Onset of neuronal differentiation is regulated by paraxial mesoderm and requires attenuation of FGF signalling

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SUMMARY

While many neuronal differentiation genes have been identified, we know little about what determines when and where neurons will form and how this process is coordinated with the differentiation of neighbouring tissues. In most vertebrates the onset of neuronal differentiation takes place in the spinal cord in a head to tail sequence. Here we demonstrate that the changing signalling properties of the adjacent paraxial mesoderm control the progression of neurogenesis in the chick spinal cord. We find an inverse relationship between the expression of caudal neural genes in the prospective spinal cord, which is maintained by underlying presomitic mesoderm and FGF signalling, and neuronal differentiation, which is repressed by such signals and accelerated by somitic mesoderm. We show that key to this interaction is the ability of somitic mesoderm to repress Fgf8 transcription in the prospective spinal cord. Our findings further indicate that attenuation of FGF signalling in the prospective spinal cord is a prerequisite for the onset of neuronal differentiation and may also help to resolve mesodermal and neural cell fates. However, inhibition of FGF signalling alone does not promote the formation of neurons, which requires still further somite signalling. We propose a model in which signalling from somitic tissue promotes the differentiation of the spinal cord and serves to co-ordinate neural and mesodermal development.

Key words: Chick, Spinal cord, Fibroblast growth factor, Neuronal differentiation

INTRODUCTION

It is a feature of the developing vertebrate nervous system that neuronal differentiation commences in different regions at different times and that within each region neurons do not differentiate simultaneously. Mechanisms must therefore exist to regulate when and where neuronal differentiation can take place and these help to ensure that neurons are generated at a time when factors that convey neuronal identity and guide axons to their targets are present in the differentiating embryo (reviewed by Edlund and Jessell, 1999; Jessell, 2000; Tannahill et al., 2000). The controlled production of neurons within a particular region has been studied in many vertebrates and has been shown to rely on cascades of basic helix-loop-helix (bHLH) transcription factors that promote neuronal differentiation (e.g. Ma et al., 1996; Cau et al., 1997) (reviewed by Guillemot, 1999) and on lateral inhibition, acting through the Notch-Delta-HES-1 signalling pathway, that antagonises such cascades and ensures that neighbouring cells do not differentiate simultaneously (reviewed by Lewis, 1996). However, much less is known about the mechanisms that control when a particular region of the nervous system begins to form neurons (although this has been addressed in the frog neural plate) (e.g. Brewster et al., 1998; Papalopulu and Kintner, 1996).

Distinct patterns of neuronal differentiation are strikingly apparent during the generation of the amniote spinal cord. Here neurons appear in the neural tube in a head to tail sequence (McConnell and Sechrist, 1980; Langman and Haden, 1970; Sechrist and Bronner-Fraser, 1991; Nornes and Carry, 1978; Nornes and Das, 1974) that reflects the progressive generation of this region (Brown and Storey, 2000; Mathis et al., 2001; Mathis and Nicolas, 2000). The first neurons born in the chick embryo are future reticular and spinal interneurons, which start their final round of DNA synthesis as the head fold forms, while the first future spinal motor neurons undergo their last cell cycle 6-8 hours later (McConnell and Sechrist, 1980; Sechrist and Bronner-Fraser, 1991). This sequential rostro-caudal wave of neuronal differentiation could be a consequence of a cell autonomous neural programme or it could be generated in a series of steps that are regulated by extrinsic factors.

A number of processes occur in parallel with the generation of the spinal cord including regression of the primitive streak, formation of the notochord and somitogenesis, and these adjacent tissues could provide extrinsic signals that regulate the onset of neuronal differentiation. The notochord has been shown to regulate cell type specification within the dorsoventral axis of the spinal cord (reviewed by Edlund and
Jessel, 1999) as have signals from paraxial tissue (Pierani et al., 1999) which also convey rostrocaudal pattern (Muhr et al., 1999; Grapin-Botton et al., 1997; Itasaki et al., 1996; Gould et al., 1998; Ensini et al., 1998; Pituello et al., 1999). In addition, somitic signals have also been shown to control the timing of neural crest migration (Sela-Donenfeld and Kalcheim, 2000). These data identify a role for somites in the regulation of neural pattern and differentiation, however, it is not clear how such signalling relates to the timing of neuron birth and to the progression of neuronal differentiation.

The chick posterior hindbrain and spinal cord are derived from a unique region, the caudal neural plate (CNP), which regresses alongside the primitive streak to the tail end of the embryo (Schoenwolf, 1992; Brown and Storey, 2000). CNP cells are distinguished by the expression of a number of genes including the proneural gene homologue, cash4 (Henrique et al., 1997) and the homeobox-containing gene Saxl (Spann et al., 1994). We have shown that these caudal neural genes are induced continuously by the regressing node (the anterior tip of the primitive streak) and that this node activity can be mimicked by FGF signalling (Henrique et al., 1997; Storey et al., 1998), a well established inducer of caudal neural character (reviewed by Doniach, 1995; Ribisi et al., 2000). By misexpressing cash4 in fly and frog embryos we have also shown that this gene promotes neural cell fates (Henrique et al., 1997) and the cellular context in which it is expressed in the chick suggests that cash4 mediates neural specification steps within the CNP (Brown and Storey, 2000). This possibility is supported by fate maps of this cell population which show that it forms neural tissue, but also that cells migrate out of the CNP and contribute to epidermal or mesodermal tissue (Brown and Storey, 2000). In contrast, cells derived from this region, but now located above the node, all contribute to the neural tube (Brown and Storey, 2000; Selleck and Bronner Fraser, 1995; Mathis et al., 2001). Cells in the CNP may therefore be specified, but not yet committed to a neural fate and, consistent with this, express early pan neural genes, such as Sox2 and 3 (Streit et al., 1997) (our observations) as well as genes that are also characteristic of mesodermal tissues, e.g. Brachyury (bra) (Kispert et al., 1995; Storey et al., 1998).

cash4 and Saxl persist in the CNP, but are both strikingly down regulated as cells form the neural plate/neural tube above the level of the primitive streak (Henrique et al., 1997; Spann et al., 1994) and it is here that the first neurons in the caudal nervous system are born (Sechrist and Bronner-Fraser, 1991). These post-mitotic cells express Delta 1, the ligand that mediates lateral inhibition via stimulation of the Notch pathway and thereby prevents the differentiation of neighbouring cells (Henrique et al., 1995). Within the later pathway and where neuronal differentiation takes place in the spinal cord. Conversely, the presomitic mesoderm represses neuronal differentiation and also maintains caudal neural genes in overlying tissue. Thus, a fine balance between somitic and presomitic signals controls when and where neuronal differentiation takes place in the spinal cord. We further identify FGF signalling as a key pathway regulating these events: we show that FGF mimics the ability of the presomitic mesoderm to maintain caudal neural genes and repress neuronal differentiation and that the somites strikingly attenuate Fgf8 transcription in the prospective spinal cord. Our findings suggest that a decrease in FGF signalling is an initial step in the differentiation of the spinal cord, which may help to resolve cell fates in this tissue and which is required, together with additional somite signalling, to promote neuronal differentiation.

MATERIALS AND METHODS

Embryo culture, grafting and mesoderm removal

Fertile hen’s eggs (High Sex × Rhode Island Red; Winter Farm, Thirplow, Herts) were incubated at 38°C for appropriate periods to yield embryos of stages 6-9 (Hamburger and Hamilton, 1951). Host embryos were set up and maintained in New culture as described previously (Storey et al., 1992) or in EC culture (Chapman et al., 2001). Following brief exposure to 0.1% trypsin in PBS the presomitic mesoderm was removed from beneath the CNP in stage 7-8 embryos, and 4-5 somites were removed from stage 8-10 embryos.

FGF beads

Heparin-coated beads (Sigma) were washed in PBS and soaked in 50 
μg/ml mouse FGF8b (R&D Systems) or human FGF4 (a gift from J. K. Heath, or R&D Systems) or PBS (see Storey et al., 1998) and implanted beneath the CNP, adjacent to the primitive streak in stage 4+ to 7+ embryos.

In vitro explant culture

CNP explants (400×300 μm2) were derived from stage 6-8 embryos (Fig. 2A), PNT explants (300×100 μm2) from stage 8-9 embryos (Fig. 5E) and neural tube explants (200×100 μm2) from HH9 (Fig. 5E), after brief treatment with 0.1% trypsin. These tissues were then cultured in collagen at 37°C in 5% CO2 in Optimum supplemented with foetal calf serum, glutamine and antibiotics using standard procedures (Placzek and Dale, 1999). Each explant was processed and scored individually and in most cases control and experimental explants were derived from the same embryo. Somites and presomitic mesoderm explants from stage 6-8 were removed following brief trypsin treatment. Two somites (the most recently formed somites at stage 7+8–) were combined with single CNP explants. Explants were treated with human FGF4 at 200 ng/ml or mouse FGF8b at 330 ng/ml (R&D Systems) in the presence of heparin (100 ng/ml). FGF signalling was inhibited with SU5402 in DMSO (Calbiochem) added to medium at 2.5 μM or 5 μM. To assess specificity of response to FGF some explants were pre-incubated with SU5402 for 20 minutes at room temperature before the addition of medium containing both FGF and SU5402. The MAP kinase pathway was blocked using
PD184352 in DMSO added to medium at 2 μM (a gift from P. Cohen) while control contralateral CNP were only treated with DMSO.

In situ hybridisation, immunocytochemistry and BrdU treatment

Standard methods for cyro-sectioning (15 μm) and whole-mount in situ hybridization technique were used. Automated in situ hybridisation was carried out on explants using a robotic InsituPro machine. Neurofilament protein was detected using the monoclonal antibody 3A10 (Furley et al., 1990) (obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, John Hopkins University School of Medicine, Baltimore, MD and the Department of Biological Sciences, University of Iowa, Iowa City, IA) and used as described (Storey et al., 1992). After incubation for 30-60 minutes in 100 μl 0.1 mM BrdU embryos were fixed and processed for BrdU detection as described previously (Henrique et al., 1995).

RESULTS

NeuroM onset coincides with somitogenesis

The onset of neuronal differentiation in the chick spinal cord was investigated by monitoring the expression pattern of NeuroM, a gene characteristic of post-mitotic cells in later neural tube (Roztocil et al., 1997). We find that the first NeuroM-expressing cells appear at stage 7+/8- in the neural plate/tube opposite the first formed somites (Fig. 1A-I). This is shortly after the appearance of single Delta 1-positive cells in this region, which identifies the first born neurons (Henrique et al., 1995; Sechrist and Bronner-Fraser, 1991) and underscores the observation that neuronal differentiation does not take place in the CNP, which expresses cash4 and Sax1 (Henrique et al., 1997; Spann et al., 1994) and genes characteristic of early mesodermal tissue, bra and Delta 1 in a homogeneous domain (Kispert et al., 1995; Henrique et al., 1995) (Fig. 1A-J-L). To confirm that this early phase of NeuroM expression is also characteristic of post-mitotic neurons we tested whether such cells undergo DNA synthesis by assessing BrdU incorporation in NeuroM-expressing cells. BrdU was not found in the majority of such cells (642/670 cells (96%) in 5 embryos of stages 9-13, following 30-60 minutes exposure to BrdU) strongly suggesting that NeuroM-positive cells have left the cell cycle (Fig. 1M-P).

As somites begin to form a new region can be defined (which we have named the ‘preneural tube’; PNT) that lies above the level of the node/primitive streak and adjacent to the emerging notochord, but below the most recently formed somite (Fig. 1B). Consistent with previous work showing that NeuroM is expressed after Delta 1 in the later neural tube (Roztocil et al., 1997), we observe here that NeuroM expression lags behind that of Delta 1 as the spinal cord is laid down in a head to tail sequence. While the PNT expresses bra, cash4, Sax1 and Delta 1 (in single cells) we show that NeuroM expression is never detected in this region (Fig. 1F,H) (cash4 and Sax1 are expressed in both CNP and PNT and not mesodermal tissues and are hereafter referred to as caudal neural genes). These observations indicate that the events of neurogenesis become increasingly spatially separated as the spinal cord is laid down. As NeuroM is characteristic of cells undergoing neuronal differentiation this striking coincidence of somitogenesis and NeuroM onset raises the possibility that somitic signals regulate neurogenesis progression.

CNP explants retain early neural characteristics and do not undergo neuronal differentiation

To test whether the onset of neuronal differentiation is regulated by extrinsic signals we assessed the specification of the CNP (as this region does not contain neurons in vivo) and the requirement for signals from neighbouring tissues for its differentiation. We first examined the gene expression profile of explants of this tissue from stage 6-8 embryos (Fig. 2A). CNP explants cultured alone for 18 or 24 hours continue to express the pan neural gene Sox2 (7/7) as well as cash4 (15/15), Sax1 (11/11), bra (12/13) and homogenous Delta 1 (7/7) (Fig. 2B-F) and only occasionally contain cells expressing paraxis, a gene transcribed as the paraxial mesoderm differentiates into somites (Barnes et al., 1997) (2/8) (data not shown). In addition, such explants fail to express NeuroM (36/39) and do not contain isolated Delta 1-expressing cells (6/7) (Fig. 2E,G), nor morphologically distinct neurons (as identified by the presence of fine processes accumulating neurofilament) (8/8) (Fig. 2H). This contrasts with explants of PNT which contain NeuroM-positive cells after only 8 hours in vitro (4/4, data not shown). CNP explants thus retain expression of genes characteristic of early neural tissue, do not routinely contain somitic mesoderm and do not undergo neuronal differentiation and are thus a useful assay for the identification of tissues and factors that regulate the progression of neurogenesis in the spinal cord.

Somitic tissue promotes neuronal differentiation

Strikingly, CNP explants cultured with the underlying presomitic mesoderm do undergo neuronal differentiation within 24 hours, as indicated by the presence of isolated Delta 1-expressing cells (4/5) (Fig. 2L) and NeuroM-positive cells (16/20) (Fig. 2K,L). This presomitic mesoderm cultured either alone (13/13) (Fig. 2M) or in contact with the CNP (8/8) (data not shown) comes to express paraxis after 24 hours. As this gene is characteristic of somitic tissue (Barnes et al., 1997) we further tested whether somites also induce NeuroM expression. CNP explants cultured with somites contain NeuroM-positive cells (16/19) (Fig. 2N,O) and also possess cells with fine processes accumulating neurofilament (4/8) (Fig. 2P). CNP cultured with the underlying presomitic mesoderm also down regulates cash4 (9/11) (Fig. 2Q, compare with Fig. 2C). The effects of differentiating presomitic mesoderm or somites on Sax1 expression, however, proved difficult to assess in this assay as this gene is expressed after cash4 and is not always present at the time of explant excision (Spann et al., 1994). Sax1 expression was not consistently altered by the presence of underlying presomitic mesoderm (n=14, data not shown) or by addition of a somite (n=6, data not shown). Together, these findings show that somitic signals promote neuronal differentiation and concomitantly repress at least one caudal neural gene.

Are somitic signals required for neuronal differentiation?

To ascertain whether CNP explants eventually form neurons in the absence of signals from other tissues we cultured them for longer periods of time. NeuroM-expressing cells are detected in CNP explants cultured for 48 hours (22/39) (Fig. 2R)
demonstrating that neurogenesis does progress within this tissue. This may indicate an intrinsic tendency to form neurons, however, some CNP explants express paraxis after 48 hours (22/32, containing at least 2-4 positive cells; Fig. 2S) raising the possibility that differentiating somitic tissue elicits neuronal differentiation in these longer-term cultures. This idea is supported by double in situ hybridization experiments detecting both paraxis and NeuroM (n=23), which revealed that paraxis negative explants (5/23) do not contain NeuroM-positive cells (5/5, data not shown).

To address whether somitic signals are required in vivo for neuronal differentiation we next generated embryos in which the neural tube forms without the underlying paraxial mesoderm and thus develops without exposure to somites. To achieve this the presomitic mesoderm was removed from beneath the CNP in stage 7-8 embryos and these were then cultured for 5-6 hours, by which time the node has regressed still further and up to 4 additional somites have formed on the contralateral non-operated side (Fig. 3A). Only a few NeuroM-positive cells are found in neural tube developing in the absence of somites (10/10) (Fig. 3B,C), while, in contrast, expression of the pan-neural marker Sox2 remains unaltered (4/4) (Fig. 3D,E), indicating that this operation is not generally deleterious. These findings therefore suggest that the onset of neuronal differentiation is impaired in the absence of somitic signals. Further, unilateral removal of the somites S2-S5 from 5 somite embryos (Fig. 3F) also results in a dramatic reduction in the number of NeuroM-positive cells in the neural tube after 6 hours (5/5) (Fig. 3G,H) while expression of Sox2 (4/4) and Delta 1 remain unaltered (4/4) (Fig. 3I-L). This indicates a continuing requirement for somite signals for neuronal differentiation in the early neural tube.

**Progression of neurogenesis is repressed by the presomitic mesoderm**

Strikingly, premature activation of NeuroM was additionally observed in the PNT in 3/7 cases in which presomitic mesoderm was still absent beneath this region following 6 hours culture (Fig. 3M,N). This observation suggests that during normal development neuronal differentiation is repressed in the CNP and PNT by signal(s) from the underlying presomitic mesoderm.
paraxial mesoderm. Removal of the paraxial mesoderm from beneath the CNP in vivo also leads to local loss of *cash4* (5/6) (Fig. 3O,P) and *Saxl* (4/4) (Fig. 3Q,R) following a 6-hour incubation. Together these findings further confirm the relationship between the loss of genes expressed in the CNP/PNT and the onset of neuronal differentiation and indicate that signals from the paraxial mesoderm maintain expression of CNP genes and repress neuronal differentiation.

**FGF signalling maintains caudal neural genes and represses neuronal differentiation**

What is the identity of the signals provided by the paraxial mesoderm? We have shown previously that FGF signalling is a key pathway involved in the induction of *cash4* and *Saxl* (Henrique et al., 1997; Storey et al., 1998). Cells in the CNP are exposed to many FGFs from the primitive streak (including FGFs 4 and 8) and *Fgf8* expression spreads into the paraxial mesoderm, CNP and PNT (Walshe and Mason, 2000; Storey et al., 1998; Mahmood et al., 1995; Shamin and Mason, 1999; Ohuchi et al., 2000; Bertrand et al., 2000) (see also Fig. 6C). *Sprouty2* (an indication of FGF8 activity) (Minowada et al., 1999) is also expressed in the paraxial mesoderm and extends into the PNT (Chambers and Mason, 2000). We show here that prolonging exposure to FGF signalling by implanting FGF4-soaked beads beneath the CNP (in stage 4+ to 7+ embryos and subsequent culture to stage 9-12; approx. 12 hours) leads to local ectopic maintenance of *cash4* (4/4) and *Saxl* (4/4) in the closed neural tube, while control PBS beads have no effect (5/5, 3/3) (Fig. 4A-E) (see also Bertrand et al., 2000). Given the inverse regulation of caudal neural genes and *NeuroM* these findings raised the possibility that exposure to FGF also inhibits neuronal differentiation. Indeed FGF4- or FGF8-soaked beads placed beneath the CNP (as described above) leads to a reduction of *NeuroM* expression in the neural tube (FGF4 4/4, FGF8 9/11) while PBS beads do not alter *NeuroM* expression (5/6) (Fig. 4F,G).

FGF presented on beads could affect many different cell types in the embryo and so to determine where FGF acts to repress neuronal differentiation we next treated explants of defined cell populations with FGF4. Explants consisting of the CNP and underlying paraxial mesoderm show a dramatic reduction in the number of *NeuroM*-expressing cells present after 24 hours (7/7) (Fig. 5A,B) and 48 hours (12/12, data not shown), as do CNP explants cultured alone with FGF4 for 48 hours (7/7) (Fig. 5C,D). Exposure to FGF4 also inhibits neuronal differentiation in PNT after 24 hours (7/7) (Fig. 5E-G). Furthermore, this effect can be prevented by the presence of the FGFR antagonist SU5402 (Mohammadi et al., 1997).

![Fig. 2. Somites elicit neuronal differentiation in CNP explants.](image-url)

(B-H,J,L,O-Q are tissue sections.) (A) Diagram of CNP explant in a stage 7+ embryo. (B-H) CNP explants continue to express *Sox2* (B), *cash4* (C), *Saxl* (D) *bra* (E) and homogenous *Delta1* (F), but do not express *NeuroM* (G) or contain cells with neurofilament (NF)-positive fine processes (H). (I-L) CNP explants cultured with differentiating PSM contain isolated *Delta1*-positive cells (I,J) and *NeuroM*-expressing cells (K,L), and this paraxial mesoderm differentiates into *paraxis*-expressing tissue (M) (note, *NeuroM*-positive cells in K,L appear at one end of the explant and are likely to be underlain by *paraxis*-positive cells, as seen in M). (N,O) CNP explants cultured with somites also express *NeuroM* and (P) contain cells with neurofilament-positive fine processes (arrowhead). (Q) *cash4* is downregulated in CNP explants cultured with differentiating PSM. (R) CNP explants cultured alone express *NeuroM* after 48 hours in culture and (S) in some cases contain *paraxis*-positive cell. Scale bars, (B-H,J,L,O,P) 50 μm; (I,K,M,N,R,S) 100 μm; (Q) 100 μm.
FGF signalling has been shown to induce early mesodermal genes, such as bra (Isaacs et al., 1994; Storey et al., 1998) which is expressed in the CNP and extends into the PNT (Kispert et al., 1995) and one explanation for the inhibition of neuronal differentiation in these FGF-treated explants could, therefore, be that all cells are transformed into mesoderm. To test this possibility PNT explants were assessed for bra expression with and without FGF treatment. In all cases untreated explants down regulated bra within 24 hours (6/6) (Fig. 5H) and also failed to express the somitic tissue marker paraxis (6/6, data not shown), indicating that the PNT does not normally form mesoderm. Following FGF treatment, only a small patch of bra expression is maintained, indicating that the whole PNT is not transformed into mesoderm after 24 hours (5/5) (Fig. 5I). This finding leaves open the possibility that FGF could still be acting indirectly by maintaining an early mesodermal cell population, which provides signals that in turn repress neuronal differentiation. We therefore finally tested whether NeuroM expression in neural tube explants (Fig. 5E) (which do not express bra, or up regulate this gene on treatment with FGF4 11/11, data not shown), is altered in the presence of FGF4. In all cases NeuroM expression is reduced or lost in FGF treated neural tube after 24 hours (8/8) (Fig. 5J,K), indicating that FGF signals can act directly on neuroepithelial cells to repress neuronal differentiation.

Somites suppress Fgf8 transcription in the CNP

These findings suggest that somitic signals that promote neuronal differentiation must act at least in part by attenuating FGF signalling. We thus next assessed the ability of somitic mesoderm to down regulate Fgf8 expression in CNP explants. Such explants cultured alone express Fgf8 (25/25) after 18-24 hours (Fig. 5L). In contrast, CNP explants cultured for 18-24 hours with differentiating presomitic mesoderm all down regulate Fgf8 (7/7) (data not shown) while culture with somites can completely abolish Fgf8 in the CNP and results in a dramatic reduction of Fgf8 transcripts in all cases (10/10) (Fig. 5M). Thus, somites provide signals that repress Fgf8, identifying this paraxial tissue as a source of signals able to attenuate FGF signalling in the prospective spinal cord.

Inhibition of FGF signalling is not sufficient to elicit neuronal differentiation

Do somites promote neuronal differentiation by simply removing FGF signalling or do they also interact with further signalling pathways to regulate caudal neural genes and the formation of neurons? To test this, CNP explants were treated with the FGFR antagonist SU5402 which acts at the conserved

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**Fig. 3.** Removal of the presomitic mesoderm elicits premature neuronal differentiation and the down regulation of CNP-specific genes. (C,E,H,J,L,N,P,R are transverse sections.) (A) Diagram illustrating removal of PSM at stage 7+ and the embryo 6 hours later. (B,C) Depletion of NeuroM-expressing cells in neural tube developing following PSM removal. S1, most recently formed somite on un-operated side. (D,E) Sox2 expression is unaffected by PSM removal. (F) Diagram of the removal of somites at stage 8/8+ and the embryo 6 hours later. (G,H) Depletion of NeuroM-expressing cells following somite removal. S, first somite formed after removal on operated side. (J) Sox2 and (K,L) Delta 1 are unaffected by somite removal. (M,N) NeuroM expression extends into the PNT in the absence of PSM. (O,P) Removal of PSM also down regulates cash4 and (Q,R) Sax1 in the PNT. Tissue between arrowheads in B,G,M,O and Q are regions of ectopic (M) or absent gene expression (B,G,O,Q). Scale bars: (B,D,G,I,K,M,O,Q) 100 μm; (C,E,H,J,L,N,P,R) 100 μm.
ATP binding site of FGFR1 and is therefore likely to inhibit signalling through all FGF receptors (Mohammadi et al., 1997). CNP explants exposed to this drug for 24 hours down regulate bra (12/13) (Fig. 5N,O), a well known FGF-regulated gene (Isaacs et al., 1994). This antagonist, however, does not alter expression of Sox2 (4/4) nor does it lead to a consistent increase in the number of NeuroM-expressing cells (n=13) (Fig. 5P-R). Further, PNT explants which express NeuroM after 8 hours, but not after 4 hours (5/5, data not shown), fail to exhibit precocious NeuroM expression after 4 hours culture with SU5402 (5/5, data not shown). In confirmation of these findings, treatment of CNP explants for 18 or 24 hours with PD184352 (a drug that can inhibit cell proliferation (Sebolt-Leopold et al., 1999) and which blocks the MAP kinase pathway downstream of FGF signalling by specifically suppressing activation of MKK1 (Davies et al., 2000), does not alter NeuroM expression in CNP explants (n=12) (data not shown). Thus, blocking FGF signalling represses the early mesodermal gene bra, but does not alter expression of the pan-neural gene Sox2, nor is it sufficient to promote neuronal differentiation in the CNP or PNT.

**DISCUSSION**

We show that neuronal differentiation begins in the caudal nervous system coincident with somitogenesis and that this pattern is controlled by the changing signalling properties of the paraxial mesoderm. A key molecular mechanism mediating this process is the attenuation of FGF signalling by somitic signals. Loss of FGF signalling is a prerequisite for neuronal differentiation and may also help to resolve early neural and mesodermal cell fates in the prospective spinal cord, but additional, somitic signalling is also required to elicit neuronal differentiation. These findings show that signals from the differentiating paraxial mesoderm regulate the differentiation of the spinal cord and may thus serve to co-ordinate neural and mesodermal development.

**Changing signalling properties of the paraxial mesoderm regulate the onset of neuronal differentiation**

We present two lines of evidence that strongly suggest that signals from the differentiating somitic mesoderm regulate the onset of neuronal differentiation in the developing spinal cord: (1) somitic signals accelerate the appearance of neurons in CNP explants, as indicated by the swift onset of Delta1 in single cells, NeuroM expression and the appearance of cells with neurofilament-positive fine processes; and (2) these signals are also required in vivo for the normal onset of neuronal differentiation, as revealed by the strikingly few NeuroM-positive cells in neural tube forming in the absence of the differentiating somitic mesoderm. Removal of somites flanking the later neural tube also depletes the number of NeuroM-expressing cells, while Sox2 and Delta1 expression remain unaltered, indicating that there is a continuing requirement for somite signals for neurogenesis progression. However, it is likely that somites become dispensable for the production of neurons at later stages, as their removal at stages 12-16 does not alter the number of motor neurons (Martin, 1971). This suggests that the influence of somite-derived factors is confined to the first born neurons (future reticular and spinal interneurons). As explants of the neural tube readily form neurons in vitro (this study; also see explant [i]) (Yamada et al., 1993; Pons and Marti, 2000) this requirement for somite signals in vivo suggests that they normally act to oppose other signals present in the neural tube that repress neuronal differentiation (see Fig. 6A).

We also present evidence that somitic signals repress the caudal neural gene cash4, consistent with its down regulation in vivo prior to neuronal differentiation. Sax1 expression was not consistently altered by such signals in our assay. This apparent difference in regulation may reflect the later onset of Sax1 expression (see Spann et al., 1994) or its regulation by other signals in the embryo. Indeed, removal of the presomitic mesoderm in vivo leads to the loss of cash4 and Sax1, suggesting that during normal development repression of caudal neural genes is mediated by a combination of signal loss, as the presomitic mesoderm differentiates, and exposure to signals from the newly formed somites. In this context it is striking that removal of the presomitic mesoderm also leads to the precocious appearance of NeuroM-positive cells in the PNT in a small number of cases. This suggests that the presomitic mesoderm not only maintains caudal neural genes, but also represses neuronal differentiation. This in vivo situation contrasts with CNP explants cultured alone in vitro (for 24 hours), which maintain cash4 and Sax1 and do not contain neurons. This difference may be explained if mesoderm cells in CNP explants (which in vivo would have been displaced during gastrulation) provide signals that maintain caudal neural genes and repress NeuroM. In addition other tissues/signals
may be present in the embryo, which normally oppose signals from the presomitic mesoderm and which act swiftly following removal of this tissue in vivo. These opposing signals could be provided by the notochord/floor plate at the ventral midline and/or abutting NeuroM-positive spinal cord (which has been exposed to somites). Together, these findings indicate that the onset of neuronal differentiation in the spinal cord is regulated by a balance between signals provided by the presomitic mesoderm and the somites and that both these tissues may act in the embryo to counter opposing signals (Fig. 6A). Evidence for the involvement of somite signalling in neuronal differentiation is also found in other vertebrate embryos. The appearance of Islet1-expressing primary neurons in Zebrafish commences before notochord formation, adjacent to the first forming somites (Korzh et al., 1993). Although in the frog some neurons differentiate before somitogenesis (e.g. Lee et al., 1995) it is interesting that neurons only appear in response to the neural inducer Noggin in circumstances when both neural tissue and muscle are formed (Lamb et al., 1993). The ability of somitic tissue to regulate neuronal differentiation may therefore be conserved across species.

**Reduction of FGF signalling initiates differentiation of the spinal cord**

Our experiments involving activation or repression of FGF signalling suggest that removal of FGF signalling is a prerequisite for neuronal differentiation and that it may also help to resolve neural and mesodermal cell fates in the prospective spinal cord (Fig. 6B,C). A first step in the differentiation of the spinal cord is the movement of cells out of the CNP, where some cells are fated to form mesodermal as well as neural tissue (Brown and Storey, 2000) and into the PNT, where they are now all fated to form neural tissue (Selleck and Bronner-Fraser, 1995; Mathis et al., 2001). This transition thus involves a loss of mesodermal potential, as reflected by the progressive down regulation of bra and the persistence of neural genes as cells move away from the FGF-expressing primitive streak. We show here that FGF treatment of PNT explants maintains a patch of bra-positive cells (see also Isaacs et al., 1994; Umbhauer et al., 1995; LaBonne et al., 1995; Dubrulle et al., 2001), and that blocking FGF signalling in the CNP leads the loss of bra, but does not alter expression of the pan-neural gene Sox2. This suggests that attenuation of FGF signalling may lead to the loss of mesodermal potential in the prospective spinal cord and that this could be a first step in the differentiation of caudal neural tissue. This idea that FGF signalling influences neural versus mesodermal cell fate decisions in the caudal neural plate appears to be supported by observations in chimeric mice in which epiblast cells mutant for FGFR1 fail to ingress through the primitive streak to form mesoderm and instead generate ectopic neural tissue (Deng et al., 1997; Ciruna et al., 1997; Ciruna and Rossant, 2001). Furthermore, mice lacking a functional Tbx6 gene form ectopic neural tubes at the expense of caudal paraxial mesoderm, indicating on-going neural versus mesodermal cell fate decisions at relatively late stages of development (Chapman and Papaioannou, 1998).

We also show that exposure to FGF signalling maintains caudal neural genes and blocks neuronal differentiation in the developing spinal cord in vivo and that such signalling suppresses this process in explants of the CNP, PNT and neural
The neural tube in contact with somites contains *NeuroM*-expressing cells (purple dots) while the CNP/PNT underlain by the PSM does not express this neuronal marker and expresses caudal neural genes. Somites provide signals that repress caudal neural genes and activate neuronal differentiation, whereas the PSM provides signals that maintain caudal neural gene expression and prevent neuronal differentiation. Removal experiments indicate that signals from other tissues in the embryo must also influence the appearance of neurons.

Somites repress Fgf8 expression in CNP/PNT. Attenuation of FGF signalling down regulates the early mesodermal gene *bra* but does not elicit neuronal differentiation (*NeuroM*); somites must therefore also interact with additional pathways to promote *NeuroM* either by inducing an activator (dashed line) and/or repressing a repressor (see text).

(Fig. 6A) Tissue level regulation of neuronal differentiation by paraxial mesoderm and levels of FGF signalling. (A) Tissue level regulation of neuronal differentiation. The neural tube in contact with somites contains *NeuroM*-expressing cells (purple dots) while the CNP/PNT underlain by the PSM does not express this neuronal marker and expresses caudal neural genes. Somites provide signals that repress caudal neural genes and activate neuronal differentiation, whereas the PSM provides signals that maintain caudal neural gene expression and prevent neuronal differentiation. Removal experiments indicate that signals from other tissues in the embryo must also influence the appearance of neurons.

(B) FGF signalling and the regulation of spinal cord differentiation. FGF8 and FGF4 are present in the primitive streak and FGF8 is also present in the PSM and the CNP/PNT where these signals promote *bra* and caudal neural genes and prevent the appearance of *NeuroM*-positive cells. Somites repress Fgf8 expression in CNP/PNT. Attenuation of FGF signalling down regulates the early mesodermal gene *bra* but does not elicit neuronal differentiation (*NeuroM*); somites must therefore also interact with additional pathways to promote *NeuroM* either by inducing an activator (dashed line) and/or repressing a repressor (see text). (C) Fgf8 expression in stage 9–embryo, showing transcripts in the CNP and PNT, aligned with B. Scale bar: 100 μm.

Recent findings further show that cells made deaf to FGF signalling in the CNP are more rapidly displaced from this region into the neural tube and suggest that this may be due to changes in cell adhesion properties (Mathis et al., 2001). The latter may also account for changes in cell behavior as cells move from the PNT to the neural tube where the first *NeuroM* cells appear opposite the somites. Comparison of the movements of neural precursors in the PNT and neural tube reveal increased restriction in cell movement in the neural tube (Stern et al., 1991; Mathis et al., 2001) and this nicely correlates with *NeuroM* expression suggesting that the onset of neuronal differentiation involves an increase in cell adhesion. In this context, it is striking that *Pax6* expression in the neural tube also coincides with the onset of somitogenesis (Pituello et al., 1999). This transcription factor is induced, like *NeuroM*, by somitic signals (Pituello et al., 1999) and is repressed by the presomitic mesoderm and FGF signalling (Bertrand et al., 2000). However, in contrast to effects on *NeuroM* in CNP explants (discussed below), inhibition of the FGF pathway does up regulate *Pax6* in the embryo (Bertrand et al., 2000) and this gene may thus mediate an earlier step in neuronal differentiation that is a direct consequence of the loss of FGF signalling. The significance of the initial widespread *Pax6* expression in the spinal cord is, however, unknown. This gene mediates cell type specification within the later spinal cord (e.g. Ericson et al., 1997) but analyses of other regions of the CNS in *Pax6*-deficient mice also indicate a role in increasing cell adhesion (Chapouton et al., 1999; Stoykova et al., 1997) and the timing of neuronal differentiation, although the direction of this regulation shows regional variation (Götz et al., 1998; Warren et al., 1999; Warren and Price, 1997; Sun et al., 1998; Scardigli et al., 2001).

**Somites attenuate FGF signalling, but this is not sufficient to elicit neuronal differentiation, which requires still further somite signalling**

A major finding of this study is that somitic signals repress Fgf8 transcription in the CNP and we have tested the possibility that inhibition of the FGF pathway mimics the somite’s ability to promote neuronal differentiation. However, blocking FGF signalling in the CNP or PNT did not accelerate neuronal differentiation and nor did treatment with a specific MAPK inhibitor, indicating that further somite signalling is required for this step. Perhaps consistent with this, it has been shown in the
frog neural plate that simply blocking the cell cycle is not sufficient to elicit neuronal differentiation (Ohnuma et al., 2001). Additional somite signalling must therefore either promote expression of an activator, or repress a further repressor of neuronal differentiation in the prospective spinal cord (Fig. 6B).

Significantly, it is possible that the loss of Fgf8 in the paraxial mesoderm itself is also regulated by somitic signals. Attenuation of FGF signalling in the presomitic mesoderm has recently been shown to be required for mesodermal differentiation and to be a key step in the definition of a ‘determination front’ that specifies somite boundaries and axial identity (Dubrulle, 2001; Zakany, 2001). Thus, the dependence of neural as well as mesodermal differentiation on attenuation of FGF levels by somitic signals may be part of a mechanism that co-ordinates the development of these two tissues. Importantly, Fgf8 expression in the PNT diminishes before the appearance of the last formed somite (Fig. 6C), suggesting that the ability to repress Fgf8 transcription is gradually acquired by the differentiating (rostral) presomitic mesoderm prior to somitogenesis. This is consistent with the position of the “determination front” in the presomitic mesoderm and with our finding that neuronal differentiation does not only rely on a reduction of FGF levels but also on a further consequence of somite signalling. This additional requirement may thus ensure that neurons differentiate when the somites are presenting cues that specify axial position and neuronal cell type within the neural tube (e.g. Itasaki et al., 1996; Ensini et al., 1998) and may thereby serve to co-ordinate the onset of neuronal differentiation with the assignment of cell identity.

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