Development 129, 1681-1691 (2002)
Printed in Great Britain © The Company of Biologists Limited 2002
DEV1772

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

Ruth Diez del Corral[1], Dorette N. Breitkreuz[2] and Kate G. Storey[1,*]

[1]Division of Cell and Developmental Biology, School of Life Sciences, University of Dundee, Wellcome Trust Biocentre, Dow Street, Dundee, DD1 5EH, UK
[2]Department of Human Anatomy and Genetics, University of Oxford, South Parks Road, Oxford OX1 3QX, UK
*Author for correspondence (e-mail: k.g.storey@dundee.ac.uk)

Accepted 8 January 2002

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

Jessell, 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 *Sax1* (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 *Sax1* 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 neural tube post-mitotic cells migrating out of the *Delta 1*-expressing ventricular zone have been shown to transiently express the basic helix-loop-helix (bHLH) transcription factor *NeuroM* (Roztocil et al., 1997). This gene is a homologue of the fly proneural gene, *atonal*, and its onset marks the progression of the neuronal differentiation pathway following down regulation of *Delta 1* (Roztocil et al., 1997). Thus, the dynamic changes in the rostrocaudal pattern of gene expression that accompany the laying down of the spinal cord appear to reflect a progressive change in cell state as cells leave the CNP where the first steps in neural specification take place, and form the neural plate/tube, where they can now exit the cell cycle and undergo neuronal differentiation.

Here we examine the role of extrinsic factors specifically in the regulation of the onset of neuronal differentiation in the chick spinal cord. We show that the appearance of *NeuroM*-expressing neurons strikingly coincides with the onset of somitogenesis in the adjacent paraxial mesoderm. Using a combination of in vitro and in vivo approaches we demonstrate that somitic tissue is required for the progression of neurogenesis 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+/–9 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 μm^2) were derived from stage 6-8 embryos (Fig. 2A), PNT explants (300×100 μm^2) from stage 8-9 embryos (Fig. 5E) and neural tube explants (200×100 μm^2) from HH9 (Fig. 5E), after brief treatment with 0.1% trypsin. These tissues were then cultured in collagen at 37°C in 5% CO_2 in Optimem 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 CNPs were only treated with DSMO.

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. 2F,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

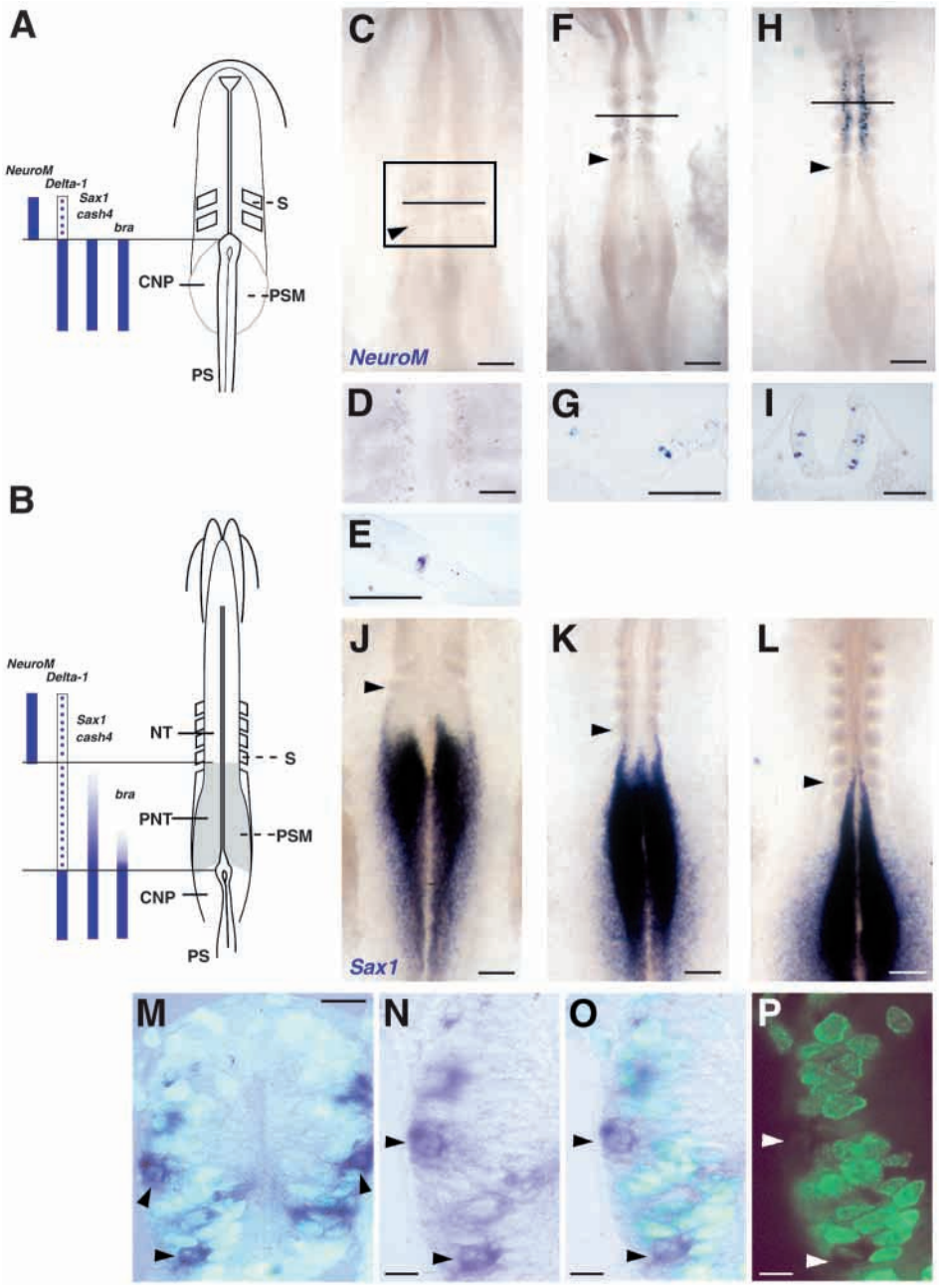
Strikingly, CNP explants cultured with the underlying presomitic mesoderm do undergo neuronal differentiation within 24 hours, as indicated by the presence of isolated *Delta 1*-expressing cells (4/5) (Fig. 2I,J) and *NeuroM*-positive cells (16/20) (Fig. 2K,L). This presomitic mesoderm cultured either 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

Fig. 1. Spatial and temporal separation of the events of neurogenesis during the generation of the spinal cord (D,E,G,I,M-P are tissue sections). (A,B) Summary of the gene expression domains in the developing embryo (medio-lateral extent of expression is not indicated). (A) Stage 7+ embryo, CNP, caudal neural plate; PS, primitive streak; S, first formed somite; PSM, presomitic mesoderm; dashed lines, underlying tissues. (B) Stage 8 embryo. PNT, preneural tube; NT, neural tube. (C-E) The first neurons appear opposite recently formed somites (arrowhead) as revealed by *NeuroM* expression in a stage 8– embryo. (F-I) *NeuroM* expression extends caudally within the neural tube and appears adjacent to the most recently formed somites (arrowheads) in stage HH8+ (F,G) and stage HH9 (H,I) embryos. (J-L) *Sax1* expression in the regressing CNP and PNT prefigures neurogenesis and is down regulated in the neural tube at about the level of the most recently formed somite (arrowheads). (M-P) *NeuroM*-expressing cells (blue, arrowheads) do not incorporate BrdU (FITC/green) in stage 12 neural tube. Double exposures (M,O), bright field (N), FITC only (P). Scale bars, (C,F,H,J,K,L) 200 μm; (D) 100 μm; (E,G,I) 50 μm; (M) 20 μm; (N,O,P) 10 μm.

presomitic mesoderm. Removal of the presomitic mesoderm from beneath the CNP in vivo also leads to local loss of *cash4* (5/6) (Fig. 3O,P) and *Sax1* (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 presomitic 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 presomitic mesoderm? We have shown previously that FGF signalling is a key pathway involved in the induction of *cash4* and *Sax1* (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 presomitic 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 presomitic 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 *Sax1* (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 presomitic 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)

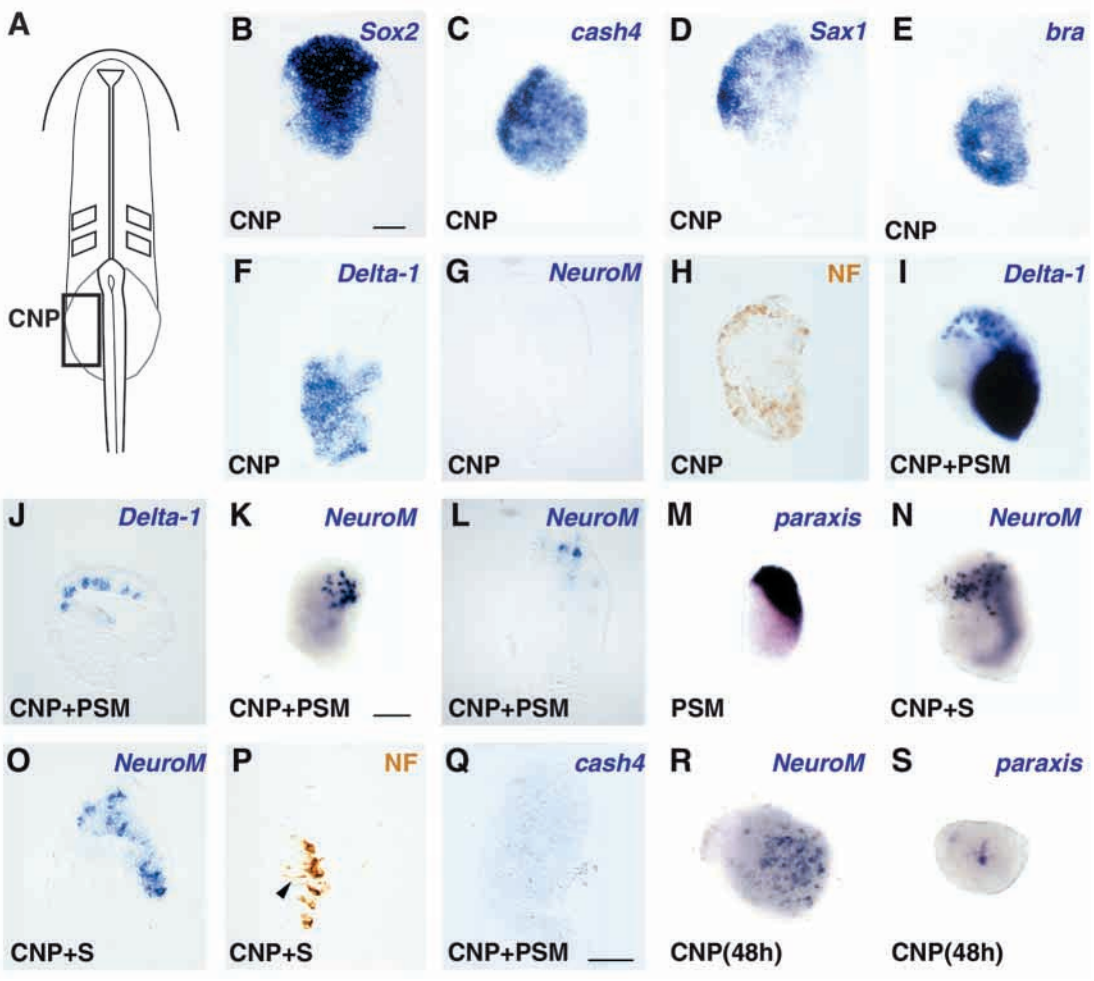


Fig. 2. Somites elicit neuronal differentiation in CNP explants. (B-H,J,L,O-Q are tissue sections.) (A) Diagram of CNP explant in a stage 7+ embryo. (B-H) CNP explants continue to express *Sox2* (B), *cash4* (C), *Sax1* (D) *bra* (E) and homogenous *Delta 1* (F), but do not express *NeuroM* (G) or contain cells with neurofilament (NF)-positive fine processes (H). (I-L) CNP explants cultured with differentiating PSM contain isolated *Delta 1*-positive cells (I,J) and *NeuroM*-expressing cells (K,L), and this presomitic 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 down regulated 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.

(6/7, data not shown) and can also be elicited by the less active protein FGF8 (6/11, data not shown). 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

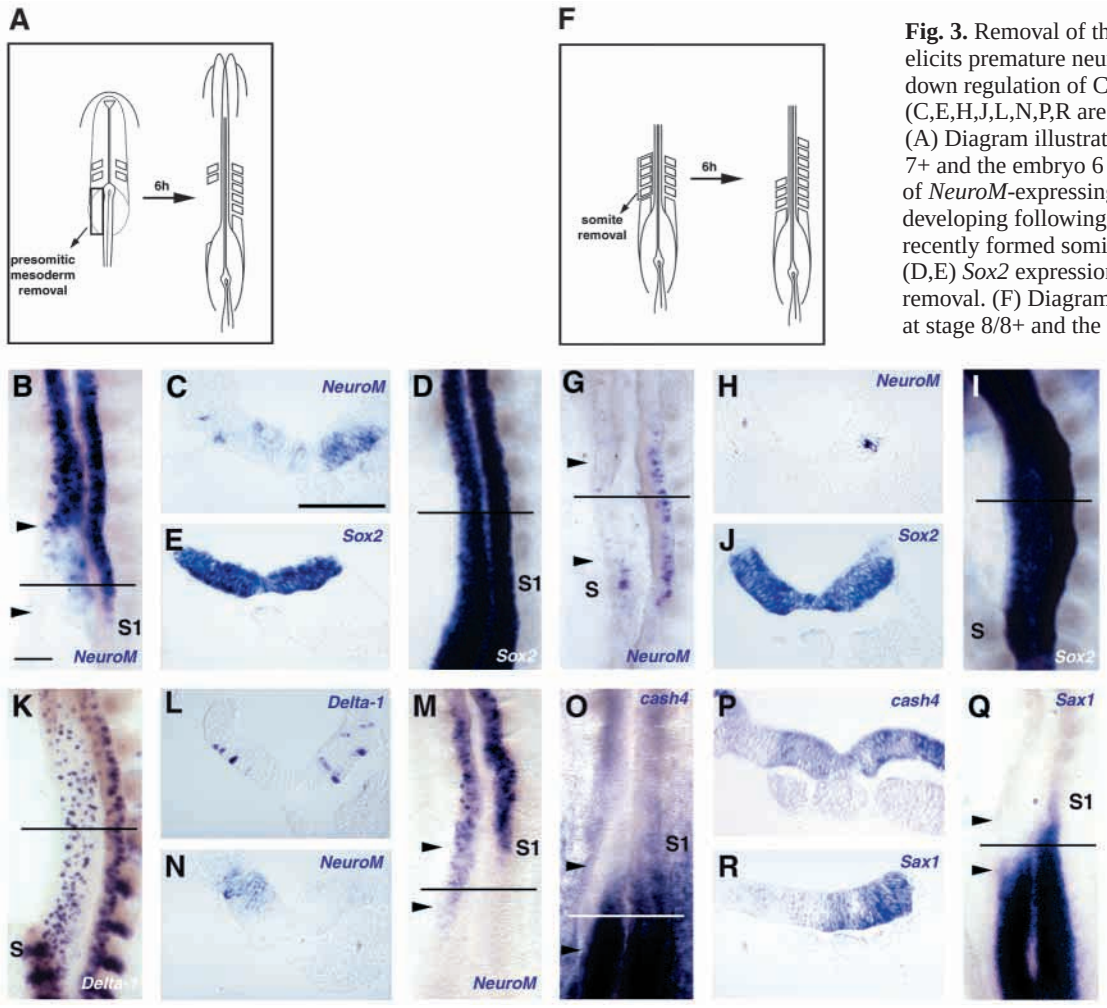


Fig. 3. Removal of the presomitic mesoderm elicits premature neuronal differentiation and the down regulation of CNP-specific genes. (C,E,H,J,L,N,P,R are transverse sections.) (A) Diagram illustrating removal of PSM at stage 7+ and the embryo 6 hours later. (B,C) Depletion of *NeuroM*-expressing cells 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. (I,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.

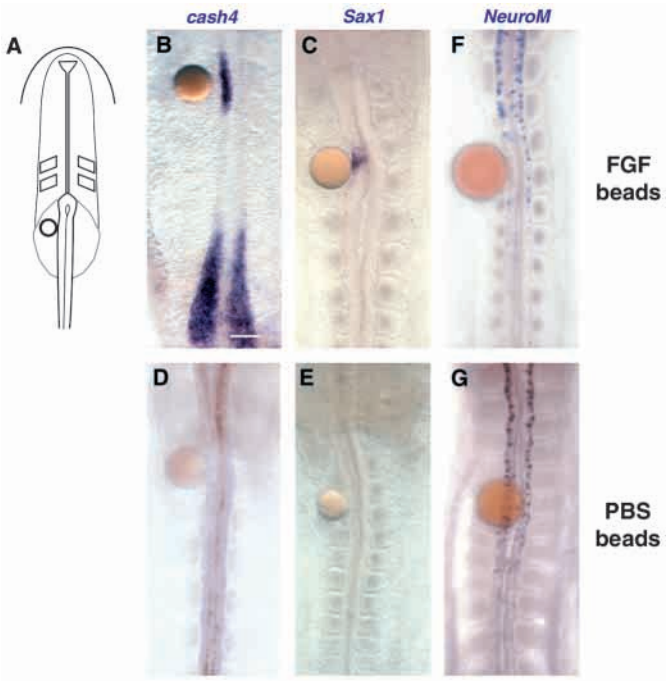


Fig. 4. FGF signalling upregulates CNP-specific genes and represses neuronal differentiation in vivo. (A) Diagram showing position of bead beneath stage 7+ CNP. (B,C) FGF4 beads placed beneath the CNP maintain ectopic expression of *cash4* (B) and *Sax1* (C), but control PBS beads (D,E) do not alter gene expression. (F,G) FGF8 beads inhibit expression of *NeuroM* (F), while PBS beads do not (G). Scale bar: (B-G) 200 μm.

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 *Delta 1* 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 *Delta 1* 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

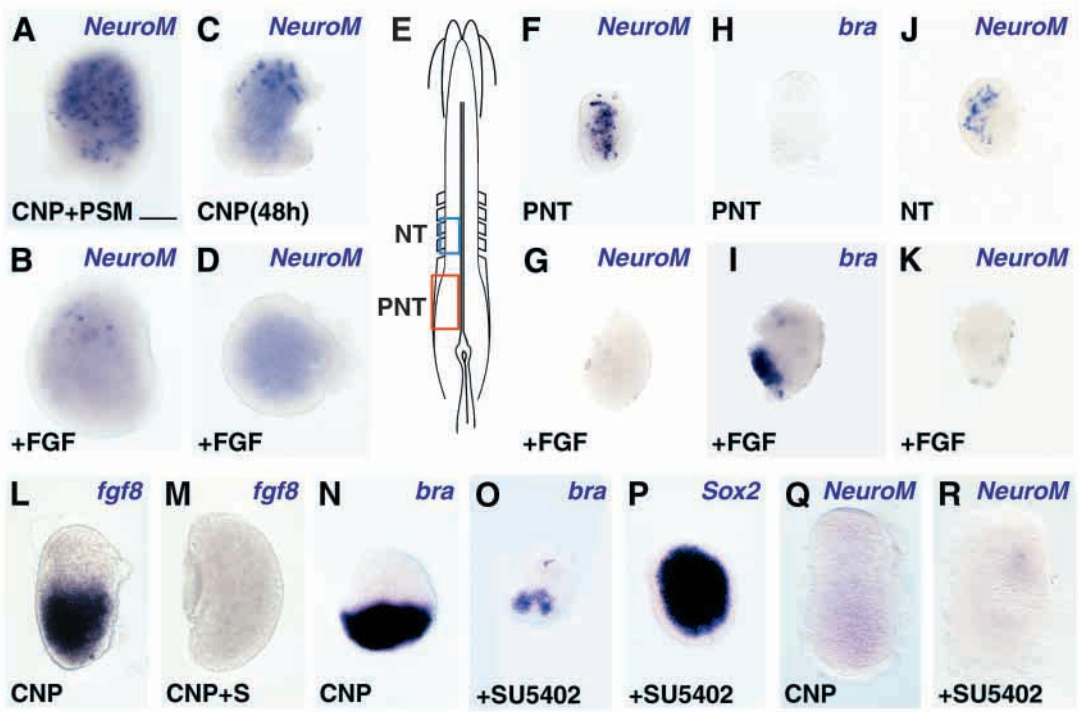


Fig. 5. FGF signalling represses neuronal differentiation and somites repress *Fgf8*, but attenuation of this pathway is not sufficient to elicit neuronal differentiation. (A) CNP explants cultured with PSM express *NeuroM* after 24 hours, but (B) only a few *NeuroM*-positive cells appear in the presence of FGF4. (C) CNP explants contain *NeuroM*-positive cells after 48 hours, but (D) fail to express this gene with FGF4. (E) Diagram to show the position of PNT and NT explants from a stage HH8 embryo. (F) PNT explants cultured for 24 hours contain *NeuroM*-positive cells but FGF4 treatment (G) inhibits *NeuroM* expression. (H) PNT explants cultured for 24 hours do not contain any *bra*-expressing cells but maintain some expression in the presence of FGF4 (I). (J) NT explants contain *NeuroM* +ve cells, while (K) FGF4 inhibits *NeuroM* expression. (L) CNP explants retain *Fgf8* after 24 hours, but (M) *Fgf8* is repressed when cultured with somites. (N) CNP explants cultured for 24 hours express *bra* but treatment with SU5402 down regulates *bra* (O) and maintains expression of *Sox2* (P). (Q) CNP explants do not contain *NeuroM*-positive cells after 24 hours even following treatment with SU5402 (R). Scale bar: 100 μm.

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 by 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

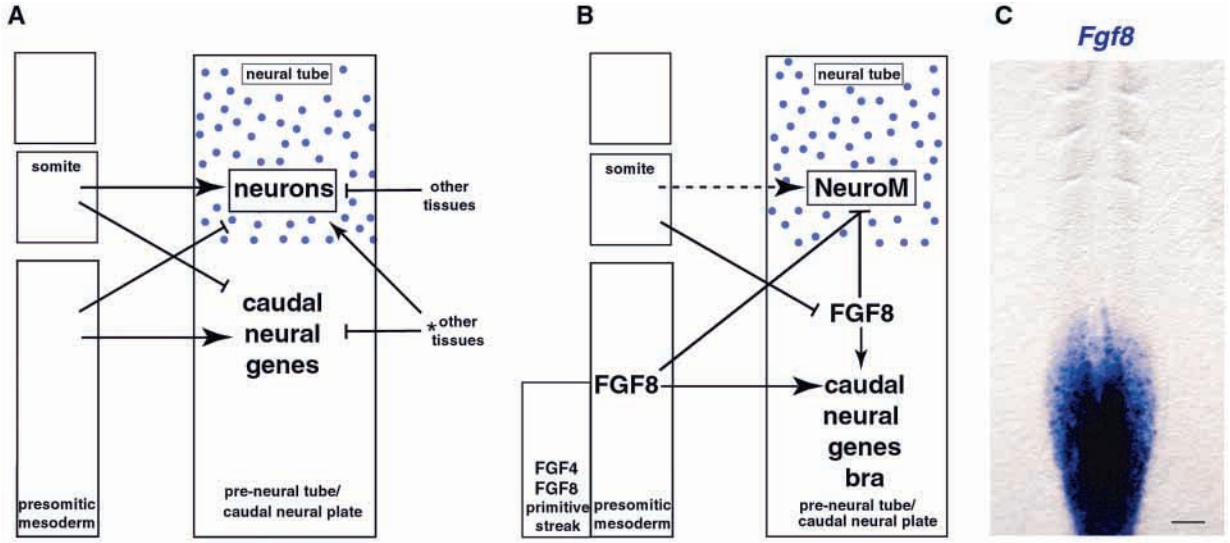


Fig. 6. Model for the regulation of neuronal differentiation by paraxial mesoderm and levels of FGF signalling. (A) Tissue level regulation of neuronal differentiation. The neural tube in contact with somites contains *NeuroM*-expressing cells (purple dots) while the CNP/PNT underlain by the PSM does 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. *Induction of neurons by other tissues at the level of the CNP/PNT may also be a consequence of the down-regulation of caudal neural genes. (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.

tube, the latter indicating that FGF signalling can therefore repress neuronal differentiation directly within the neuroepithelium (Fig. 6B). We note that this inhibition can be mediated in vivo and in vitro by both FGF4 and FGF8 and although weaker effects are observed with the less active FGF8 (see also Dubrulle et al., 2001) it is likely that FGF8 is the endogenous factor acting in the PNT. FGF signalling has been shown to stimulate proliferation of other neural precursor populations in vivo (Lee et al., 1997) and in vitro (e.g. Vescovi et al., 1993; Kilpatrick and Bartlett, 1993; Kalyani et al., 1997; Learish et al., 2000) and attenuation of this pathway may thus be a prerequisite for cell cycle exit. Continued FGF signalling in caudal regions of the chick may therefore serve to maintain cells in an undifferentiated and proliferative cell state and thereby provide a mechanism for the progressive generation of the spinal cord over an extended period.

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 (Gotz 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.

We thank P. Alexander, M. Ballivet, Y. Gruenbaum, D. Henrique, G. Martin, and J. Smith for plasmids containing *paraxis*, *NeuroM*, *Sax1* (*Sox2, cash4, Delta 1*), *Fgf8* and *bra*, respectively, and John K. Heath for FGF4 and Philip Cohen for PD 184352. We are also grateful to Declan Lunny, Julie Adam, Jenny Corrigan and Pam Halley for technical assistance, and Cheryll Tickle and Domingos Henrique for helpful comments. R. D. C. was supported by an EMBO Long Term Fellowship and D. N. B. by a Wellcome Trust Prize Studentship. Work in the Storey lab. is supported by the MRC and Human Frontiers Science Programme (RG00452/98B). K. G. S. is an MRC Senior Research Fellow.

REFERENCES

Barnes, G. L., Alexander, P. G., Hsu, C. W., Mariani, B. D. and Tuan, R. S. (1997). Cloning and characterization of chicken Paraxis: a regulator of paraxial mesoderm development and somite formation. *Dev. Biol.* **189**, 95-111.

Bertrand, N., Medevielle, F. and Pituello, F. (2000). FGF signalling controls the timing of Pax6 activation in the neural tube. *Development* **127**, 4837-4843.

Brewster, R., Lee, J. and Ruiz i Altaba, A. (1998). Gli/Zic factors pattern the neural plate by defining domains of cell differentiation. *Nature* **393**, 579-583.

Brown, J. M. and Storey, K. G. (2000). A region of the vertebrate neural plate in which neighbouring cells can adopt neural or epidermal cell fates. *Curr. Biol.* **10**, 869-872.

Cau, E., Gradwohl, G., Fode, C. and Guillemot, F. (1997). Mash1 activates a cascade of bHLH regulators in olfactory neuron progenitors. *Development* **124**, 1611-1621.

Chambers, D. and Mason, I. (2000). Expression of sprouty2 during early development of the chick embryo is coincident with known sites of FGF signalling. *Mech. Dev.* **91**, 361-364.

Chapman, D. L. and Papaioannou, V. E. (1998). Three neural tubes in mouse embryos with mutations in the T-box gene Tbx6. *Nature* **391**, 695-697.

Chapman, S. C., Collignon, J., Schoenwolf, G. C. and Lumsden, A. (2001). Improved method for chick whole-embryo culture using a filter paper carrier. *Dev. Dyn.* **220**, 284-289.

Chapouton, P., Gartner, A. and Gotz, M. (1999). The role of Pax6 in restricting cell migration between developing cortex and basal ganglia. *Development* **126**, 5569-5579.

Ciruna, B. and Rossant, J. (2001). FGF signalling regulates mesoderm cell fate specification and morphogenetic movement at the primitive streak. *Developmental Cell* **1**, 37-49.

Ciruna, B. G., Schwartz, L., Harpal, K., Yamaguchi, T. P. and Rossant, J. (1997). Chimeric analysis of fibroblast growth factor receptor-1 (Fgfr1) function: a role for FGFR1 in morphogenetic movement through the primitive streak. *Development* **124**, 2829-2841.

Davies, S. P., Reddy, H., Caivano, M. and Cohen, P. (2000). Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem. J.* **351**, 95-105.

Deng, C., Bedford, M., Li, C., Xu, X., Yang, X., Dunmore, J. and Leder, P. (1997). Fibroblast growth factor receptor-1 (FGFR-1) is essential for normal neural tube and limb development. *Dev. Biol.* **185**, 42-54.

Doniach, T. (1995). Basic FGF as an inducer of anteroposterior neural pattern. *Cell* **83**, 1067-1070.

Dubrulle, J., McGrew, M. J. and Pourquie, O. (2001). FGF signalling controls somite boundary position and regulates segmentation clock control of spatiotemporal Hox gene activation. *Cell* **106**, 219-232.

Edlund, T. and Jessell, T. M. (1999). Progression from extrinsic to intrinsic signaling in cell fate specification: a view from the nervous system. *Cell* **96**, 211-224.

Ensini, M., Tsuchida, T. N., Belting, H. G. and Jessell, T. M. (1998). The control of rostrocaudal pattern in the developing spinal cord: specification of motor neuron subtype identity is initiated by signals from paraxial mesoderm. *Development* **125**, 969-982.

Ericson, J., Rashbass, P., Schedl, A., Brenner-Morton, S., Kawakami, A., van Heyningen, V., Jessell, T. M. and Briscoe, J. (1997). Pax6 controls progenitor cell identity and neuronal fate in response to graded Shh signaling. *Cell* **90**, 169-180.

Gotz, M., Stoykova, A. and Gruss, P. (1998). Pax6 controls radial glia differentiation in the cerebral cortex. *Neuron* **21**, 1031-1044.

Gould, A., Itasaki, N. and Krumlauf, R. (1998). Initiation of rhombomeric Hoxb4 expression requires induction by somites and a retinoid pathway. *Neuron* **21**, 39-51.

Grapin-Botton, A., Bonnin, M. and Le Douarin, M. (1997). Hox gene induction in the neural tube depends on three parameters: competence, signal supply and paralogue group. *Development* **124**, 849-859.

Guillemot, F. (1999). Vertebrate bHLH genes and the determination of neuronal fates. *Exp. Cell Res.* **253**, 357-364.

Hamburger, V. and Hamilton, H. L. (1951). A series of normal stages on the development of the chick embryo. *J. Morphol.* **88**, 49-92.

Henrique, D., Adam, J., Myat, A., Chitnis, A., Lewis, J. and Ish-Horowicz, D. (1995). Expression of a Delta homologue in prospective neurons in the chick. *Nature* **375**, 787-790.

Henrique, D., Tyler, D., Kintner, C., Heath, J. K., Lewis, J. H., Ish Horowicz, D. and Storey, K. G. (1997). cash4, a novel achaete-scute homolog induced by Hensen's node during generation of the posterior nervous system. *Genes Dev.* **11**, 603-615.

Isaacs, H. V., Pownall, M. E. and Slack, J. M. (1994). eFGF regulates Xbra expression during Xenopus gastrulation. *EMBO J.* **13**, 4469-4481.

Itasaki, N., Sharpe, J., Morrison, A. and Krumlauf, R. (1996). Reprogramming Hox expression in the vertebrate hindbrain: influence of paraxial mesoderm and rhombomere transposition. *Neuron* **16**, 487-500.

Jessell, T. M. (2000). Neuronal specification in the spinal cord: inductive signals and transcriptional codes. *Nat. Rev. Genet.* **1**, 20-29.

Kalyani, A., Hobson, K. and Rao, M. S. (1997). Neuroepithelial stem cells from the embryonic spinal cord: isolation, characterization, and clonal analysis. *Dev. Biol.* **186**, 202-223.

Kilpatrick, T. J. and Bartlett, P. F. (1993). Cloning and growth of multipotential neural precursors: requirements for proliferation and differentiation. *Neuron* **10**, 255-265.

Kispert, A., Ortner, H., Cooke, J. and Herrmann, B. G. (1995). The chick Brachyury gene: Developmental expression pattern and response to axial induction by localized activin. *Dev. Biol.* **168**, 406-415.

Korzh, V., Edlund, T. and Thor, S. (1993). Zebrafish primary neurons initiate expression of the LIM homeodomain protein Isl-1 at the end of gastrulation. *Development* **118**, 417-425.

LaBonne, C., Burke, B. and Whitman, M. (1995). Role of MAP kinase in

mesoderm induction and axial patterning during Xenopus development. *Development* **121**, 1475-1486.

Lamb, T. M., Knecht, A. K., Smith, W. S., Stachel, S. E., Economides, A. N., Stahl, N., Yancopolous, G. D. and Harland, R. M. (1993). Neural induction by the secreted polypeptide noggin. *Science* **262**, 713-718.

Langman, J. and Haden, C. C. (1970). Formation and migration of neuroblasts in the spinal cord of the chick embryo. *J. Comp. Neurol.* **138**, 419-425.

Learish, R. D., Bruss, M. D. and Haak-Frendscho, M. (2000). Inhibition of mitogen-activated protein kinase kinase blocks proliferation of neural progenitor cells. *Brain Res. Dev. Brain Res.* **122**, 97-109.

Lee, J. E., Hollenberg, S. M., Snider, L., Turner, D. L., Lipnick, N. and Weintraub, H. (1995). Conversion of Xenopus ectoderm into neurons by NeuroD, a basic helix-loop-helix protein. *Science* **268**, 836-844.

Lee, S. M., Danielian, P. S., Fritzsch, B. and McMahon, A. P. (1997). Evidence that FGF8 signalling from the midbrain-hindbrain junction regulates growth and polarity in the developing midbrain. *Development* **124**, 959-969.

Lewis, J. (1996). Neurogenic genes and vertebrate neurogenesis. *Curr. Opin. Neurobiol.* **6**, 3-10.

Ma, Q., Kintner, C. and Anderson, D. J. (1996). Identification of neurogenin, a vertebrate neuronal determination gene. *Cell* **87**, 43-52.

Mahmood, R., Kiefer, P., Guthrie, S., Dickson, C. and Mason, I. (1995). Multiple roles for FGF-3 during cranial neural development in the chicken. *Development* **121**, 1399-1410.

Martin, A. H. (1971). Mitotic patterns in the neural tube of the chick after unilateral removal of 1-3 somites. *Acta Embryol. Exp.* **1**, 9-16.

Mathis, L., Kulesa, P. M. and Fraser, S. E. (2001). FGF receptor signalling is required to maintain neural progenitors during Hensen's node progression. *Nat. Cell Biol.* **3**, 559-566.

Mathis, L. and Nicolas, J. F. (2000). Different clonal dispersion in the rostral and caudal mouse central nervous system. *Development* **127**, 1277-1290.

McConnell, J. A. and Sechrist, J. W. (1980). Identification of early neurons in the brainstem and spinal cord: I. An autoradiographic study in the chick. *J. Comp. Neurol.* **192**, 769-783.

Minowada, G., Jarvis, L. A., Chi, C. L., Neubuser, A., Sun, X., Hacohen, N., Krasnow, M. A. and Martin, G. R. (1999). Vertebrate Sprouty genes are induced by FGF signaling and can cause chondrodysplasia when overexpressed. *Development* **126**, 4465-4475.

Mohammadi, M., McMahon, G., Sun, L., Tang, C., Hirth, P., Yeh, B. K., Hubbard, S. R. and Schlessinger, J. (1997). Structures of the tyrosine kinase domain of fibroblast growth factor receptor in complex with inhibitors. *Science* **276**, 955-960.

Muhr, J., Graziano, E., Wilson, S., Jessell, T. M. and Edlund, T. (1999). Convergent inductive signals specify midbrain, hindbrain, and spinal cord identity in gastrula stage chick embryos. *Neuron* **23**, 689-702.

Nornes, H. O. and Carry, M. (1978). Neurogenesis in spinal cord of mouse: an autoradiographic analysis. *Brain Res.* **159**, 1-6.

Nornes, H. O. and Das, G. D. (1974). Temporal pattern of neurogenesis in spinal cord of rat. I. An autoradiographic study – Time and sites of origin and migration and settling patterns of neuroblasts. *Brain Res.* **73**, 121-138.

Ohnuma, S., Philpott, A. and Harris, W. A. (2001). Cell cycle and cell fate in the nervous system. *Curr. Opin. Neurobiol.* **11**, 66-73.

Ohuchi, H., Kimura, S., Watamoto, M. and Itoh, N. (2000). Involvement of fibroblast growth factor (FGF)18-FGF8 signaling in specification of left-right asymmetry and brain and limb development of the chick embryo. *Mech. Dev.* **95**, 55-66.

Papalopulu, N. and Kintner, C. (1996). A posteriorising factor, retinoic acid, reveals that anteroposterior patterning controls the timing of neuronal differentiation in Xenopus neuroectoderm. *Development* **122**, 3409-3418.

Pierani, A., Brenner-Morton, S., Chiang, C. and Jessell, T. M. (1999). A sonic hedgehog-independent, retinoid-activated pathway of neurogenesis in the ventral spinal cord. *Cell* **97**, 903-915.

Pituello, F., Medevielle, F., Foulquier, F. and Duprat, A. M. (1999). Activation of Pax6 depends on somitogenesis in the chick embryo cervical spinal cord. *Development* **126**, 587-596.

Placzek, M. and Dale, K. (1999). Tissue recombinations in collagen gels. In *Molecular Embryology* (ed. P. Sharpe and I. Mason), pp. 305-318. NJ, USA: Humana Press.

Pons, S. and Marti, E. (2000). Sonic hedgehog synergizes with the extracellular matrix protein vitronectin to induce spinal motor neuron differentiation. *Development* **127**, 333-342.

Ribisi, S. Jr, Mariani, F. V., Aamar, E., Lamb, T. M., Frank, D. and Harland, R. M. (2000). Ras-mediated FGF signaling is required for the formation of posterior but not anterior neural tissue in Xenopus laevis. *Dev. Biol.* **227**, 183-196.

Roztocil, T., Matter Sadzinski, L., Alliod, C., Ballivet, M. and Matter, J. M. (1997). NeuroM, a neural helix-loop-helix transcription factor, defines a new transition stage in neurogenesis. *Development* **124**, 3263-3272.

Scardigli, R., Schuurmans, C., Gradwohl, G. and Guillemot, F. (2001). Crossregulation between Neurogenin2 and pathways specifying neuronal identity in the spinal cord. *Neuron* **31**, 203-217.

Schoenwolf, G. C. (1992). Morphological and mapping studies of the paranodal and postnodal levels of the neural plate during chick neurulation. *Anat. Rec.* **233**, 281-290.

Sebolt-Leopold, J. S., Dudley, D. T., Herrera, R., Van Becelaere, K., Wiland, A., Gowan, R. C., Tecle, H., Barrett, S. D., Bridges, A., Przybranowski, S. et al. (1999). Blockade of the MAP kinase pathway suppresses growth of colon tumors in vivo. *Nat. Med.* **5**, 810-816.

Sechrist, J. and Bronner-Fraser, M. (1991). Birth and differentiation of reticular neurons in the chick hindbrain: Ontogeny of the first neuronal population. *Neuron* **7**, 947-963.

Sela-Donenfeld, D. and Kalcheim, C. (2000). Inhibition of noggin expression in the dorsal neural tube by somitogenesis: a mechanism for coordinating the timing of neural crest emigration. *Development* **127**, 4845-4854.

Selleck, M. A. and Bronner-Fraser, M. (1995). Origins of the avian neural crest: the role of neural plate-epidermal interactions. *Development* **121**, 525-538.

Shamin, H. and Mason, I. (1999). Expression of FGF4 dring early development of the chick embryo. *Mech. Dev.* **85** 189-192.

Spann, P., Ginsburg, M., Rangini, Z., Fainsod, A., Eyal Giladi, H. and Gruenbaum, Y. (1994). The spatial and temporal dynamics of Sax1 (CHox3) homeobox gene expression in the chick's spinal cord. *Development* **120**, 1817-1828.

Stern, C. D., Jaques, K. F., Lim, T. M., Fraser, S. E. and Keynes, R. J. (1991). Segmental lineage restrictions in the chick embryo spinal cord depend on the adjacent somites. *Development* **113**, 239-244.

Storey, K. G., Crossley, J. M., De Robertis, E. M., Norris, W. E. and Stern, C. D. (1992). Neural induction and regionalisation in the chick embryo. *Development* **114**, 729-741.

Storey, K. G., Goriely, A., Sargent, C. M., Brown, J. M., Burns, H. D., Abud, H. M. and Heath, J. K. (1998). Early posterior neural tissue is induced by FGF in the chick embryo. *Development* **125**, 473-484.

Stoykova, A., Gotz, M., Gruss, P. and Price, J. (1997). Pax6-dependent regulation of adhesive patterning, R-cadherin expression and boundary formation in developing forebrain. *Development* **124**, 3765-3777.

Streit, A., Sockanathan, S., Perez, L., Rex, M., Scotting, P. J., Sharpe, P. T., Lovell Badge, R. and Stern, C. D. (1997). Preventing the loss of competence for neural induction: HGF/SF, L5 and Sox-2. *Development* **124**, 1191-1202.

Sun, T., Pringle, N. P., Hardy, A. P., Richardson, W. D. and Smith, H. K. (1998). Pax6 Influences the time and site of origin of glial precursors in the ventral neural tube. *Mol. Cell Neurosci.* **12**, 228-239.

Tannahill, D., Britto, J. M., Vermeren, M. M., Ohta, K., Cook, G. M. and Keynes, R. J. (2000). Orienting axon growth: spinal nerve segmentation and surround-repulsion. *Int. J. Dev. Biol.* **44**, 119-127.

Umbhauer, M., Marshall, C. J., Mason, C. S., Old, R. W. and Smith, J. C. (1995). Mesoderm induction in Xenopus caused by activation of MAP kinase. *Nature* **376**, 58-62.

Vescovi, A. L., Reynolds, B. A., Fraser, D. D. and Weiss, S. (1993). bFGF regulates the proliferative fate of unipotent (neuronal) and bipotent (neuronal/astroglial) EGF-generated CNS progenitor cells. *Neuron* **11**, 951-966.

Walshe, J. and Mason, I. (2000). Expression of FGFR1, FGFR2 and FGFR3 during early neural development in the chick embryo. *Mech. Dev.* **90**, 103-110.

Warren, N., Caric, D., Pratt, T., Clausen, J. A., Asavaritikrai, P., Mason, J. O., Hill, R. E. and Price, D. J. (1999). The transcription factor, Pax6, is required for cell proliferation and differentiation in the developing cerebral cortex. *Cereb. Cortex* **9**, 627-635.

Warren, N. and Price, D. J. (1997). Roles of Pax-6 in murine diencephalic development. *Development* **124**, 1573-1582.

Yamada, T., Pfaff, S. L., Edlund, T. and Jessell, T. M. (1993). Control of cell pattern in the neural tube: motor neuron induction by diffusible factors from notochord and floor plate. *Cell* **73**, 673-686.

Zakany, J., Kmita, M., Alarcon, P., De la Pompa, J. L. and Duboule, D. (2001). Localised and transient transcription of Hox genes suggests a link between patterning and the segmentation clock. *Cell* **106**, 207-217.