Development 130, 147-159

doi:10.1242/dev.00200

A conserved role for the MEK signalling pathway in neural tissue specification and posteriorisation in the invertebrate chordate, the ascidian *Ciona intestinalis*

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Accepted 15 October 2002

SUMMARY

Ascidians are invertebrate chordates with a larval body plan similar to that of vertebrates. The ascidian larval CNS is divided along the anteroposterior axis into sensory vesicle, neck, visceral ganglion and tail nerve cord. The anterior part of the sensory vesicle comes from the a-line animal blastomeres, whereas the remaining CNS is largely derived from the A-line vegetal blastomeres. We have analysed the role of the Ras/MEK/ERK signalling pathway in the formation of the larval CNS in the ascidian, *Ciona intestinalis*. We show evidence that this pathway is required, during the cleavage stages, for the acquisition of: (1) neural fates in otherwise epidermal cells (in a-line cells); and (2) the posterior identity of tail nerve cord precursors that otherwise adopt a more anterior neural character (in A-line cells). Altogether, the MEK signalling pathway appears to play evolutionary conserved roles in these processes in ascidians and vertebrates, suggesting that this may represent an ancestral chordate strategy.

Key words: Ciona, MEK, FGF, ERK, Ascidian, Tunicate, Neural patterning, Neural induction

INTRODUCTION

In chick embryos, FGF signalling is implicated in the earliest inductive events of neural tissue, which occur before the onset of gastrulation (Wilson et al., 2000; Streit et al., 2000). Upon binding to its receptor, FGF activates the Ras-, MEK-, ERK-signalling cascade through a receptor tyrosine kinase, FGFR (reviewed by Szebenyi and Fallon, 1999). This early neural induction step, which generates tissue of anterior character, is followed by a stabilisation step to give committed neural precursors (reviewed by Stern, 2001). Finally, a posteriorisation step is required to generate posterior neural tissue. Although a conserved role for the Ras/MEK/ERK signalling pathway in early vertebrate neural induction remains controversial, particularly in *Xenopus* (Weinstein and Hemmati-Brivanlou, 1999; Harland, 2000), this cascade has been implicated in posteriorisation of neural tissue in both chick and *Xenopus* (reviewed by Gamse and Sive, 2000; Stern, 2001). In this study, we analysed whether there was a conserved role for this signalling pathway in the development of the ascidian nervous system.

Ascidians belong to the subphylum Urochordata of the phylum Chordata (reviewed by Satoh, 1994), which arose during the Cambrian before the emergence of cephalochordates and vertebrates (Shu et al., 2001). Thus, studying ascidians may help pinpoint the key mechanisms that led to the emergence of the chordate body plan.

The swimming larvae of ascidians have a tadpole morphology, are very simple and develop with a small cell number and fixed cell lineage (Satoh, 1994). Their central nervous system (CNS) consists of a sensory vesicle, which contains two sensory pigment cells, followed by a constriction termed the neck, then a further swelling, the visceral ganglion, and finally a simple tail nerve cord of ependymal cells which is just four cells in cross section (for reviews, see Meinertzhagen and Okamura, 2001; Lemaire et al., 2002). The bilaterally symmetrical eight-cell stage embryo contains four lineages, a4.2 (anterior animal), b4.2 (posterior animal), A4.1 (anterior vegetal) and B4.1 (posterior vegetal). The anterior part of the sensory vesicle comes from the a4.2 lineage, whereas the majority of the remaining CNS derives from the A4.1 lineage except for dorsal-most cells, which originate from the b4.2 lineage (Nishida, 1987).

The formation of neural tissue from the a4.2 lineage requires inductive interactions with vegetal cells and can be induced by recombination with the natural A4.1 inducer blastomere or by

treatment with bFGF (reviewed by Lemaire et al., 2002). The A4.1-derived neural lineages emerge at the 44-cell stage following the division of precursor blastomeres, which contain both neural and notochord fates. In this division, neural fate is the default fate (Minokawa et al., 2001). When a precursor blastomere is cultured in isolation, both daughters will adopt neural fate. By contrast, treatment of notochord/neural precursors with bFGF causes both daughter cells to adopt a notochord fate at the expense of neural fate (Nakatani et al., 1996; Minokawa et al., 2001). Thus, FGF signalling is implicated in both the binary decision between notochord and neural fates and in the specification of the anterior neural fates in a4.2 derivatives.

Recent studies have analysed the role of the FGF signalling pathway during ascidian embryogenesis by blocking the function of various components of this pathway. This included the use of a dominant-negative form of Ras (Nakatani and Nishida, 1997; Kim and Nishida, 2001), pharmaceutical inhibitors of MEK or FGFR (Kim and Nishida, 2001) or dominant-negative FGFR (Shimauchi et al., 2001). All of these treatments generated a similar phenotype, whereby gastrulation was perturbed and notochord, mesenchyme and anterior neural fates were lost. These data confirmed the crucial, direct or indirect, requirement of the FGF/MEK signalling pathway for these cell fate decisions. However, these studies did not address in detail the role of FGF/MEK signalling during the formation of the nervous system.

In this study we used a dominant negative form of Ras (dnRas) ($p21^{N17}$) (Whitman and Melton, 1992) and two specific pharmacological inhibitors of MEK (Davies et al., 2000) to block this signalling pathway. We then examined in these embryos the formation and patterning of the neural tissue.

MATERIALS AND METHODS

Embryos and inhibitor treatment

Embryology, FGF treatment, in situ hybridisation and acetylated tubulin antibody staining were as described (Hudson and Lemaire, 2001). U0126 (Calbiochem) and PD184352 (Upstate Biochemicals) were diluted to the indicated concentration in artificial sea water. U0126 irreversibly blocks MEK signalling in ascidians (Kim and Nishida, 2001) and the same was found for PD184352. Therefore, once applied at the stage indicated in the text, embryos were continuously cultured in the presence of the inhibitor. We found that 2 μM PD184352 produced the most robust effect on gene expression over several experiments, whereas concentrations higher than 4 μM affected embryonic cleavages. U0126 gave the same phenotype at concentrations ranging from 2 μM to 10 μM without affecting cleavages. We therefore chose to use 2 μM as a working concentration for both inhibitors. Cytochalasin (Sigma C6762) was applied at a concentration of 4 μg/ml.

Injection of dnRas mRNA

mRNA was synthesised using mMessage mMachine kits (Ambion). Microinjections were carried out as described (Yasuo and Satoh, 1998). dnRas mRNA (Whitman and Melton, 1992) was injected into fertilised eggs at a concentration of 2 μg/μl. Lower concentrations tested (0.25, 0.5, 1 μg/μl) did not produce a strong phenotype (data not shown). An ~0.25×egg diameter-sized drop was injected into each egg, resulting in an injection of approximately 16 pg of mRNA.

Fate-mapping experiments

Fate-mapping was carried out by depositing a tiny drop of colza oil saturated with DiI [$DiIC_{18}(3)$, Molecular Probes] on the surface of the appropriate blastomere as described previously (Nakatani et al., 1999). For Fig. 6, individual labelled embryos were photographed before and after in situ hybridisation, as the DiI staining is lost during this procedure. Comparison of DiI and in situ pictures was facilitated by the use of the nuclear dye Hoechst 33342 (2 μg/ml). Overlays of pictures were created using Adobe Photoshop.

Molecular analysis

Probes used for in situ were *Ci-ETR-1* (cluster no. 01087) (Satou et al., 2001); *Ci-gsx* and *Ci-otx* (Hudson and Lemaire, 2001); *Ci-bra* (Corbo et al., 1997); *Cihox5* (Gionti et al., 1998); and *Ci-HB9/Mnx* (AF499007, C. H. and P. L., unpublished). The following EST clones were used: *Ci-α tubulin* (AF499644, AF499645); *Ci-TRP* (AL666750, AL663481); *Ci-Epi-1* (AL666060, AL663140, which are the same as AB037395 entered by M. A. Kobayashi and T. Nishikata, unpublished); *24H09* (AF499646) and *08C09* (AL666565, AL666006) (D. C. and P. L., unpublished); and *Citb8a22* (cluster number 00124) and *Cilv38e16* (cluster number 01427) (Satou et al., 2001).

Anti-ERK staining

For activated-ERK antibody (anti-Diphosphorylated ERK1/2; Sigma M8159) staining, embryos were fixed for 30 minutes at room temperature in 4% paraformaldehyde, 0.1% glutaraldehyde in artificial sea water buffered with 50 mM EPPS (N-[2-hydroxyethyl]piperazine-N′-3-ethanesulfonic acid) (Sigma E7758). Endogenous avidin/biotin was blocked using Avidin/Biotin Blocking Kit (Zymed). Antibody was used at 1/750 in PBS-0.1% Tween, 10% goat serum, 1% Blocking Reagent (Roche). Signal was amplified using the Vectastain ABC POD Kit (Vector Laboratories) according to the manufacturer's instructions.

RESULTS

Dominant negative RAS and two inhibitors of MEK block FGF-dependent signalling events in *Ciona* embryos

In embryos cultured in either inhibitor, or injected with *dnRas* mRNA, gastrulation was perturbed and no morphological signs of notochord or pigment cells were observed (Fig. 1A-C). These embryos looked strikingly similar to those observed in previous studies carried out in *Halocynthia* embryos, where FGF/MEK signalling was blocked at the level of either the FGFR, Ras or MEK (Nakatani and Nishida, 1997; Kim and Nishida, 2001; Shimauchi et al., 2001).

Blocking FGF signalling via inhibition of Ras, FGFR or MEK (using U0126) has previously been shown to inhibit notochord formation in *Halocynthia* (Nakatani and Nishida, 1997; Kim and Nishida, 2001; Minokawa et al., 2001; Darras and Nishida, 2001a; Shimauchi et al., 2001). Likewise, *Ciona* embryos injected with *dnRas* mRNA showed a strong downregulation of expression of ascidian *brachyury*, *Ci-bra* (Corbo et al., 1997), a causal gene in notochord formation (Yasuo and Satoh, 1998; Takahashi et al., 1999) (Fig. 1D-E). Similarly, in embryos cultured in the presence of PD184352 *Ci-bra* expression was completely blocked (Fig. 1F,G). Hence, *dnRas* mRNA injection or incubation in either MEK inhibitor blocked a known FGF-dependent event in whole *Ciona* embryos.

To further confirm that the inhibitors could block an FGF-dependent MEK signalling event in *Ciona*, we tested the effect of their application on the induction of neural fates by FGF in isolated animal explants. Animal blastomeres were isolated at the

Table 1. Expression of *Ci-otx* and *Ci-ETR-1* in animal explant assays

										+FGF							
			Concentration of inhibitor (μM)														
			0		1		2		4		0		1		2		4	
Probe	Stage	Inhibitor	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*
Ci-otx	ne	PD	**0**	41	**11**	36	**0**	58	**0**	45	**76**	42	**2**	42	**6**	36	**3**	33
Ci-ETR-1	tb	PD	**0**	22	**0**	14	**0**	21	-	-	**94**	16	**0**	17	**0**	15	-	-
Ci-otx	ne	U0	**0**	28	**3**	39	**7**	41	-	-	**83**	29	**2**	43	**5**	44	-	-
Ci-ETR-1	ne	U0	**6**	34	**6**	32	**15**	46	-	-	**95**	42	**8**	53	**0**	31	-	-

The percentages of explants showing expression is indicated.
ne, neurula; tb, tailbud stage; PD, PD184352; U0, U0126.

eight-cell stage and cultured until neurula stage when they were assayed for expression of neural markers (Table 1; Fig. 1I-H). When the isolated blastomeres were treated with bFGF in the presence of the inhibitors, no expression of neural markers was observed (Fig. 1K).

The similarity of the phenotypes of embryos, in this and previous studies, in which the FGF/Ras/MEK signalling cascade is blocked by a variety of different approaches, together with the demonstration that they can block known MEK-dependent events in *Ciona* embryos, strongly suggest that *Ciona* and *Halocynthia* embryos share similar developmental mechanisms and that the Ras/MEK/ERK pathway can be specifically blocked using PD184352 and U0126 in these embryos.

MEK signalling is required for the formation of a-line neural tissue

In order to analyse the role of MEK-signalling in the formation of neural tissues, we chose to concentrate our analysis on the inhibitor-treated embryos. These pharmacological inhibitors are more efficient than mRNA injection, which can be mosaic. In addition, we could establish the timing of the requirement of MEK signalling during neural induction and patterning events, by applying the inhibitor at different developmental time points. However, as indicated below, the expression profiles of a number of the various markers used to study the MEK inhibitor treated embryos was confirmed in dnRas mRNA-injected embryos.

We first looked at the formation of pigment cells and neurones in embryos treated with MEK inhibitors from the eight-cell stage onwards. In most subsequent experiments, both U0126 and PD184352 were tested (see figure legends). However, because in all cases both inhibitors gave very similar results, only results with PD184352 are presented. Pigment cells are easily recognisable because of their melanised granules. Neurones in the sensory

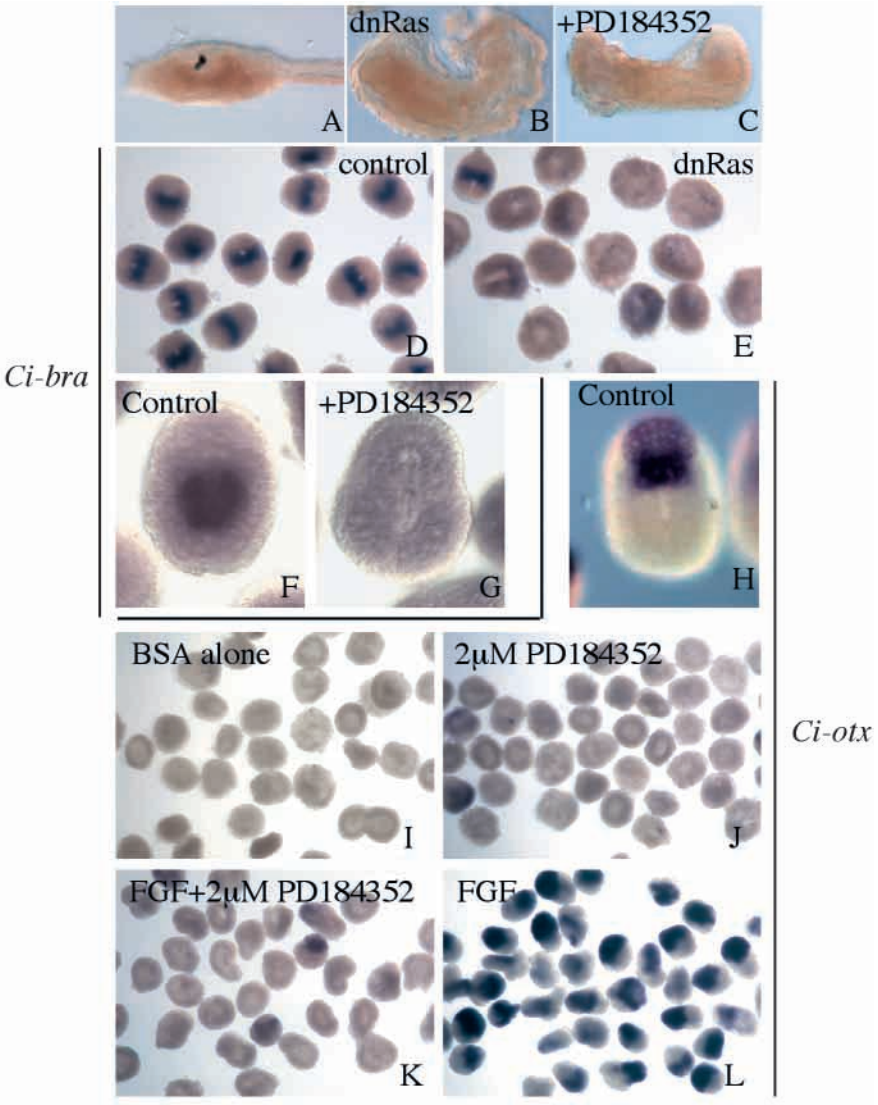

Fig. 1. PD184352 and dnRAS mRNA injections block FGF/MEK-dependent events in *Ciona* embryos. (A) control embryo at larval stage. (B) Overall morphology of a dnRAS mRNA injected embryo. (C) Overall morphology of a PD184352-treated embryo. (D-G) *Ci-bra* in situ hybridisation. (D) Control embryos; (E) embryos injected with dnRas mRNA; F) control embryo, dorsal view with anterior upwards; (G) embryo cultured in PD184352 from the eight-cell stage onwards (100% negative, *n*=37). (H-L) *Ci-otx* in situ hybridisation. (H) Control embryo dorsal view with anterior upwards; (I-L) a4.2 explants cultured in (I) BSA/sea water, (J) BSA/sea water plus 2 μM PD184352, (K) 2 μM PD184352+100 ng/ml bFGF or (L) FGF alone.

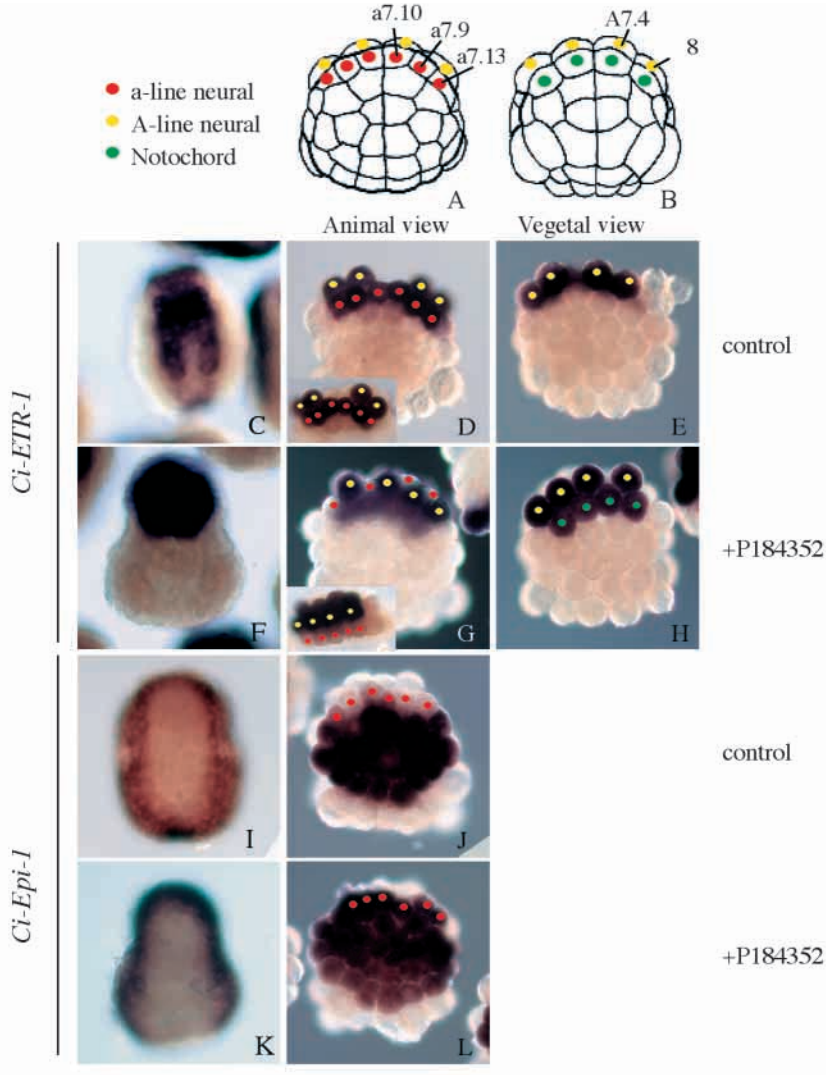

Fig. 2. Expression of *Ci-ETR-1* and *Ci-Epi-1* at the neurula stage in embryos treated with or without PD184352 from the eight-cell stage onwards. (A,B) Schematic representation of 64-cell stage embryo showing fates of each blastomere with a spot of colour, key on left. Neural blastomere names are shown. Blastomere identities are also indicated on the cleavage-arrested embryos with an appropriate coloured dot. (C-L) In situ hybridisation was carried out for the genes indicated on the left of the panels. Cleaving embryos are shown in the left-hand panel. Embryos in which cleavage has been arrested from the 64-cell stage are shown in the middle and right hand panels. Application of PD184352 is indicated on the right: left-hand panels, dorsal views with anterior upwards (PD184352 treated embryos are effectively a vegetal view); middle panels, animal views; right-hand panels, vegetal views. Only animal views are shown for *Ci-Epi-1* because this gene is not expressed in vegetal cells. (C-H) *Ci-ETR-1*. (C) Control, 100% positive, n>100. (D,E) Cleavage arrested control, n=156. (D) a-line, 100% positive. (E) A-line, 100% positive. (F) Cleaving embryo+PD184352, 100% positive, n=268. (G,H) Cleavage-arrested embryo+PD184352, n=271. (G) a-line, 0% expression. (H) A-line: 100% in A-line neural precursors; 96% in notochord. Expression of neural markers is detected in the notochord because of the conversion of notochord into neural cells in the absence of FGF or MEK signalling, as previously reported (see Introduction). Expression of *Ci-α tubulin* was similar, except low levels of expression were also detected in the anterior endoderm (not shown). (I-L) *Ci-Epi-1*. (I) Control, n>100. (J) Control cleavage-arrested embryo, n=55. (K) Cleaving embryo+PD184352, n=71. (L) Cleavage arrested+PD184352, n=42. Embryos treated with U0126 produced similar results.

vesicle and nerve cord and epidermal sensory neurones (ESNs) in the head and tail can be revealed with anti-acetylated tubulin antibodies (see Fig. 3G) (Crowther and Whittaker, 1994; Nakatani et al., 1999; Hudson and Lemaire, 2001). Pigment cells, head neurones and head ESNs are derived from a-line cells and the tail ESNs from b-line cells. The lineage of the remaining neurones labelled by α-tubulin is not yet clear. Embryos placed in inhibitor at the eight-cell stage were devoid of pigment cells and lacked neurones, except for the tail ESNs (see Fig. 3A).

To extend these findings, we analysed the expression of two general neural markers, *ETR-1* (Yagi and Makabe, 2001; Satou et al., 2001) and *Ci-α tubulin*, which are expressed in both a-line (a4.2 derivatives) and A-line (A4.1 derivatives) neural cells (Fig. 2C). As both markers gave similar results, only results obtained with *Ci-ETR-1* are shown (Fig. 2). Expression of these markers persisted in neurulae treated with the inhibitor from the eight-cell stage onwards (Fig. 2F). To determine from which lineage the cells expressing *Ci-ETR-1* and *Ci-α tubulin* came from in the MEK inhibitor-treated embryos, we analysed their expression in embryos in which cleavage had been arrested at the 64-cell stage with cytochalasin B. In such embryos, blastomeres do not undergo cytokinesis, but continue to express marker genes consistent with the main fates that they would adopt during normal development. High levels of *Ci-ETR-1* and *Ci-α tubulin* were seen in both a- and A-line neural lineages of control cleavage-arrested embryos (Fig. 2D,E). However, expression was abolished in the a-line lineage (Fig. 2G) of embryos treated with MEK inhibitor from the eight-cell stage, while it persisted in the A-line lineage (Fig. 2H, discussed in more detail later). These results suggest that a-line cells fail to adopt a neural fate in MEK inhibitor-treated embryos. Expression of *Ci-otx*, a marker of sensory vesicle and dorsoanterior epidermis, was also inhibited in a-line cells of most PD184352-treated cleavage-arrested embryos (see Fig. 6C-F). This was confirmed in cleaving embryos in which the a4.2 blastomeres had been DiI labelled (see Fig. 6K,K′,L,L′). Furthermore, two markers of a-line neural tissue, *Ci-gsx* (Hudson and Lemaire, 2001) and *Ci-msxb* (Aniello et al., 1999), were lost in MEK inhibitor treated neurulae (0% expression, n=37, 85, respectively; data not shown).

Formation of a-line neural tissue involves a fate choice between epidermal and neural fates. To test whether lack of MEK signalling leads to the adoption of an epidermis fate in the presumptive neural precursors, we analysed the expression of the epidermal marker *Ci-Epi-1*, in control and MEK inhibitor treated embryos at the neurula stage. In embryos

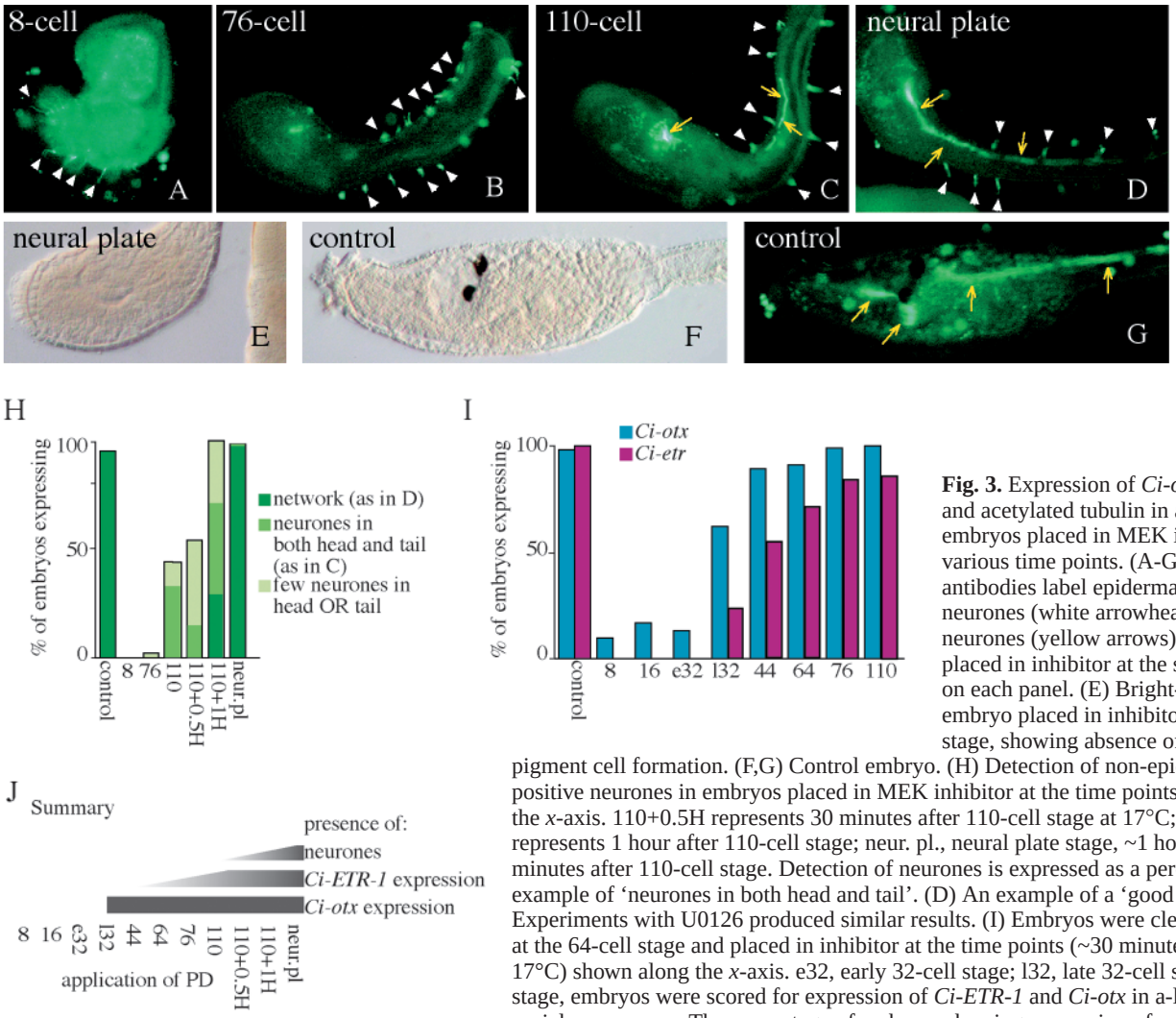

Fig. 3. Expression of *Ci-otx*, *Ci-ETR-1* and acetylated tubulin in animal cells of embryos placed in MEK inhibitor at various time points. (A-G) Anti-tubulin antibodies label epidermal sensory neurones (white arrowheads) and neurones (yellow arrows). Embryos were placed in inhibitor at the stages indicated on each panel. (E) Bright-field view of embryo placed in inhibitor at neural plate stage, showing absence of palp and pigment cell formation. (F,G) Control embryo. (H) Detection of non-epidermal tubulin-positive neurones in embryos placed in MEK inhibitor at the time points shown along the *x*-axis. 110+0.5H represents 30 minutes after 110-cell stage at 17°C; 110+1H represents 1 hour after 110-cell stage; neur. pl., neural plate stage, ~1 hour and 45 minutes after 110-cell stage. Detection of neurones is expressed as a percentage. (C) An example of 'neurones in both head and tail'. (D) An example of a 'good network'. Experiments with U0126 produced similar results. (I) Embryos were cleavage-arrested at the 64-cell stage and placed in inhibitor at the time points (~30 minute intervals at 17°C) shown along the *x*-axis. e32, early 32-cell stage; l32, late 32-cell stage. At neurula stage, embryos were scored for expression of *Ci-ETR-1* and *Ci-otx* in a-line sensory vesicle precursors. The percentage of embryos showing expression of each marker is indicated on the graph. At least 100 embryos were scored for each time point. (J) Summary of the gradual acquisition of neural fates in a-line neural precursors. Time of application of PD184352 shown.

treated with PD184352 but dividing normally, the expression domain of *Ci-Epi-1* was not noticeably altered (Fig. 2I,K). However, the perturbed gastrulation movements makes this experiment difficult to interpret. Therefore, we analysed the expression of *Ci-Epi-1* in cleavage-arrested embryos. In such embryos, in the absence of PD184352, *Ci-Epi-1* expression was seen throughout the non-neural animal cells, but was much lower or absent in the a-line neural precursors (Fig. 2J). However, in embryos treated with MEK inhibitor, strong expression of *Ci-Epi-1* was found in the prospective a-line neural precursors (Fig. 2L), suggesting that these cells have adopted an epidermal fate.

The results presented in this section indicate that MEK signalling is crucial for a-line cells to adopt a neural instead of epidermal fate. We next addressed when MEK signalling was required for the acquisition of neural fate in a-line cells.

Temporal requirement for MEK signalling for the acquisition of neural fates in a-line blastomeres

To determine the temporal requirement for MEK signalling, we analysed the effect of blocking this signalling pathway at progressively later developmental times. As in the previous section, we analysed the formation of neurones and pigment cells at larval stages (Fig. 3A-H). We also analysed the expression at the neurula stage of *Ci-ETR-1* and *Ci-otx* in the a-line lineage of embryos that had been cleavage-arrested from the 64-cell stage (Fig. 3I).

Incubation of the embryos at the 8-, 16- or early 32-cell stages led to a loss of all the above markers in the a-line cells. However, when embryos were treated from the late 32-cell stage, *Ci-otx* expression was found in a-line cells in 62% of cases. a-line *Ci-ETR-1* expression could also be, albeit faintly, detected in 25% of embryos. Treatment of embryos from the 44-cell stage or later had little effect on a-line *Ci-otx* expression, whereas the percentage of embryos expressing *Ci-ETR-1* increased progressively in embryos treated from the 44-, 64-, 76- and 110-cell stages. However, this expression appeared generally weaker than a-line expression in control embryos (not shown). Despite the expression of neural markers in a-line cells of embryos treated with PD184352 before the onset of gastrulation (110-cell

stage), terminal differentiation of acetylated-tubulin-positive neurones (including a-line derived neurones) or pigment cells did not occur (Fig. 3B,H). A few neurones started to be detected in embryos placed in the inhibitor at the 110-cell stage (Fig. 3C,H). The number of neurones detected increased gradually until a good network of neurones was observed in larvae obtained from embryos placed in MEK-inhibitor at the neural plate stage (Fig. 3D,H). This time window fits well with that shown for neuronal induction in a4.2 by inductive interaction with A4.1 (Okado and Takahashi, 1990). Interestingly, embryos treated in PD184352 from neural plate stages, or those placed in inhibitor 1 hour later, during neurulation, still did not form the a-line-derived structures, head epidermal sensory neurones, palps or pigment cells (Fig. 3E; data not shown).

These data show that there is an ongoing requirement for MEK signalling from before the late 32-cell stage until the gastrula stage, for the specification of generic neural fate and at least until the neurula stage for the differentiation of specific neuronal cell types in a-line neural tissue (Fig. 3J).

The MEK signalling pathway acts during the earliest step of the induction of neural tissue in a-line cells

Loss of neural marker expression at neurula stages in embryos in which MEK signalling is inhibited may indicate a loss of gene activation or a loss of maintenance of an initiated neural programme. In order to distinguish between these two possibilities, we analysed the earliest markers of a-line neural fates.

Restriction of a-line precursors to a neural plate fate starts at the 32-cell stage. At this stage, the a6.5 blastomere becomes restricted to an anterior sensory vesicle and palp fate (Nishida, 1987). This fate restriction is accompanied by the onset of expression of *Ci-otx*, which requires an inductive interaction between a6.5 and vegetal blastomeres (Hudson and Lemaire, 2001).

In embryos cultured in the presence of PD184352 from the eight-cell stage and analysed at the 44-cell stage, expression of *Ci-otx* was completely lost in a6.5 (a-line neural) (Fig. 4A,B). Inhibition of a-line *Ci-otx* expression was also observed following dnRas mRNA injection (Table 2). This was not simply a delay of expression, because analysis one cleavage later at the 64-cell stage also revealed a loss of expression of *Ci-otx* in the a-line sensory vesicle precursors in PD184352-treated embryos (Fig. 4C,D). Expression of *Ci-ETR-1* begins at the 110-cell stage in the A-line nerve cord lineage and in the a-line neural plate precursors (Fig. 4E). In PD184352-treated embryos, expression of *Ci-ETR-1* is found in two rows of the A-line cells (the prospective notochord and nerve cord), but is lost from the a-line cells (Fig. 2F).

In summary, in this and the previous sections, we have shown that MEK signalling is required for the earliest events in the induction of a-line neural progenitors and that it is required throughout early cleavage and gastrula stages for full neural induction and specification of differentiated cell types. We next analysed the role of the MEK signalling pathway in the formation of the A-line-derived neural tissue.

Patterning of the A-line derived neural tissue requires an intact Ras/MEK signalling pathway

Consistent with an autonomous specification of the A-line neural tissues (Minokawa et al., 2001), in embryos treated with

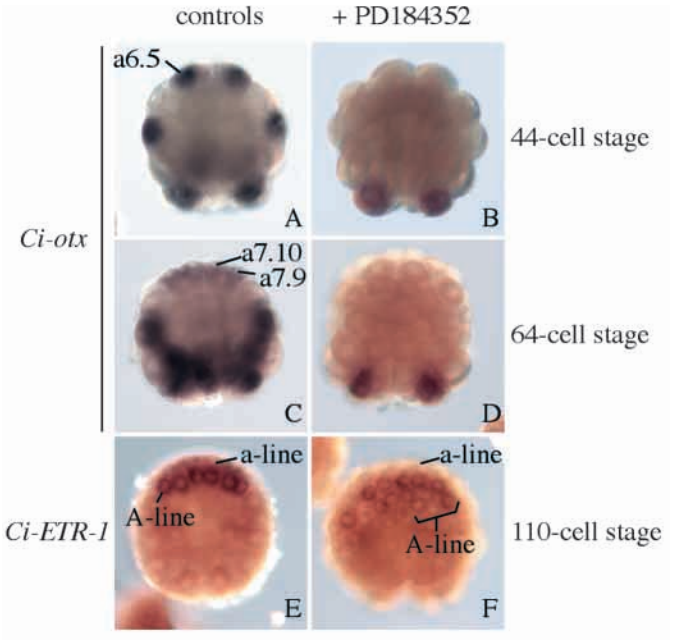

Fig. 4. Loss of expression of early neural markers from embryos treated with MEK inhibitor from the eight-cell stage onwards. The developmental stage is indicated on the right of the panels. All panels show vegetal views, anterior upwards. (A-D) *Ci-otx* in situ hybridisation. (A,C) Control embryos. (B,D) +PD184352. *Ci-otx* expression is lost from a6.5 at the 44-cell stage (0% expression, *n*=269) (B) and from a-line neural cells at the 64-cell stage (0%, *n*=208) (D). *Ci-otx* expression is also lost in b6.5 derivatives (dorsal nerve cord, muscle, endoderm fates) and B-line endoderm. The latter may represent the loss of posterior endoderm reported previously (Kim and Nishida, 2001). The same results were observed with U0126. (E,F) *Ci-ETR-1* in situ hybridisation. (E) Control embryo. (F) PD184352-treated embryos, expression of *Ci-ETR-1* is lost in a-line cells, but persists in A-line (100%, *n*=30).

Table 2. Expression of various markers in dnRas-injected embryos

		Control				dnRAS injected			
Probe	Stage	+	+/–	–	*n*	+	+/–	–	*n*
Ci-bra	l-gast	**94**	6	0	112	**10**	22	68	31
Ci-otx[a]	44 cell	**83**	4	13	75	**6**	6	88	36
Ci-otx	tb	**100**	0	0	71	**100**	0	0	22
08C09	l-tb	**99**	0	1	107	**98**	0	2	44
24H09	l-tb	**97**	0	3	194	**4**	0	96	45
Ci-Hox5	tb	**92**	5	3	108	**36**	9	55	53
Cilv38e16	e-tb	**97**	2	1	88	**17**	15	68	47
Citb8a22	e-tb	**90**	4	6	102	**41**	7	52	29
Ci-HB9/Mnx	l-gast	**100**	0	0	65	**15**	13	72	39

n, number of embryos pooled from at least two independent experiments; +, positive for expression; +/– weak expression or very small domain of expression; –, no expression; gast, gastrula; tb, tailbud; e- and l-, early and late; *Ci-otx*[a], expression of *Ci-otx* in a6.5 blastomere.

MEK inhibitor and cleavage arrested at the 64-cell stage, expression of the neural markers *Ci-ETR-1* and *Ci-α tubulin* persisted in the A-line neural lineage precursors at the neurula stage (Fig. 2H). Expression of neural markers also appeared in the notochord precursors (Fig. 2H) because of the conversion of notochord into neural when FGF/MEK signalling is blocked

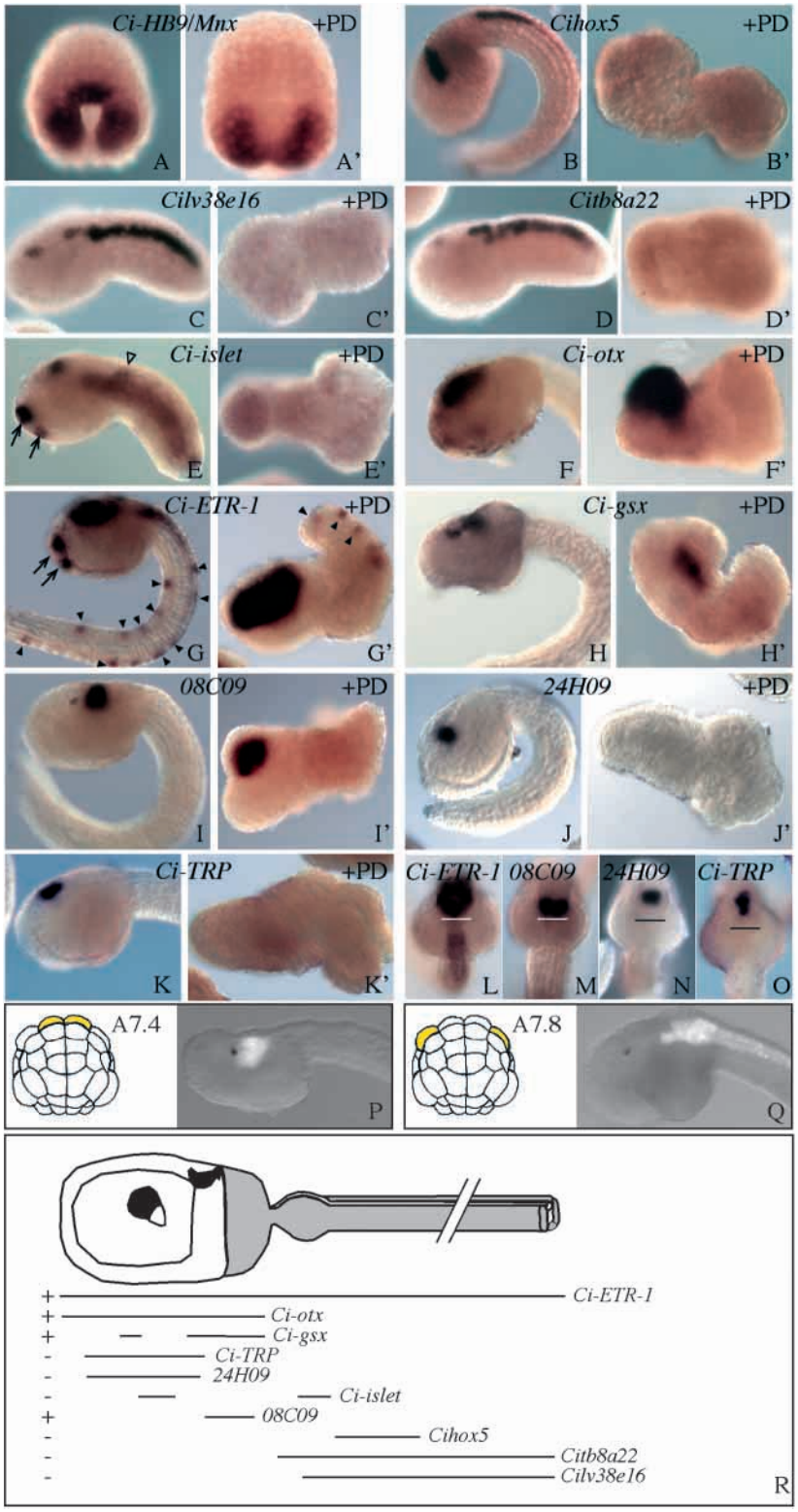

Fig. 5. Expression of neural markers in embryos treated with MEK inhibitor from the eight-cell stage onwards. Arrows indicate gene expression in palps. (A,A′) *Ci-HB9/Mnx*. Late gastrula stage embryo; dorsal view, anterior upwards. Expression is lost in PD184352-treated embryos (+PD) in neural plate cells (*n*=174). (B-K) Control embryo; lateral view, dorsal upwards. (B′-K′) PD184352-treated embryos. (B,B′) *Cihox5* (+PD, 0% expression, *n*=116). (C,C′) *Cilv38e16* (+PD, 0% expression, *n*=139). (D,D′) *Citb8a22* (+PD, 0% expression, *n*=36). (E,E′) *Ci-islet* (+PD, 0% expression, *n*=20). Arrowhead indicates the visceral ganglion expression. (F,F′) *Ci-otx* (+PD, 100% expression, *n*=80). (G,G′) *Ci-ETR-1* (+PD, 100% expression, *n*=108). Expression in tail epidermal sensory neurones was not affected (arrowheads). (H,H′) *Ci-gsx* (control, 72% expression; +PD,46% expression, *n*=278). (I,I′) *08C09* (+PD, 91% expression, *n*=103). (J,J′) *24H09* (+PD, 0% expression, *n*=103). (K,K′) *Ci-TRP* (+PD, 0% expression, *n*=30). In all cases where gene expression was lost, this was not simply a delay of expression, as the onset of gene expression was either much earlier than the stage shown, or loss of expression was verified at a later developmental stage. Similar results were obtained with U0126. (L-O) Dorsal views of embryos showing expression of *Ci-ETR-1*, *08C09*, *24H09* and *Ci-TRP* to indicate relative expression domains along the anteroposterior axis. A bar indicates the posterior limit of the sensory vesicle. (P) DiI labelling of A7.4 at the 64-cell stage. At tailbud stages, label is seen in the posterior sensory vesicle (posterior to the pigment cells) and in the ventral tail nerve cord (not seen in this focal plane). (Q) DiI labelling of A7.8 at the 64-cell stage. At tailbud stages, label is seen in the visceral ganglion, lateral tail nerve cord and a few muscle cells (not visible in picture). (R) A schematic summary of the data shown in this figure. The central nervous system consists of a sensory vesicle, visceral ganglion and tail nerve cord. In black are the pigment cells. A-line cells are coloured grey. The relative expression domains along the anteroposterior axis of genes used in this figure is indicated. + and – indicate the expression status of the genes in PD184352-treated embryos.

(see Introduction). Consistently, at tailbud stages, we observed a large domain of expression of *Ci-ETR-1* in embryos treated with PD184352 from the eight-cell stage onwards (Fig. 5G,G′). Thus, the neural fate of A-line cells appears not to be negatively affected by PD184352 treatment. However, in vertebrates, FGF/MEK signalling has also been implicated in neural tube patterning (reviewed by Gamse and Sive, 2000). In order to test whether MEK signalling may be playing a role in the regionalisation of the A-line autonomously forming neural tissue in ascidians, we analysed a range of region specific neural markers (Fig. 5R) in embryos incubated in PD184352 from the eight-cell stage onwards.

First of all, in order to determine whether these markers were expressed in a-line or A-line-derived neural tissue, we carried out DiI labelling of neural precursors. Nishida (Nishida, 1987) has reported in *Halocynthia* embryos that A7.4 blastomeres contribute to a region posterior to the pigment cells referred to as the brain stem (Nishida, 1987) or the trunk nerve cord (Minokawa et al., 2001). In *Ciona* embryos, a similar region is generally referred to as the posterior sensory vesicle (Nicol and Meinertzhagen, 1991; Takamura, 1998; Meinertzhagen and Okamura, 2001) and we shall refer to it as such. In order to determine which lineage

gives rise to this region in *Ciona*, we first labelled A7.4. Descendants of the labelled cells could be seen in the ventral tail nerve cord and also in the posterior part of the sensory vesicle posterior to the pigment cells (Fig. 5P). Consistently, a6.5 derivatives contributed to the anterior sensory vesicle (not shown) and A7.8 gives rise to the more posterior visceral ganglion and lateral tail nerve cord (Fig. 5Q). Therefore, markers expressed in the anterior part of the sensory vesicle, including the pigment cells, should be expressed in the a-line neural lineages, whereas those expressed in more posterior territories should be in the A-line neural lineages.

We first analysed expression of tail nerve cord markers in embryos treated with MEK inhibitor. *Ci-HB9/Mnx* (C. H. and P. L., unpublished) is normally expressed at neural plate stages in muscle and four nerve cord precursors (Fig. 5A). Expression of *Ci-HB9/Mnx* was unaffected in the muscle lineage, but was completely abolished from the neural cells in PD184352-treated embryos (Fig. 5A′). *Cihox5* (Gionti et al., 1998) and the EST genes *Cilv38e16* and *Citb8a22* (Satou et al., 2001) are expressed in the tail nerve cord of tailbud embryos, with expression of *Citb8a22* extending into the visceral ganglion (Fig. 5B-D). Expression of these markers was lost in embryos treated with MEK inhibitor (Fig. 5B′,C′,D′). Consistently, *Ci-islet* (Giuliano et al., 1998), which is expressed in the visceral ganglion of normal embryos, was also lost (Fig. 5E,E′).

We next analysed markers of anterior and posterior sensory vesicle. At tailbud stages, *Ci-otx* is expressed throughout the sensory vesicle (Fig. 5F) and persists in MEK-inhibited embryos (Fig. 5F′). At tailbud stages, expression of *Ci-gsx* is found in a small anterior and large posterior domain of the sensory vesicle including the area posterior to the pigment cells (Fig. 5H). Expression of *Ci-gsx* persists in MEK inhibitor treated embryos (Fig. 5H′). Consistently, expression of the EST gene *08C09* (D. C. and P. L., unpublished) which is also expressed in the posterior sensory vesicle (Fig. 5I,M) is still expressed in MEK inhibited embryos (Fig. 5I′). By contrast, markers that are expressed in the anterior sensory vesicle, *Ci-islet*, *24H09* and *Ci-TRP* (D. C. and P. L., unpublished), are lost (Fig. 5).

When a subset of the above markers were tested in dnRas mRNA injected embryos, exactly the same trend was observed (Table 2).

These data suggest that the only neural tissue identity remaining in embryos deficient for Ras/MEK signalling is that of the posterior sensory vesicle.

Ci-otx is ectopically expressed in the tail nerve cord lineage in MEK inhibitor treated embryos

In the previous section we showed that only markers of posterior sensory vesicle could be detected in MEK inhibitor-treated embryos, whereas those of anterior sensory vesicle, visceral ganglion and tail nerve cord are lost. The posterior sensory vesicle comes from the A7.4 blastomere (Fig. 5P), whereas the visceral ganglion and tail nerve cord derives largely from the A7.8 blastomere (Fig. 5Q). In order to examine whether the fate of the A7.8 blastomere had been converted to more anterior tissue, we analysed *Ci-otx* expression in PD184352-treated embryos using two approaches. Embryos were cleavage arrested at the 64-cell stage and cultured until the neurula stage, or individual blastomere pairs were labelled at the 64-cell stage with DiI and cultured until the neurula stage when gene expression was analysed. In control embryos, expression of *Ci-otx* is found in A7.4 derivatives in both cleaving embryos (Fig. 6M,M′) and those arrested at the 64-cell stage (Fig. 6H), whereas A7.8 did not express *Ci-otx* (Fig. 6H,O,O′). However, in embryos treated with the MEK inhibitor, expression of *Ci-otx* can be detected in both A7.4 and A7.8 derivatives (Fig. 6I,J,N,N′,P,P′). In MEK inhibitor-treated embryos, gastrulation is affected and A7.8 blastomeres do not move towards the posterior (compare Fig. 6O with 6P). Therefore, the expression of *Ci-otx* in these cells could be a result of their improper position in the embryo. However, in cleavage-arrested embryos, the A7.8 blastomere occupies the same position in control and MEK inhibitor-treated embryos (Fig. 6H,I), yet A7.8 expresses *Ci-otx* only in the MEK inhibitor-treated embryos. Therefore, the altered fate in A7.8 is unlikely to be due to the lack of cell migration and more likely reflects loss of signalling between this lineage and its neighbours. Occasionally, expression of *Ci-otx* could also be seen in the notochord cells of PD184352-treated embryos (Fig. 2J), suggesting that not only had the notochord blastomeres adopted neural fate, but that they had acquired a more anterior character.

These results show that the posterior CNS fates (tail nerve cord and visceral ganglion), which derive largely from A7.8, are lost and that expression of the anterior marker *Ci-otx* is expanded into this territory. This suggests that, in the absence of MEK signalling, all A-line neural blastomeres acquire a more anterior neural fate, corresponding to the anterior most A-line neural fate, the posterior sensory vesicle.

MEK signalling is required prior to gastrulation to suppress *Ci-otx* expression in tail nerve cord precursors

We next analysed when MEK signalling was required during embryogenesis in order to keep expression of *Ci-otx* out of the A7.8 tail nerve cord lineage. In the same experiments described in Fig. 3I, embryos were scored for expression of *Ci-otx* in A7.8 blastomere (Fig. 7A). Expression of *Ci-otx* was seen in A7.8 in embryos placed in MEK inhibitor before the late 32-cell stage, but was lost in all cases in embryos placed in MEK inhibitor from the late 32-cell stage or at later developmental stages. Previous reports show that MEK dependent notochord induction begins at the 32-cell stage (Kim and Nishida, 2001). Similar to these reports, we found that the signal required to suppress neural fate in the notochord was completed around the end of the 32-cell stage (Fig. 7B). In embryos placed in PD184352 at the late 32-cell stage, one experiment showed 99% expression of *Ci-ETR-1* in the notochord precursors and the other experiment showed 3% expression. Embryos placed in inhibitor at the 44-cell stage, or later, showed no *Ci-ETR-1* expression in notochord precursors.

These data show that two MEK-dependent events taking place before the end of the 32-cell stage act to (1) suppress neural fate in notochord precursors and (2) suppress anterior fate in A7.8 blastomere derivatives. The latter event may be completed slightly before the former.

Spatial pattern of ERK activation at the 32-cell stage

We have shown that MEK signalling is required by the end of the 32-cell stage for both the onset of neural fate specification in a-line cells and also for the cell fate choices of A-line neural

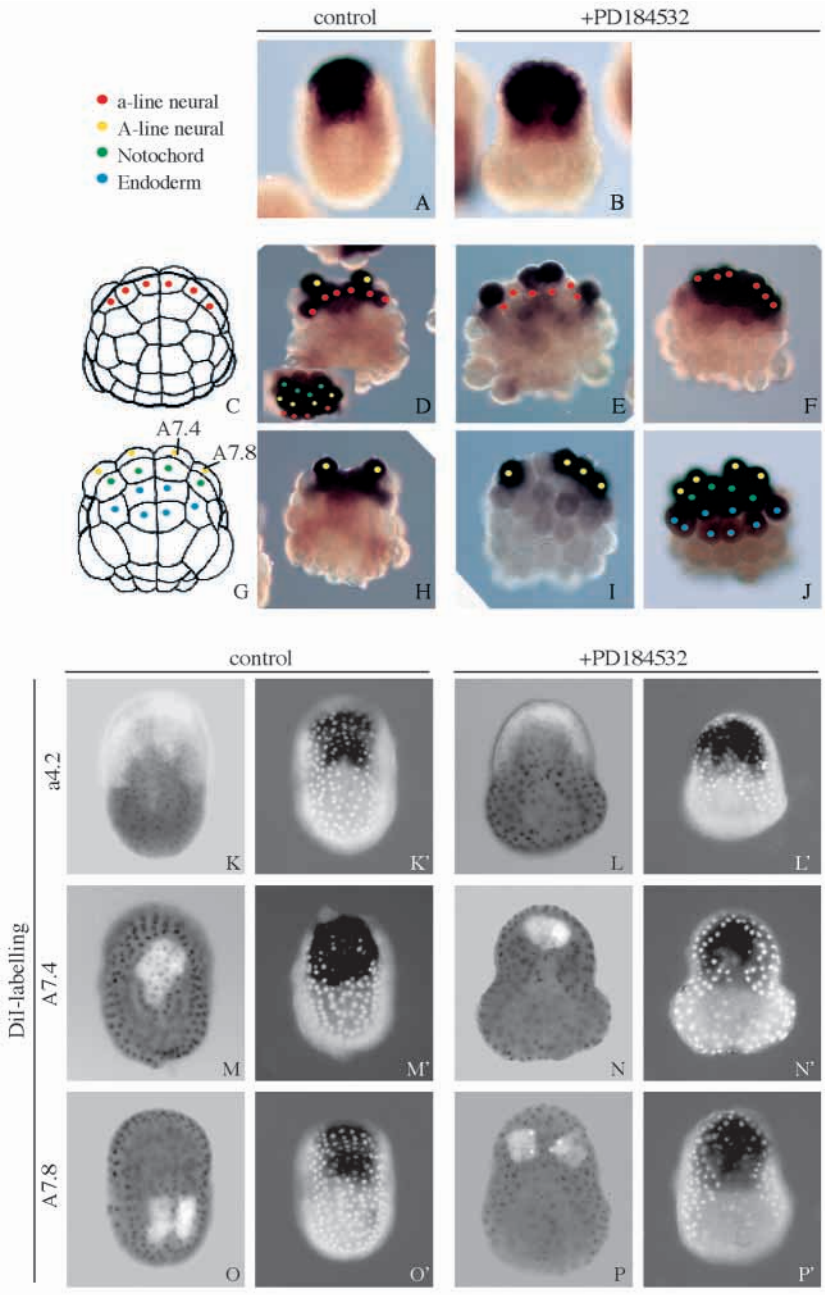

Fig. 6. Expression of *Ci-otx* in different lineages in control and PD184352-treated embryos. (A) Control embryo; dorsal view, anterior upwards; 100% positive, $n \geq 100$. (B) PD184352-treated embryo; 100%, $n=302$. The size of the expression domain was variable. (C-J) cytochalasin cleavage-arrested embryos. (C,G) Schematic representation of 64-cell stage embryo showing fates of each blastomere with a spot of colour, key is in the top left-hand corner. Blastomeres are also indicated on the cleavage arrested embryos with an appropriate coloured dot. (D,H) Control-cleavage arrested, $n=418$. (E,F,I,J) Cleavage-arrested embryo+PD184352, $n=379$. (D) Control a-line (100% expression). Expression was also seen in the anterior epidermis precursor, a7.11 (99%). (E,F) +PD. Expression was generally lost in a-line 60% (E), persisting in 40% of cases (F). Individual percentages for four independent experiments are 85%, 21%, 18%, 0% positive (experiments 1-4 respectively). Thus, in three out of four experiments, expression of *Ci-otx* in a-line cells was largely inhibited. In the experiment where embryos expressed *Ci-otx* in a-line in 85% of cases, the same batch of embryos expressed neither *Ci-ETR-1* nor *Ci-α tubulin* in a-line cells. (H) Control A-line: 99% positive in A7.4; 0% positive in A7.8. (I,J) +PD A-line: 100% positive in A7.4; 75% positive for A7.8. Notochord expression: 40% (individual percentages for experiments 1-4; 85%, 20%, 19%, and 2%). Embryos with notochord expression also had low levels of expression in anterior endoderm. The variability of the *Ci-otx* expression domain in PD184352-treated embryos in a-line and notochord was batch dependent and higher concentrations of inhibitor had little effect on the type of phenotype found. Embryos treated with U0126 gave similar results. (K-P′) DiI labelling showing difference in *Ci-otx* expression in control embryos and embryos treated with PD184352 from the eight-cell stage onwards. (K-P) DiI label; (K′-P′) in situ hybridisation. Nuclear stain facilitates comparison between K-P and K′-P′. Embryos in K′-P′ appear slightly smaller because of shrinkage during in situ hybridisation. The blastomere that was labelled with DiI is indicated on the left of the panels. In all cases both left and right blastomeres were labelled. Controls on left, PD184352-treated embryos on right panels. $n=10$-20 for each. It can be seen that embryos treated with PD184352 fail to gastrulate, with cells maintaining their relative positions from before the gastrula stage.

precursors. MEK directly activates ERK by phosphorylation of an evolutionary conserved sequence (Payne et al., 1991). The *Ciona* genome contains one ERK in which this sequence is completely conserved (Chambon et al., 2002). The availability of an antibody specific to the phosphorylated form of ERK allows direct visualisation of the activation of this pathway in situ (Yao et al., 2000). Therefore, we looked to see if this pathway is activated in the neural cell precursors at the 32-cell stage. We found that activated ERK was detectable at the early 32-cell stage in all A-line vegetal blastomeres, including the notochord/neural precursors (Fig. 8A) and at the late 32-cell stage in the a-line neural precursors (Fig. 8C). Consistently, the activation of ERK was not detectable in PD184352-treated embryos (Fig. 8B,D). Thus, in this study we have shown that both A-line and a-line neural cells require an intact MEK signalling pathway in order to adopt their correct fates and that, using antibodies against phosphorylated ERK as a read out for pathway activation, this pathway is active in these lineages.

DISCUSSION

We have shown that the Ras/MEK signalling pathway is required during early embryogenesis, directly or indirectly, for the formation of both the anterior- and posterior-most neural tissue (except for the posterior epidermal sensory neurones). In the absence of this signalling pathway, the a-line nervous system adopts an epidermal fate, whereas the A-line neural tissue is transformed towards anterior-most A-line neural fate, that is posterior sensory vesicle. MEK signalling is required

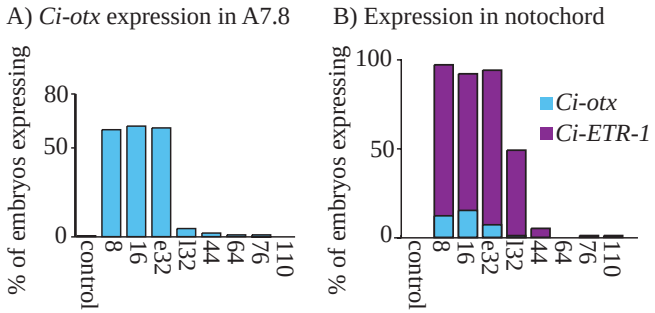

Fig. 7. Expression of *Ci-ETR-1* and *Ci-otx* in vegetal cells of embryos placed in MEK inhibitor at various time points. Embryos were placed in cytochalasin B to arrest cell cleavage at the 64-cell stage. (A) Graph showing the percentage of embryos with expression of *Ci-otx* in A7.8 blastomeres in embryos placed in inhibitor at the time points shown along the *x*-axis. (B) Graph showing the percentage of expression in notochord precursors in embryos placed in inhibitor at the developmental time points shown along the *x*-axis. At the late 32-cell stage, one experiment showed 3% notochord expression, the other 99% expression. This is most likely to be due to slight differences in timing between the experiments.

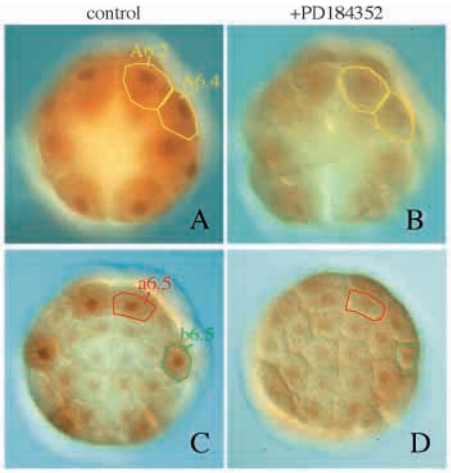

Fig. 8. In situ immunohistochemical analysis of ERK activation in 32-cell stage *Ciona* embryos. (A,B) Vegetal view of early 32-cell stage. (C,D) Animal view of late 32-cell stage. (A,C) Control embryos. (B,D) Embryos treated with PD184352. A-line vegetal blastomeres (A6.4, A6.2) are shown outlined in yellow; a-line neural precursors (a6.5) in red; and b6.5, which also contains neural fate, in green.

during early cleavage stages to restrict *Ci-otx* expression to anterior territories and there is an ongoing requirement for the full acquisition of neural fate in a-line neural lineages. Finally, our analysis of the spatial pattern of activation of ERK is compatible with a direct requirement of this pathway in the neural lineages. The effect of the MEK inhibitor on a-line cells will be discussed first, followed by the effect on the A-line neural cells. Finally, we will compare the role of MEK in ascidian and vertebrate neural tissue formation.

Neural induction in animal cells

In vertebrates, dorsal mesoderm (organiser) has been implicated in the induction of neural tissue (reviewed by Harland, 2000; Stern, 2001). Furthermore, it has been shown in ascidians that recombination of notochord blastomeres with a4.2 explants results in the induction of the a-line derived pigment cells (Reverberi et al., 1960). Therefore, one possibility, which could account for the observed loss of the a-line neural tissue upon MEK inhibition, is that this is a direct result of the loss of notochord in these embryos. However, there is a wealth of evidence that the notochord is not fully specified until the 64-cell stage (Nakatani and Nishida, 1994; Darras and Nishida, 2001a; Minokawa et al., 2001). This is after the MEK-dependent activation of ERK in a-line neural precursors (Fig. 8) and the MEK-dependent expression of ascidian *otx* in these cells (Wada et al., 1996; Hudson and Lemaire, 2001) (Fig. 4). Hence, it is possible that there is no requirement for a fully specified notochord for the onset of a-line neural induction. Consistently, in one experiment where embryos were placed in PD184352 at the late 32-cell stage, *Ci-ETR-1* was expressed in prospective notochord precursors (100%) and *Ci-otx* was expressed in a-line neural precursors (58%) (this study). However, this does not rule out that the notochord precursors secrete neural inducing signals.

FGF constitutes a good candidate for the neural inducing signal. It can induce expression of *Ci-otx* in animal pole explants at the 44-cell stage (Hudson and Lemaire, 2001). Furthermore, it has recently been shown in *Ciona savigni* that *Cs-FGF4/6/9* is expressed in the vegetal cells of 16-32-cell stage embryos, thus at a time and in a place consistent with a role in a-line neural induction (Imai et al., 2002).

There is an ongoing requirement for MEK signalling in order for a-line neural precursors to fully adopt neural fate, express *Ci-ETR-1* and generate differentiated neurones and pigment cells, suggesting MEK may also be required for maintenance. The observation that FGF treatment of animal explants can induce neural and neuronal markers in the absence of the induction of non-neural tissues, suggests that a MEK-dependent signal may be acting directly to instruct these fates, though this requires further investigation (Inazawa et al., 1998; Hudson and Lemaire, 2001; Darras and Nishida, 2001b).

In summary, our results support the idea that the acquisition of neural fate and the differentiation of specific neural cell types in a-line neural cells may involve multiple MEK-dependent steps.

Specification of head and tail epidermal sensory neurones involved different mechanisms

An interesting observation made was that head and tail epidermal sensory neurones appear to be specified using different mechanisms. It has been shown that formation of tail epidermal sensory neurones depend upon interaction of b4.2 with B4.1-line cells, whereas formation of head epidermal sensory neurones depend upon interactions of a4.2 with A4.1 (Hudson and Lemaire, 2001; Ohtsuka et al., 2001). This suggested that different signals may be required for the two neuronal cell types to form. Despite both of these neurone types being inducible by bFGF in animal explants, it appears that only the head epidermal sensory neurones are dependent upon embryonic MEK signalling (Fig. 3). This suggests that induction of anterior and posterior epidermal sensory neurones involve different signal transduction cascades.

Regionalisation of A-line neural cells along the anterior-posterior axis

We have shown that MEK signalling is required before the late 32-cell stage in order to suppress *Ci-otx* expression in posterior neural territories. These embryos also lack a notochord and therefore it is possible that the notochord is required to posteriorise the nerve cord. However, the MEK-dependent signal required to keep *Ci-otx* out of the tail nerve cord lineage has been completed at the late 32-cell stage. Additionally, at this stage, activated ERK can be detected in the notochord/nerve cord precursors (Fig. 8). This is before the notochord lineage becomes restricted. Furthermore, we observed in the same experiment, both expression of *Ci-ETR-1* in the notochord (100%) and restriction of *Ci-otx* to the A7.4 blastomere (95%). Thus, the posteriorisation signal appears to predate the appearance of committed notochord precursors.

Interestingly, by the end of the 32-cell stage, three MEK-dependent processes have been initiated: (1) induction of a-line neural fates; (2) induction of notochord; and (3) posteriorisation of the nerve cord lineage. It is possible that a general vegetal FGF-like signal, as proposed by Kim et al. (Kim et al., 2000), is responsible for all three events. This is supported by the widespread activation of ERK in 32-cell stage embryos (Fig. 8).

If a general vegetal signal is indeed responsible for the tail nerve cord to suppress anterior fates, a remaining question would be why A7.4 (posterior sensory vesicle) derivatives do not respond to this signal. A7.4 blastomere derivatives may receive another, MEK-independent signal, instructing some of them to adopt an anterior fate. Alternatively, A7.4 could be intrinsically different from A7.8 because of the inheritance of specific maternal determinants.

How does this compare to what we know about neural development in vertebrates?

The adoption of neural fate in ascidian a-line sensory vesicle precursors appears similar to the process of neural induction in vertebrates, occurring as a result of a binary choice between neural and epidermal fates. At first glance, the decision of A-line cells to adopt neural fate during a neural/notochord fate decision appears far removed from the generally accepted view in vertebrates of neural induction from an otherwise epidermal fate. However, this phenomenon is not such a peculiarity of ascidians as it might first seem. In zebrafish embryos mutant for Nodal signalling, the dorsal mesoderm (prechordal plate and notochord) changes its fate to that of neural (for reviews, see Harland, 2000; Schier and Talbot, 2001). Indeed, it has been proposed that restriction of mesoderm-inducing signals (notably Nodals) in frog and fish embryos, or localised inhibition of broadly dispersed mesoderm-inducing signals in mice, is vital for allowing formation of a neural plate (Harland, 2000). Thus a decision between dorsal mesoderm and neural tissue is not unprecedented and may be part of a general mechanism of neural tissue formation in chordates. Therefore, it seems that in ascidians, both mechanisms that specify neural fate in vertebrates, induction from an otherwise epidermal fate and protection from mesoderm-inducing signals, are used, the former in a-line neural cells, the latter in A-line neural cells.

In vertebrates, neural tissue was thought to occur by a mechanism, during gastrulation, that inhibited epidermal-promoting factors, allowing neural fates to develop. Recent data suggests neural induction begins much earlier and that there are multiple steps involved in this process (for reviews, see Weinstein and Hemmati-Brivanlou, 1999; Harland, 2000; Stern, 2001; Wilson and Edlund, 2001). A revised model has been proposed that incorporates the more recent evidence from chick studies. This new model proposes that (at least) three steps can be identified during neural induction (Stern, 2001): (1) activation – a step prior to gastrulation that induces non-committed neural forebrain character in cells that can revert to non-neural fates; (2) stabilisation – whereby these cells become committed to a neural (forebrain) fate; and finally, (3) transformation – during which some of these neural cells are transformed to a posterior neural identity. It seems likely that these latter two events temporally overlap during the gastrula stages (Gamse and Sive, 2000).

There is strong evidence in chick embryos that the first step – activation – is dependent upon FGF signalling (Wilson et al., 2000; Streit et al., 2000). This is still somewhat controversial in *Xenopus*, but at least some studies have reported a requirement for FGF/MEK signalling in neural induction (reviewed by Mason, 1996; Uzgare et al., 1998; Hongo et al., 1999). This first step is reminiscent of the situation reported here in *Ciona* embryos for the initial induction of the a-line neural progenitors, and thus may be an ancestral chordate strategy.

In chick embryos, step 2 – stabilisation of neural fate – gives rise to committed neural precursors. This second step requires an active FGF signalling pathway, and in both chick and *Xenopus*, inhibitors of BMP, such as Chordin, are also involved in this step (reviewed by Wilson and Edlund, 2001). In *Ciona* there is an ongoing requirement for MEK signalling, from before the late 32-cell stage until the gastrula stage, for the adoption of neural fate in a-line neural cells. This may represent a similar stabilisation mechanism. Despite the conserved role for MEK involvement in this step, there is evidence that BMP:Chordin antagonism is not a crucial mechanism involved in neural induction or maintenance in ascidians (Darras and Nishida, 2001b).

Finally, MEK signalling has also been implicated in the transformation step (reviewed by Mason, 1996; Pownall et al., 1996; Henrique et al., 1997; Holowacz and Sokol, 1999; Ribisi et al., 2000). A similar process appears to occur in ascidians as the nerve cord precursors lose their posterior identity and express *Ci-otx* in the absence of MEK signalling. The timing of this posteriorisation signal appears to differ in ascidians and vertebrates. In vertebrates the posteriorising signal appears to act during gastrula stages (Gamse and Sive, 2000), although recent data in zebrafish suggests it begins earlier, at late blastula stages (Kudoh et al., 2002). In ascidians it seems to act even earlier, during the early cleavage stages. This may reflect the fact that, in ascidians, cell-fate decisions are generally taken much earlier in development than in vertebrates. This, in turn, may reflect the different modes of development in vertebrates and ascidians, the former with a large number of cells and late fate restriction, the latter with few cells and earlier restriction (reviewed by Lemaire and Marcellini, 2003).

In summary, a requirement for MEK in (1) the early specification, (2) later stabilisation of neural fates during neural induction and (3) specification of posterior neural territories is conserved in vertebrates and ascidians. This may,

therefore, represent part of the core programme of nervous system formation in chordates and suggests that the ancestral chordate may have already used these mechanisms during neural tissue specification. It will be interesting to determine if the role of MEK signalling is also conserved in non-chordate deuterostomes, or whether this strategy is a chordate innovation.

Many thanks to the following people for kindly sending us materials: N. Satoh and the Japanese In situ Consortium; P. Cohen, M. Levine, R. Di Lauro and M. Branno. We thank V. Bertrand, K. Dale, L. Kodjabachian, M. Maroto, Y. Okamura and U. Rothbacher for many discussions and their helpful comments on the manuscript. Also, many thanks to H. Nishida for kindly sharing data with us prior to publication. C. H. thanks the people of the UMR7009 for their hospitality and kindness while experiments were carried out there. S. D. thanks H. Nishida and T. Minokawa for teaching him DiI labelling techniques. This work was supported by the Centre National de la Recherche Scientifique (CNRS), the Association pour la Recherche contre le Cancer, the French Research Ministry (ACI Programme), the Human Frontier Science Program Organisation and the European Union (TMR programme). Work carried out in the laboratory of H. Y. was supported by a CNRS ATIPE grant. C. H. was a EU-TMR fellow and is currently supported by the Association Française contre les Myopathies (AFM).

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