Early Development of Glycine- and GABA-mediated Synapses in Rat Spinal Cord

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Motoneuron responses to the inhibitory amino acids glycine and GABA, and the contribution of inhibitory synapses to developing sensorimotor synapses were studied in rat spinal cords during the last week *in utero*. In differentiating motoneurons, glycine and GABA induced Cl^- -dependent membrane depolarizations and large decreases in membrane resistance. These responses gradually decreased during embryonic development, and at birth they were significantly smaller than in embryos. In motoneurons of embryos and neonates, dorsal root stimulation produced only depolarizing potentials, some of which reversed at -50 mV membrane potential. Reduction of extracellular Cl^- concentrations increased the amplitude of these potentials, suggesting that they are generated by Cl^- current. Contribution of Cl^- -dependent potentials to compound dorsal root-evoked potentials was studied by determining the effects of glycine and GABA antagonists on them. In motoneurons of embryos at days 16–17 of gestation (D16–D17), strychnine or bicuculline blocked dorsal root-evoked potentials. This suppression was neither the result of a decrease in neuronal excitability nor the inhibition of glutamate receptors. Strychnine-evoked depression was not blocked by atropine, indicating that it was not due to disinhibition of muscarinic synapses. By D19, strychnine and bicuculline significantly increased dorsal root-evoked potentials rather than blocking them. This reversed function did not result from an increase in neuronal excitability or changes in the specificity of strychnine and bicuculline antagonism.

The number of glycine- and GABA-immunoreactive cells increased 20% between D17 and D19. The number of immunoreactive cells and fibers significantly increased in the motor nuclei and dorsal horn laminae. These morphological changes may contribute to establishment of new synaptic contacts on motoneurons, thus changing the actions of strychnine and bicuculline on dorsal root-evoked potentials.

The amino acids glycine and GABA act as inhibitory neurotransmitters in the adult spinal cord (Werman et al., 1968; Krnjević et al., 1977, reviewed by Curtis and Johnston, 1974; Davidoff and Hackman, 1983; Young and MacDonald, 1983). Glycine and GABA are found in high concentrations in mammalian spinal cord (Aprison and Werman, 1965; Berger et al., 1977), and the amino acids coexist within cell bodies in the dorsal horn of adult rats (Todd and Sullivan, 1990). Glycine- and GABA-gated channels are permeable to Cl^- ions, and although the subunits of these receptors are different (Pritchett et al., 1989; Grenningloh et al., 1990), glycine- and GABA-activated channels have similar multiple levels of conductance (Hamill et al., 1983; Bormann et al., 1987).

The complex influence of inhibitory synapses on spinal cord excitation has been examined in adult mammals (reviewed by Burke and Rudomin, 1977; Davidoff and Hackman, 1984; Rudomin, 1990), but little is known about the interactions between inhibitory and excitatory synaptic pathways in developing rat spinal cords (Saito, 1979; Seno and Saito, 1985; Miyata et al., 1987), and in dissociated spinal neurons (Nelson et al., 1977; Jackson et al., 1982). Recent physiological and morphological studies have described the pattern and time course of development of excitatory sensorimotor synapses in rat spinal cords (Kudo and Yamada, 1987; Ziskind-Conhaim, 1990), but the time course of formation of inhibitory synapses is unknown.

Electrical activity is believed to play an important role in neuronal differentiation and synaptogenesis (Nelson et al., 1990). The excitation level depends on the temporal activation of both excitatory and inhibitory pathways. However, stimulation of immature inhibitory pathways can evoke excitation rather than inhibition (reviewed by Cherubini et al., 1991). For example, Cl^- -mediated depolarizing potentials have been recorded in spinal motoneurons of newborn rats (Fulton et al., 1980; Takahashi, 1984; Jahr and Yoshioka, 1986) and embryonic chicks (Obata et al., 1978; Velumian, 1984; O'Donovan, 1989).

The objectives of our study were to (1) examine motoneuron response to glycine and GABA during the establishment of sensorimotor synapses, (2) determine the influence of newly formed inhibitory synapses on dorsal root-evoked potentials, and (3) study the changes in the distribution of glycinergic and GABAergic cells in lumbar spinal cord during the last week *in utero*.

A brief report of some of these results has been published previously (Wu and Ziskind-Conhaim, 1991).

Materials and Methods

Lumbar spinal cords of embryonic and neonatal rats at days 16–21 of gestation (D16–D21; birth is at D21–D22) and 1–2 d after birth were used. The procedures for preparing the spinal cord, for intracellular

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Figure 1. GABA- and glycine-induced depolarizations in D19 motoneurons. *a*, GABA (1 mM) induced depolarization of 14 mV and resistance decrease of 54%, which was completely reversible. Similar depolarization (14 mV) was induced in the presence of TTX (0.5 μ M) and 10 mM Mg^{2+} /1 mM Ca^{2+} , which blocked synaptic activity. After the removal of GABA (*wash*) and membrane repolarization to resting potential, membrane resistance did not change during transient depolarization by intracellular current injection. *b*, Glycine (2 mM) produced depolarization of 5 mV and resistance decrease of 57% were recorded in the same motoneuron, in the presence of TTX (0.5 μ M) and high- Mg^{2+} /low- Ca^{2+} solution. Glycine responses were reversible (not shown). *Upper traces* are digitized records of the membrane potentials in response to continuous applications of GABA or glycine. The *downward deflections* in the upper traces are the electrotonic potentials evoked by intracellularly injected constant-current hyperpolarizing pulses (500 msec duration at 0.2 Hz; *bottom traces*). Membrane resistance was estimated during the brief hyperpolarization to the resting potential value (*dashed line*).

recording, and for stimulation were similar to those described previously (Ziskind-Conhaim, 1988a, 1990). In summary, the medial surface of a hemisected spinal cord was pinned to a Sylgard-coated recording chamber and perfused with recording solution at 28–30°C. Motoneurons were impaled with microelectrodes filled with 3 M potassium acetate, and identified by antidromic potentials evoked by ventral root stimulation. Compound synaptic potentials were evoked by brief (0.5 msec) dorsal root stimuli.

To shift Cl^- equilibrium potential to a more positive value, 50% of the extracellular Cl^- was substituted with either isethionate or glucuronate. Junction potentials of about -12 mV developed during the perfusion with low- Cl^- solution. An alternative method for changing Cl^- concentrations across the membrane was to increase intracellular Cl^- concentrations with KCl-filled microelectrodes (Barker and Ran-

som, 1978). In embryonic motoneurons, however, only unstable resting potentials of less than -40 mV were recorded with KCl-filled electrodes.

Immunocytochemistry

Segments of lumbar spinal cord were fixed with 2.5% glutaraldehyde and 1% paraformaldehyde in 0.1 M phosphate buffer. Embryonic spinal cords were lightly stained with cresyl violet and embedded in gelatin-albumin mixture (Ziskind-Conhaim, 1988a, 1990). After storing overnight in 30% sucrose solution at 4°C, 50 μ m transverse sections were incubated for 1 hr in 1 M ethanolamine to reduce nonspecific staining.

Glycine. Sections were incubated for 40–45 hr in the primary antibody, rabbit anti-glycine serum diluted 1:500 (Wentholt et al., 1987). The diluent was a solution of 0.2% gelatin (Difco), 0.3% Triton X-100,

Table 1. Glycine- and GABA-induced dose-dependent depolarizations in D19–D21 motoneurons

Concentration (mM)	Glycine		GABA	
	Membrane depolarization (mV)	Resistance decrease (%)	Membrane depolarization (mV)	Resistance decrease (%)
0.5	1.2 $\pm$ 1.0 (<i>n</i> = 3)	16.9 $\pm$ 5.6	5.4 $\pm$ 1.5*** (<i>n</i> = 5)	17.2 $\pm$ 12.1
2	10.4 $\pm$ 4.0* (<i>n</i> = 7)	53.1 $\pm$ 21.7**	18.2 $\pm$ 2.4*,*** (<i>n</i> = 7)	46.5 $\pm$ 18.9**

This table shows dose-dependent depolarization and decrease in membrane resistance induced by glycine and GABA in D19–D21 motoneurons (mean $\pm$ SEM; *n* = number of motoneurons). Higher concentrations of glycine and GABA significantly increased the depolarization and decreased membrane resistance. At concentrations of 0.5–10 mM, GABA-mediated depolarizations were larger than those evoked by glycine. Glycine- and GABA-induced responses were recorded in the presence of TTX (0.5 μ M) and 10 mM Mg^{2+} /1 mM Ca^{2+} .

* Significantly larger than at 0.5 mM (*p* < 0.01).

** Significantly larger than at 0.5 mM (*p* < 0.05).

*** Significantly larger than glycine-induced depolarizations (*p* < 0.01).

Table 2. The amplitude of glycine- and GABA-mediated depolarizations increased in low concentrations of extracellular Cl^-

Substance	Membrane depolarization (mV)	Resistance decrease (%)
Glycine (2 mM)	10.4 $\pm$ 4.0 (n = 7)	53.1 $\pm$ 21.7
Glycine in low Cl^-	16.2 $\pm$ 2.3* (n = 5)	37.2 $\pm$ 10.0
GABA (1 mM)	11.3 $\pm$ 3.5 (n = 13)	35.1 $\pm$ 12.2
GABA in low Cl^-	18.2 $\pm$ 2.8** (n = 4)	35.8 $\pm$ 10.5

Larger depolarizations were induced by glycine and GABA when 50% of the extracellular Cl^- was replaced by isethionate or glucuronate. Data are from D19–D21 motoneurons, in the presence of TTX (0.5 μM) and 10 Mg^{2+} /1 mM Ca^{2+} , presented as mean $\pm$ SDM; n = number of motoneurons.

* Significantly larger than depolarization produced by glycine in normal Cl^- concentration ($p < 0.05$).

** Significantly larger than depolarization produced by GABA in normal Cl^- concentration ($p < 0.01$).

1% goat serum (Sigma), and 0.01% Na azide. The sections were washed in phosphate-buffered saline and treated according to the avidin–biotin method (Hsu et al., 1981). The immunocytochemical reaction product was developed in 0.1 M Tris buffer containing 0.035% 3,3-diaminobenzidine hydrochloride (Sigma) and 0.003% H_2O_2 .

The specificity of glycine antibody was determined by incubating the sections with antiserum preabsorbed with glycine–bovine serum albumin conjugate.

GABA. The procedure for staining with GABA antiserum was similar to that of glycine. Rabbit anti-GABA serum was diluted 1:3000 (Incstar).

Data analysis

To determine the distribution of glycinergic and GABAergic immunoreactive cells in various laminae, stained cells were counted in more than seven sections/spinal cord (n = 3 or 4 spinal cords), and at least 60 cells were counted per section. At early developmental ages, it was difficult to distinguish between laminae II and III; therefore, cells in dorsal laminae I–III were pooled. The statistical significance of differences between the means was determined using independent *t* test.

Solutions

The recording solution contained (in mM) NaCl, 116.4; KCl, 5.4; CaCl_2 , 4.0; MgSO_4 , 1.3; NaHCO_3 , 26.2; NaH_2PO_4 , 0.92; glucose, 11.0 (pH 7.2–7.4). The solution was gassed with 95% O_2 , 5% CO_2 . In solutions in which extracellular Cl^- was reduced, isethionic or glucuronic acid was used to replace 50% of NaCl. The following substances were added to the recording solution at known concentrations: 2-amino-5-phosphonovaleric acid (APV), baclofen, bicuculline methchloride, and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) from Research Biochemicals, Inc., and GABA, glycine, glucuronic acid, isethionic acid, muscimol, and strychnine from Sigma.

Results

Glycine- and GABA-mediated depolarizations

In embryonic and neonatal motoneurons, application of GABA and glycine induced membrane depolarizations that were associated with large decreases in membrane resistance (Fig. 1). These depolarizations were not mediated by synaptic activity, because similar depolarizations were produced when synaptic activity was blocked by TTX (0.5 μM) and 10 mM Mg^{2+} /1 mM Ca^{2+} (Fig. 1). At these concentrations, either TTX or high Mg^{2+} blocked dorsal root-evoked potentials.

The evoked depolarizations were dose dependent at glycine and GABA concentrations of 0.5–10 mM, and at all ages the average amplitude of GABA-mediated depolarization was sig-

Figure 2. The amplitude of glycine- and GABA-induced depolarizations decreased during embryonic development. The depolarizations produced by glycine and GABA (1 mM) in neonatal motoneurons were significantly smaller than those produced in D19–D21 embryonic motoneurons. Similar age-dependent reduction was apparent for the decrease in membrane resistance produced by the agonists. Data points are the means (n = 4–12 motoneurons); error bars are SDM. *, significantly smaller than at D19–D21 ($P < 0.05$); **, significantly smaller than at D16–D17 ($P < 0.02$); ***, significantly smaller than at D19–D21 ($P < 0.005$).

nificantly larger than that induced by glycine (Table 1). For example, at D19–D21, 0.5 mM glycine generated an average depolarization of 1.2 mV compared to the 5.4 mV depolarization generated by GABA. Despite the larger depolarization produced by a given concentration of GABA, both amino acids induced a similar decrease in membrane resistance (Table 1). Baclofen, a GABA_B agonist, did not change motoneuron membrane potential (1–2 mM; n = 3), indicating that GABA_B receptors do not contribute to motoneuron depolarization.

To determine whether increased Cl^- conductances generated motoneuron depolarizations, the concentration of extracellular Cl^- was reduced by 50% to shift the Cl^- equilibrium potential to a more positive value. In low concentration of extracellular Cl^- , both glycine and GABA induced significantly larger depolarizations than those evoked in normal recording solution (Table 2).

Motoneuron responses to glycine and GABA decreased during embryonic development

The depolarizations produced by glycine and GABA gradually decreased during the last week *in utero* (Fig. 2). For example, motoneuron responses to 1 mM glycine were reduced from 8.6 mV at D16–D18 to 5.4 mV at D19–D21 and to 2.0 mV at birth. Those depolarizations were associated with apparent decreases of 42.2%, 28.6%, and 7.0% in membrane resistance. Similar reductions were recorded for GABA-mediated depolarizations and resistance decreases.

The reduced responses to GABA were probably not due to the development of a GABA uptake mechanism, which could have reduced GABA concentrations at the receptor sites. A similar age-dependent decrease was recorded with muscimol, an agonist of GABA_A that is not taken up effectively by GABA uptake carriers. The depolarization produced by muscimol (0.2 mM) was reduced from 18.9 mV at D17–D18 [± 0.6 standard

Figure 3. Membrane depolarization reversed some of the dorsal root-evoked potentials in D16 and D21 motoneurons. Transient depolarizing current injected intracellularly depolarized the motoneurons from about -65 mV resting membrane potentials (dashed lines) to -49 mV (dotted lines). At D16, some depolarizing potentials (arrows) reversed after latencies of more than 150 msec, while at D21 they reversed at less than 50 msec. The records at D21 are averages of five dorsal root-evoked potentials generated at 0.1 Hz.

deviation of the mean (SDM; $n = 3$) to 11.9 mV at D19–D21 (± 3.7 SDM; $n = 5$).

Dorsal root-evoked inhibitory potentials

During embryonic and neonatal development, the compound dorsal root-evoked potentials consisted of short- and long-latency depolarizing potentials. The short-latency responses (< 10 msec) were monosynaptic potentials, while the long-latency potentials (> 10 msec) were polysynaptic potentials (Ziskind-Conhaim, 1990). These synaptic potentials were mediated by both NMDA and non-NMDA receptors.

Because glycine and GABA induced membrane depolarizations in embryonic motoneurons, it is possible that glycine- and GABA-mediated potentials constituted some of the depolariz-

ing potentials evoked by dorsal root stimulation. The finding that membrane depolarization to around -50 mV reversed some of the dorsal root-evoked potentials (Fig. 3) indicates that their reversal potential is between -50 mV and the membrane resting potential (-62 mV; $n = 89$). These, therefore, were not glutamate-mediated potentials. It was difficult to examine the amplitude of hyperpolarizing potentials as a function of membrane depolarization, because it required large depolarizing currents that could not be injected through the high-resistance micro-electrodes.

At D16–D17, dorsal root stimulation produced prolonged (> 150 msec) depolarizing potentials, and some of the potentials reversed after latencies of more than 100 msec (Fig. 3, D16). During membrane depolarization, the average amplitude of dorsal root-evoked hyperpolarizing potentials was 2.9 mV (± 0.6 SDM), compared to 2.1 mV depolarization (± 0.5 SDM; $n = 6$) of the same potentials evoked at resting membrane potential (Fig. 3, arrows). Although some potentials were reversed only after long latencies, a decrease in the amplitude of earlier polysynaptic potentials was also apparent. The duration of dorsal root-evoked potentials decreased during embryonic development, and by D20–D21 reversed potentials with latencies shorter than 50 msec were recorded (Fig. 3, D21). Motoneuron depolarization to -50 mV produced dorsal root-evoked hyperpolarizing potentials with an average amplitude of 1.9 mV (± 0.1 SDM), compared to 0.5 mV (± 0.25 SDM; $n = 7$) for the same potentials recorded at resting membrane potentials. The amplitude of the monosynaptic potentials did not change significantly during motoneuron depolarization (see also Ziskind-Conhaim, 1990).

The polysynaptic potentials were blocked by either APV (20 μ M) or CNQX (10 μ M), antagonists of NMDA and non-NMDA receptors, respectively. These antagonists also blocked the potentials that reversed at -50 mV, suggesting that glutamate-mediated neurons synapse on the interneurons that generate the hyperpolarizing potentials.

To determine whether the reversed potentials were Cl^- dependent, extracellular Cl^- concentration was reduced. Changing

Figure 4. *a*, At all ages, the amplitude of poly- but not monosynaptic potentials (star) was increased in low concentrations of extracellular Cl^- . The records at D20 are averages of five dorsal root-evoked potentials produced at 0.1 Hz. Resting membrane potentials are marked by the dashed lines (see Fig. 3). *b*, Polysynaptic potentials that were reversed at -50 mV in normal recording solution (arrows) could not be reversed at that potential in low concentrations of extracellular Cl^- . Recordings are from the same D16 motoneuron shown in Figure 3.

Figure 5. Strychnine and bicuculline blocked dorsal root-evoked potentials in D17 motoneurons. The effects of strychnine (10 μ M; *a*) and bicuculline (20 μ M; *b*) were reversible.

to a low- Cl^- solution increased the amplitude of the polysynaptic potentials (Fig. 4*a*). Moreover, membrane depolarization to -50 mV failed to reverse these potentials (Fig. 4*b*). These findings suggest that some of the depolarizing potentials were generated by Cl^- current (see Discussion).

Strychnine and bicuculline blocked dorsal root-evoked potentials in D16–D17 motoneurons

The effects of strychnine and bicuculline were studied to determine whether glycine- and GABA-activated synapses produced the Cl^- -dependent potentials. At D16–D17, strychnine (10 μ M) or bicuculline (20 μ M) blocked dorsal root-evoked potentials (Fig. 5; $n = 33$). At these concentrations, the antagonists significantly reduced the amplitude of glycine- and GABA-induced depolarizations (Table 3). This inhibitory action was slow, reaching its maximal effect more than 15 min after the antagonists were added to the solution. Most substances affect motoneurons, which are located laterally in the ventral horn, within 5 min after their application. The effect of strychnine and bicuculline may be slow to develop because of their diffusion to deeper, medial zones of the spinal cord where interneurons may be located. The effect of the antagonists was largely reversible, and dorsal root-evoked potentials were recorded within 20–30 min after the removal of the antagonists. Picrotoxin (100 μ M), a noncompetitive antagonist of GABA_A receptors, was as effective as bicuculline and strychnine in blocking those potentials ($n = 3$).

The inhibitory effects of strychnine and bicuculline are probably not due to a decrease in neuronal excitability, because the antagonists did not increase the threshold for action potential produced by depolarizing current injected intracellularly ($n = 19$). Strychnine and bicuculline did not depress dorsal root-evoked potentials by blocking glutamate receptors, because the antagonists did not block glutamate-induced motoneuron depolarizations ($n = 3$).

It is possible that the inhibitory actions of strychnine and bicuculline were caused by disinhibition of cholinergic synapses. ACh can block monosynaptic potentials by acting on presynaptic muscarinic receptors located on primary afferent fibers (Evans, 1978; Yoshioka et al., 1990; Jiang and Dun, 1991).

However, atropine (5 μ M), an antagonist of muscarinic receptors, did not prevent the inhibition produced by strychnine (Fig. 6; $n = 3$).

Strychnine and bicuculline increased the amplitude of dorsal root-evoked potentials in D19–D21 motoneurons

At late embryonic ages (D19–D21), strychnine and bicuculline significantly increased the amplitude of the polysynaptic potentials, which resulted in high-frequency firing (Fig. 7; $n = 30$). Their potentiating action developed slowly, reaching a maximal effect more than 20 min after their application. The firing rate

Table 3. The specificity of strychnine and bicuculline antagonism did not change during embryonic development

	Membrane depolarization (mV)	
	D16–D18	D19–D21
Glycine ^a	8.6 $\pm$ 3.1 ($n = 6$)	10.4 $\pm$ 4.0 ($n = 7$)
Glycine + strychnine ^b	3.6 $\pm$ 0.8* ($n = 4$)	1.0 $\pm$ 1.3** ($n = 8$)
Glycine + bicuculline ^c	7.5 $\pm$ 4.2 ($n = 7$)	7.6 $\pm$ 2.8 ($n = 11$)
GABA ^d	9.2 $\pm$ 2.3 ($n = 9$)	11.3 $\pm$ 3.5 ($n = 13$)
GABA + strychnine	7.1 $\pm$ 1.7 ($n = 4$)	9.8 $\pm$ 2.7 ($n = 5$)
GABA + bicuculline	3.7 $\pm$ 1.2** ($n = 8$)	2.4 $\pm$ 0.9** ($n = 5$)

At all ages, strychnine significantly reduced glycine- but not GABA-induced depolarizations. Bicuculline, however, was effective in decreasing GABA- but not glycine-mediated depolarizations. Data are presented as mean $\pm$ SEM; n = number of motoneurons.

^a 1 mM glycine at D16–D18, and 2 mM glycine at D19–D21.

^b 20–50 μ M strychnine.

^c 50 μ M bicuculline.

^d 0.5 mM GABA at D16–D18, and 1 mM GABA at D19–D21.

* Significantly smaller than depolarizations produced by glycine ($p < 0.05$).

** Significantly smaller than depolarizations produced by glycine and GABA ($p < 0.01$).

Figure 6. The inhibition induced by strychnine was not mediated via muscarinic receptors. In a D17 motoneuron, atropine (5 μ M) did not prevent the suppression produced by strychnine. The records are averages of five dorsal root-evoked potentials generated at 0.1 Hz.

declined to control levels within 30 min after the removal of the antagonists from the recording solution. Strychnine-induced increases in dorsal root-evoked synaptic potentials were independent of the increases produced by bicuculline. The firing rate generated in the presence of strychnine (after 25 min) was further increased by bicuculline (not shown).

The induction of a prolonged burst of action potentials was not due to an increase in motoneuron excitability. Strychnine and bicuculline had no effect on action potential threshold and input resistances ($n = 29$).

The reversed function of strychnine and bicuculline from inhibitory to excitatory action at D19–D21 did not result from a change in their specific antagonism. At all ages, strychnine significantly reduced the amplitude of glycine- but not GABA-evoked depolarizations. Similarly, bicuculline effectively decreased GABA-mediated but not glycine-induced depolarizations (Table 3).

Distribution of glycine- and GABA-containing cells

In embryonic spinal cords, glycinergic and GABAergic cells were diffusely distributed in various laminae, and numerous immunoreactive fibers were also apparent in the ventral and lateral funiculi (Figs. 8, 9; Table 4). Intensely stained, small cells were located only in laminae I–III of the dorsal horn, and only a few immunoreactive cells were present in the motor nuclei (lamina IX). Immunoreactive cells and fibers were not apparent in sections of spinal cords that were incubated with antisera preabsorbed with glycine- and GABA-bovine serum albumin conjugates.

Between D17 and D19, the number of immunoreactive cells significantly increased (Table 4). The increase in glycinergic cells

was apparent in laminae IV, V, and IX (Fig. 8, Table 4), and more GABAergic cells were counted in laminae I–III and lamina IX (Fig. 9, Table 4). Furthermore, during this period, more glycinergic fibers and GABAergic cells were in close apposition to motoneuron somata (Figs. 8, 9). We cannot rule out the possibility that the number of glycine- and GABA-containing cells did not change, but the concentration of glycine and GABA increased with age making the existing cells more visible.

Discussion

In spinal motoneurons of rat embryos, glycine and GABA produced Cl^- -dependent membrane depolarizations similar to those described in dissociated spinal neurons (Barker and Ransom, 1978; Mandler et al., 1990) and other developing central neurons (reviewed by Cherubini et al., 1991). In spinal motoneurons, glycine and GABA generated membrane depolarizations until at least 1–2 d after birth, but it is not known at what age the agonists began to produce membrane hyperpolarization. In rat CA3 and CA1 hippocampal neurons, the transition from GABA-induced Cl^- -dependent depolarizations to hyperpolarizations occurs at the second week after birth (Ben-Ari et al., 1989; Zhang et al., 1990).

The ionic mechanism underlying glycine- and GABA-induced depolarizations in embryonic motoneurons is unknown. However, because these depolarizations are Cl^- -dependent, they may be produced by an outward Cl^- current that is generated by modified Cl^- concentrations across developing membranes (Andersen et al., 1980). It has been suggested that the internal Cl^- concentration in hippocampal neurons is regulated by inward and outward Cl^- transport mechanisms (Misgeld et al., 1986). The inward pump mechanism can increase intracellular Cl^-

Figure 7. Strychnine and bicuculline increased the amplitude of dorsal root-evoked potentials in D19 and D20 motoneurons. Strychnine (10 μ M) and bicuculline (20 μ M) did not affect the monosynaptic potentials (stars). At least some of the repetitive firing occurred at constant latencies as illustrated by the averaged records (five dorsal root stimuli at 0.1 Hz). The effect of the antagonists was completely (bicuculline) or partially reversible (strychnine).

Figure 8. Light micrographs of transverse sections of D17 (left) and D19 (right) lumbar spinal cords, stained with antiserum to glycine. At both ages, most immunoreactive cells were distributed in laminae V, VI, and VII. Immunoreactive fibers were densely distributed throughout the ventral and lateral funiculi. By D19, the number of glycinergic cells increased in laminae IV, V, and IX (Table 4). At this age, glycinergic fibers surrounded large somata in the motor nuclei (lamina IX). The lower four micrographs are enlargements of dorsal (D) and ventral (V) areas marked by the dotted lines. Laminae II–IV and IX are marked. Ventral is down; lateral is right. Scale bar: 100 μ m for top two micrographs; 60 μ m for bottom four micrographs.

Figure 9. Micrographs of transverse sections of D17 (left) and D19 (right) lumbar spinal cords, stained with antiserum to GABA. By D19, the number of GABAergic cells increased in laminae I–III and IX. At this stage, GABAergic cells were close to large somata in motor nuclei. The bottom four micrographs are enlargements of areas marked by the dotted lines (D and V). Laminae II–IV and IX are marked. Ventral is down; lateral is right at D17 and left at D19. Scale bar: 100 μ m for top two micrographs; 60 μ m for bottom four micrographs.

Table 4. The number of immunoreactive cells in certain laminae increased between D17 and D19

	Glycine		GABA	
	D17	D19	D17	D19
Laminae				
I–III	12.4 ± 0.7 (19.5)	12.6 ± 1.4 (15.8)	40.6 ± 7.6 (23.3)	79.4 ± 2.4* (32.5)
IV	10.6 ± 1.4 (16.5)	17.2 ± 2.0* (21.6)	29.0 ± 6.6 (16.7)	34.6 ± 1.0 (16.7)
V	9.4 ± 1.4 (14.6)	18.8 ± 2.8* (23.5)	26.2 ± 4.4 (15.1)	34.0 ± 3.6 (15.9)
VI	10.6 ± 1.6 (16.8)	12.4 ± 1.2 (15.4)	29.8 ± 5.4 (17.1)	33.2 ± 2.4 (15.5)
VII	17.6 ± 2.8 (27.4)	13.8 ± 1.2 (17.3)	39.2 ± 10.8 (22.5)	30.8 ± 0.6 (14.4)
VIII	1.2 ± 0.6 (1.9)	1.2 ± 0.2 (1.5)	5.6 ± 1.0 (3.2)	3.6 ± 1.0 (1.7)
IX	2.0 ± 0.6 (3.3)	4.0 ± 0.6** (4.9)	3.6 ± 0.6 (2.1)	7.2 ± 1.4** (3.3)
Cells/section	64.0 ± 7.2	80.2 ± 6.0***	174.0 ± 14.2	213.8 ± 8.2**

Data are counts of immunoreactive cells in lumbar spinal cord of D17 and D19 embryos, presented as mean ± SEM; average percentage of immunoreactive cells is given in parentheses.

* Significantly larger than at D17 ($p < 0.01$).

** Significantly larger than at D17 ($p < 0.02$).

*** Significantly larger than at D17 ($p < 0.04$).

concentration and shift Cl^- equilibrium potential to a more positive value. Active accumulation of intracellular Cl^- persists in sensory neurons of adult frog (Alvarez-Leefmans et al., 1988). An alternative explanation for the induced depolarization is that other permeable anions with equilibrium potentials more positive than membrane resting potential, are the carriers of glycine- and GABA-gated currents (Araki et al., 1961; Eccles et al., 1977; Bormann et al., 1987). This has been shown in crayfish muscle fibers, in which an outward HCO_3^- current through GABA-gated channel produces membrane depolarization (Kaila et al., 1989). In these fibers, the increase in intracellular Cl^- concentration during GABA-induced depolarization is due to passive redistribution of Cl^- .

GABA-induced depolarization may be important for neuronal differentiation, because it activates voltage-sensitive Ca^{2+} channels and increases intracellular Ca^{2+} in neonatal but not mature cortical (Yuste and Katz, 1991) and cerebellar neurons (Connor et al., 1987). The elevated intracellular Ca^{2+} can act as a modulator that increases neuronal survival (Collins et al., 1991; reviewed by Miller, 1988).

Our data illustrate that the depolarization, but not the resistance decrease produced by GABA, is significantly larger than that evoked by glycine. It is possible that glycine- and GABA-gated channels are different in their relative ionic conductances to small anions. GABA may produce larger depolarization if its channel is more permeable to HCO_3^- than glycine-gated channel. However, it is unlikely that glycine generated smaller potentials because its receptors are inserted onto membranes of more distal dendrites than GABA receptors. Attenuation due to activation of remote receptors should affect the resistance and potential changes similarly.

It is not clear why, at D16–D17, membrane depolarization reversed some dorsal root-evoked potentials after latencies of more than 100 msec while potentials with shorter latencies were reversed only at later ages. One possibility is that early Cl^- -

activated synapses are located on distal dendrites while later synapses are established more proximal to the soma. Another possibility is that the early inhibitory inputs that synapse on motoneurons are activated by a chain of synapses similar to the cutaneous pathways while pathways with fewer synapses, such as the Ia inhibition, become more prominent later in development (Fig. 10). The conductance velocity of intercostal nerves increases twofold during embryonic development (Ziskind-Conhaim, 1988b), but it is unlikely that such an increase could account for the difference in the latencies.

This is the first study to illustrate that the actions of glycine and GABA antagonists on dorsal root-evoked potentials change during development. The mechanisms underlying strychnine and bicuculline inhibition of dorsal root-evoked potentials are unknown. The simplest explanation is that at the onset of synaptogenesis (D16–D17) most of the synaptic contacts are formed by glycinergic and GABAergic neurons (Fig. 10, D17). Therefore, dorsal root-evoked potentials are Cl^- -dependent and are blocked by strychnine and bicuculline. NMDA-mediated synapses are important for neuronal excitation of such glycinergic and GABAergic pathways, because at this age APV blocks the long-latency polysynaptic potentials (Ziskind-Conhaim, 1990). At D16–D17, short-latency monosynaptic potentials were recorded in 35% of motoneurons. Although those potentials were not Cl^- -dependent, they were also blocked by strychnine and bicuculline. It is possible that early in development of synaptic pathways an inhibitory neurotransmitter other than glycine, GABA, or ACh acts presynaptically to block synaptic transmission in primary afferent fibers. Such an inhibitory pathway, which is generally suppressed by glycinergic and GABAergic synapses, may be unmasked when the inhibitory synapses are blocked.

Excitatory synapses are established on all motoneurons by D19, and the relative strength of the inhibitory and excitatory synapses determines the amplitude of compound dorsal root-

Figure 10. Schema of possible inhibitory and excitatory synapses between dorsal root afferents, interneurons, and motoneurons of D17 (left) and D19 (right) embryos. At D17, strong inhibitory connections are formed on motoneurons (Mn) while excitatory synapses are either weak or are not yet established (D17, dashed line). This explains the inhibitory action of strychnine and bicuculline on dorsal root-evoked potentials. At this age, the reversal of the long-duration potentials after long latencies may be due to innervation of inhibitory neurons by multisynaptic pathways (broken circle, D17). At the onset of synaptogenesis, NMDA-activated synapses (asterisk) play an important role in neuronal excitation. By D19, the duration of dorsal root-evoked potentials is shorter, and some potentials reverse after shorter latencies, probably due to the elimination or weakening of some multisynaptic contacts (D19, broken line). At this age, mono- and polysynaptic excitatory pathways are established, and the amplitude of dorsal root-evoked potentials increases when the inhibitory synapses are blocked. Furthermore, suppressing multiple inhibitory pathways (broken circle, D19) can explain the observed prolonged and rhythmic firing of motoneurons.

evoked potentials (Fig. 10, D19). Although Cl^- -dependent potentials depolarize rather than hyperpolarize motoneurons, their inhibitory action is attributed to the large decrease in input resistance. A decrease of 50% in membrane resistance can probably shunt the membrane to incoming excitatory potentials. Therefore, inhibition of glycine- and GABA-activated synapses increases the amplitude of the depolarizing potentials.

At D19, strychnine and bicuculline produced repetitive firing that occurred at almost constant intervals, suggesting that the disinhibition unmasks neuronal pathways that are effectively blocked by glycine- and GABA-mediated synapses (Fig. 10, D19). Our finding that the number of glycinergic and GABAergic cells increases in the dorsal horn and motor nuclei during the last week *in utero* suggests that new synaptic contacts are formed during this period. In rat embryos, numerous synaptic connections are formed between D15 and D19 of gestation. At D16, dorsal root afferents do not extend into the motoneuron dendritic field and in most motoneurons dorsal root stimulation produces only polysynaptic potentials (Ziskind-Conhaim, 1990). By D18–D19, however, monosynaptic contacts are formed on all motoneurons. It is possible, therefore, that the apparent reversed action of strychnine and bicuculline by D19 is due to reorganization of synaptic contacts.

During the last week *in utero*, the gradual decrease in the duration of dorsal root-evoked potentials may result from weakening or elimination of multisynaptic pathways (Fig. 10, D19). The gradual decrease in motoneuron responses to glycine and GABA may also be related to the rearrangement of synaptic contacts. Elimination of multisynaptic pathways can initiate a decrease in the number of glycine and GABA receptors, resulting in reduced responses to the amino acids. However, other mech-

anisms can contribute to the attenuated responses to glycine and GABA. For example, developmental changes in the molecular structures of glycine and GABA receptors (Akagi and Miledi, 1988; Becker et al., 1988; Hoch et al., 1989) or establishment of inhibitory synapses on more distal dendrites can result in smaller depolarizations. It is unlikely that the decrease in motoneuron responses is due to a shift in Cl^- equilibrium potential, because during the last week *in utero* the change in membrane resistance was also reduced.

Our study shows that during embryonic development, GABA and glycine induce Cl^- -dependent depolarizing potentials. The data suggest that inhibitory synapses are first formed when dorsal root afferents begin to establish synaptic contacts with spinal neurons. At this age, dorsal root stimulation evokes Cl^- -mediated depolarizing potentials that are blocked by strychnine and bicuculline. Strong excitatory synaptic inputs are formed by D19, and disinhibition by strychnine and bicuculline induces large depolarizing synaptic potentials. The changes in the relative contribution of inhibitory inputs to dorsal root-evoked potentials may be the result of both reorganization of neuronal pathways and changes in the location and structure of glycine and GABA receptors.

References

- Akagi H, Miledi R (1988) Heterogeneity of glycine receptors and their messenger RNAs in rat brain and spinal cord. *Science* 242:270–273.
- Alvarez-Leefmans FJ, Gamino SM, Giraldez F, Noguero I (1988) Intracellular chloride regulation in amphibian dorsal root ganglion neurones studied with ion-selective microelectrode. *J Physiol (Lond)* 406:225–246.
- Andersen P, Dingledine R, Gjerstad L, Langmoen IA, Laursen AM (1980) Two different responses of hippocampal pyramidal cells to application of gamma-aminobutyric acid. *J Physiol (Lond)* 305:279–296.
- Aprison MH, Werman R (1965) The distribution of glycine in cat spinal cord and roots. *Life Sci* 4:2075–2083.
- Araki T, Ito M, Oscarsson O (1961) Anion permeability of the synaptic and non-synaptic motoneurone membrane. *J Physiol (Lond)* 159:410–435.
- Barker JL, Ransom BR (1978) Amino acid pharmacology of mammalian central neurones grown in tissue culture. *J Physiol (Lond)* 280:331–354.
- Becker C-M, Hoch W, Betz H (1988) Glycine receptor heterogeneity in rat spinal cord during postnatal development. *EMBO J* 7:3717–3726.
- Ben-Ari Y, Cherubini E, Corradetti R, Gaiarsa J-L (1989) Giant synaptic potentials in immature rat CA3 hippocampal neurones. *J Physiol (Lond)* 416:303–325.
- Berger ST, Carter JG, Lowry OH (1977) The distribution of glycine, GABA, glutamate and aspartate in rabbit spinal cord, cerebellum and hippocampus. *J Neurochem* 28:149–158.
- Bormann J, Hamill OP, Sakmann B (1987) Mechanism of anion permeation through channels gated by glycine and γ -aminobutyric acid in mouse cultured spinal neurones. *J Physiol (Lond)* 385:243–286.
- Burke RE, Rudomin P (1977) Spinal neurons and synapses. In: *Handbook of physiology* (Kandel ER, ed), pp 877–944. Baltimore: Williams and Wilkins.
- Cherubini E, Gaiarsa JL, Ben-Ari Y (1991) GABA: an excitatory transmitter in early postnatal life. *Trends Neurosci* 14:515–519.
- Collins F, Schmidt MF, Guthrie PB, Kater SB (1991) Sustained increase in intracellular calcium promotes neuronal survival. *J Neurosci* 11:2582–2587.
- Connor JA, Tseng H-Y, Hockberger PE (1987) Depolarization- and transmitter-induced changes in intracellular Ca^{2+} of rat cerebellar granule cells in explant culture. *J Neurosci* 7:1384–1400.
- Curtis DR, Johnston GAR (1974) Amino acid transmitters in the mammalian central nervous system. *Rev Physiol* 69:97–188.
- Davidoff RA, Hackman JC (1983) Drugs, chemicals and toxins: their effects on the spinal cord. In: *Handbook of the spinal cord* (Davidoff RA, ed), pp 409–476. New York: Dekker.

- Davidoff RA, Hackman JC (1984) Spinal inhibition. In: Handbook of spinal cord (Davidoff RA, ed), pp 385–459. New York: Dekker.
- Eccles J, Nicoll RA, Oshima T, Rubia FJ (1977) The anionic permeability of the inhibitory postsynaptic membrane of hippocampal pyramidal cells. *Proc R Soc Lond [Biol]* 198:345–361.
- Evans RH (1978) Cholinceptive properties of motoneurons of the immature rat spinal cord maintained *in vitro*. *Neurophysiology* 17:277–279.
- Fulton BP, Miledi R, Takahashi T (1980) Electrical synapses between motoneurons in the spinal cord of newborn rat. *Proc R Soc Lond [Biol]* 208:115–120.
- Grenningloh G, Pribilla I, Prior P, Multhaup G, Beyreuther K, Taleb O, Betz H (1990) Cloning and expression of the 58 kd β subunit of the inhibitory glycine receptor. *Neuron* 4:963–970.
- Hamill OP, Bormann J, Sakmann B (1983) Activation of multiple-conductance state chloride channels in spinal neurones by glycine and GABA. *Nature* 305:805–808.
- Hoch W, Betz H, Becker C-M (1989) Primary cultures of mouse spinal cord express the neonatal isoform of the inhibitory glycine receptor. *Neuron* 3:339–348.
- Hsu SM, Raine L, Fanger H (1981) Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques. *J Histochem Cytochem* 29:577–580.
- Jackson MB, Lecar H, Breneman DE, Fitzgerald S, Nelson PG (1982) Electrical development in spina cord cell culture. *J Neurosci* 2:1052–1061.
- Jahr CE, Yoshioka K (1986) Ia afferent excitation of motoneurons in the *in vitro* new-born rat spinal cord is selectively antagonized by kynurenate. *J Physiol (Lond)* 370:515–530.
- Jiang ZG, Dun NJ (1991) Presynaptic suppression of excitatory postsynaptic potentials in rat ventral horn neurons by muscarinic agonists. *Brain Res* 381:182–186.
- Kaila K, Pasternack M, Saarikoski J, Voipio J (1989) Influence of GABA-gated bicarbonate conductance on potential, current and intracellular chloride in crayfish muscle fibres. *J Physiol (Lond)* 416:161–181.
- Krnjević K, Puil E, Werman R (1977) GABA and glycine actions on spinal motoneurons. *Can J Physiol Pharmacol* 55:658–669.
- Kudo N, Yamada T (1987) Morphological and physiological studies of development of the monosynaptic reflex pathway in the rat lumbar spinal cord. *J Physiol (Lond)* 389:441–459.
- Mandler RN, Schaffner AE, Novotny EA, Lange GD, Smith SV, Barker JL (1990) Electrical and chemical excitability appear one week before birth in the embryonic rat spinal cord. *Brain Res* 522:46–54.
- Miller RJ (1988) Calcium signalling in neurons. *Trends Neurosci* 11:415–419.
- Misgeld U, Deisz RA, Dodt HU, Lux HD (1986) The role of chloride transport in postsynaptic inhibition of hippocampal neurons. *Science* 232:1413–1415.
- Miyata Y, Nakano S, Yasuda H (1987) Postnatal transient expression of long-lasting descending inhibitory effect on spinal reflexes in the rat. *Neurosci Res* 4:268–278.
- Nelson PG, Ransom BR, Henkart M, Bullock PN (1977) Mouse spinal cord in cell culture. IV: Modulation of inhibitory synaptic function. *J Neurophysiol* 40:1178–1187.
- Nelson PG, Fields RD, Yu C, Neale EA (1990) Mechanisms involved in activity-dependent synapse formation in mammalian central nervous system cell cultures. *J Neurobiol* 21:138–156.
- Obata K, Oide M, Tanaka H (1978) Excitatory and inhibitory actions of GABA and glycine on embryonic chick spinal neurons in culture. *Brain Res* 144:179–184.
- O'Donovan MJ (1989) Motor activity in the isolated spinal cord of the chick embryo: synaptic drive and firing pattern of single motoneurons. *J Neurosci* 9:943–958.
- Pritchett DB, Sontheimer H, Shivers BD, Ymer S, Kettenman H, Schofield PR, Seeburg PH (1989) Importance of a novel GABA_A receptor subunit for benzodiazepine pharmacology. *Nature* 338:582–585.
- Rudomin P (1990) Presynaptic inhibition of muscle spindle and tendon organ afferents in the mammalian spinal cord. *Trends Neurosci* 13:499–505.
- Saito K (1979) Development of spinal reflexes in the rat fetus studied *in vitro*. *J Physiol (Lond)* 294:581–594.
- Seno N, Saito K (1985) The development of the dorsal root potential and the responsiveness of primary afferent fibers to γ -aminobutyric acid in the spinal cord of rat fetuses. *Dev Brain Res* 17:11–16.
- Takahashi T (1984) Inhibitory miniature synaptic potentials in rat motoneurons. *Proc R Soc Lond [Biol]* 221:103–109.
- Todd AJ, Sullivan AC (1990) Light microscope study of the coexistence of GABA-like and glycine-like immunoreactives in the spinal cord of the rat. *J Comp Neurol* 296:496–505.
- Velumian AA (1984) Direct evidence for postsynaptic inhibition in the embryonic chick spinal cord. *Dev Brain Res* 14:229–239.
- Wentholt RJ, Huie D, Altschuler RA, Reeks KA (1987) Glycine immunoreactivity localized in the cochlear nucleus and superior olivary complex. *Neuroscience* 22:897–912.
- Werman R, Davidoff RA, Aprison MH (1968) Inhibitory action of glycine on spinal neurons in the cat. *J Neurophysiol* 31:81–95.
- Wu W-L, Ziskind-Conhaim L (1991) Age-dependent effects of bicuculline and strychnine on dorsal root-evoked potentials. *Soc Neurosci Abstr* 17:88.
- Yoshioka K, Sakuma M, Otsuka M (1990) Cutaneous nerve-evoked cholinergic inhibition of monosynaptic reflex in the neonatal rat spinal cord: involvement of M₂ receptors and tachykinergic primary afferents. *Neuroscience* 38:195–203.
- Young AB, MacDonald RL (1983) Glycine as a spinal cord neurotransmitter. In: Handbook of the spinal cord (Davidoff RA, ed), pp 1–43. New York: Dekker.
- Yuste R, Katz LC (1991) Control of postsynaptic Ca²⁺ influx in developing neocortex by excitatory and inhibitory neurotransmitters. *Neuron* 6:333–344.
- Zhang L, Spigelman I, Carlen PL (1990) Whole-cell patch study of GABAergic inhibition in CA1 neurons of immature rat hippocampal slices. *Dev Brain Res* 56:127–130.
- Ziskind-Conhaim L (1988a) Electrical properties of motoneurons in the spinal cord of rat embryos. *Dev Biol* 128:21–29.
- Ziskind-Conhaim L (1988b) Physiological and morphological changes in developing peripheral nerves of rat embryos. *Dev Brain Res* 42:15–28.
- Ziskind-Conhaim L (1990) NMDA receptors mediate poly- and monosynaptic potentials in motoneurons of rat embryos. *J Neurosci* 10:125–135.