Co-release of GABA does not occur at glycinergic synapses onto lumbar motoneurons in juvenile mice

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INTRODUCTION

Fast inhibition in the nervous system is mediated by γ-aminobutyric acid (GABA) and glycine. Histological studies have shown that both neurotransmitters are present at synaptic terminals in the spinal cord (Bohlhalter et al., 1994; Taal and Holstege, 1994; Todd et al., 1996; Örnung et al., 1996). Recordings of miniature inhibitory post-synaptic currents (mIPSCs) from motoneurons (Jonas et al., 1998) have demonstrated that a high proportion of currents are mediated by both glycine and GABA. Since each mIPSC constitutes the post-synaptic response to an individual quantum of transmitter, the observation suggests that vesicles are loaded with both neurotransmitters which are released together; the notion of co-release was supported by similar observations in recordings from connected interneuron-motoneuron pairs.

Evidence of co-release has been reported in the brainstem of juvenile rats (O’Brien and Berger, 1999) and in the dorsal horn of adult rats (Chéry and De Koninck, 1999). GABA even modulates the glycineergic current and shortens the time constant of the decay phase (Lu et al., 2008) through its action as a partial agonist. Vesicular filling of both neurotransmitters is possible since the vesicular inhibitory amino acid transporter (VIAT) is shared between GABA and glycine albeit with higher affinity for GABA (Wojcik et al., 2006).

Electrophysiological evidence of co-release on motoneurons (Jonas et al., 1998) however is based on experiments using preparations obtained mainly from young (P6–7) rats. Recordings from neurons in the dorsal horn (Keller et al., 2001) and from interneurons in the ventral horn (González-Forero and Alvarez, 2005) have identified populations of cells in which the extent of co-release decreases with age. In the adult rat, the majority of inhibitory inputs from Ia interneurons and Renshaw cells onto motoneurons are glycineergic (>80%) but a third of glycineergic terminals are also immunoreactive for the glutamic acid decarboxylase (GAD) enzyme (Alvarez et al., 2005). Since GAD is a marker for GABA synthesis, co-release may be a physiologically important mechanism of inhibition by a population of interneurons in the mature spinal cord.

In the present study, we recorded from motoneurons in spinal cord preparations obtained from mice of an age range (P8–14) in which they are almost fully weight-bearing. This is the latest developmental stage at which such recordings can be reliably obtained. The principal aim of the study was to ascertain whether GABA is released from pre-motor glycineergic interneurons and if it has any detectable effect on glycineergic inhibition of motoneurons. We thus performed experiments to detect the contribution of any GABAergic component in glycineergic inhibitory post-synaptic currents (IPSCs). Subsequently we manipulated the relative content of pre-synaptic GABA and glycine to determine if this had any modulatory effect on the kinetics of post-synaptic responses.

MATERIALS AND METHODS

Spinal preparations were extracted from P8–14 mice in which the enhanced green fluorescent protein (EGFP) is expressed under the control of the promoter of the neuronal glycine transporter GlyT2...
Whole-cell voltage-clamp recordings from motoneurons were performed using an Axopatch 200B amplifier (Molecular Devices) and filtered with an eight-pole Bessel filter at 5 kHz. Both voltage and current signals were sampled at 50 kHz using an Axon 1440A interface device (Molecular Devices) and the data were acquired and current signals were sampled at 50 kHz using an Axon 1440A interface device (Molecular Devices) and the data were acquired using Clampex 10 software (Molecular Devices). Electrodes were filled with an internal solution of composition (in mM) 113 NaCl, 3 KCl, 25 NaHCO3, 1 NaH2PO4, 2 CaCl2, 2 MgCl2 and 11 d-glucose. The typical motoneuron whole-cell capacitance of ~200 pF gave a corner frequency of 0.2–0.8 kHz.

During all recordings, the aCSF composition included 3 mM kynurenic acid (Sigma) to block excitatory glutamatergic activity. Drugs were bath applied through the perfusion system as detailed below and in the Results section. Applied drugs included 5 μM SR-95531 (Sigma), 0.3–2 μM strychnine (Sigma), 1 μM diazepam (Sigma), 100 nM tetrahydrodeoxy cortisol (THDOC, Sigma), 20 mM isoniazid (Sigma), 4 mM α-(methylamino) isobutyric acid (MeAIB, Sigma), 1 μM (3S)-3-[[4-[(Trifluoromethyl)benzoyl]amino]phenyl]methoxy]-α-aspartic acid (TFB-TBOA, Tocris), and 2 mM nipeptic acid (Tocris). Whenever we superfused isoniazid, 1 μM CGP-55584-HCl was included in the perfusate to isolate glycinergic or GABAergic mIPSCs respectively.

For experiments employing extracellular stimulation, we applied an electrical current through a patch pipette filled with normal aCSF using a constant current DS3 isolated stimulator (Digitimer). After patching a motoneuron in transverse slices, the stimulation electrode was maneuvered within the ventral region of Rexed lamina VIII, in the Renshaw cell area until a response could be elicited. Having established the minimum stimulation intensity required to evoke inhibitory post-synaptic currents (IPSCs) reliably, it was fixed at ~1.5× threshold. For experiments performed on the coronal preparation, the stimulation electrode was placed in the ipsilateral lateral white matter at least two or three segments rostral or caudal to the motoneuron which was always recorded from the dorsolateral motor nucleus of L5.

**Paired recordings**

For paired recordings, we used an optical configuration that allowed simultaneous visualization of motoneurons and EGFP positive interneurons in transverse slices. Infrared-DIC transmitted light was collected through the back port of a beam splitter, while a laser scanning confocal D-Eclipse C1 camera (Nikon) mounted on the front port was used to collect light emitted from EGFP positive cells. Having established a stable whole-cell patch on a motoneuron, a second electrode of ~6 MΩ filled with normal aCSF was introduced into the Renshaw area. Putative pre-synaptic neurons were patched in a loose cell-attached voltage-clamp configuration to stimulate the membrane and record evoked spikes (Barbour and Isop, 2000) using a 1–1.5 V voltage step of 20 μs applied from an ELC-03X (NPI) amplifier.

**Figure 1** illustrates how a connection was identified from post-synaptic responses of the motoneuron as IPSCs time-locked to evoked spikes. Typically, 1/100 of tested interneurons were
connected to the recorded motoneuron. Since a number of synaptic connections exhibited a high failure rate, double stimulations were applied to induce paired-pulse facilitation to potentiate responses. Upon finding a connection, the pre-synaptic interneuron was re-patched using a ~4 MΩ electrode containing an internal solution of composition (in mM) K-glucuronate 125, KCl 6, CaCl$_2$ 2, HEPES 10, EGTA 10, Mg-ATP 2, pH 7.3 with KOH, and osmolarity of 290–310 mOsM. In whole-cell current clamp, pre-synaptic cells were stimulated periodically every 9 s using an ELC-03X amplifier (NPI) by application of the minimum positive current required to evoke an action potential reliably.

**CONCENTRATION JUMPS**

Effects of the GABA-depleting agents on glycine receptors were investigated by performing concentration jump experiments on recombinant rat α1β glycine receptors, the adult isoform. Receptors were expressed in HEK293 cells using standard culture and transfection procedures (Burzomato et al., 2003). Concentration jumps were performed in an extracellular solution of composition (in mM) K-glucuronate 125, KCl 6, CaCl$_2$ 2, HEPES 10, EGTA 10, Mg-ATP 2, pH 7.3 with KOH, and osmolarity of 290–310 mOsM. In whole-cell current clamp, pre-synaptic cells were stimulated periodically every 9 s using an ELC-03X amplifier (NPI) by application of the minimum positive current required to evoke an action potential reliably.

**ANALYSIS OF INHIBITORY CURRENTS**

Successfully evoked IPSCs were discriminated offline using Clampfit 10.2 (Molecular Devices) whereas mIPSCs were detected using WinEDR 3.2.4 (Strathclyde Electrophysiology Software). Both evoked IPSCs and mIPSCs were subjected to exponential fitting analysis using MATLAB 7 software (MathWorks). Events were excluded from the analysis if their amplitude was less than 3 standard deviations of the baseline noise, if there were overlapping events within 50 ms, or if the asymptotic decay did not reach 10% of baseline. The Levenberg-Marquardt least-squares iterative algorithm was used to fit one or two exponential components to the decay phase of each post-synaptic current from 95 to 5% of peak amplitude:

$$\hat{I}_1(t) = I_0 + A_1 e^{-t/\tau_1}$$

$$\hat{I}_2(t) = I_0 + A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2}$$

The fit was selected on the basis of the $F$-test statistics for the two curves with $F > 4.8$ as a threshold for favoring the second. Since the decay constants were normally distributed, they were amenable to parametric test statistics. Comparisons across treatments were undertaken using one-way ANOVA $F$ statistics, with *post-hoc* tests based on Student’s $t$ statistics employing Bonferroni’s correction for multiple comparisons. Summary data are presented as mean ± SEM. To confirm successful detection of mixed inhibitory currents, we performed exponential fitting analysis on mIPSCs recorded from motoneurons ($n = 5$) of neonatal (P0–3) mice. Under control conditions, bi-exponential fits consistently represented a proportion of events (14.5 ± 4.5%) that was substantially reduced (to 4.7 ± 1.0%) in the presence of 5 μM SR-95531.

Since mIPSCs were always recorded in the presence of kynurenic acid, inward currents could only have resulted from GABAergic or glycineric events. We thus quantified the overall inhibitory drive for each sweep by evaluation of the integral of
the entire current trace per unit of time. Using pharmacological isolation of GABAergic or glycinergic events, we used the integral to estimate their relative contributions to overall inhibitory drive.

Prior to integral estimation however we first corrected for slow baseline drifts. We used a low-pass filter at 0.5 Hz that canceled completely the fast-rising events associated with synaptic activity and subtracted the result from the original signal to obtain a drift-free trace. Since the subtraction could impose a non-zero center for the baseline, it was necessary to eliminate any offsetting effects on the integral evaluation by a further subtraction of this bias. We estimated the bias using the mode of the data, which was evaluated by convolution with a Gaussian kernel of a standard deviation of $\sigma = 0.3$, where $n$ is the size and $\sigma^2$ is the variance of the data (Bhumbra and Dyball, 2010).

RESULTS

EVOKE D IPSCs EXHIBITED NO CO-DETECTION

Simultaneous recordings of interneurons and motoneurons were obtained from eight connected pairs. In all cases, strychnine completely abolished evoked IPCSs (data not shown). In a subset of four, responses were tested with bath application of diazepam and gabazine for exponential fitting analysis of the decay phase. Evoked IPSCs were best-fitted with a single exponential in all cases under control conditions ($n = 203$) and in the presence of diazepam ($n = 143$). Across all four pairs, the mean of the averaged time constant $\tau$ fitted for all sweeps was $3.3 \pm 0.2$ ms. The lack of a second component to the exponential fits and the very short mean time constant are consistent with a purely glycinergic component to evoked currents.

Pharmacological modulation of any GABAergic component with diazepam and gabazine confirmed the lack of co-detection of GABA with glycine. A representative example of a paired recording for the different drug treatments is illustrated in Figure 2A. Responses of the motoneuron showed no effect by application of 1 $\mu$M diazepam or 5 $\mu$M SR-95531 of evoked IPSCs. By contrast, responses were abolished after application of 2 $\mu$M strychnine.

Group data for the four connected pairs are illustrated in Figure 2B. For three of the pairs, one-way ANOVA statistics showed no statistically significant effect of the treatments on the decay constant ($F \leq 0.11$, $P \geq 0.523$). In the fourth pair, the test statistic was significant ($F = 19.3$, $P < 0.001$) as result of a small decrease in the time constant in the presence of diazepam ($\tau = 3.2 \pm 0.1$ ms) compared to control ($\tau = 3.9 \pm 0.1$ ms, $t = 4.64$, $P < 0.001$). A decrease in the time constant however is not consistent with the effect of diazepam enhancing and prolonging the GABA component of evoked IPSCs.

Comparisons of mean amplitudes to those observed in control conditions showed a small decrease in the presence of diazepam ($82 \pm 11\%$) and SR-95531 ($93 \pm 8\%$). Since superfusion of either agent resulted in modest decreases, we attributed the progressive.

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**FIGURE 2** Paired recordings showed no contribution of GABA to evoked IPSCs. Traces in (A) illustrate responses from a connected pair representing a single spike elicited in the interneuron above and the evoked IPSCs recorded from the motoneuron below with the mean current overlaid in black. Bath application of neither 1 $\mu$M diazepam (purple) nor 5 $\mu$M SR-95531 (red) modulated the time constant of evoked IPSCs whereas 2 $\mu$M strychnine (blue) abolished all responses. Group data from four connected pairs are represented in graph (B), which illustrates no significant effect of diazepam (purple) or SR-95531 (red) on the time constant (see text). Error bars indicate mean ± SEM.
attenuation of IPSCs to the inevitable run down of neurotransmitter due to dialysis inherent to paired recordings (Diana and Marty, 2003). Analysis of the paired recordings thus indicated that there is no detectable GABAergic component the evoked IPSCs.

Paired recordings were performed on transverse slices and thus were selective for horizontal rather than vertical connections. It is possible that axon terminals from horizontal and vertical projections differ in their neurotransmitter content (Liu et al., 2010) or are apposed to post-synaptic membranes with different compositions of receptors. We thus investigated the effects of extracellular stimulation of ascending or descending connections on evoked IPSCs using the coronal preparation.

Seven motoneuronal recordings were obtained from coronal preparations while stimulating ascending or descending projections. Evoked IPSCs were best-fitted with a single exponential in all cases under control conditions ($n = 223$) and in the presence of diazepam ($n = 223$). Across all cells, the mean of the averaged time constant $\tau$ fitted for all sweeps was $5.8 \pm 0.9$ ms. The lack of a second component to the exponential fits and the very short mean time constant are consistent with a purely glycinergic component to evoked currents.

Superfusion of diazepam or gabazine confirmed the lack of co-detection of GABA with glycine. A representative example of a evoked IPSCs recorded during different drug treatments is illustrated in Figure 3A. Responses of the motoneuron showed no effect by application of $1 \mu M$ diazepam or $5 \mu M$ of evoked IPSCs. By contrast, responses were abolished after application of $1 \mu M$ strychnine.

Group data for the seven motoneurons are illustrated in Figure 3B. Paired t-test statistics evaluated from the average fitted time constant for each cell across all seven motoneurons confirmed no significant effect on the decay kinetics of evoked IPSCs by diazepam ($\tau = 6.1 \pm 0.9$ ms, $t = -1.51, P = 0.183$) or SR-95531 ($\tau = 5.7 \pm 1.0$ ms, $t = -1.04, P = 0.346$). Analysis of the recordings obtained from coronal preparations thus indicated that there is no detectable GABAergic component in IPSCs evoked by simulation of ascending or descending projections.

To investigate putative GABAergic components of currents mediated by diazepam-insensitive GABAA receptors, we recorded evoked responses from four motoneurons in transverse slices during bath application of the $200 \text{ nM THDOC}$. Group results are illustrated in Figure 3C. In comparison to control conditions ($\tau = 5.5 \pm 0.3$ ms), paired t-test statistics showed no significant effect of THDOC on the time constant ($\tau = 5.6 \pm 0.3$ ms, $t = -0.47, P = 0.672$).

**RECORDINGS OF mIPSCs SHOWED NO CO-DETECTION**

Since neither paired recordings nor extracellular stimulation showed demonstrable GABA co-detection in evoked responses, we investigated whether there were any GABAergic components

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**FIGURE 3 | Extracellular stimulation of ascending and descending projections showed no contribution of GABA to evoked IPSCs.** Traces in (A) illustrate responses of a motoneuron with the mean current overlayed in black. Bath application of neither $1 \mu M$ diazepam (purple) nor $5 \mu M$ SR-95531 (red) modulated the time constant of evoked IPSCs whereas $2 \mu M$ strychnine (blue) abolished all responses. Group data for all motoneurons are represented in graph (B), which illustrates no significant effect of diazepam (purple) or SR-95531 (red) on the time constant. Graph (C) shows no significant effect of $200 \text{ nM THDOC}$ (purple) on the time constant of responses evoked in transverse slices (see text).
in miniature IPSCs. Miniature currents were recorded from seven motoneurons of which six were tested with 5 μM SR-95531 and four were tested with 1 μM diazepam. Exponential fitting analysis showed that the decay phase of currents were best-fitted with a single exponential in control conditions (n = 1398) and in the presence of diazepam (n = 992). Across all motoneurons, the mean of the averaged time constant $\tau$ fitted for all mIPSCs under control conditions was 6.0 ± 0.9 ms. The lack of a second component to the exponential fits and the short mean time constant are consistent with purely glycinergic miniature currents.

Pharmacological modulation of any GABAergic component with diazepam and gabazine confirmed the lack of co-detection of GABA with glycine. A representative example of mIPSCs recorded from a motoneuron during different drug treatments is illustrated in Figure 4A. Exponential fitting analysis showed no effect of diazepam or SR-95531 on the evoked IPSCs.

Group data for the mIPSCs recorded from all the cells are illustrated in Figure 4B. The mean relative amplitude of the currents compared to control conditions was not changed in the presence of diazepam (99.4 ± 5.0%) or SR-95531 (99.1 ± 6.9%). There was also no significant change on the mean fitted time constant with diazepam ($\tau = 6.2 ± 1.3$ ms, paired $t = -0.57$, $P = 0.607$) or SR-95531 ($\tau = 5.9 ± 0.8$ ms, paired $t = 0.50$, $P = 0.618$).

Since the experiments were performed at room temperature, the apparent lack of a second component in mIPSCs could have resulted from a reduction in GABA synthesis from glutamate due to a decrease in the activity of glutamate transporters. We thus recorded mIPSCs from five motoneurons at 32˚C under control conditions and in the presence of diazepam. Exponential fitting analysis showed that the decay phase of currents were best-fitted with a single exponential before ($n = 4111$) and after ($n = 2972$) diazepam administration. As expected for recording at higher temperatures, the mean decay constant was markedly short in control conditions ($\tau = 2.7 ± 0.2$ ms). Superfusion of diazepam nevertheless had no significant effect on the fitted time constant ($\tau = 3.1 ± 0.5$ ms, paired $t = -1.06$, $P = 0.348$).

The short time constant and the minimal effects of diazepam and SR-95531 suggested that glycinergic contribution to inhibitory inputs were substantially greater than the GABAergic component notwithstanding the absence of co-detection. Their relative contributions in mIPSC activity at room temperature were evaluated using integral analysis for the recordings in which each component was pharmacologically isolated. A representative example of such a recording during different drug administrations is illustrated in Figure 4C. While application of 5 μM SR-95531 had no demonstrable effect on the activity of mIPSCs, they were virtually abolished by 0.3 μM strychnine. The example thus illustrates a substantially greater contribution of a glycinergic inputs compared to inhibition mediated by GABA.

Group data for the mIPSC integral analysis for the six cells recorded are illustrated in Figure 4D. In comparison to control conditions, the overall inhibitory drive did not change in presence of SR-95531 in five cells (99.0 ± 6.4% of control) whereas it was significantly reduced in only one (to 45.0 ± 25.7%).
contrast, out all the seven cells tested with strychnine, activity was completely abolished in three cells and the remaining four exhibited a profound suppression of inhibitory activity following drug administration (to 24.9 ± 4.1%). The integral analysis thus demonstrates a substantial predominance of a glycinergetic component to inhibitory inputs in comparison to GABAergic contributions.

**GABA DEPLETION DID NOT AFFECT EVOKED IPSCs**

While the single exponential profile of IPSCs and pharmacological isolation of a GABAergic component demonstrated no co-detection, it did not preclude possible co-release onto a post-synaptic membrane with a dearth of GABA receptors. Since GABA however is a partial agonist of post-synaptic glycine receptors, its co-release could attenuate glycinergetic currents and would shorten their time constant (Lu et al., 2008). Glutamate uptake contributes to GABA synthesis (Mathews and Diamond, 2003) by the action of GAD. Glutamate is either transported into cells or synthesized from glutamine by glutaminase.

In order to deplete GABA, we used 4 mM MeAIB to block glutamine uptake (Varoqui et al., 2000), 1 μM TFB-TBOA to block glutamate uptake (Shimamoto et al., 1998), and 20 mM isoniazid to inhibit GAD (De Koninck and Mody, 1997) thus depleting upstream substrates of GABA and directly inhibiting its synthesis from glutamate. GABA uptake into cells was blocked using 2 mM nipecotic acid. During recordings of glycinergetic currents from voltage-clamped motoneurons, responses evoked from extracellular stimulation in the Renshaw cell area were attenuated by ~70% following administration of 20 mM isoniazid (data not shown). Since the decrease in peak current was observed within 5 min of drug application, the reduction in response was unlikely to have resulted from perturbations in metabolic processes but as a consequence of direct action of the four drugs on the receptors mediating post-synaptic currents. We thus sought to identify which of the GABA-depleting agents confer the least direct action on glycinergetic receptors using concentration jumps.

**Figure 5** illustrates responses to fast glycine applications on outside-out patches pulled from HEK293 cells expressing recombinant α1β. Under control conditions (**Figure 5A**), concentration jumps showed uniform responses. In the illustrated example, bath application of 20 mM isoniazid resulted in an attenuation of the current.

Group data illustrated in **Figure 5B** illustrates that all GABA-depleting agents decreased the peak current, which were observed within 5 min of administration. Reductions in mean amplitude were substantial following application of 4 mM MeAIB (25.5 ± 3.9%, n = 7), 1 μM TFB-TBOA (34.4 ± 9.8%, n = 5), and 2 mM nipecotic acid (29.0 ± 4.2%, n = 7). The comparatively modest attenuation associated with isoniazid (14.0 ± 3.0%, n = 5) thus identified this agent as our choice of drug for depleting GABA.

We confirmed the efficacy of GABA depletion by isoniazid using extracellular stimulation to evoke IPSCs recorded from whole-cell voltage-clamped motoneurons during administration of 20 mM isoniazid in the presence of 1 μM strychnine to isolate GABAergic currents. A representative example of the progressive attenuation in currents is illustrated in **Figures 6A–C**. A plot of the corresponding changes in amplitude (**Figure 6D**) shows that the attenuation was substantial and occurred over a period of 30–45 min, demonstrating an effect more compatible with influences on metabolic processes rather than a direct action on post-synaptic receptors.

**Group data represented in Figure 6E** illustrates the relative change in evoked IPSC amplitude when comparing the peak currents observed 30–45 min after isoniazid administration with those recorded during the control period. In all cases (n = 4) there was an attenuation in the response, with an average reduction of

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**FIGURE 5** Fast glycine application experiments showed that isoniazid administration produced the least attenuation in current among the GABA-depleting agents. Traces in (A) illustrate an example in which a small reduction in the current is observed in the presence of 20 mM isoniazid. Graph (B) illustrates isoniazid attenuated the current to the least extent (see text) compared to 4 mM MeAIB, 1 μM TFB-TBOA, and 2 mM nipecotic acid.
FIGURE 6 | Application of 20 mM isoniazid depleted vesicular GABA over a period of 30–45 min. In the presence of 1 μM strychnine, IPSCs were evoked by extracellular stimulation. The traces illustrate an example of a recording of post-synaptic responses during the control period [trace (A)] and two epochs following isoniazid superfusion [traces (B) and (C)]. Mean responses are overlayed in black. Graph (D) plots the changes in amplitude, illustrating the time periods corresponding to (A–C), and shows a gradual but substantial reduction in current. The group data represented in graph (E) illustrates that evoked IPSCs in all motoneurons were progressively attenuated during the ∼40 min of isoniazid superfusion.

of 61 ± 5%. The results thus confirm that application of 20 mM isoniazid depletes vesicular GABA over a period of 30–45 min.

Since co-released vesicular GABA would hasten the decay of glycincergic currents (Lu et al., 2008), then any GABA-mediated effect on evoked IPSCs would be perturbed by isoniazid administration over a time scale of 30–45 min during which the decay would become progressively longer. In the absence of strychnine, evoked IPSC recordings were obtained during application of 20 mM isoniazid. A representative example is illustrated in Figures 7A–C, which shows only a modest reduction in the size of the current. The plot of corresponding changes in amplitude (Figure 7D) however shows the attenuation was modest and occurred over a period of ∼10 min, demonstrating an effect more compatible with direct action on post-synaptic receptors rather than on metabolic processes.

Group data (n = 6) are illustrated as graphs showing the effect on the relative amplitude (Figure 7E) of isoniazid superfusion after 10 min and after an hour. While a modest attenuation of 13.5 ± 6.6% in the current amplitude was observed after 10 min, the reduction in amplitude after an hour was only 15.8 ± 7.6%. Since there was only a small fast attenuation, the changes in relative amplitude demonstrate an effect more compatible with direct action on post-synaptic receptors rather than on metabolic processes. Comparison of time constants of evoked responses in control conditions with those after an hour of isoniazid superfusion (Figure 7F) showed no significant change from $\tau = 5.8 \pm 0.8$ to $\tau = 5.8 \pm 0.8$ ms (paired $t = −0.05$, $P = 0.963$). The results indicate that little or no GABA is co-released in glycincergic synapses investigated in the present study.

GLYCINE LOADING DID NOT AFFECT EVOKED IPSCs

Since GABA depletion did not affect the kinetics of evoked IPSCs, we investigated the effects of loading glycine into pre-synaptic vesicles using paired recordings. Addition of 20 mM glycine to the internal solution of the pipette used to patch the interneuron would favor glycine loading compared to GABA. Any changes to the neurotransmitter content of pre-synaptic vesicles would be detected as a progressive change in post-synaptic responses over time after establishing a whole-cell configuration for the interneuron. After stabilization of the current clamp on the pre-synaptic neuron, trains of 1000 spikes at 50 Hz were delivered to deplete vesicles every 10–15 min.

There were however no progressive changes in evoked currents recorded from connected pairs (n = 4). In the representative example illustrated in Figure 8A, IPSCs evoked immediately upon establishing a whole-cell configuration were not manifestly
FIGURE 7 | Effects on glycinergic IPSCs by application of 20 mM isoniazid were not compatible with vesicular GABA depletion. The traces illustrate an example of a recording of post-synaptic responses during the control period (trace (A)) and two epochs following isoniazid superfusion (traces (B) and (C)). Mean responses are overlayed in black. Graph (D) plots the changes in amplitude, illustrating the time periods corresponding to (A–C), and shows a modest but rapid reduction in current. The group data for all motoneurons shows that the attenuation of evoked IPSCs (graph (E)) after 1 h was no greater than that observed after 10 min, and that isoniazid superfusion had no systematic effect on the decay time constant (graph (F)).

DISCUSSION

Analysis of our initial paired recordings identified individual glycinergic synaptic connections in which GABA is not co-detected. Co-detection was also absent in responses to extracellular stimulation of glycinergic projections in the coronal preparation. There was no demonstrable GABAergic contribution to the substantially larger number of inputs associated with the mIPSCs recorded from motoneurons in transverse slices. Our initial results therefore show that glycinergic synapses onto lumbar motoneurons have no ostensible GABA\textsubscript{R}-mediated component.

Mixed evoked and miniature IPSCs with pharmacologically isolable GABAergic and glycinergic components have however been observed in recordings from lumbar motoneurons (Jonas et al., 1998). While our results may appear to contrast with those previously reported, the apparent discrepancy is most likely to have arisen as a result of differences in the age and species used for recordings. We used P8–14 mice whereas (Jonas et al., 1998) recorded mainly from P6–7 rats. Mice are already weight-bearing at P8 whereas rats are not until P12. The dissimilar findings may thus reflect the different stages of maturation of the spinal cord for the two animals.

While mIPSC recordings from interneurons in the ventral horn have exhibited co-detection in neonatal rats (González-Forero and Alvarez, 2005), the GABA\textsubscript{A}-mediated component is diminished specifically in non-Renshaw cells in juveniles (from P9). In the dorsal horn of the rat (Keller et al., 2001), the extent of co-detection also decreases with age and is absent altogether in the mature state (≥P21). Since our exponential fitting analysis showed neither a second component in evoked or miniature IPSCs nor any pharmacologically isolable GABAergic contribution in evoked responses, co-detection must already be negligible in lumbar motoneurons of P8–14 mice.

Notwithstanding the absence of co-detection, the mIPSC analysis demonstrates that the glycinergic contribution to inhibitory inputs in juvenile mice is substantially greater than the GABAergic
component. In the ventral horn there is a postnatal shift in the distribution of mIPSCs from GABAergic to glycinergic inhibition (Gao et al., 2001), a reduction in immunoreactivity of GABA and GAD (Ma et al., 1992), and a decrease in GABA_A receptor expression (Ma et al., 1993).

We investigated whether the lack of a GABAergic component in the evoked and miniature IPSCs resulted from a paucity of co-released GABA or post-synaptic GABA_A receptors by experimental manipulation of the relative pre-synaptic content of GABA and glycine. Currents evoked by extracellular stimulation were not affected by pre-synaptic depletion of GABA. Paired recordings showed no modulatory effect on post-synaptic responses while glycine was loaded into pre-synaptic vesicles in preference to GABA. We thus infer that GABA is not co-released in physiologically relevant amounts at glycinergic synapses onto lumbar motoneurons in juvenile mice.

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