Inhibitory Postsynaptic Potentials in Lumbar Motoneurons Remain Depolarizing After Neonatal Spinal Cord Transection in the Rat

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Jean-Xavier, Céline, Jean-François Pfieger, Sylvie Liabeuf, and Laurent Vinay. Inhibitory postsynaptic potentials in lumbar motoneurons remain depolarizing after neonatal spinal cord transection in the rat. J Neurophysiol 96: 2274–2281, 2006. First published June 28, 2006; doi:10.1152/jn.00328.2006. GABA and glycine are excitatory in the immature spinal cord and become inhibitory during development. The shift from depolarizing to hyperpolarizing inhibitory postsynaptic potentials (IPSPs) occurs during the perinatal period in the rat, a time window during which the projections from the brain stem reach the lumbar enlargement. In this study, we investigated the effects of suppressing influences of the brain on lumbar motoneurons during this critical period for the negative shift of the reversal potential of IPSPs (EIPSP). The spinal cord was transected at the thoracic level on the day of birth [postnatal day 0 (P0)]. EIPSP, at P4–P7, was significantly more depolarized in cord-transected than in cord-intact animals (EIPSP above and below resting potential, respectively). EIPSP at P4–P7 in cord-transected animals was close to EIPSP at P0–P2. K-Cl cotransporter KCC2 immunohistochemistry revealed a developmental increase of staining in the area of lumbar motoneurons between P0 and P7 in cord-intact animals; this increase was not observed after spinal cord transection. The motoneurons recorded from cord-transected animals were less sensitive to the experimental manipulations aimed at testing the functionality of the KCC2 system, which is sensitive to [K+]i, and blocked by bumetanide. Although bumetanide significantly depolarized EIPSP, the shift was less pronounced than in cord-intact animals. In addition, a reduction of [K+]i decreases, leading to a shift of the chloride equilibrium potential toward further negative values and thereby to a change in glycine and GABA-evoked potentials from depolarization to hyperpolarization (Gao and Ziskind-Conhaim 1995; Takahashi 1984). Up-regulation of a transporter that regulates [Cl−], the neuron specific K-Cl cotransporter, KCC2, underlies this shift (Delpire and Mount 2002; Payne et al. 2003; Rivera et al. 1999, 2004a; Stein et al. 2004). Cortical neurons lacking KCC2 expression fail to show a developmental decrease in [Cl−], (Zhu et al. 2005). KCC2 knockout mice exhibit severe motor deficits and an inability to breathe, which results in perinatal death (Hübner et al. 2001). The upstream mechanisms responsible for the developmental up-regulation of KCC2 are unknown. There is a need to understand these mechanisms because inhibitory amino acids become excitatory again, and KCC2 is down-regulated in some pathological conditions (i.e., neuronal damage, peripheral nerve-induced chronic pain, human temporal lobe epilepsy; Cohen et al. 2002; Coull et al. 2003; Nabekura et al. 2002; Payne et al. 2003; Toyoda et al. 2003). This down-regulation of KCC2 may be a general early response involved in various kinds of neuronal trauma (Rivera et al. 2004b) and seems to reflect a recapitulation of early developmental mechanisms (Rivera et al. 2004a). Counteracting or preventing the down-regulation of KCC2 may be useful in the treatment of these pathological states.

Maturation of inhibitory synaptic transmission in the rat spinal cord occurs during the perinatal period (Gao and Ziskind-Conhaim 1995; Wu et al. 1992), in a time window during which the projections from the brain stem reach the lumbar enlargement (Brocard et al. 1999; Lakke 1997; Vinay et al. 2002). This raises the question of the influence of the brain on the maturation of spinal cord inhibition. In this study, we investigated the effects of a complete spinal cord transection, made at birth (Norreel et al. 2003), on IPSPs recorded in vitro from lumbar motoneurons at the end of the first postnatal week (4th to 7th days). We report that the typical developmental shift from depolarizing to hyperpolarizing IPSPs does not occur in the absence of descending modulatory inputs. Preliminary results have been presented in abstract form (Jean-Xavier et al. 2004).

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INTRODUCTION

Network operation in the CNS relies on the proper balance between excitatory and inhibitory drives. Glycine and GABA are the major inhibitory transmitters in the adult mammalian spinal cord. The activation of GABA_A- and glycine-receptor–gated chloride ([Cl−]) channels results in an inward flux of Cl− and membrane potential hyperpolarization. Therefore the inhibitory action of glycine and GABA consists in both shunting and membrane potential hyperpolarization. Therefore the inhibitory postsynaptic potentials (IPSPs) occurs during the perinatal period in the rat, a time window during which the projections from the brain stem reach the lumbar enlargement. In this study, we investigated the effects of suppressing influences of the brain on lumbar motoneurons during this critical period for the negative shift of the reversal potential of IPSPs (EIPSP). The spinal cord was transected at the thoracic level on the day of birth [postnatal day 0 (P0)]. EIPSP, at P4–P7, was significantly more depolarized in cord-transected than in cord-intact animals (EIPSP above and below resting potential, respectively). EIPSP at P4–P7 in cord-transected animals was close to EIPSP at P0–P2. K-Cl cotransporter KCC2 immunohistochemistry revealed a developmental increase of staining in the area of lumbar motoneurons between P0 and P7 in cord-intact animals; this increase was not observed after spinal cord transection. The motoneurons recorded from cord-transected animals were less sensitive to the experimental manipulations aimed at testing the functionality of the KCC2 system, which is sensitive to [K+]i, and blocked by bumetanide. Although bumetanide significantly depolarized EIPSP, the shift was less pronounced than in cord-intact animals. In addition, a reduction of [K+]i decreases, leading to a shift of the chloride equilibrium potential toward further negative values and thereby to a change in glycine and GABA-evoked potentials from depolarization to hyperpolarization (Gao and Ziskind-Conhaim 1995; Takahashi 1984). Up-regulation of a transporter that regulates [Cl−], the neuron specific K-Cl cotransporter, KCC2, underlies this shift (Delpire and Mount 2002; Payne et al. 2003; Rivera et al. 1999, 2004a; Stein et al. 2004). Cortical neurons lacking KCC2 expression fail to show a developmental decrease in [Cl−], (Zhu et al. 2005). KCC2 knockout mice exhibit severe motor deficits and an inability to breathe, which results in perinatal death (Hübner et al. 2001). The upstream mechanisms responsible for the developmental up-regulation of KCC2 are unknown. There is a need to understand these mechanisms because inhibitory amino acids become excitatory again, and KCC2 is down-regulated in some pathological conditions (i.e., neuronal damage, peripheral nerve-induced chronic pain, human temporal lobe epilepsy; Cohen et al. 2002; Coull et al. 2003; Nabekura et al. 2002; Payne et al. 2003; Toyoda et al. 2003). This down-regulation of KCC2 may be a general early response involved in various kinds of neuronal trauma (Rivera et al. 2004b) and seems to reflect a recapitulation of early developmental mechanisms (Rivera et al. 2004a). Counteracting or preventing the down-regulation of KCC2 may be useful in the treatment of these pathological states.

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The spinal cord was transected at the thoracic (T) T8–T10 level, and et al. 2003). Briefly, rats were deeply anesthetized by hypothermia. Interventions were performed similar to those described previously (Norreel et al. 2005). Intracellular recordings were made to minimize animal suffering and conformed to the guidelines from the French Ministry for Agriculture and Fisheries, Division of Animal Rights.

Neonatal spinal cord transection and preparation of in vitro experiments were performed similar to those described previously (Norreel et al. 2003). Briefly, rats were deeply anesthetized by hypothermia. The spinal cord was transected at the thoracic (T) T8–T10 level, and one to two segments of the cord were removed. The completeness of spinal cord transection was verified postmortem by visual inspection of lack of continuity between the spinal stumps. For in vitro experiments, the spinal cord and ventral roots were removed from sacral segments up to T8–T10. The preparation was pinned down, ventral side up, in the recording chamber. All dissection and recording procedures were performed under continuous perfusion with saline solution (in mM: 130 NaCl, 4 KCl, 3.75 CaCl2, 1.3 MgSO4, 0.58 NaH2PO4, 25 NaHCO3, and 10 glucose; oxygenated with 95% O2–5% CO2; pH = 7.4, temperature of 24–27°C). In CO2/HCO3-, free solution (oxygenated with 100% O2), NaHCO3 was replaced by HEPES (25 mM). The external concentration of K+ ([K+]o) was decreased from 4 to 2 mM in some experiments; the NaCl concentration was raised to 132 mM to keep the Cl− concentration unchanged. All compounds were purchased from Sigma (St. Louis, MO).

Ventral roots were usually dissected into two rootlets. Monopolar stainless steel electrodes were placed in contact with the roots and insulated with petroleum jelly for stimulation. Glass suction electrodes were used to stimulate the ventral funiculus at the L2–L3 level, on the recording side. After the pia had been removed, lumbar motoneurons were recorded intracellularly using glass microelectrodes filled with 2 M K-acetate (90–150 MΩ). Intracellular potentials were recorded in the discontinuous current-clamp (DCC) mode (Axoclamp 2B amplifier; Digidata 1200 interface, pClamp8 software, Axon Instruments). Only neurons exhibiting a stable (>15 min) resting membrane potential were considered for analysis. Motoneurons [n = 142 in control (n = 69), sham-operated (n = 11), and cord-transected animals (n = 65)] in the L2–L4 segments were identified by the antidromic response to stimulation of one of the ventral rootlets. Stimulation of either the other rootlets or the ventral funiculus usually induced IPSPs in the presence of 2-amino-5-phosphonovaleric acid (AP5, 30–100 μM) and 6-cyano-7-nitroquin-oxaline-2,3-dione (CNQX, 3–10 μM). IPSPs were recorded at various holding potentials (500 ms-long current pulses). The input resistance of motoneurons was measured by injecting moderate (0.2–0.5 nA) hyperpolarizing current pulses. Amplitudes of IPSPs were measured and plotted against holding potentials. At least 22 values were collected for each motoneuron. The EIPSP was given by the intercept of the regression line with the x-axis (Graphpad Prism 4 Software). Stimulation of the ventral funiculus was used to determine EIPSP in most of the cells (76%). When the two inputs were tested in individual neurons, EIPSP was similar (P > 0.05, paired t-test) for the two inputs and the average was considered for subsequent statistical analysis. Because EIPSP, VREST, and input resistance were very similar in control and sham-operated animals at P4–P7 (Table 1), we pooled the data (cord-intact animals) for statistical comparison with cord-transected animals of the same age and controls at P0–P2.

Results are presented in the form of means ± SE. The statistical tests used are given in the text and figure legends.

**KCC2 immunohistochemistry**

Three animal groups were used: cord-intact (n = 6) and cord-transected (n = 5) animals at P7 and neonates (P0, n = 3). After fixation in 4% paraformaldehyde, the lumbar spinal cords were embedded in 3% agarose. Each experiment was conducted on one cord-intact and one cord-transected animals from the same litter. Cords were cut, transversally, at 30 μm on a vibratome (Leica VT1000S). Sections were preincubated with normal goat serum (1% PBS +3% BSA; Sigma) and Triton-X (0.2%). They were incubated overnight with an affinity-purified rabbit anti-KCC2 polyclonal antibody (diluted 1:200; Upstate Biotech, Lake Placid, NY; Fiumelli et al. 2005; Grob and Mouginot 2005; Lohrke et al. 2005; Vale et al. 2003, 2005) followed by goat anti-rabbit IgG coupled to Alexa-546 (diluted 1:400; Molecular Probes). Sections from different animal groups (cord-intact vs. cord-transected animals at P7 and cord-intact P7 vs. neonates) were processed simultaneously and mounted on the same slides. Sections were observed on a confocal microscope (Olympus). To quantify the immunohistochemical pattern, intensity measures were acquired (FluoView Software). Two regions were delineated in the pools of motoneurons (Fig. 2A, nm) in the ventral horn and white matter (Fig. 2A, wm) in the L4 segment for analysis. The same areas were considered in successive sections (n = 145). The mean areas of the mm and wm regions were 35,682 ± 523 and 81,899 ± 1,546 μm2, respectively. The ratio of mean pixel intensities in the gray and white matter (nm/wm) was calculated for each section.

**RESULTS**

**IPSPs remain depolarizing after neonatal spinal cord transection**

IPSPs were elicited by electrical stimulation of either ventral roots (Renshaw cell-mediated recurrent inhibition; Mentis et al. 2005; Nishimaru et al. 2005) or the ventral funiculus of the

| Table 1. VREST, EIPSP, Rin, and EIPSP−VREST for motoneurons recorded at different postnatal (P) ages in control, sham-operated, and cord-transected animals (HCO3−-containing saline when not specified and HCO3−-free saline for HEPES) |
| P4-7 | Control (25) | −72.36 ± 0.82 | −74.50 ± 0.93 | 17.15 ± 1.72 | −2.14 ± 1.05 |
| Sham (11) | −74.36 ± 0.93 | −74.04 ± 1.27 | 15.74 ± 1.68 | 0.32 ± 0.98 |
| Spinal (31) | −71.62 ± 0.65 | −67.19 ± 1.54 | 17.10 ± 1.45 | 4.52 ± 1.39 |
| Spinal HCO3−-free (7) | −70.14 ± 1.30 | −67.12 ± 4.19 | 17.52 ± 1.24 | 2.89 ± 3.47 |
| P0-2 | Control (15) | −71.96 ± 1.25 | −69.12 ± 1.61 | 26.12 ± 2.56 | 2.83 ± 1.22 |

Values are means ± SE. Number of motoneurons is shown in parentheses. VREST, resting membrane potential; EIPSP, reversal potential of IPSP; Rin, input resistance; EIPSP−VREST, driving force.
spinal cord, in the presence of the glutamate receptor antagonists CNQX and AP5. These IPSPs are mediated by glycine and GABA_A receptors (Marchetti et al. 2002; Pinco and Lev-Tov 1994; Schneider and Yfffe 1992). Varying membrane potential by injection of depolarizing or hyperpolarizing currents altered the amplitude of IPSPs (Fig. 1, A and B). The reversal potential of IPSPs (E_IPSP) was significantly more depolarized (+7 mV on average) in cord-transected than in cord-intact animals (Table 1; Fig. 1, A–C). Synaptic responses were abolished by picrotoxin (10–20 μM) and strychnine (2–4 μM), showing that they were still mediated by GABA_A and glycine receptors after spinal cord transection (n = 3; data not shown). Resting membrane potential (V_REST) and input resistance were not significantly affected by spinal cord transection (Table 1). As a result, E_IPSP was, on average, below and above V_REST in animals with intact and transected spinal cords, respectively (Fig. 1, A, B, and D). In cord-intact animals, motoneurons exhibiting hyperpolarizing IPSPs at V_REST were more numerous than those with depolarizing responses (Fig. 1E, left, column). These proportions were reversed in cord-transected animals (Fig. 1E, right, column). When IPSPs were hyperpolarizing at V_REST, E_IPSP was significantly more negative in cord-intact than in cord-transected animals (Fig. 1F, lower part of columns). Conversely, E_IPSP, when above V_REST, was more depolarized in cord-transected than in cord-intact animals (Fig. 1F, upper part of columns).

To determine the effect of the transection on the developmental switch from depolarizing to hyperpolarizing IPSPs, we measured E_IPSP early after birth (P0–P2, control animals). E_IPSP at P0–P2 was above V_REST (Table 1; Fig. 1D). It was close to E_IPSP measured at P4–P7 in cord-transected animals and significantly more negative than in cord-intact animals at P4–P7 (Fig. 1C). Altogether, these results suggest that the spinal cord transection at birth stopped the developmental shift of E_IPSP toward further hyperpolarizing values.

**Outward Cl^− transport system is less efficient in cord-transected animals**

Efflux of HCO_3^− through the anion pore may contribute to the depolarizing IPSPs (Kulik et al. 2000). To study this issue, we recorded IPSPs in cord-transected animals after CO_2/ HCO_3^− had been removed from the superfusate. E_IPSP was similar in HCO_3^−-free (HEPES) and HCO_3^−-containing salines (Table 1; P > 0.05, t-test), indicating that HCO_3^− does not contribute to the depolarizing IPSPs after the transection. These experiments suggest that chloride homeostasis was affected in cord-transected animals.

Because the developmental negative shift in E_IPSP is attributable to an increased efficacy of Cl^− extrusion and the up-regulation of KCC2 (Hummer et al. 2001; Lu et al. 1999), we studied the presence and functionality of this transporter in cord-transected and cord-intact animals at P7. Anti-KCC2 antibody was used to localize the KCC2 cotransporter in the lumbar spinal cord. Specificity of the antibody was confirmed by lack of signal when the secondary antibody was applied in the absence of primary antibody (data not shown). KCC2 staining in cord-intact animals was abundant in the ventral horn (vh). A dense matrix of processes was heavily stained around large cell bodies, presumably motoneurons (mn, Fig. 2, A and C). Some labeled neurites stretched out of the gray matter in all directions into the lateral and ventral funiculi. These labeled neurites in both the gray and white matters are likely dendrites because KCC2 is not expressed in axons (Hummer et al. 2001). In addition, dendrites are known to temporarily extend into the white matter in neonates (Curfs et al. 1993). At higher magnification, KCC2 immunolabeling clustered around large cell bodies, suggesting a preferential plasma membrane localization (Fig. 2C, inset), although this issue cannot be clarified with certainty at this resolution level. In cord-
FIG. 2. Differences in KCC2 immunolabeling during normal development and after spinal cord transection. A and B: confocal images showing immunohistochemical staining with anti-KCC2 antibody in the transverse hemi-sections of lumbar L4 segments of a cord-intact (A) and a cord-transected (B) animals at P7. Six optical sections (5 μm) are superimposed. cc, central canal; mn, area of motoneuron cell bodies; wm, white matter; dh, dorsal horn; vh, ventral horn. Areas considered for analysis are delineated in A. C–E: 10 optical sections (1 μm) are superimposed to show KCC2 staining at higher magnification in the ventral horn of P7 cord-intact (C) and cord-transected (D) animals and neonates (P0, E). Insets: confocal images of 4 (C), 1 (D), and 2 (E) optical sections (0.5 μm) superimposed showing KCC2 staining at the cellular level. Note intracellular staining in large cell bodies, likely motoneurons, and processes (*) in the ventral horn of cord-transected animals and neonates. Arrowheads point to unlabeled nucleoli of large cells. Scale bars: 200 (A and B), 100 (C–E), and 20 μm (insets). F: ratio (means ± SE) of mean pixel intensities in gray and white matter (mn/wm) at P0 and P7 in cord-intact (white columns) and cord-transected (gray columns) animals. **P < 0.01; ***P < 0.001; 1-way ANOVA with Tukey post-test. Number of sections analyzed in each group indicated in columns.
transected animals, these clusters of stained spots were not present around large cells (Fig. 2D, inset), and diffuse cytoplasmic staining was commonly observed in somas around unlabeled nuclei and in processes (Fig. 2D, inset, arrowheads and asterisk, respectively). A similar KCC2 immunolabeling was present at P0 in cord-intact animals (Fig. 2E).

At low magnification, a lower level of KCC2 immunoreactivity was observed in the ventral horn at P7 after spinal cord transection compared with cord-intact animals of the same age (cf. Fig. 2, A and B). The immunohistochemical data were quantified in two areas of the ventral horn and white matter in the L4 segment (Fig. 2A). The ratio of mean pixel intensities in the gray and white matter (mn/wm) was significantly larger at P7 in cord-intact animals (Fig. 2F) than in both cord-transected animals of the same age and neonates, indicating that the relative KCC2 targeting to the somatic and dendritic compartments changed with age and that this development was affected by neonatal spinal cord transection. Interestingly, P7 cord-transected and P0 animals, which exhibit similar $E_{\text{IPSP}}$ values (Fig. 1C) have similar ratios. The larger ratio in cord-intact animals at P7 was caused, at least partly, by the clusters of KCC2 labeling surrounding motoneurons (Fig. 2C). These results suggest that the developmental upregulation of KCC2 staining in the ventral horn was affected by neonatal spinal cord transection.

We tested the functionality of KCC2 cotransporters at P4–P7 by either applying the loop diuretic bumetanide or decreasing [K+]o. Bumetanide blocks both KCC2 and the Na-K-2Cl cotransporter, NKCC1, which mediates Cl− uptake (Payne et al., 2003; Yamada et al., 2004). Because of the lack of data on the effect of bumetanide on the whole spinal cord in vitro, we tested the effect of bath applications (10–20 min) of bumetanide at different concentrations (100, 250, and 500 μM) on motoneurons recorded from control animals at P4–P7 (Fig. 3A). Applications of bumetanide always induced a depolarizing shift of $E_{\text{IPSP}}$ (+0.84 ± 0.35 mV, $n = 5$ at 100 μM; +4.16 ± 2.33 mV, $n = 6$ at 250 μM; +11.78 ± 2.50 mV, $n = 9$ at 500 μM); this effect was significant only for the highest concentration tested. Effects were reversible (Fig. 3A). Bumetanide was then tested on cord-transected animals at this concentration of 500 μM, shown to be efficient to block the Cl− transport system in cord-intact animals. Bumetanide depolarized $E_{\text{IPSP}}$ significantly (Fig. 3B, left histogram), the shift was, however, smaller than in cord-intact animals (Fig. 3B, right histogram).

Bumetanide has a greater affinity for NKCC than for KCC (Payne et al., 2003) and is usually used at concentrations <100 μM to be specific for NKCC1. Bumetanide, at 50 μM, had no significant effect on $E_{\text{IPSP}}$ in cord-transected animals (Fig. 3C). If the regulation of neuronal [Cl−]i in cord-transected animals is dominated by the NKCC1 cotransporters, application of bumetanide should cause a hyperpolarizing shift of $E_{\text{IPSP}}$. However, this loop diuretic always depolarized $E_{\text{IPSP}}$, whatever the concentration used in this study. Therefore, the inward Cl− transport system, NKCC1, does not appear to play a significant role in setting $E_{\text{IPSP}}$ at more depolarized values after spinal cord transection.

KCC2 is sensitive to $[\text{K}^+]_o$; the direction and the driving force of the K-Cl cotransporter result from the difference in the transmembrane ionic gradient of both K+ and Cl− (Alvarez-Leefmans, 2001; Payne, 1997; Thompson and Gahwiler, 1989; Woodin et al., 2003). Thus [Cl−]i is affected by changing $[\text{K}^+]_o$ (Woodin et al., 2003; Zhang et al., 1991). In cord-intact animals, decreasing $[\text{K}^+]_o$ from 4 to 2 mM caused a rapid negative shift of the $E_{\text{IPSP}}$ in motoneurons (Fig. 3D); effects were reversible. The negative shift of $E_{\text{IPSP}}$ was significant for motoneurons recorded from cord-intact animals but not for those recorded after spinal cord transection (Fig. 3D). Note that neither $V_{\text{REST}}$ nor the slope of IPSPs was significantly affected by reducing $[\text{K}^+]_o$ ($P > 0.05$; Table 2). The Cl− extrusion mechanisms therefore seem to be less efficient in cord-transected than in cord-intact animals.

**DISCUSSION**

These findings suggest that a neonatal spinal cord transection, thereby removing the influences of the brain on the lumbar spinal cord, prevents—or at least delays—the typical ontogenetic switch from depolarizing to hyperpolarizing IPSPs...
that occurs during postnatal development. This switch relies on
the decrease in [Cl\(^-\)]i, (Ehrlich et al. 1999; Kakazu et al. 1999; Owens et al. 1996; Rivera et al. 1999).

The down-regulation of the inward-directed Cl\(^-\) pumps or
vice versa, the increased efficacy of the Cl\(^-\) extruding system can,
in theory, contribute to the age-related reversal of trans-
membrane Cl\(^-\) gradients. NKCC1 has been proposed to be
important in active accumulation of intracellular Cl\(^-\) in immature
neurons (Dzhala et al. 2005; Ikeda et al. 2003; Plotkin et al.
1997; Sun and Murali 1999; Vardi et al. 2000). However,
the time window of NKCC1 expression does not match that of
depolarizing effects in all neuronal systems (Balakrishnan et al.
2003; Clayton et al. 1998; Yan et al. 2001). This is the case of
the spinal cord, in which NKCC1 transcripts are detected at
E12 but have almost disappeared at E18.5 (Hübner et al. 2001)
and are absent at P0 (Balakrishnan et al. 2003) at a time when
the Cl\(^-\) reversal potential is above \(V_{\text{REST}}\), as shown in our
experiments. Therefore NKCC1 may not be the major trans-
porter leading to GABA- and glycine-evoked depolarizations
in neonatal motoneurons, and its down-regulation does not
provide the molecular basis of the ontogenetic switch from
depolarizing to hyperpolarizing IPSPs in motoneurons (Hübner
et al. 2001). This is supported by our experiments using bumetanide, which blocks both NKCC1 and KCC2 cotrans-
porters: the relatively high concentration of bumetanide re-
quired to affect \(E_{\text{IPSP}}\) and the net depolarizing effect of this
loop diuretic on \(E_{\text{IPSP}}\), even at the lowest concentrations used,
suggest that KCC2 are responsible for the setting of neuronal
[Cl\(^-\)]i, in both cord-intact and cord-transected animals at the end
of the first postnatal week. However, our experiments do
not rule out the possibility that an inward-directed Cl\(^-\) system
is functional after neonatal spinal cord transection. This issue
will require further study when the nature of this system in the
spinal cord becomes clear.

In contrast to NKCC1, changes—increases in this case—in
KCC2 levels have been associated with the developmental
transition from GABA/glycine-induced depolarization to hy-
perpolarization in several regions of the CNS including the
spinal cord so that it is widely accepted that KCC2 is an
“indicator of neuronal maturation” (Li et al. 2002; Lu et al.
1999; Mikawa et al. 2002; Rivera et al. 1999, 2004a; Shibata
mRNA expression can be detected in the ventral part of the
rostral spinal cord as early as E12.5 in rodent embryos (Hübner
et al. 2001; Li et al. 2002). KCC2 protein levels increase until
P3–P7 (Stein et al. 2004). In addition to the up-regulation of
KCC2 gene expression or protein synthesis, posttranslational
modifications occur that affect the functionality of the trans-
porter (Kelsch et al. 2001; Stein et al. 2004). An important
event in the development of the chloride-extruding system is
the translocation of the KCC2 protein from intracellular com-
partments to the plasma membrane at a position of an active
cotransporter (Balakrishnan et al. 2003). There are, in addition,
posttranslational modifications that activate an initially inac-
tive form of the protein (Kelsch et al. 2001; Vale et al. 2003
2005). In our experiments, the overall KCC2 staining in the
ventral horn—where motoneuron cell bodies are located—was
faunter after cord transection compared with cord-intact ani-
mals of the same age. The comparison with P0 animals sug-
gests that the developmental upregulation of KCC2 in the
somatic—relative to dendritic—compartment that occurs dur-
ing the first postnatal week has been prevented by neonatal
spinal cord transection (Fig. 2F). Some cytoplasmic staining
was clearly observed in cell bodies from cord-transected ani-
mals, whereas the labeling in cord-intact rats seemed to be
associated with the plasma membrane. Both the qualitative
and quantitative differences in KCC2 immunolabeling between
the three animal groups strongly support the observation that \(E_{\text{IPSP}}\)
was more depolarized in neonates and at the end of the first
postnatal week after spinal cord transection.

The motoneurons recorded from cord-transected animals
were less sensitive to the two experimental manipulations
aimed at testing the functionality of the KCC2 system. Al-
though bumetanide significantly depolarized \(E_{\text{IPSP}}\), the shift
was less pronounced than in cord-intact animals. In addition,
a reduction of \([K^+]_o\) affected \(E_{\text{IPSP}}\) significantly only in controls
(Fig. 3). These results suggest a lower efficacy of the cotrans-
port mechanisms in cord-transected animals, caused likely by
fewer KCC2 transporters being present in the plasma mem-
brane, as indicated by immunohistochemistry, and possibly
an alteration of the functional status. Further experiments will be
required to compare the regulation of KCC2 protein by phos-
phorylation/dephosphorylation events in cord-intact and cord-
transected animals (Kelsch et al. 2001; Vale et al. 2003, 2005).

Afferen t activity is important for the ontogenetic switch
from depolarizing to hyperpolarizing glycine responses in the
auditory brain stem (Shibata et al. 2004). The possibility that
inputs from the brain stem and the neurotransmitters they
contain (serotonin, noradrenaline, . . .) may play a similar
critical role in the overall maturation of spinal cord inhibitory
synaptic transmission, for instance, by up-regulating KCC2
and enabling it to become functional, is a hypothesis that
deserves to be tested further. The first supraspinal projections
reach the lumbar enlargement before birth (E17; Vinay et al.
2000, 2002), when \(E_{\text{IPSP}}\) is around \(-50/-55\) mV (Wu et al.
1992). The number of descending axons reaching the lumbar
cord increases gradually until the end of the second postnatal
week, when inhibitory synaptic transmission is fully mature.
Whether the effect of descending pathways is direct, on intra-
acellular events leading to the upregulation of KCC2, or indirect,
through a modulation of lumbar network activity, is unknown.

What are the functional consequences of an \(\sim 10\)-mV posi-
tive shift of \(E_{\text{IPSP}}\) in cord-transected animals? Several studies
performed on various regions of the CNS (Chen et al. 1996;
Gao and van den Pol 2001; Guledde and Stuart 2003; Stein and Nicoll 2003), including the spinal cord (Jean-Xavier et al. 2005) have shown that small depolarizing IPSPs ($E_{\text{IPSP}}$ 5–10 mV above $V_{\text{REST}}$) can, depending on timing and location, promote action potential firing in response to subthreshold excitatory inputs. In addition, a shift of $E_{\text{IPSP}}$ toward more depolarized values causes a reduction of the inhibitory synaptic strength (Vale et al. 2003). The basic rhythmic activity underlying locomotion is generated by spinal cord interneuronal networks, termed central pattern generators (CPGs; Grillner and Wallén 1985). The alternation of muscle activities between the two hindlimbs relies on mutual glycinergic inhibition of the networks on the two sides of the cord (Butt et al. 2002; Hinckley et al. 2005; Kjaerulff and Kiehn 1997; Kremer and Lev-Tov 1997; Pfieger et al. 2002). We examined recently, in animals cord-transected at birth, the capacity of the CPGs to produce locomotor-like activity (Norreel et al. 2003). The excitability of CPGs was increased after the transection and the left/right alternating locomotor pattern was lost at P6–P7. How $E_{\text{IPSP}}$ develops in interneurons during the perinatal period and whether $E_{\text{IPSP}}$ is more depolarized also in interneurons after neonatal spinal cord transection are important questions that require further exploration. However, the more depolarized $E_{\text{IPSP}}$ observed in motoneurons and possibly in interneurons in cord-transected animals may be partly responsible for both of these observations.

The nonsignificant trend for $E_{\text{IPSP}}$ to be more positive in cord-transected animals at P4–P7 than at P0–P2 suggests that some dedifferentiation processes may also occur and raises the important question as to whether inhibition becomes excitatory again after a spinal cord injury in adults (Vinay et al. 2006), as shown in other pathological conditions such as peripheral nerve injury–induced neuropathic pain (Coull et al. 2003), axonal injury (Nabekura et al. 2002; Toyoda et al. 2003), and human temporal lobe epilepsy (Cohen et al. 2002). In conclusion, we show that pathways descending from the brain stem are critical for inhibition to become and possibly to remain hyperpolarizing in lumbar motoneurons. These results shed light on a new upstream mechanism responsible for the developmental regulation of KCC2, a protein that plays a key role in both ontogeny and neurological diseases.

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