Sonic Hedgehog and Retinoic Acid are not sufficient to induce motoneuron generation in the avian caudal neural tube

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Abstract

The caudal neural tube (CNT) of the avian embryo is devoid of both dorsal and ventral roots. We show that the lack of ventral roots in the CNT, from somite 48 caudalwards, is due to an absence of post-mitotic motoneurons (MNs). The absence of MNs is not due to a defective notochordal induction since Sonic Hedgehog (SHH) signaling is intact and the caudal notochord is able to induce ectopic MNs when grafted laterally to a host neural tube. The transcription factors involved in MN specification (Pax6, Nkx6.1, and Olig2) are all expressed in the CNT, despite the lower expression level of Pax6, but an overlap between Olig2 and the ventrally expressed transcription factor Nkx2.2 is observed in the CNT. Grafting a quail CNT into the cervical level of a chick host rescues MN generation, demonstrating both the CNT potential for MN generation and the key role of the caudal environment in the MN differentiation blockade. The transplantation of the CNT-flanking somites into the cervical level does not inhibit MN generation. Furthermore, implantation of a retinoic-acid-soaked bead laterally to the CNT does not rescue MN generation. Together, these data indicate that the rostral environment contains a signal different from both SHH and Retinoic Acid that acts on MN differentiation.

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Introduction

The vertebral central nervous system (CNS) is organized according to both anterior–posterior (AP) and dorso-ventral (DV) axes. The spinal cord represents the most caudal compartment of the CNS and is usually considered as a single morphological entity. However, several histological features allow one to delineate different spinal regions, such as the Lateral Motor Column that innervates the skeletal muscle only at the limb level. In birds, the caudalmost part of the spinal cord displays rudimentary features of development with neither sensory nor motor nerves. The reason for the absence of ventral roots has never been studied, in contrast to the sensory nerves whose absence is due to intrinsic limited developmental potentials of the neural crest cells at this AP level (Catala et al., 2000).

The ventral patterning of the neural tube has been extensively studied in the vertebrate embryo (Jessell, 2000). Sonic Hedgehog (SHH), a glycoprotein secreted from both the notochord and the floor plate, specifies the motoneurons (MNs) and ventral interneurons in a concentration-dependent manner (Briscoe and Ericson, 2001; Ericson et al., 1997a). This signaling pathway controls the expression of key transcription factors that are expressed in defined domains of the DV axis of the neural tube. Their combinatorial expression subdivides the ventral spinal cord into five progenitor domains (p0, p1, p2, pMN, and p3), each of which gives rise to a distinct class of post-mitotic neurons (Jessell, 2000). The motoneuron (MN) progenitor domain is characterized by the concomitant expression of three transcription factors, Pax6, Nkx6.1, and Olig2 (Marquardt and Pfaff, 2001). These proteins upregulate in
turn MNR2, whose expression is restricted to committed MN progenitors and begins during their ultimate cell division (Tanabe et al., 1998). Furthermore, the proneural gene Ngn2 plays an essential and synergistic role with Olig2 in promoting MN specification by inducing MNR2 expression. Their combined activity drives MN progenitors out of the cell cycle (Mizuguchi et al., 2001; Novitch et al., 2001). After cell cycle exit, MNs express the transcription factor Islet1 (Isl1) (Erićson et al., 1992). In addition, it has been recently shown that somite-secreted RA plays a key role in the ventral patterning of the spinal cord (Diez del Corral et al., 2003; Novitch et al., 2003; Pierani et al., 2001; Wilson et al., 2004). RA controls the expression of several of the homeodomain and bHLH proteins referred above that are involved in ventral neuron specification in the spinal cord (Diez del Corral and Storey, 2004).

In this study, we have analyzed the lack of motor nerves in the avian caudal neural tube (CNT) flanked by somites 48 to 53. We show that both post-mitotic MNs and MN progenitors are absent in the CNT. To understand this particular characteristic, we have studied several hypotheses: (1) an inductive/ventralizing signaling defect; (2) programmed cell death selectively eliminating MN progenitors; (3) a general lack of neurogenesis affecting not only MNs but all neurons; (4) an alteration in the DV patterning. We show that the notochord underneath the CNT is able to induce ectopic MNs when grafted laterally to a host neural tube. Neither programmed cell death nor a general lack of neurogenesis can account for the absence of MNs in the CNT. Nevertheless, the DV patterning is altered, accounting for a blockade of MN differentiation. We show that the CNT has the potential to generate MNs. Indeed, the presence of MNs in a quail CNT grafted at the brachial level of a chick host confirms that these progenitors have the intrinsic potential to generate MNs revealing a pivotal role of the environment in the blockade. The somites flanking the CNT do not impede MN generation when transplanted at the brachial level. Furthermore, RA restoring experiments do not rescue MN generation suggesting the existence of an additional secreted molecule involved in MN differentiation.

Embryos were fixed in 4% paraformaldehyde for 1 to 2 h at room temperature (RT) for immunostaining and TUNEL assay and overnight at 4°C for in situ hybridization.

**CNT transplantation into brachial levels**

The CNT transplantation was performed as described previously (Catala et al., 2000), using the quail-chick chimera technique. Briefly, a two to three somite-long segment of neural tube (corresponding to the region between the 48th and 53rd somite) was dissected by pancreatin (1/4 dilution in PBS) dissociation, transferred to a 10% (v/v) fetal calf serum solution in DMEM for 10 min at RT and then kept in DMEM during host preparation (10 to 15 min). Neural tube segments were grafted heterotopically and heterochronically into the cervical region flanked by somites 10 to 15 of a recipient chick embryo (HH stages 10–11). These chimeras were incubated for 24 or 48 h, and then fixed as described previously for chick embryos.

**CNT underlying notochord transplantation**

The procedure for notochord dissection was identical to that of the CNT, described above. The transplantation was performed as described by van Straaten et al. (1985).

**Caudal somites transplantation into brachial levels**

The procedure for caudal somite transplantation was similar to that of the CNT transplantation. Two rows of three caudal somites were dissected from an E5 quail embryo. After presomitic mesoderm ablation in a 10–12-somite stage chick embryo, from both sides of the embryo, the somites were grafted into the site of ablation. The surviving chimeras were fixed 24 to 36 h later.

**Retinoic acid bead implantation**

Beads (AG1-X2, BioRad) were soaked in all-trans RA (Sigma) (0.3 and 3 μM diluted in DMSO) made by agitation for 1 to 2 h before the experiment. After opening the amniotic membrane the E5 chick embryo, the tail was pulled up and a slit was made between the CNT and the neighboring somite in ovo. Beads were bisected into two halves and implanted at the CNT level in ovo, the shell was sealed and the egg incubated at 38°C. The embryos were fixed 24 to 48 h later. These concentrations lead to an induction of an ectopic ZPA in the chick limb bud when inserted at its rostral pole (data not shown).

**Immunofluorescence, in situ hybridization (ISH), and TUNEL assay**

After fixation, chick embryos and chimeras were incubated in a solution with 30% sucrose in PBS overnight,
embedded in gelatin and frozen. Serial 7–15 μm sections were then cut on a cryostat and processed for immunofluorescence, ISH, or TUNEL assay. The antibodies which were used are as follows: mouse anti-ISLET-1/2 [4D5 and 2D6; Developmental Studies Hybridoma Bank (DSHB), University of Iowa], mouse anti-SHH (5E1; DSHB), mouse anti-Nkx2.2 (74.5A5; DSHB), mouse anti-QCPN (QCPN; DSHB), mouse anti-FoxA2/HNF3beta (4C7; DSHB), mouse anti-Nkx6.1 (polyclonal antibody; a gift from Dr. Ole Ladson), rabbit anti-OLIG2 (DF308, polyclonal antibody; a gift from Dr. Charles Stiles), rabbit anti-Raldh2 (polyclonal antibody; a gift from Dr. David Anderson). The anti-HuD mouse antibody (mAb) was obtained from Molecular Probes (Catalog number A21271). The primary antibodies were detected using species-specific conjugated secondary antibodies AF488 or AF546 (Molecular Probes), diluted 1/2000 in PBS. For double staining of chimeras with Isl1 and QCPN or Not1, and Pax6/Nkx2.2, subtype-specific secondary antibodies anti-IgG1 and anti-IgG2b were used (Molecular Probes). The immunostained sections were examined using an Olympus BX60 fluorescent microscope coupled to a monochrome cooled camera (Coolsnap-Photometrics) or a Leitz TCS confocal microscope.

ISH on sections of embryos was performed as described (Ravassard et al., 1997). The Patched1 and cDelta1 riboprobes were provided by Drs. Cliff Tabin and David Ish-Horowitz respectively.

TUNEL (TdT-mediated dUTP nick end labeling) assay was performed on cryostat sections following the manufacturer instructions (Roche, Cat. No. 2 156 792). Control experiments were performed using DNase induced creation of 3'-OH terminals on DNA (data not shown).

**Results**

The most caudal domain of the avian spinal cord is devoid of motor roots, which represent the emergence of axons growing from the motoneuronal cell bodies. In order to understand this particular anatomical feature, we analyzed the presence of both MNs and their precursors at these precise levels (corresponding to the level of somites 48 to 53) that we will name here caudal neural tube (CNT).

Post-mitotic and progenitor motor neurons are absent at the most caudal level of the spinal cord

In order to analyze the presence or absence of postmitotic MNs in the caudalmost region of the spinal cord, we used fluorescence immunohistochemistry for the detection of Isl1, a transcription factor expressed in MNs after their withdrawal from the cell cycle (Ericson et al., 1992). Analyzes of its expression shows that MNs are absent in the CNT (Fig. 1B) in contrast to more rostral levels at E5.5 (Fig. 1A, arrowhead).

We then investigated the presence of MN progenitors (pMN), throughout the whole CNT, at various developmental stages after CNT formation starting at E4. For this purpose, we performed serial cross-sections from the level of somite 48 to the tip of the tail. It has been previously

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**Fig. 1.** Both post-mitotic MNs and MN progenitors are absent in the caudal neural tube (CNT), flanked by somites 48 to 53 (A–H). In the rostral neural tube, before the level of somite 48, MNs are detected by immunofluorescent staining using anti-Isl1 antibody at E5.5 (arrowhead, A). In the CNT, post-mitotic MNs are not generated at E5.5 (B) or at later stages (data not shown). MN progenitors are present in the rostral neural tube, both at E4 (data not shown) and E5 (C, E, G). MN progenitors are detected by immunofluorescent staining and co-express MNR2 (C) and Ngn2 (E). Arrows indicate MN progenitors (double stained in G). In contrast, at E5 (data not shown) or E6 (D, F, H), corresponding to 24 and 48 h after the generation of the CNT, respectively, MNs are never detected. n, Notochord.
shown that MN progenitors are characterized by the expression of MNR2, a homeodomain transcription factor, whose expression is restricted to MN committed progenitors (Tanabe et al., 1998). This transcription factor is normally expressed down to the 47th somite level in the pMN domain (Fig. 1C). In contrast, in the CNT, MNR2 is not expressed (Fig. 1D). Furthermore, Ngn2, a proneural bHLH transcription factor involved on MN specification and necessary for MNR2 upregulation (Mizuguchi et al., 2001; Novitch et al., 2001) is not expressed in the CNT (Fig. 1F) in contrast to the more rostral levels (Fig. 1E). Nuclei co-expressing MNR2 and Ngn2 are detected in rostral levels of the NT at E4 (Fig. 1G, arrows) but never in the CNT at E5 (data not shown), E6 (Fig. 1H) or at later stages (data not shown). This indicates that committed MN progenitors are absent in the CNT.

Since both postmitotic MN and MN progenitors are absent at these levels, we formulated several hypotheses that could account for this absence: (1) a defective ventralizing/inductive signaling; (2) programmed cell death events selectively eliminating MN progenitors; (3) a general lack of neurogenesis.

Ventral polarizing signals are present down to the caudalmost spinal cord

SHH is a secreted factor expressed by both the notochord and the floor plate in the vertebrate embryo. It plays a key role in the ventral patterning and in the specification of ventral spinal cord neurons, including MNs, in a concentration-dependent manner (Ericson et al., 1996, 1997a). We have observed that SHH is expressed in the floor plate and the notochord down to the tip of the tail of the E5.5 chick embryo (Figs. 2A, D).

We, then, analyzed the expression of the SHH membrane receptor Patched, which is a twelve-transmembrane protein that transduces SHH signaling, expressed in the chick neurepithelium in a decreasing ventral-to-dorsal gradient (Marigo and Tabin, 1996). We have confirmed that Patched mRNA is detected in a decreasing ventral-to-dorsal gradient down to the tip of neural tube (Figs. 2B, E). We have finally analyzed the expression of the ventral polarization marker FoxA2/HNF3beta, a transcription factor expressed by the floor plate in the chick embryo. We found that FoxA2 protein is present down to the tip of the tail of the fully elongated chick embryo both at E5.5 (Figs. 2C, F) or even later as E9 (data not shown).

The absence of MNs at the most caudal part of the spinal cord could be due to a defect in the general process of ventralization and MN induction. It has been previously shown that secreted signals emanating from both the floor plate and the notochord drive this process (Placzek et al., 1990; van Straaten et al., 1985; van Straaten et al., 1988; Yamada et al., 1991, 1993). The isochronic graft of an ectopic notochord laterally to the neural tube of an E2 chick host induces the specification of ectopic MNs at dorsal levels. In order to analyze the potentials of the notochord underlying the CNT for similar capacities, we

![Fig. 2. The CNT has the molecular conditions for motoneurons induction. The expression of Shh was assessed by immunofluorescent staining at E5.5 with anti-SHH showing it is expressed in the whole extension of the CNT (D) as well as in the rostral neural tube (A). The expression of the Shh receptor, Patched, was assessed by ISH at E5.5 (B, E). It is expressed in a decreasing ventral-to-dorsal gradient in the neurepithelium of rostral (B) and caudal (E) neural tubes. The ventralization of the neural tube was confirmed by immunofluorescent staining with the anti-FoxA2/HNF3beta antibody (a floor plate established marker, C, F). FoxA2 is expressed down to the tip of the tail in the floor plate at E5.5, including the whole extension of the CNT (C, F). In order to test the capacity of the notochord underneath the CNT on inducing ectopic MN, we used the transplantation technique. After dissecting the notochord of the CNT level from an E5 chick donor embryo, we grafted it laterally to an E1.5 chick host cervical neural tube (n = 4). In figure G, several ectopic MNs are detected by the Isl1 expression (arrows), 48 h after surgery, confirming the capacity of the CNT underlying notochord (indicated in the host by an arrowhead) to induce MN. n, Notochord. FP, floor plate.](image-url)
dissected it from an E5 chick donor and grafted it, in ovo, laterally to the neural tube (NT) of a chick host embryo at the 10-somite stage (stage 10 HH). After 24 to 48 h, the chimeras (n = 4) were fixed and analyzed for Isl1 and Not1 expression. Not1 is a specific notochord marker that allows the identification of both endogenous and grafted notochords. In Fig. 2G (arrows), ectopic MNs expressing Isl1 can be observed in the dorsal part of the NT, demonstrating that the notochord underneath the CNT is able to induce ectopic MNs.

Together, these results suggest that ventral polarization signaling, including MN specification-dependent signaling, is present in the CNT region, even in the absence of MNs.

**Programmed cell death is not eliminating motoneurons**

Another hypothesis that could explain the absence of both MNs and their progenitors could be programmed cell death events selectively eliminating these cells in the CNT. To address this, we used the TUNEL assay, which assesses the 3’-OH nicked ends formed in the DNA, one of the hallmarks in cells undergoing apoptosis. We performed the TUNEL assay on sections of the caudal spinal cord of E5, E6, and E7 embryos. Cells undergoing programmed cell death are seen in the dorsal part of the spinal cord, but only sporadically in the ventral part of the spinal cord. Consequently, programmed cell death does not account for the selective elimination of MN or their progenitors in the CNT (Fig. 3).

**Neurogenesis does occur in the caudal neural tube**

A general lack of neurogenesis could account for the lack of MNs at caudal levels. In order to address this question, we looked for the presence of neurepithelial cells exiting the cell cycle in the CNT. For this purpose, we assessed the expression pattern of cDelta1 at these levels, since cDelta1 is upregulated in neuroblasts that withdraw from the cell cycle during neurogenesis (Henrique et al., 1995). cDelta1 mRNA is expressed throughout the entire DV axis of the CNT, except in the roof plate and the floor plate, both at E5 (data not shown) and E6 (Fig. 4D). In contrast, at more rostral levels, cDelta1 expression presents a “salt and pepper” pattern in the neurepithelium (Fig. 4A). We then analyzed the fate of these post-mitotic cells. In order to further confirm that neurons could arise from these progenitors, we used the mouse antibody (mAb) HuD as a selective marker of all neuronal cytoplasms (Wakamatsu and Weston, 1997). We observed very few HuD-positive cells in the CNT at E6 (Fig. 4E; arrowheads) in contrast to more rostral levels where HuD-positive cells are present in higher numbers (Fig. 4B). In addition, neither Ngn2 (Fig. 1F) nor NeuroM (data not shown) (a bHLH protein downstream of Ngn2 expressed in newly differentiating neurons) (Roztocil et al., 1997) is expressed in the CNT at E6. However, the total number of cells expressing HuD in the CNT is dramatically increased in the E9 embryo (Fig. 4F; arrows) demonstrating that neurons other than MN are eventually generated in the caudalmost NT. These data not only rule out the possibility that a
complete lack of neurogenesis accounts for the absence of post-mitotic MN in the CNT but also suggests a delay in neurogenesis at these levels.

**MN specification code is altered in the caudal neural tube from the earlier steps of DV patterning**

The lack of MN progenitors in the CNT in the presence of inductive signaling for MN differentiation prompted us to analyze the status of dorso-ventral patterning of the NT at the caudal level in comparison to more rostral levels. The specification of the neuronal precursors, localized in the ventral spinal cord, has been shown to be mainly under the control of a transcriptional code of homeobox and bHLH transcription factors (Jessell, 2000; Lee and Pfaff, 2001). Using immunohistochemistry, we examined the expression pattern of a set of these transcription factors that were shown to be critical for MN specification. A first series of analysis was performed on E5–E5.5 embryos, a developmental timing that corresponds to the early DV patterning of the CNT.

We first analyzed Pax6 expression, a homeobox transcription factor that exhibits a ventral-low–dorsal-high gradient in the ventral spinal cord, presenting a low concentration in the MN precursors domain (Ericson et al., 1997b). This Pax6 expression pattern was confirmed for rostral levels (Fig. 5A). However, from the 48th somite caudalwards, this protein presents a decreasing rostral-to-caudal gradient of expression (Figs. 5D, G). We then analyzed the expression of Pax6, Nkx6.1, and Olig2. Pax6 expression level is progressively reduced along the CNT (D, G). Nkx6.1 and Olig2 are expressed down to the tip of the E5 chick embryo with no major change along the CNT (rostral, E, F, and caudal, H, I). Co-repressors Pax6 and Nkx2.2 expression is compared by double immunofluorescent staining (J–O) at both E5.5 (J, L, N) and E6 (K, M, O). At E5.5, the Nkx2.2 expression domain is slightly shifted dorsally, abutting the low levels of expression domain of Pax6 (L) corresponding to the MN progenitors domain, in the rostral CNT. Importantly, at more caudal levels, accompanying the disappearance of Pax6 expression, Nkx2.2 is expressed in the most dorsal part of the neural tube (N, arrows) and this domain is enlarged at later stages, E6 (O, arrows), when Pax6 expression is weakly detected. Double immunofluorescent staining for Olig2 and Nkx2.2 (P–U) shows overlapping domains. Olig2 and Nkx2.2 expression domains do not overlap at E5 or at E6 in the neural tube of rostral level (P, Q). In contrast, in the CNT, these two proteins are co-expressed from E5 (R, T, arrowheads). Confocal imaging shows a complete overlap of Nkx2.2 and Olig2 expression domains along almost the entire DV axis of the CNT at E6 (S, U, arrows). n, Notochord.
patterns of Nkx6.1 and Olig2, both of which play a critical role on MN specification (Briscoe et al., 2000; Mizuguchi et al., 2001; Novitch et al., 2001; Sander et al., 2000). None of these markers present significant differences between rostral and caudal levels (Figs. 5B, C, E, F, H, I). Thus, despite Nkx6.1 and Olig2 expression down to the last somite, MN progenitors are not generated in the CNT. These data suggest a blockade of MN differentiation after the expression onset of Nkx6.1 and Olig2.

In order to analyze the ventral boundary of the MN progenitor domain, which corresponds to the expression boundary of the co-repressors, Pax6 and Nkx2.2 (Briscoe et al., 1999, 2000), we assessed Nkx2.2 expression. At the rostral levels of the spinal cord, Nkx2.2 expression is restricted to the V3 interneuron precursor domain (p3), during the period of MN specification (Fig. 5J). In the CNT, Nkx2.2 is also expressed in the same region of the DV axis. However, it should be noted that its expression domain presents a slight dorsal shift, abutting directly the Pax6\textsuperscript{high} expression domain at E5.5 (Fig. 5L). Furthermore, at the caudal level of the CNT, several cells localized in the dorsal part of the neural tube also express Nkx2.2 (Fig. 5N, arrows) and their number increases at later stages (E6, Fig. 5O, arrows).

Since Nkx2.2 is shifted dorsally in the CNT, it was important to compare the expression patterns of Nkx2.2 and Olig2, as they do not normally overlap during MN specification (Marquardt and Pfaff, 2001; Zhou et al., 2001). The expression domains of Olig2 and Nkx2.2 partially overlap in the CNT from E5 (Figs. 5R, T, arrowheads) in contrast to more rostral levels (Fig. 5P) where the expression domains of these two proteins do not overlap. Furthermore, at E6, the overlapping expression domains are enlarged to cover almost the whole DV axis of the CNT (Figs. 5S, U, arrows) in contrast to more rostral levels (Fig. 5Q).

Together, these results suggest that the ventral patterning of the CNT is altered from the earliest steps of development, as compared to more rostral levels. This alteration is temporally dynamic since only at E6 the Nkx2.2 and Olig2 expression domains overlap completely, which is incompatible with MN specification (Zhou et al., 2001).

**Does the caudal neural tube have the potential to give rise to motoneurons in different environmental conditions?**

Our results show that despite an altered DV patterning in the CNT, including a lower expression level of Pax6, the three transcription factors that constitute the transcriptional code in an MN precursor cell (i.e., Pax6, Nkx6.1, and Olig2) are present in the CNT. Even though MNs do not arise at these levels. This observation prompted us to analyze if these precursor cells have the intrinsic potentiality to generate MN. To address this, we used the quail-chick chimera technique. A segment of E5 quail CNT, corresponding to the level of the newly formed caudalmost somites, was grafted, after endogenous neural tube removal, at the brachial level of a chick host embryo (10-somite stage), which is permissive for MN differentiation. The chimeras were incubated at 38°C and five chimeras were analyzed at 24 or 48 h after transplantation. The grafted segments of the spinal cord were detected by the QCPN mAb. Five out of the five chimeras show MNR2-positive cells (data not shown). Furthermore, these cells express Isl1, a post-mitotic MN marker (Fig. 6, arrows). Together, these results confirm that the CNT has the potential to carry out the final division cycle and generate somatic MN. This result indicates also that the environmental tissues play a crucial role in the MN differentiation blockade at the caudalmost level of the chick spinal cord.

![Fig. 6](image-url). The CNT has the potential for MN generation despite the differentiation blockade governed by the environment. The potential of the CNT to generate MNs was challenged by transplanting it into a permissive environment. The quail-chick chimera technique was used. After dissecting the CNT from an E5 quail donor embryo, it was transplanted heterotopically and heterochronically into the cervical region of a 10–12-somite stage chick host. After 48 h, post-mitotic MNs are generated in the grafted CNT (identified with an antibody against the quail specific epitope, QCPN). The MNs generated are detected with the antibody anti-Isl1 (arrows) (n = 5/5).
The somites flanking the caudal neural tube do not block MN generation

Our results show that the caudal environment plays a key role in the MN differentiation blockade. To analyze this, two hypotheses were considered: (1) an inhibitory signal emanated by the surrounding tissues of the CNT; (2) the lack of an instructive signal in the environment of the CNT, which would be present at more rostral levels. In order to analyze the environment of the CNT and the mechanism that could be involved in the MN differentiation blockade, we have studied the role of the caudal somites. It is now firmly established that the somites play an instructive role in the DV patterning of the NT and support the emergence of MNs (Bertrand et al., 2000; Diez del Corral et al., 2003; Novitch et al., 2003; Wilson et al., 2004). One of our hypotheses is that the caudal somites behave differently to the rostral ones, either by secreting somite-derived inhibitory signals or by lacking an instructive signal, as mentioned above. In order to address the former possibility, we performed in ovo transplantation of E5-quail somites flanking the CNT (caudal somites) at the presomitic mesoderm level of a chick host (10–12-somite stage). This transplantation was performed bilaterally in order to rule out the possibility of a diffusing molecule coming from the contralateral side and abolishing a hypothetical effect on MN generation. After 24 to 34 h of incubation, surviving chimeras had developed MNs (n = 4/4) despite the neighboring caudal somites, as demonstrated by Isl1 expression in Fig. 7 (arrows). These results indicate that an inhibitory signal is not secreted by the caudal somites. Thus, some instructive signal could be lacking in the MN inductive system.

The role of Retinoic Acid (RA) in the control of the DV patterning and MN generation has been recently reported (Diez del Corral et al., 2003; Novitch et al., 2003; Pierani et al., 1999; Wilson et al., 2004). To analyze the status of RA signaling at the CNT level, we used immunofluorescence to investigate the expression pattern of retinaldehyde dehydrogenase 2 (Raldh2), the RA synthesizing enzyme in the vertebrate paraxial mesoderm (Berggren et al., 1999; Diez del Corral et al., 2003; Novitch et al., 2003). We investigated the Raldh2 expression at E4 and E5, the stages of DV patterning induction in the CNT. At E4, the enzyme is expressed in the somites at rostral levels (Fig. 8A) but much less in the somites at the CNT level (Fig. 8C). At E5, the expression of Raldh2 is detected in the somite derivatives as well as in the neural tube (MNs and roof plate) at rostral levels (Fig. 8B). At the CNT level, the Raldh2 expression is restricted to the lateral part of the somite-derived dermomyotome (Fig. 8D). This observation suggests that RA does not reach the CNT to participate in MN induction, arguing in favor of a lack of instructive/inductive signaling. To further analyze this possibility, we restored RA signaling by implanting a bead soaked with RA between the CNT and the flanking somite in ovo (Fig. 8E). After 24 to 48 h, no MN were detected in surviving embryos whatever the concentration used (n = 6/6), as analyzed by the expression of MNR2 (Fig. 8F) and Isl1 (data not shown), in contrast to the CNT grafting experiments (Fig. 6) where MNR2-expressing MNs are detected 24 h after transplantation. These results suggest that, at the CNT level, RA is not sufficient, together with SHH emanated from ventral polarizing centers, to restore MN generation.

Discussion

In the present study, we have tried to determine why there is an absence of motor nerves in the caudal region of the chick embryo, corresponding to the level of somites 48 to 53. We have characterized the particular cellular and molecular features that underlie this absence at this
particular AP level. We have shown that the absence of ventral roots at these levels is due to an absence of postmitotic MNs (by using the Isl1 mAb) as well as their committed precursors (by using the MNR2 mAb), accompanied by an altered DV patterning of the CNT. The ventralizing/motoneuron-inductive machinery is set up down to the caudalmost part of the chick embryo.

We have shown that the CNT does not generate MNs or MN precursors (Fig. 1). Moreover, we have demonstrated that this is not due to a defective ventralizing/induction system, which is mainly directed by the underlying notochord (Fig. 2). The notochord has been shown to play a key role in both floor plate and MN specification, either endogenous or ectopically by grafting a supplementary notochord laterally to the neural tube (Placzek et al., 1990; van Straaten et al., 1985, 1988; Yamada et al., 1991, 1993).

The notochord underlying the CNT was examined for the same ability and we saw no difference between the notochord from rostral or caudal levels. The caudal notochord is able to induce ectopic MN when grafted laterally to a rostral neural tube mimicking the result obtained by Yamada et al. (1991). These data suggest that the property of motoneuronal induction played by the notochord is homogeneously distributed along the AP axis. Furthermore, since the caudal notochord does induce ectopic MNs at more rostral levels, it can be concluded that the CNT cannot respond to notochordal induction since no MNs are generated. Nevertheless, some ventralizing signals are active at these levels.

Among the secreted factors involved in the patterning of the ventral spinal cord, several studies indicate that the secreted factor SHH induces the specification of MNs, as well as other subtypes of ventral neurons of the embryonic spinal cord (Ericson et al., 1997a). Genetic studies, in the mouse, have shown that in the absence of Shh, MNs are not generated (Chiang et al., 1996), reinforcing the important properties inherent to SHH signaling in the ventral neurepithelium. Our results show that SHH is expressed in the notochord and floor plate at caudal levels in the chick embryo. The SHH membrane receptor, Patched, is also expressed down to the tip of the tail, as well as the downstream target FoxA2/HNF3beta (Sasaki et al., 1997), which has been extensively used as a floor plate marker (Catala et al., 2000; Ding et al., 1998; Teillet et al., 1998), indicating that the neural tube is ventrally polarized throughout the AP axis. These results indicate that even though the ventralizing signaling is assembled and functional, MNs cannot be generated at caudal levels.

Lack of temporal coordination between neuron generation and DV patterning

A suitable hypothesis to account for the absence of MNs in this region would be a general lack of neurogenesis. We searched through the CNT for the presence of post-mitotic neurons using both cDelta1 as a neuronal cell cycle withdrawal marker (Henrique et al., 1995) and HuD expression as a marker of all neuronal cytoplasms (Wakamatsu and Weston, 1997). The analysis shows that neurogenesis does occur in the CNT since at E9 the anti-HuD antibody stains several post-mitotic neurons (Fig. 4F). Nevertheless, it is striking that at E6, despite the expression of cDelta1 in the majority of the CNT cells, few of them are positive for HuD. Moreover, at these levels, the proneural protein Ngn2, as well as NeuroM, are not expressed (Fig. 1F), suggesting that in the E6 CNT, not all components of the neurogenic program are present. Indeed, the homogeneous distribution cDelta1 mRNA in the CNT at E6 (Fig. 4D), in contrast to more rostral levels (Fig. 4D), could explain the reduced number of neurons. One could hypothesize that the neurepithelial progenitors that contact with Delta1, expressed in the neighboring cell,
undergo the process of lateral inhibition and are impeded from differentiating.

Neurons are eventually generated, at E9, but a gradual decrease in neuronal number is observed in the CNT. This could be related to the decreasing rostral-to-caudal gradient of Pax6 expression. It is possible that Pax6 plays a role in the control of neurogenesis, as suggested by Bertrand et al. (2000). Moreover, it has been shown that Ngn2 elements are under the direct control of Pax6, in several regions of the DV axis and that Ngn2 expression is Pax6-dose dependent (Scardigli et al., 2001, 2003). Our results show that Ngn2 is absent in the CNT, which could account for the rostral-to-caudal gradient of Pax6 expression observed at these levels. Furthermore, Pax6 expression in the neural tube has been shown to be under the control of retinoid signaling (Diez del Corral et al., 2003) emanating from the paraxial mesoderm. Retinoid signaling does not seem to be sufficient in the CNT local environment to induce Pax6 expression (discussed below). It is possible that the low level of expression of Pax6, from the 48th somite caudalwards, is limiting for Ngn2 expression, accounting for the lack of MNR2 expression. Nevertheless, neurogenesis proceeds in the CNT since, at E9, several HuD-positive cells can be seen along the CNT DV axis (Fig. 4F, arrows). These results suggest that the neurogenic program is likely to be delayed in the CNT thus not allowing the MN generation before the alteration in DV pattern (discussed below). In these conditions, the timing for MN generation would have been overcome when neurogenesis is achieved and MNs cannot be generated. Together, these data reveal a novel physiological system that reinforces the importance of a tight temporal coordination between neurogenesis and DV patterning in the vertebrate neural tube, in order to generate adequate neurons in a given position at a given time. Furthermore, the observations into this novel physiological system are in agreement with those reported by Novitch et al. (2001) and Mizuguchi et al. (2001). The authors showed that neurepithelial progenitors expressing MNR2 are generated in higher numbers when coupling DV patterning and neurogenesis by co-expressing Olig2 (a DV patterning gene) and Ngn2 (a proneural gene).

Molecular regulation of the dorso-ventral patterning in the caudal neural tube

In the vertebrate ventral spinal cord, each neuronal progenitor domain is characterized by the concomitant expression of specific sets of transcription factors that direct the specification of each neuronal subtype (Briscoe and Ericson, 2001). The MN progenitor domain is characterized by the concomitant expression of three of these transcription factors, Pax6, Nkx6.1, and Olig2, all of which are expressed in the CNT (Fig. 5). Nonetheless, Pax6 is significantly downregulated in this region (gradual decrease from rostral to caudal in the CNT, Figs. 5A, D, G). Importantly, the decreasing gradient of Pax6 expression from rostral to caudal in the CNT is accompanied by an increasing expression of Nkx2.2, a Pax6 co-repressor (Briscoe and Ericson, 2001). At E5.5, Nkx2.2 is slightly shifted dorsally and, at very caudal levels, it is also expressed in the most dorsal cells, including the roof plate (Fig. 5N, arrows). Later, at E6, only a small region in the intermediate NT does not express Nkx2.2 (Figs. 5L–O). We assume that the pattern described above is only achieved since Pax6 presents little or no expression at these levels, thus allowing the dorsal shift in Nkx2.2 expression. However, the intermediate region of the CNT does not express Nkx2.2 at E6 (Figs. 5S, U). The reason for this inhibition is not understood but it suggests the existence of an inhibitor expressed at this precise level. The dorsal shift of Nkx2.2 expression prompted us to compare its expression domain with that of Olig2. Nkx2.2 represses the latter during MN specification, which constitutes an important step for the establishment of a precise boundary between the p3 and pMN domains (Mizuguchi et al., 2001; Novitch et al., 2001; Sun et al., 2001; Zhou et al., 2001). Our results show that the expression domains of these proteins partially overlap from the earlier steps of differentiation and patterning of the CNT (E5, Figs. 5R, T). At E6, these domains overlap completely and occupy almost the whole DV axis of the CNT, which both mimic a hyperventralization of the CNT and is incompatible with MN generation. Together, these data suggest that the alteration of DV patterning in the CNT plays a key role in the blockade of MN generation, together with the putative neurogenesis delay (discussed above).

The caudal neural tube has the potential to generate motoneurons but its environment prevents their development

The MN differentiation blockade in the CNT might be explained by two distinct scenarios: (1) an intrinsic cell programming, which does not allow the CNT progenitors to undergo MN differentiation; (2) an environmental conditioning that prevents MN differentiation. In order to analyze which of these hypotheses is responsible for the blockade, we grafted the CNT in a more rostral environment, which is permissive for MN differentiation. MNR2 and Isl1-positive cells are observed from 24 h after grafting (Fig. 6), indicating that the environment plays a role in the blockade of MN differentiation. The total number of MN generated in the grafted neural tube progressively increased during the 48 h after grafting. Hence, even if MNs do not normally differentiate in the caudalmost spinal cord, these progenitors have the intrinsic potential to generate this cell type indicating that the environment plays a role in the MN generation blockade.

The contribution of the caudal environment in the motoneuron specification

The study of the CNT environment led to an analysis of the paraxial mesoderm contribution to the blockade
Fig. 9. Model suggesting that in addition to SHH and RA, another secreted signal (X) may be involved in MN generation. Our experiments with RA-soaked beads suggest the existence of another secreted signal, in addition to SHH and RA, involved in MN generation. The CNT region has normal Shh signaling but not RA signaling. In RA gain-of-function experiments, MNs are not generated in presence of SHH and RA; thus, an additional molecule, X, lacking at caudal levels, is likely to be involved in MN specification in the vertebrate spinal cord.

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