inhibition of MNs requires that the synaptic inputs are sufficiently strong to keep the membrane potential below threshold. Although intracellular recordings indicated that the IPSPs in PBSt MNs were already large by P1, they rapidly became depolarizing during the recording. We therefore sought a functional test of reciprocal inhibition that did not require intracellular recording. In adult cats, reciprocal inhibition from Q sensory afferents reduces the strength of the PBSt monosynaptic reflex (Lloyd 1946). We adapted this test to neonatal mice by recording the burst of APs in motor
axons of the L5 VR evoked by stimulation of sensory afferents in the PBSt nerve, and then measuring the inhibition of these responses produced by co-stimulation of Q afferents (refer to Fig. 8A). Representative traces from one experiment at P7 are shown in Fig. 8B. The relative timing of PBSt and Q nerve stimulation was varied to determine when inhibition was maximal (Fig. 8C). At the optimal timing (~3 ms), the reflex response in P7 mice was inhibited by approximately 50% (54 ± 9% of control, p<.001, n = 7; Fig. 8D).

Reciprocal inhibition was also functional at earlier developmental times. At P3, the optimal timing of Q stimulation was ~4 ms, producing nearly as much inhibition as at P7 (58 ± 6% of control, p<0.001, n = 7). At P1, it was difficult to evoke a monosynaptic reflex in the L5 ventral root, but in those preparations possessing a reflex response, stimulation of Q 3-4 ms before PBSt also produced a small attenuation of the reflex (88 ± 4% of control, p<0.05, n = 5). The maximum inhibition we observed at P1 was 20%. The strength of inhibitory synapses in the ventral horn is known to increase postnatally (Gonzalez-Forero and Alvarez 2005), so the weaker inhibition seen at P1 may result from weaker inhibitory inputs from Ia INs. Surprisingly, there was no corresponding increase in the amplitude of Ia IN IPSPs recorded intracellularly from PbST MNs during the first postnatal week (Fig 7B). The reversal potential of glycine/GABA-mediated synaptic potentials, which becomes more negative during embryonic development, plateaus at ~-70 mV by P0 (Delpy et al. 2008), and the resting potential in spinal MNs is also constant during the first postnatal week(Stein et al. 2004 and results reported here). It is possible that an increase in IPSP amplitude is obscured by the strong nonlinear summation of synaptic currents, because the reversal potential is close to the peak amplitude of the
synaptic response. This possibility can be addressed in future experiments by measuring synaptic current at a controlled membrane potential rather than potential, as in the current experiments.

**Ia inhibitory interneurons in Pax6-/- mice**

Earlier studies have demonstrated that Ia INs are likely to be members of the V1 class of ventral INs (Sapir et al. 2004), a group of cells that differentiate into multiple local circuit inhibitory neuron cell types including RCs. Previously, it has been shown that the precursors of the V1 class express the transcription factor Pax6. These cells also transiently express En1 after they become postmitotic (Burrill et al. 1997; Ericson et al. 1997). One group of V1 INs, the RCs, fail to develop in *Pax6*-mutant embryos, as determined by anatomical and functional criteria (Sapir et al. 2004). To address whether Pax6 is required for the development of Ia INs we looked for the presence of a functional reciprocal Ia inhibitory pathway in *Pax6* mutant mice at E18 and P0.

Synaptic responses in PBSt MNs were evoked by stimulation of the Q nerve in all 14 cells examined (four mutant mice). In five of these recordings, the synaptic potentials were initially hyperpolarizing, but then became depolarizing as the resting potential became more negative. Their latencies varied between 12 and 14.5 ms (average of 13.2 ± 0.3 ms) and their average amplitude was 4.9 ± 0.9 mV. While these latencies are slightly shorter than for this same pathway in the normal P1 mice reported above, they were the same as those recorded from a wild-type littermate in the same experimental series (Fig. 9). A probable explanation for the shorter latency is that in these experiments, conducted as part of an earlier study (Sapir et al. 2004), the bath temperature was maintained at 30°
instead of 22°, used for all other experiments reported here. Thus, in contrast to the absence of functional RCs in Pax6 mutant mice, la INs are present and have developed normal synaptic inputs and outputs.
Discussion

Reciprocal inhibition is believed to play a central role in facilitating limb movements, during both locomotion and reflex actions mediated by Ia afferents (Jankowska 2001). The reciprocal pathway from quadriceps (Q) Ia afferents onto posterior biceps semitendinosis (PBS) MNs (Araki et al. 1960; Eccles et al. 1956) has been studied extensively. In adult cats, this pathway is particularly robust (Eccles et al. 1956) and reciprocal inputs to PBS MNs are highly specific; Q Ia afferents project strongly but adductor (Add) Ia afferents do not. We therefore chose this pathway to characterize reciprocal Ia inhibition in neonatal mice, focusing on the first week of postnatal development, as several important features of maturation of spinal circuitry are known to occur at this time (Baccei and Fitzgerald 2004; Mentis et al. 2006; Seebach and Ziskind-Conhaim 1994).

Characteristics of reciprocal inhibition in neonatal mice

Our results show that the basic characteristics of reciprocal inhibition mediated by Ia INs are the same as those described in adult cats. Throughout the first postnatal week, 2 µM strychnine blocked virtually all of the disynaptic components of the postsynaptic potential in PBS MN’s, implying that Ia inhibition is mediated principally by glycine and/or GABA (Curtis 1959; Curtis et al. 1968a). This concentration of strychnine blocks glycine-mediated transmission completely, but it also blocks ~50% of GABAₐ-mediated transmission (Jonas et al. 1998). IPSPs in neonatal rat MNs are mediated by both glycine and GABA (Jonas et al. 1998; Schneider and Fyffe 1992), with the GABA-mediated synaptic currents having a slower falling phase (Gao et al. 2001). In our experiments, the
Ia-mediated synaptic potentials were not completely blocked by low (0.4 µM) concentrations of strychnine that are specific for glycine receptors (Fig. 2C), suggesting that GABA may also contribute to these IPSPs in neonatal mice. Nevertheless, a major portion of the disynaptic inhibitory response is mediated by glycine.

Ia afferents contribute most of the inhibitory response evoked in PBSt MNs by stimulation of the Q nerve in adult cats (Eccles et al. 1956). In the neonatal mouse preparation, we activated Ia axons selectively by small stretches of the Q muscle (see Methods), which avoids activation of Ib afferents. This selective Ia activation elicited IPSPs in PBSt MNs, which were blocked by strychnine (Fig. 2D), demonstrating that Ia inputs contribute a significant fraction of reciprocal inhibition in mice, as in cats. In adult cats, the majority of Ib projections to synergistic MNs are excitatory (Eccles et al. 1957; Jankowska 1992), whereas all disynaptic Q inputs to PbST MNs in the current study were blocked by 2 µM strychnine. Nevertheless, Ib afferents may contribute to the electrically evoked inhibitory responses in synergistic MNs in the neonatal murine cord.

Several lines of evidence demonstrate that Ia reciprocal inhibition in neonatal mice involves a disynaptic linkage between Ia afferents and MNs. Based on the observation that both CNQX and strychnine each block the IPSPs, the pathway involves at least two synapses. Two features of the latency of Ia IPSPs also support the existence of a polysynaptic pathway. Repetitive stimulation of a polysynaptic pathway causes an increase in latency, and the latencies of individual IPSPs fluctuate from trial to trial. Whereas monosynaptic pathways display neither of these characteristics (Hippenmeyer et al. 2007; Mentis et al. 2006; Wang et al. 2007), the Ia IPSPs we measured displayed both features (Fig. 2 A & B). We also found that the total latency of the Ia IPSP was too short
to include more than two synaptic linkages (Fig. 7A). Together, these results provide strong evidence that reciprocal inhibition in mice is mediated via a disynaptic pathway, as in adult cats.

A further characteristic of mature Ia INs in cats is that they are inhibited by RCs. Stimulation of the L5 and L6 VRs, which activates RCs in these segments, reduces the amplitude of IPSPs elicited by activation of Ia INs (Hultborn et al. 1971a; c). We found a similar reduction in reciprocal IPSP amplitude when the L3-4 VRs were stimulated a few ms before the Q nerve (Fig 4A). The extent of inhibition is similar to that reported in cats (Hultborn et al. 1971a).

**Specificity of synaptic connections in the neonatal reciprocal inhibitory pathway**

A major finding from this study is that the disynaptic Ia projections to MNs mediating reciprocal inhibition are already highly specific at birth. In cats, Ia INs project to MNs supplying functional antagonists, as for the Q Ia projection to PBSt MNs, but not to MNs supplying other muscles, including synergists. A specific example is the absence of an inhibitory projection from Add muscles to PBSt MNs (Eccles et al. 1956). Although Add and PBSt muscles are not close synergists, some elements in both muscles do cause hip extension and lateral rotation (Martini et al. 2000). In the mouse, Ia afferents from the Q and Add muscles also project monosynaptically to their homonymous MNs but not to functionally unrelated MNs (Add and Q MNs, respectively, (Mears and Frank 1997). We therefore chose Q and Add nerves as inputs to test the specificity of the Ia IN pathway to PBSt MNs. Using the narrow latency range of the strong Q inputs to PBSt MNs to define disynaptic potentials, we measured the amplitudes of inputs from the Add
nerve. Add inputs were small at all three stages tested (Fig. 6B), and the ratio of the amplitudes of Q to Add inputs was as high at P1 as at P7. Because Ia inputs to MNs are established only a few days before birth in rats (Kudo and Yamada 1985), a highly specific pattern of reciprocal inhibitory connections must be established either soon after or coincident with the formation of functional Ia-MN connections.

The selective disynaptic inhibition of PBSt MNs by Q Ia afferents but not Add Ia afferents implies that both synaptic stages must be specific. Q Ia INs must be selectively innervated by Q but not Add afferents, and PBSt MNs must be selectively innervated by Q INs but not Add Ia INs. Although it is not clear how this synaptic specificity is encoded, the first stage might involve the same mechanisms that are responsible for determining selectivity at Ia-MN synapses. These synapses are known to be highly specific soon after they form (Frank and Westerfield 1983; Mears and Frank 1997), and it is likely that the selectivity of these synapses is determined by the differential and specific expression of recognition molecules on Ia afferents and MNs (Chen et al. 2003). If specific subgroups of Ia INs and MNs expressed a similar cohort of recognition molecules then their innervation by Ia afferents would be specific as well.

Less is known about the specificity of interneuronal projections at early stages. Indirect evidence suggests that local pre-motor interneurons project appropriately to motoneurons at an early stage of development (Glover 2000). Avian RCs, however, project more caudally at E8 than they do at E10 (Xu et al. 2007), suggesting a rearrangement of synaptic connections in this system. In the present study, the absence of synaptic inputs to PBSt MNs from VR stimulation at more rostral segments at P7 suggests that RCs do not project directly to more caudal segments in neonatal mice.
These VR inputs to PBSt MNs do exist at P3, but they have a significantly longer latency than would be expected for a disynaptic response. It is therefore unlikely that these responses represent direct input from RCs. The mis-projections at E8 in chick embryos occur at a significantly earlier stage in development than the first postnatal week in mice, so if a similar rearrangement occurs during murine development, it has probably already occurred by birth.

Reflex testing in humans has revealed a change in the sign of reciprocal connections between antagonist muscles at birth (Mc Donough et al. 2001). Stimulation of triceps Ia sensory fibers by tendon taps evokes excitatory responses in the antagonist biceps muscle in neonates, but these reflexes become inhibitory during the ensuing nine months. A similar change in sign of reciprocal reflex responses is seen shortly before birth in rats (Nakayama et al. 2002), but this reversal is likely caused by the change in the reversal potential for GABA/glycine-mediated synaptic transmission. Because of the protracted fetal development in humans, it appears unlikely that GABA/glycine-mediated synaptic potentials are still depolarizing at birth, although to our knowledge this has not been tested directly. We saw no evidence of these reversed reflexes in postnatal mice.

**Role of Pax6 in the development of Ia inhibitory interneurons**

An unexpected finding was that the Ia reciprocal inhibitory pathway is functional in Pax6⁻/⁻ mice at E18 and P0. In these mutants, stimulation of the Q muscle nerve evokes initially hyperpolarizing synaptic potentials in PBSt MNs, just as in normal mice. This result is intriguing in the light of other studies showing that Pax6 is required for the development of all V1 INs including RCs (Gosgnach et al. 2006; Sapir et al. 2004) and presumably parvalbumin-expressing Ia INs (Alvarez et al. 2005). In view of the
widespread loss of V1-derived cell types in Pax6−/− mice, it is unlikely that if Ia INs are derived from V1 precursors, they are selectively spared, although this possibility cannot be entirely ruled out. Alternatively, Ia INs may represent a developmentally diverse population of neurons, of which the V1-derived cells are only a subset. It will be interesting to characterize more fully other interneuron cell types in Pax6 mutant mice to determine if there are additional inhibitory cells that may contribute to Ia reciprocal inhibition in the spinal cord.
Conclusions

1. The development of reciprocal inhibition was studied in neonatal mice using intracellular and extracellular recordings, focusing on the projections from quadriceps (Q) and adductor (Add) sensory fibers onto posterior biceps–semitendinosis (PBSt) motoneurons (MNs).

2. Synaptic potentials in PBSt MNs evoked by Q nerve stimulation are mediated disynaptically and glycine is the major inhibitory transmitter.

3. The specificity of neuronal connections in this reflex pathway is already high at birth; Q afferents evoke inhibition in PBSt MNs but Add afferents do not.

4. Reciprocal inhibition functionally inhibits the monosynaptic excitatory reflex in PBSt MNs by postnatal day 3 but is weak at birth.

5. Deletion of the transcription factor Pax6, which is required for the development of V1-derived Renshaw cells, does not block the development of this reciprocal inhibitory pathway.

Acknowledgments

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References


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Figure Legends

Figure 1. Synaptic responses elicited in a PBSt MN by stimulation of Q sensory axons at P7. The averaged responses, initially hyperpolarizing, were obtained sequentially (from bottom to top) as the resting potential became more negative. Dashed vertical lines indicate the beginning and peak of the potentials. Latencies and amplitudes were measured after the responses were stable, at which time all responses were depolarizing.

Figure 2. Characteristics of Ia inhibitory responses in PBSt MNs at P7. A. Repetitive stimulation of the Q nerve at 3 Hz resulted in greater attenuation of responses in PBSt MNs than in Q MNs, a characteristic of polysynaptic pathways. B. The latencies of Q → PBSt responses are longer and more variable than those of the monosynaptic Q → Q responses. The latency of each of 50 individual responses was measured (see Methods); the resulting latency histograms are shown below each response in red. C. Strychnine (0.4 µM) blocks a major portion of Q → PBSt responses. D. Selective activation of Q Ia axons by muscle taps elicits responses in PBSt MNs that are blocked by 2 µM strychnine. The large artifact at the beginning of each trace is caused by the high voltage needed to activate the piezoelectric bimorph used to tap the muscle. Dashed vertical lines in C and D indicate the beginning and peak of the potentials.

Figure 3. Method used for measuring disynaptic input to PBST MNs. Upper trace is a typical input from Q sensory axons; lower four black traces show Add inputs to four different PBST MNs. For each black trace, the superimposed red trace is a Q → PBST
disynaptic potential used as a model to measure the amplitude of the disynaptic component. The red trace has been scaled so that the peak of the synaptic potential matches the amplitude of the black trace. Dashed line indicates the latency of disynaptic inputs.

Figure 4. Latency (A, C) and amplitude (B, D) frequency histograms of synaptic potentials in PBSt MNs following stimulation of Q or Add nerves in P7 mice. Latencies of IPSPs elicited by Q Ia axons are clustered around 8 ms (A), but latencies of Add inputs are highly variable (C, see examples in Fig. 3). Amplitude histograms show the amplitudes of the disynaptic components of Q (B) and Add (D) inputs, measured as described in Fig. 3.

Figure 5. Ventral root stimulation can attenuate reciprocal inhibition in PBSt MNs at P7. Renshaw cells were activated by stimulation of motor axons in the L3 and L4 VRs. A. The synaptic response from Q sensory axons in PBSt MNs (Q → PBSt) was attenuated by prior VR stimulation (VR + Q → PBSt), which is shown in red superimposed on the Q → PBSt response. VR stimulation alone did not elicit any response in PBSt MNs. B. Variable attenuation of Q → PBSt synaptic potentials produced by prior L3 – L4 VR stimulation. Each point indicates the percent attenuation in one PBSt MN.

Figure 6. Representative Q → PBSt responses in P1 and P3 mice. A & B. Initially hyperpolarizing responses became depolarizing at both stages as the resting potential increased. C & D. Responses at both stages were largely blocked by 2 μM strychnine.
Figure 7. Postnatal development of the reciprocal inhibitory pathway. A. The latency of Q → PBSt connections decreased from P1 to P7, as axonal conduction velocities increased. The total synaptic latency at each stage is accurately predicted by the sum of the total Q → Q latency plus one additional synaptic delay, taken as the central latency of the Q → Q connection. B. The amplitudes of these responses elicited both by the Q and Add nerves do not change during the first postnatal week.

Figure 8. Reciprocal inhibition from Q sensory axons reduces the excitability of PBSt MNs. A. The excitability of PBSt MNs was measured by recording the burst of APs elicited in motor axons of the L5 VR (which contains PBSt motor axons) elicited by stimulation of PBSt sensory axons. Prior stimulation of Q sensory axons attenuated the reflex response. B. Population APs recorded in the L5 VR elicited by stimulation of PBSt, Q and PBSt+Q sensory axons. Arrowheads and arrows mark the time of PBSt and Q stimulation respectively. C. Time course of Q inhibition of the PBSt reflex response. Individual cases are indicated by short blue bars, and the average excitability at each time point is indicated in red. *, p<0.05; ** p<0.01. D. Development of functional reciprocal inhibition during the first postnatal week, expressed as the average percent inhibition of the PBSt reflex response caused by prior Q stimulation.

Figure 9. Reciprocal Ia inhibition persists in Pax6−/− mutant mice. Stimulation of the Q nerve elicits synaptic potentials in PBSt MNs that are initially hyperpolarizing in both mutant and wild type littermate mice at E18.
A. 

- Q → PBSt
- Q → Q
- 0.3 Hz
- 3 Hz
- 5 mV
- 10 ms

B. 

- Q → Q
- 5 mV
- 5 ms

C. 

- 0.4 µM strychnine
- 2 mV
- 5 ms

D. 

- 2 µM strychnine
- 2 mV
- 5 ms
-64 mV
-50 mV

2 µM strychnine

5 mV
10 ms

-67 mV
-43 mV

5 mV
10 ms
Q-central latency
Q-total synaptic latency
Predicted disynaptic latency
Measured Q
PBSt synaptic latency

A

Latency (ms)

0  4  8  12  16

P0  P3  P7

B

IPSP Amplitude (mV)

0  2  4  6  8

P0  P3  P7

Q-PBSt
Add-PBSt

Measured Q
PBSt synaptic latency
Predicted disynaptic latency
Q-total synaptic latency
Q-central latency
$Pax6$ mutant

-83 mV
-60 mV

Wild Type

-75 mV
-63 mV

5 mV
10 ms