The Journal of Neuroscience, April 1, 2002, 22(7):2598–2606

Descending 5-Hydroxytryptamine Raphe Inputs Repress the Expression of Serotonergic Neurons and Slow the Maturation of Inhibitory Systems in Mouse Embryonic Spinal Cord

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Spontaneous synchronous rhythmic activities are a common feature of immature neuronal networks. Although the mechanisms underlying such activities have been studied extensively, whether they might be controlled by modulatory information remains questionable. Here, we investigated the role of descending serotonergic (5-HT) inputs from the medulla to the spinal cord in the maturation of rhythmic activity. We found that in spinal cords maintained, as a whole, in organotypic culture without the medulla, the maturation of spontaneous activity is similar to that found in spinal cords developed *in utero*. Interestingly, in organotypic cultures without the medulla (i.e., devoid of descending inputs), numerous intraspinal neurons expressed 5-HT, unlike in spinal cords cultivated in the presence of the medulla or matured *in utero*. We demonstrated that this 5-HT expression was specifically dependent on the absence of 5-HT fibers and was repressed by 5-HT itself via activation of 5-HT_{1A} receptors. Finally, to verify whether the expression of 5-HT intraspinal neurons could compensate for the lack of descending 5-HT fibers and play a role in the development of spontaneous activity, we blocked the 5-HT synthesis using *p*-chlorophenylalanine methyl ester in cultures devoid of the medulla. Surprisingly, we found that this pharmacological treatment did not prevent the development of spontaneous activity but accelerated the maturation of intraspinal inhibition at the studied stages. Together, our data indicate that descending 5-HT raphe inputs (1) repress the expression of spinal serotonergic neurons and (2) slow the maturation of inhibitory systems in mouse spinal cord.

Key words: neuronal phenotype; development; modulatory neurons; serotonin; disinhibition; GABA; glycine; neural networks

Early in development, various parts of the CNS express spontaneous synchronous rhythmic activity involving large ensembles of neurons (O'Donovan, 1999). For example, such activity has been found in the retina (Wong et al., 1995), brainstem (Fortin et al., 1999), hippocampus (Garaschuk et al., 1998), cochlear ganglion (Jones et al., 2001), auditory cortex (Lippe, 1994), thalamus (Itaya et al., 1995), and spinal cord (Hamburger and Balaban, 1963; Bekoff, 1976; O'Donovan and Landmesser, 1987; Nishimaru et al., 1996). In the rat spinal cord, spontaneous rhythmic activity has been found from embryonic day 13.5 (E13.5) to E18.5 (Nakayama et al., 1999) as mediated by glutamatergic and GABA–glycinergic synaptic transmission (Nishimaru et al., 1996). In the chick embryonic spinal cord, cholinergic, glutamatergic, and GABAergic synaptic transmission has also been described as mediating the generation of spontaneous bursts of activity (Milner and Landmesser, 1999). More recently, a large body of studies has focused on mechanisms by which spontaneous activity is generated (Tabak et al., 2000; Chub and O'Donovan, 2001). In contrast, how these spontaneous activities are modulated by extrinsic modulatory inputs and how these modulatory inputs contribute to the ontogenic plasticity of these activities remains elusive.

Received Sept. 14, 2001; revised Dec. 3, 2001; accepted Dec. 18, 2001.

This work was supported by grants from the Région Aquitaine. We thank V. Fénelon and A. Hill for valuable comments on this manuscript.

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Here, we investigated the role of descending modulatory inputs in the ontogenesis of spontaneous rhythmic activity in the embryonic mouse spinal cord. Serotonergic (5-HT) neurons projecting from the medulla raphe nuclei are among the first to innervate the embryonic rat spinal cord (Rajaofetra et al., 1989), suggesting an influence for 5-HT on brain development (Lauder, 1990). Therefore, we focused on the role played by these 5-HT descending modulatory inputs in the maturation of the spinal neural networks. We maintained the entire embryonic mouse spinal cord in organotypic culture for several days to investigate the ontogenic evolution of spontaneous activity, with or without the medulla (i.e., with or without the descending inputs). Using this new experimental approach, we show that the maturation of spontaneous rhythmic activity produced by spinal embryonic neuronal networks was dependent on the development of the intraspinal inhibitory system as reviewed recently (Ben-Ari, 2001). We also demonstrate that the inhibitory system was established early in development in the absence of descending 5-HT inputs. But more surprisingly, our results provide the first experimental evidence indicating that 5-HT descending inputs play a modulatory role in the development of the inhibitory spinal system, leading to slowing the establishment of this inhibitory network. Finally, our data show that the 5-HT descending modulatory inputs might repress the expression of the intraspinal 5-HT phenotype via 5-HT_{1A} receptors.

MATERIALS AND METHODS

Organotypic cultures. Embryos at E12.5 (day of fertilization = E0) were surgically removed under sterile conditions from pregnant OF1 mice

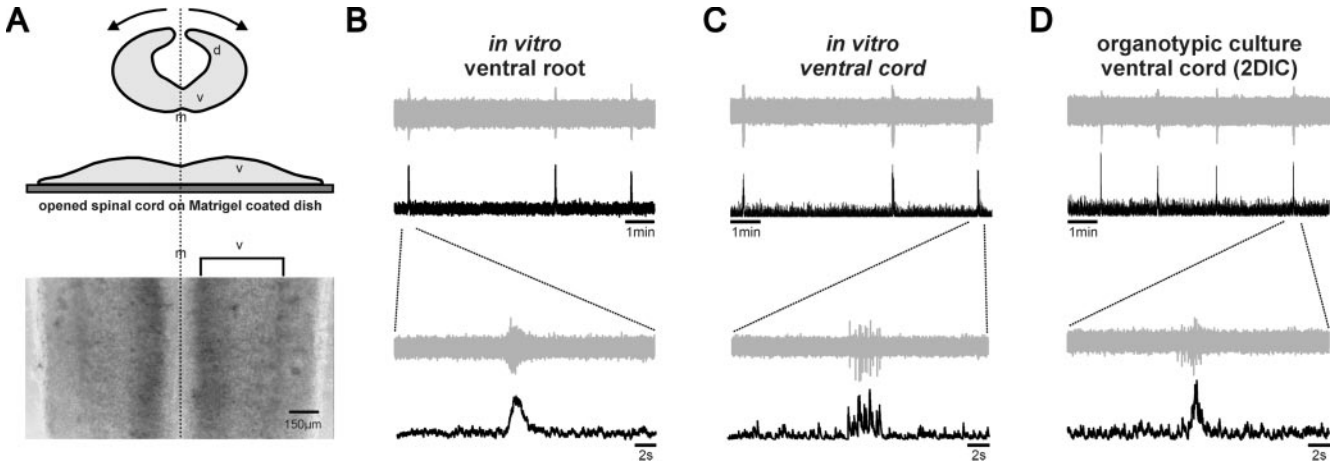

Figure 1. Spinal spontaneous rhythmic activities in acute (*in vitro*) and organotypic culture preparations. *A*, Schematic of the spinal cord organotypic culture, illustrating the opening of the dorsal side at E12.5 (*top*), and a photomicrograph of a 24 hr cultured spinal cord preparation (lumbar part). *d*, Dorsal; *m*, midline (*dotted line*); *v*, ventral. *B*, Spontaneous activities can be recorded extracellularly in acute *in vitro* preparations from ventral roots. *C*, Spontaneous activities are also recorded *in vitro* using pipettes closely apposed to the ventral gray matter. *D*, In an organotypic culture devoid of ventral roots, ventral gray matter recordings were used to monitor spontaneous rhythmic activities. *Gray traces* are raw data of activity; *black traces* are integrated activity. In *B–D*, the *two bottom traces* show one burst (delineated by *dotted lines*) on an expanded time scale.

(Iffa-Credo, L'Arbresle, France) previously anesthetized with ether. Embryos were rinsed out with Dulbecco's PBS at 6–8°C, and spinal cords with dorsal root ganglions and meninges were removed. The cords were then positioned into 400 µl of Matrigel (BD, Le Pont de Claix, France; diluted 1:4 with culture medium) in 35 mm Falcon dishes previously coated with Sylgard (Dow Corning, Midland, MI). Spinal cords were placed ventral-side down on the substrate to allow the opening of the dorsal side at the level of the dorsal fissure (Fig. 1*A*). They were maintained for 2–6 d in culture (DIC) at 37°C with 6–8% CO_2 and 100% humidity atmosphere into the following culture medium: 50% DMEM (Sigma, St. Louis, MO) containing 25 mM glucose, 25% HBSS (Sigma), 15% distilled water, 10% horse serum (Sigma), to which was added just before use 0.002% H_2O_2, 0.03% L-glutamine (Poly-Labo, Strasbourg, France), and 1‰ penicillin–streptomycin (10,000 U/10,000 µg) (Sigma).

Electrophysiology and pharmacology. In vitro spinal cords from E12.5, E14.5, and E18.5 embryos (*in utero* maturation) were placed into a recording chamber continuously perfused (1.5–2 ml/min) with Ringer's solution containing (in mM): 113 NaCl, 4.5 KCl, 1 $MgCl_2 \cdot 7\ H_2O$, 2 $CaCl_2$, 1 NaH_2PO_4, 25 $NaHCO_3$, and 11 glucose, gassed with 95% O_2–5% CO_2, pH 7.4, at 25–28°C. Spontaneous activity was recorded from lumbar ventral root (L2–L5) aspirated into a suction pipette and connected to a high-gain AC amplifier. Filtered (bandwidth 30 Hz to 3 kHz) raw signals were integrated off-line and analyzed using Spike2 software (Cambridge Electronics Design, Cambridge, UK). Spontaneous activity was extracellularly recorded from spinal cord organotypic cultures (2–6 DIC) using a glass electrode placed into the ventral cord (Fig. 1*A*) and connected to the same amplifier.

Kynurenic acid was obtained from Fluka Chemie AG (Buchs, Switzerland). NMDA, 5-HT, (−)-bicuculline methiodide, strychnine nitrate salt, and DL-*p*-chlorophenyl-alanine methyl ester were obtained from Sigma. Spiroxatrine was obtained from Research Biochemicals (Natick, MA).

Immunofluorescence. Spinal cords were fixed with 2% paraformaldehyde and 10% sucrose in 0.2 M PBS, pH 7.2, for 90 min at 4°C. Spinal cords were incubated with rabbit anti-5-HT (for specificity, see Tramu et al., 1983) (1/5000, gift from Pr. G. Tramu, University of Bordeaux 1, Talence, France) for 3 hr at room temperature in 0.2 M PBS containing 0.2% BSA and 0.1% saponin. After rinsing, they were incubated with the fluorescein-conjugated anti-rabbit IgG, and the preparations were then observed under confocal microscope after a few rinses. As a control for the specificity of the immunochemical reactions, some cultures were processed without primary ($n = 2$) or secondary ($n = 1$) antibody. No labeled elements were observed under these latter conditions.

Confocal microscopy. Treated spinal cords were transferred from the culture dish to a slide and viewed with a Leica (Nussloch, Germany) TCS 4D laser-scanning confocal microscope equipped with a krypton/argon mixed-gas laser. A total of 15–30 sections (1–3 µm thick) were recorded with a 25× or 40× oil objective. Images presented were obtained using the maximal projection provided by Scanware (Leica).

Quantitative analysis. For each experiment, periods of spontaneous activity were measured across 10–30 bursts and coefficients of variation (CV, SD/mean) were calculated; the latter were considered an index of regularity (rhythmic activity when the CV was <0.5). Burst durations were calculated in controls and after bath application of bicuculline–strychnine. The percentage of change of burst duration induced by the bicuculline–strychnine treatment was then calculated. Cumulative results were expressed as mean ± SEM. The statistical significance of the difference was assessed by a paired two-tailed Student's *t* test or by one-way ANOVA followed by a pairwise multiple comparison procedure (Tukey test).

RESULTS

Spontaneous activity in spinal cords developed *in utero* or in organotypic cultures

We performed our experiments on the embryonic mouse brainstem–spinal cord. We used this preparation either isolated *in vitro* at different embryonic stages from E12.5 to E18.5 as reported previously (Branchereau et al., 2000) or in organotypic cultures specifically developed to follow the maturation of this preparation from E12.5 plus 2–6 DIC. Then, using these two spinal cord preparations, we were able to make a direct comparison of the evolution of the maturation of embryonic spinal networks between acute *in vitro* and culture preparations at two different times: E14.5/E12.5 plus 2 DIC and E18.5/E12.5 plus 6 DIC.

Similar to results described in the embryonic rat (Nishimaru et al., 1996), we found that the mouse embryonic brainstem–spinal cord *in vitro* expressed, as early as E12.5, spontaneous rhythmic activities in all of the preparations tested ($n = 14$). These activities could be monitored from lumbar ventral roots (Fig. 1*B*) or directly from ventral gray matter with extracellular electrodes (Fig. 1*C*). The temporal features of these activities were similar with both methods (Fig. 1, compare *B,C*). We used extracellular recordings from ventral gray matter to monitor the spontaneous activities in organotypic cultures of spinal cord (Fig. 1*D*) in which the ventral roots became inaccessible. Indeed, at embryonic stages, the dorsal part of the cord has not yet completely closed, allowing us to flatten it on the coated bottom of a dish (Fig. 1*A*,

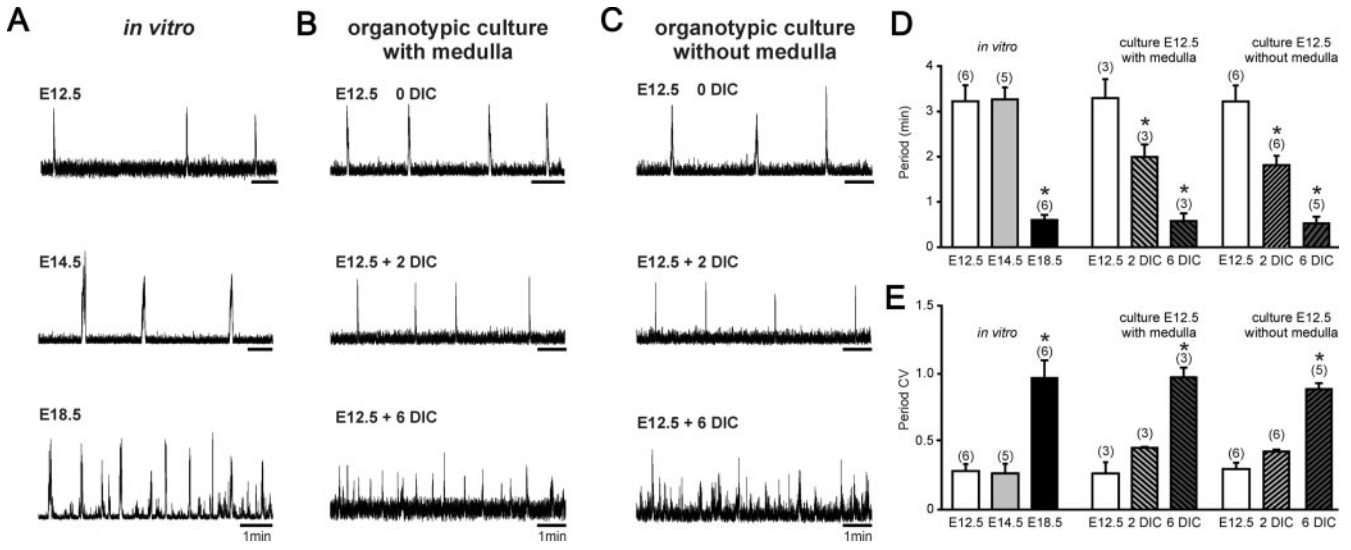

Figure 2. Spontaneous rhythmic bursts of activity recorded from lumbar spinal cords *in vitro* or in organotypic cultures, with or without the medulla, exhibited a dramatic reduction of the interburst period accompanied by a large increase of variability. Note that these changes occurred between E14.5 and E18.5 *in vitro* and between E12.5 plus 2 DIC and E12.5 plus 6 DIC in organotypic culture. *A*, Spontaneous bursts of activity recorded *in vitro* recurred with a regular period of spontaneous activity of ~3.2 min at E12.5 and E14.5 but became very erratic at E18.5. *B*, In spinal cords maintained in organotypic cultures at E12.5 with the medulla (E12.5; start of culture, 0 DIC), bursts of activity also recurred with a regular spontaneous activity period of ~2 min after 2 DIC and became very irregular after 6 DIC. *C*, Cultures without the medulla also exhibited an identical evolution in their spontaneous bursts of action potentials. *D*, *E*, Quantitative analysis of the interburst periods in each experimental condition and index of variability of these periods given by the CV (SD/mean of periods). Values represent the mean ± SEM of three to six experiments (*number* in *parentheses*). $^*p < 0.05$ (one-way ANOVA followed by Tukey test).

top) to expose the ventral gray matter (Fig. 1*A*, *bottom*) and record extracellularly any spontaneous rhythmic activities.

Role of the medulla in the maturation of spontaneous activity

To assess whether descending pathways of the medulla play a major role in the maturation of spontaneous rhythmic activities, we used our organotypic preparation of embryonic spinal cord, with or without the medulla. Beginning with E12.5 brainstem–spinal cords, the evolution of the maturation of the rhythmic activity was compared between *in vitro* preparations at different stages of *in utero* development (E14.5 and E18.5) and in organotypic cultures at different days in culture (E12.5 plus 2 DIC and E12.5 plus 6 DIC). We found that all *in vitro* preparations tested at E12.5 ($n = 9$) and E14.5 ($n = 5$) exhibited a similar period of spontaneous rhythmic activities (Fig. 2*A,D*). For example, at E12.5, these activities consisted of bursts of action potentials (5.1 ± 0.7 sec duration; mean ± SEM; $n = 6$) that occurred with a period of 3.2 ± 0.3 min ($n = 6$). In contrast, at E18.5, 46% (6 of 13) of preparations express a dramatic decrease in the period of spontaneous activity (0.6 ± 0.1 min; $n = 6$) (Fig. 2*A,D*), as described previously in embryonic rat spinal cord (Nakayama et al., 1999). The remaining preparations became silent, although rhythmic activities could be induced by bath application of NMDA/5-HT. Moreover, although the CV (i.e., SD/mean; index of relative dispersion) of period measurements was rather low (<0.4) and identical in both E12.5 and E14.5 embryonic stages, it was significantly increased at E18.5 to almost 1 (0.97 ± 0.13) ($p < 0.05$; Tukey test) (Fig. 2*E*). These data indicate that during the course of development the mouse spinal generator of spontaneous rhythmic activities undergoes ontogenic alteration that is similar to that described in rats (Nishimaru et al., 1996).

A comparable evolution of spontaneous activities was observed in organotypic cultures with the medulla (Fig. 2*B*). First, at E12.5 plus 2 DIC, all of the preparations tested ($n = 9$) expressed spontaneous rhythmic activities consisting of bursts of action potentials (1.0 ± 0.2 sec duration). Second, between E12.5 and E12.5 plus 6 DIC, 66% of the preparations (8 of 12) exhibited a dramatic reduction in the period of spontaneous activity (Fig. 2*B,D*), accompanied by a significant increase in the variability of this period (Fig. 2*E*). Moreover, similar to the E18.5 *in vitro* preparation, 33% of the E12.5 plus 6 DIC preparations remained silent but responded to NMDA/5-HT. We concluded that the general evolution of rhythmic activities was similar in the *in vitro* preparation and the organotypic culture.

In the absence of the medulla, the evolution of spontaneous rhythmic activities remained similar to the one observed in either *in vitro* or organotypic preparations with the medulla (Fig. 2*C*). The period of spontaneous activity underwent a large decrease from E12.5 to E12.5 plus 6 DIC, and its CV also expressed an important increase (Fig. 2*D,E*). Together, these data indicate that the main features of the maturation of spontaneous activities *in utero* are retained in organotypic cultures and do not depend on the presence of the medulla.

To further characterize the maturation of these rhythmic activities expressed spontaneously in the three experimental conditions, we used a pharmacological approach on *in vitro* and organotypic preparations without the medulla. Because glutamatergic and GABA–glycinergic synaptic transmission is involved in the generation of spontaneous activities during the rat spinal cord ontogeny between E12.5 and E18.5 (Nishimaru et al., 1996), we compared the effect of selective antagonists at E12.5, E18.5 *in vitro*, and E12.5 plus 6 DIC (Fig. 3).

At E12.5 *in vitro*, bath application of 4 mM kynurenate (large-spectrum glutamatergic receptor antagonist) did not affect the ongoing spontaneous rhythmic activity (Fig. 3*A*) but completely blocked these activities at E18.5 as well as in E12.5 plus 6 DIC

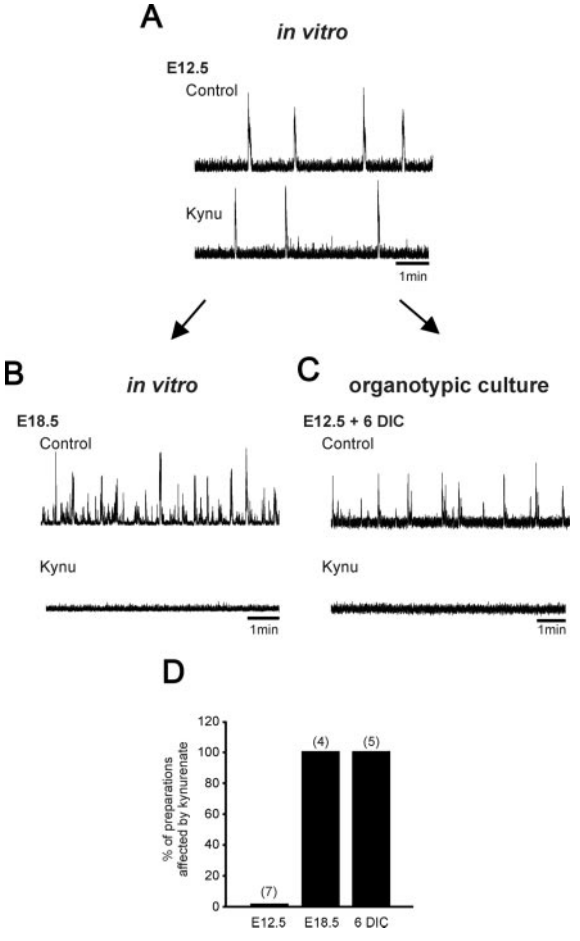

Figure 3. Similar maturation of the excitatory glutamatergic synaptic transmission *in utero* and in organotypic cultures. *A*, At E12.5, 4 mM kynurenate did not alter the ongoing spontaneous activity. *B*, *C*, Same treatment at E18.5 and E12.5 plus 6 DIC resulted in complete abolition of the spontaneous rhythmic activities. *D*, Quantitative analysis of kynurenate effects; the number of preparations is in *parentheses*.

organotypic preparations without the medulla (Fig. 3*B–D*). The same results were obtained in organotypic cultures with the medulla ($n = 2$; data not shown). The kynurenate effect was fully reversible after ~30–60 min (data not shown).

We subsequently tested whether the ontogenic switch of the GABA–glycine system from excitatory to inhibitory synaptic influence, as described in the rat embryonic spinal cord system (Wu et al., 1992), also occurs in mouse embryos. *In vitro*, the combined bath application of 30 μM bicuculline and 5 μM strychnine ($GABA_A$ and glycine receptor antagonists, respectively) decreased the duration of the bursts at E12.5 (Fig. 4*A*). This result indicates that at E12.5, the $GABA_A$–glycinergic synaptic transmission is likely to be involved as an excitatory component in the generation of spontaneous rhythmic bursts. In contrast, later in development, at E14.5 and E18.5, bath application of these antagonists always increased the burst duration (Fig. 4*B*), suggesting the blockage of inhibitory transmission (disinhibition) (Cowley and Schmidt, 1995; Bracci et al., 1996; Tscherter et al., 2001) that therefore allowed the expression of prolonged bursts of action potentials. This increase became significantly greater ($p < 0.01$; *t* test) between E14.5 and E18.5 (Fig. 4*B*, *gray area*, *D*). The effect could be reversed after 1–2 hr in control Ringer's solution (data not shown). In organotypic cultures without the medulla, the

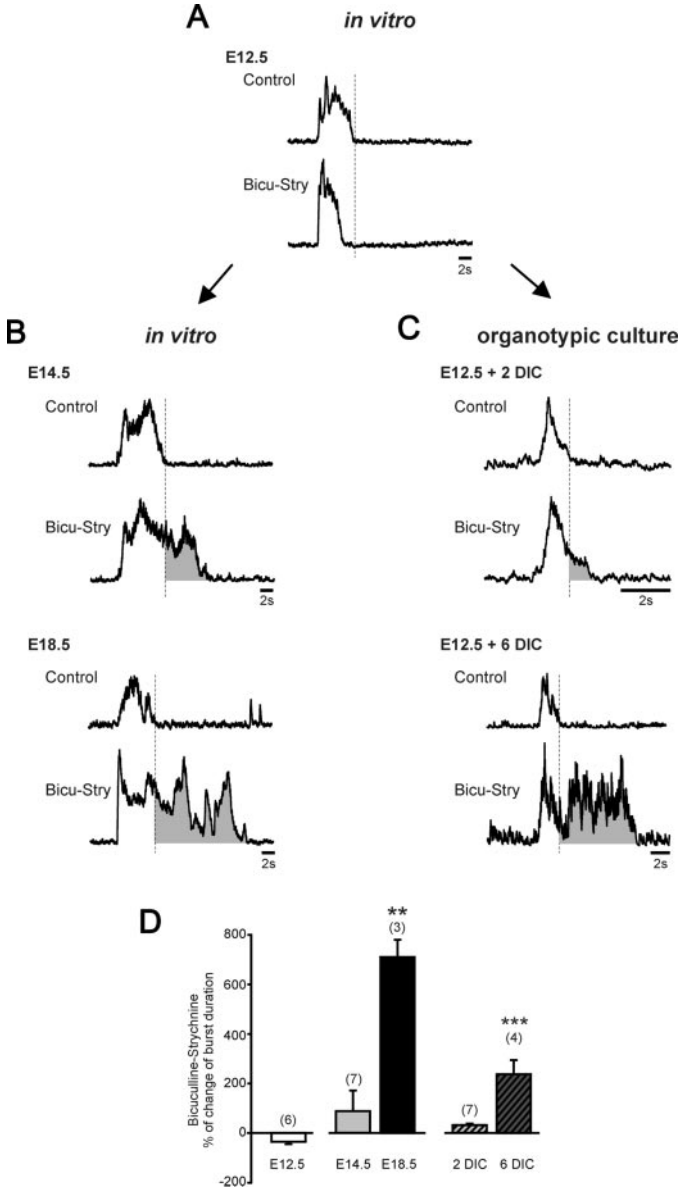

Figure 4. Similar maturation of the inhibitory synaptic transmission *in utero* and in organotypic cultures. *A*, $GABA_A$–glycinergic receptor blockage [30 μM bicuculline and 5 μM strychnine (*Bicu-Stry*)] reduced the duration of spontaneous bursts at E12.5 *in vitro* (see *vertical dotted line*). *B*, *C*, Adding the same $GABA_A$–glycinergic synaptic transmission antagonists 2 d later either at E14.5 *in vitro* or after 2 DIC induced an opposite effect leading to an increase of the duration of spontaneous bursts; this increase became larger at E18.5 *in vitro* and after 6 DIC. *Gray areas*, Extension of burst duration during drug application. The end of the burst was considered to occur when the integrated signal fell below zero. *D*, Quantitative analysis of bicuculline–strychnine effects measured as the percentage of decrease or increase of the burst duration. $^{**}p < 0.01$ (*t* test between E14.5 and E18.5); $^{***}p < 0.001$ (*t* test between E12.5 plus 2 DIC and E12.5 plus 6 DIC).

same experimental procedure also revealed a reversible increase in burst duration after 2 d in culture, which became much larger at E12.5 plus 6 DIC (Fig. 4*C,D*) ($p < 0.001$, *t* test). Similar data have been obtained in cultures with the medulla ($n = 2$; data not shown). Together, these data indicate that the spinal cord in organotypic cultures, with or without the medulla, shares an evolution in its maturation for the parameters tested (see above) similar to the one found in the embryonic *in vitro* preparation.

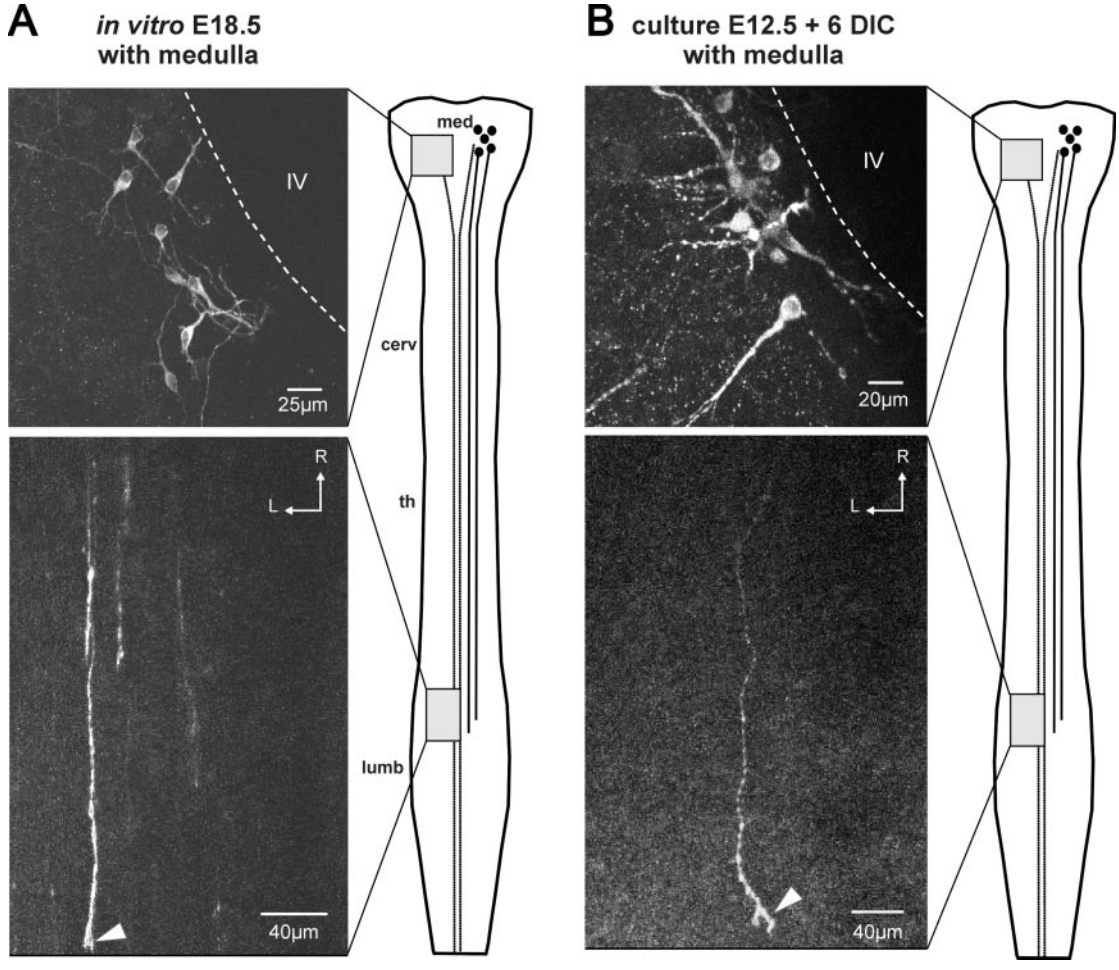

Figure 5. Caudal raphe neurons sent descending fibers into the spinal cord *in utero* or in organotypic culture. *A*, In an acute E18.5 preparation, 5-HT labeled neurons were detected at the medulla level (*top*), and their 5-HT descending axons reached the lumbar level of the spinal cord (*bottom*). *B*, In organotypic culture preparation, a similar nucleus containing 5-HT-immunostained neurons was found at the medulla level; these neurons sent fibers into the caudal levels of the cultured spinal cord. The *white dashed line* delimits the fourth ventricle (*IV*); *white arrowheads* point to the terminal end (growth cone) of a serotonergic axon. The schematic on the *right* represents the descending 5-HT inputs (*lines*) from raphe somata (*blacks dots*). *cerv*, Cervical; *med*, medulla; *lumb*, lumbar; *th*, thoracic; *L*, lateral; *R*, rostral. As in Figures 6 and 7, the *vertical dotted lines* on the *right* schematic drawings represent the location of the central canal.

Immunocytochemical analysis of intraspinal 5-HT innervation

The main features of the maturation of rhythmic activities were expressed in organotypic cultures, with or without the medulla. This result raises the following question: what role do the descending modulatory inputs play during the development of the spinal cord? Among these inputs, we examined the role of 5-HT descending pathways from the raphe nuclei during maturation, because 5-HT is known to play an important role during ontogeny (Azmitia and Whitaker-Azmitia, 1997). To address this question, we first compared the 5-HT innervation of the spinal cord in *in vitro* preparations and organotypic cultures with the medulla. The immunocytochemical analysis revealed 5-HT-stained somata in the caudal raphe nuclei at E12.5 without any labeling all along the cord (data not shown; $n = 7$). After 6 d, at E18.5, descending 5-HT fibers originating from raphe somata have reached the lumbar region (Fig. 5*A*). Moreover, no 5-HT-labeled cell bodies were detected all along the cervico-thoraco-lumbar regions ($n = 4$). Interestingly, the same pattern of 5-HT innervation was observed in E12.5 plus 6 DIC preparations in which the medulla was kept intact ($n = 3$) (Fig. 5*B*), indicating a similar evolution *in utero* and in culture.

In contrast and unexpectedly, in organotypic cultures without the medulla (i.e., devoid of 5-HT projecting neurons) after 2–3 DIC ($n = 3$), we were able to visualize 5-HT-immunoreactive somata throughout the spinal cord (Fig. 6*A*). These somata exhibited widespread neuritic processes after 6 DIC ($n = 7$; Fig. 6*B*). Moreover, these immunoreactive cell bodies were preferentially located in the ventral intermediate areas, in which they were located throughout the entire thickness of this area. To assess whether the expression of these intraspinal 5-HT neurons is controlled by 5-HT itself, we maintained in culture E12.5 embryonic spinal cord preparations without the medulla ($n = 3$) during 6 d in a 5 μM 5-HT-enriched medium. Under these conditions, we did not detect any 5-HT immunoreactivity in the spinal cord (Fig. 7*A*). Because much of the trophic effect of 5-HT on target tissues is elicited through 5-HT_{1A} receptors (Azmitia and Whitaker-Azmitia, 1997), we performed organotypic cultures of E12.5 embryonic spinal cord with the medulla in the presence of the 5-HT_{1A} receptor antagonist spiroxatrine (10 μM). This latter

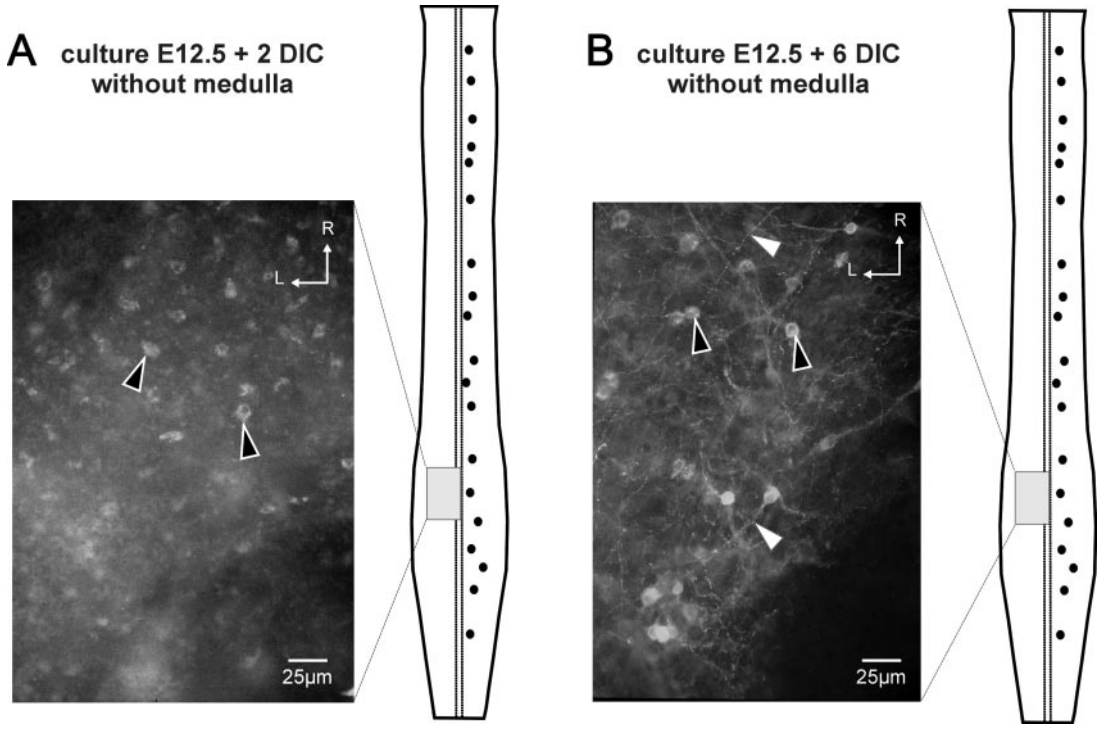

Figure 6. Expression of 5-HT intraspinal neurons in organotypic cultures without the medulla. *A*, After 2 DIC, the immunostaining procedure allowed detection of 5-HT-labeled somata (*black arrowheads*). *B*, After 6 DIC, the same protocol revealed a stronger staining of 5-HT neurons extending into their neuritic processes (*white arrowheads*). *L*, Lateral; *R*, rostral. On the *right* schematic drawings, *black dots* indicate the presence of intraspinal 5-HT-immunoreactive somata.

experimental condition ($n = 5$) revealed the presence of numerous 5-HT-immunoreactive somata in the ventral gray matter among long 5-HT fibers that can be followed along the main axis of the spinal cord (Fig. 7*B*). Together, these data show that serotonin represses the 5-HT phenotype in a subpopulation of intraspinal mouse neurons through 5-HT_{1A} receptors. Moreover, because the spinal neural network undergoes normal development without the medulla, this suggests that intraspinal 5-HT neurons may compensate the lack of 5-HT from descending raphe inputs.

Role of 5-HT in the maturation of spontaneous activity

Finally, because in all cases the spinal tissue may be exposed to 5-HT (either from descending inputs or from intraspinal 5-HT neurons), we prevented its synthesis using 10 µM *p*-chlorophenylalanine methyl ester (pCPA) (Koe and Weissman, 1966) to assess its role in the maturation of rhythmic activities. In all organotypic spinal cord preparations in the continuous presence of pCPA ($n = 7$), the overall sequences of events that characterize the maturation of the spinal cord activities (i.e., evolution of the period and CV of rhythmic activities, maturation of the glutamatergic synaptic transmission; data not shown) were unexpectedly retained. Such results seem to indicate that 5-HT does not play a role in the maturation of the spinal network activity. We found, however, that this is not the case, because in the absence of 5-HT, the maturation of functional inhibitory synaptic interactions seems to be boosted (Fig. 8). In fact, as illustrated above (Fig. 4*A*), at E12.5, the bath application of bicuculline–strychnine decreased the duration of spontaneous bursts (Fig. 8*A,D*). After 2 d, the blockage of $GABA_A$–glycine inhibitions in pCPA-treated cultures (E12.5 plus 2 DIC) disclosed a significantly ($p = 0.008$; *t* test) longer burst duration of spontaneous activity (Fig. 8*C*, *gray area*) compared with E12.5 plus 2 DIC untreated cultures (Fig. 8*B*, *gray area*). These results indicate that 2 d of 5-HT synthesis blockade reveals a stronger intraspinal inhibition. Thus, 5-HT seems to slow the maturation of inhibitory systems in mouse spinal cord.

DISCUSSION

Our results have revealed two major effects of 5-HT projecting neurons from raphe nuclei in the embryonic mouse spinal cord. Descending 5-HT inputs (1) repress the expression of the 5-HT phenotype within a subpopulation of spinal neurons and (2) slow the maturation of the intraspinal GABA–glycine inhibitory system.

Although the role of modulatory inputs has been extensively studied in invertebrates (for review, see Harris-Warrick and Marder, 1991; Nusbaum et al., 2001), in which they can be easily experimentally manipulated in the adult as well as in the immature nervous system (Meyrand et al., 1991; Le Feuvre et al., 1999), in vertebrates, it still remains extremely difficult to selectively change the modulatory information acting on specific neuronal networks during the ontogeny or adulthood. To this end, we developed a new organotypic culture preparation of the entire brainstem–spinal cord. Here, we have shown that the brainstem–spinal cord in culture expresses spontaneous rhythmic activities that undergo a sequential maturation similar to the ones expressed *in utero* (Figs. 2–4). The only exceptions were a reduction in burst duration (probably a result of the reduced neuronal population recorded in the ventral gray matter in organotypic cultures) and a slight reduction of the spontaneous activity period that occurred after 2 d in culture. These results suggest that isolated brainstem–spinal cord preparations possess all of the elements required for the maturation of spinal cord networks.

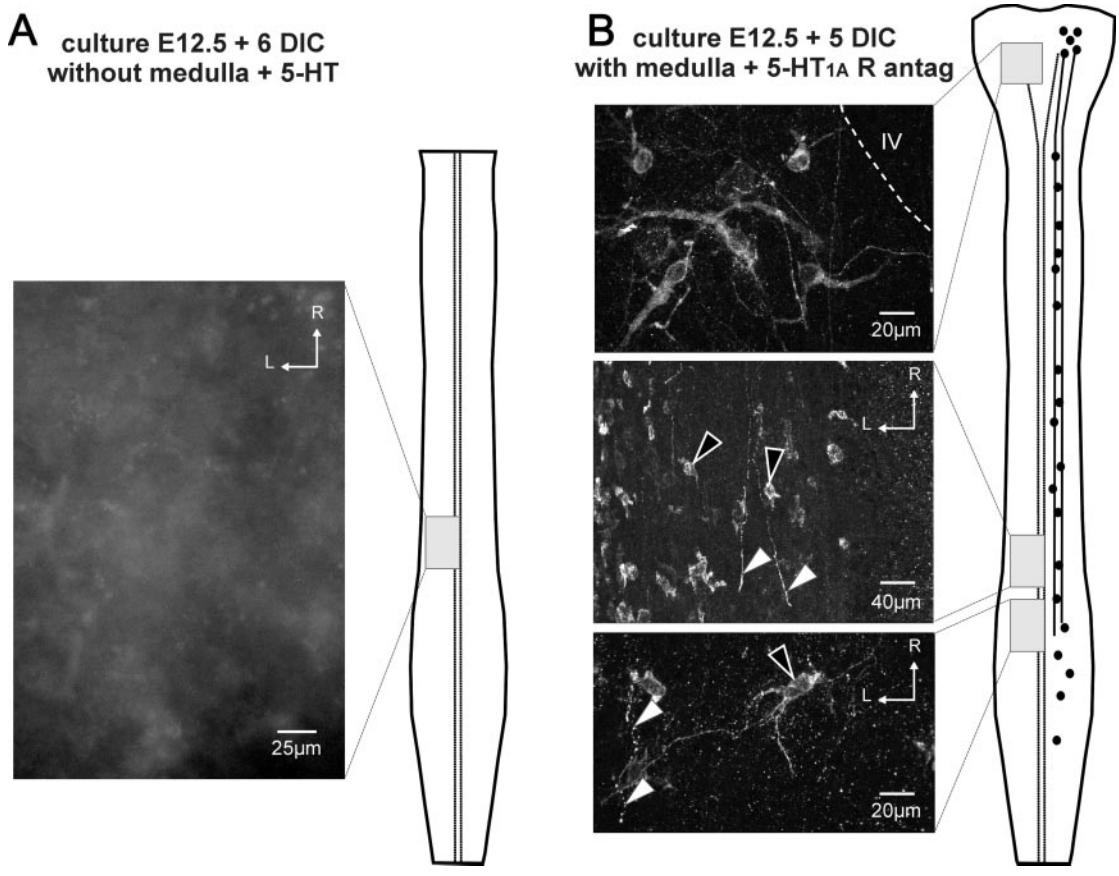

Figure 7. Experimental evidence for a repressive role of 5-HT descending inputs on the expression of 5-HT intraspinal neurons. *A*, Serotonin (5 μM) added to the culture medium resulted in the absence of 5-HT staining in the preparation without medulla. *B*, Blockade of 5-HT_{1A} by 10 μM spiroxatrine revealed 5-HT intraspinal labeled somata (*black arrowheads*) and long 5-HT fibers (*white arrowheads*). *antag*, Antagonist. *L*, lateral; *R*, rostral. The *dashed line* in the *top panel* in *B* delimits the fourth ventricle. On the *right* schematic drawings, *vertical solid lines* represent the descending 5-HT axons and *black dots* represent the 5-HT raphe and intraspinal somata.

5-HT phenotype plasticity within the embryonic spinal cord

The suppression of descending 5-HT pathways to the spinal cord tissue revealed that some intraspinal neurons could express a 5-HT phenotype. Serotonin is known to exert complex short-term excitatory and inhibitory modulation of adult networks such as locomotor (Schmidt and Jordan, 2000) or respiratory (Bianchi et al., 1995) networks. 5-HT also acts in a long-term manner. For example, 5-HT upregulates the neurogenesis as well as the long-term plasticity in the immature CNS and maintains a neuronal phenotype in the mature brain (Azmitia and Whitaker-Azmitia, 1997). Here, we show that 5-HT may act as a long-term downregulator of the 5-HT neurotransmitter phenotype. Although the mechanisms by which such downregulation occurs are still unknown, we may postulate that serotonin acts through two different mechanisms. Either 5-HT prevents the genesis of a new population of 5-HT that would be incorporated into the spinal cord, or it alters the neurotransmitter phenotype within a subpopulation of cells already present in the spinal cord. Additional experiments must be conducted to identify the source of cells giving rise to 5-HT neurons. Concerning the phenotypic plasticity, a large body of work indicates that serotonin is able to control the transmitter phenotype of various populations of neurons. In general, 5-HT upregulates the monoaminergic neuronal phenotype (Galter and Unsicker, 2000; Zhou and Iacovitti, 2000). Such upregulation is mediated by the 5-HT_{1A} receptor (Galter and Unsicker, 2000), the principal receptor involved in neurotrophic effects, which is expressed early in development (for review, see Azmitia and Whitaker-Azmitia, 1997). However, the downregulation of the spinal neuronal 5-HT phenotype by 5-HT via 5-HT_{1A} receptors, as shown in the present study, to the best of our knowledge has never been described, although a downregulation of the GABAergic phenotype by 5-HT was reported recently (Dumoulin et al., 2000).

Finally, because only a subpopulation of spinal neurons expresses the 5-HT phenotype after removal of descending inputs, the identification of this population remains to be determined. The fact that intraspinal 5-HT neurons are located at all depths of the ventral intermediate areas and that no 5-HT immunolabeling is revealed in the neuritic processes expanding outside the cultured spinal cord (our unpublished observations), however, suggests that these neurons may be ventral gray matter interneurons rather than motoneurons.

Control of the maturation of GABA–glycine inhibition by serotonin

A common feature of the maturation of embryonic networks is the change in the action of GABA–glycine amino acids from excitatory to inhibitory. For example, in the hippocampus, GABA acts as an excitatory transmitter early in development, whereas in the adult, it is the main inhibitory transmitter (Cherubini et al., 1991). In a similar manner, GABA is the primary transmitter

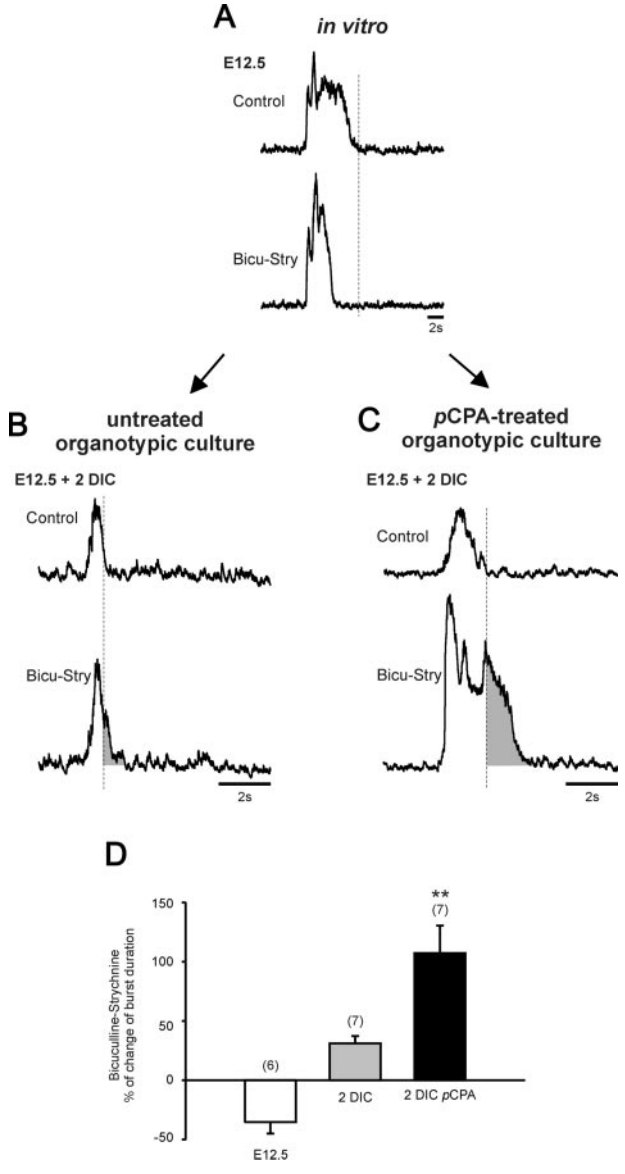

Figure 8. The blockade of $GABA_A$–glycine receptors revealed an increase in the duration of the spontaneous rhythmic bursts in pCPA-treated organotypic cultures after 2 DIC. *A*, As shown in Figure 4*A*, at E12.5, an application of 30 μM bicuculline and 5 μM strychnine (*Bicu-Stry*) induced a decrease of spontaneous burst duration. *B*, After 2 d of culture in control medium (E12.5 plus 2 DIC, untreated cultures), the same application of bicuculline–strychnine induced an increase in burst duration (*gray area*). *C*, pCPA-treated E12.5 plus 2 DIC cultures (10 μM pCPA) exhibited a larger increase of burst duration (*gray areas*) after $GABA_A$–glycine receptor blockage. *D*, Quantitative analysis revealed a significant difference between E12.5 plus 2 DIC controls and pCPA-treated preparations. $^{**}p < 0.01$ (*t* test).

driving action potentials in embryonic hypothalamic neurons (Gao and Van Den Pol, 2001). Moreover, in the rat spinal cord, a switch of the GABA–glycine response from excitatory to inhibitory occurs between embryonic days 17 and 19 (Wu et al., 1992).

Although the mechanisms underlying these changes have already been well investigated (Ehrlich et al., 1999), no data are available concerning modulatory control of this switch from excitation to inhibition. However, our results indicate that 5-HT is involved in this process, because 5-HT seems to act on the maturation of the inhibitory system in early developmental stages in the mouse embryo. Indeed, at least at one given developmental stage, the lack of 5-HT reveals a stronger intraspinal inhibition. Interestingly, it must be noted that the functional reversed effect of $GABA_A$–glycinergic synaptic transmission from excitatory to inhibitory described in the present study (Fig. 4) is concomitant with the invasion of lumbar parts of the cord by 5-HT descending terminals in mice (data not shown). The cellular mechanisms by which 5-HT controls the establishment of the inhibitory $GABA_A$–glycinergic synaptic transmission within spinal networks remain unclear. 5-HT may either act on the presynaptic GABAergic–glycinergic neuronal population or regulate the ontogeny of postsynaptic $GABA_A$–glycine receptor subunits. Finally, 5-HT may regulate changes in the regulation of intracellular $[Cl^-]$ that may be responsible for the switch of the GABA–glycinergic transmission from excitatory to inhibitory (Owens et al., 1996).

Role of modulatory inputs in the ontogeny of neural networks

In late development, modulatory inputs play a crucial role in the final developmental tuning of neural networks. For example, it has been shown that descending serotonergic spinal projections exert a modulatory action that controls the maturation of sensorimotor networks in amphibian embryos (Sillar et al., 1993; Woolston et al., 1994) and neonatal rats (Vinay et al., 2000). In contrast, in early development, studies performed on embryonic chick spinal cords indicate that the suppression of central descending modulatory inputs from the medulla does not prevent ongoing spinal rhythmic activities (Hamburger and Balaban, 1963; Bekoff, 1976; O'Donovan and Landmesser, 1987). Such data seem to indicate that modulatory inputs play a minor role in the maturation of networks that generate these spontaneous rhythmic activities. In contrast, although our data indicate that the absence of descending modulatory information does not prevent spontaneous rhythmic activities, we show that the absence of 5-HT inputs triggers the expression of an intraspinal 5-HT system. This local 5-HT system may compensate for the lack of 5-HT released from raphe descending inputs. Therefore, to fully understand the role of 5-HT, it is necessary not only to remove the 5-HT descending pathways but also to block the biogenic amine synthesis. In this condition, it is possible to reveal an additional role of 5-HT during early stages of the development that consists of a repressive role in the maturation of the inhibitory spinal network. Although it was shown recently that central modulatory input may exert repressive control on the expression of adult networks in the invertebrate embryo (Le Feuvre et al., 1999), such ontogenic repressive control has never been reported in mammals. Finally, because it was shown recently that spinal transplantation of embryonic 5-HT neurons may help functional recovery after spinal cord injury (Ribotta et al., 2000), our findings shed a new light on possibilities to explore new trends in functional recovery of spinal network operation.

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