Ebf gene function is required for coupling neuronal differentiation and cell cycle exit

Mario Garcia-Dominguez, Christophe Poquet, Sonia Garel and Patrick Charnay*

Unité 368 de l'Institut National de la Santé et de la Recherche Médicale, Ecole Normale Supérieure, 46 rue d'Ulm, 75230 Paris Cedex 05, France

*Author for correspondence (e-mail: charnay@wotan.ens.fr)

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Summary

Helix-loop-helix transcription factors of the Ebf/Olf1 family have previously been implicated in the control of neurogenesis in the central nervous system in both *Xenopus laevis* and the mouse, but their precise roles have remained unclear. We have characterised two family members in the chick, and have performed a functional analysis by gain- and loss-of-function experiments. This study revealed several specific roles for Ebf genes in the spinal cord and hindbrain regions of higher vertebrates, and enabled their precise positioning along the neurogenic cascade.

During neurogenesis, cell cycle exit appears to be tightly coupled to migration to the mantle layer and to neuronal differentiation. We show that antagonizing Ebf gene activity allows the uncoupling of these processes. Ebf gene function is necessary to initiate neuronal differentiation and migration toward the mantle layer in neuroepithelial progenitors, but it is not required for cell cycle exit. Ebf genes therefore appear to be master controllers of neuronal differentiation and migration, coupling them to cell cycle exit and earlier steps of neurogenesis.

Mutual activation between proneural and Ebf genes suggests that besides their involvement in the engagement of differentiation, Ebf genes may also participate in the stabilisation of the committed state. Finally, gain-of-function data raise the possibility that, in addition to these general roles, Ebf genes may be involved in neuronal subtype specification in particular regions of the CNS.

Key words: Ebf1/Ebf genes, Neurogenesis, Cell cycle, Neuronal differentiation, HLH domain

Introduction

The construction of the vertebrate central nervous system (CNS) involves the coordinated production and differentiation of specific neuronal and glial cell populations according to developmental stage and location. Neurogliogenesis occurs from the ventricular zone, a proliferative neuroepithelium whose cells have properties of stem cells. Neuroepithelial cells can generate committed progenitors that are more restricted in their fate and proliferation potential, and whose progeny leave the cell cycle, migrate into the mantle layer and differentiate. As development proceeds, the mantle layer thickens, while the ventricular zone becomes thinner. During recent years, important insights into the genetic control of vertebrate neurogenesis have been gained, revealing an increasing complexity. These studies, building on observations initially carried out in *Drosophila*, first revealed the existence of so-called proneural genes, which encode transcription factors of the basic helix-loop-helix (bHLH) family, are expressed in the proliferative neuroepithelium, and are necessary and sufficient to promote the generation of the committed progenitors (Bertrand et al., 2002). They include genes of the achaete-scute (*Mash1* in mouse and *Cash1* in chicken) and neurogenin (*Ngn1* and *Ngn2*) families. Proneural genes are subjected to repression by inhibitors (Hes/Her/Esr), which are themselves under the control of Notch signalling. Other bHLH genes are associated with differentiation of the committed progenitors and are expressed in early post-mitotic cells migrating toward the mantle layer (*NeuroM*, *NSCL1*), or in cells which have reached the mantle layer (*NeuroD*) (Begley et al., 1992; Roztocil et al., 1997). However, a specific gene type may not be restricted to a particular function during neurogenesis, as proneural genes have recently been shown to play additional roles, integrating positional information in the process and contributing to the specification of neuronal subtype identity (Fode et al., 2000; Gowan et al., 2001).

Another class of genes, those of the Ebf/Olf1 family (reviewed by Dubois and Vincent, 2001; Liberg et al., 2002), has been involved in the control of neuronal differentiation. *Ebf*/*Olf1* was independently identified as a transcription factor implicated in mouse B-lymphocyte differentiation (*Ebf*) (Hagman et al., 1993; Travis et al., 1993) and in transcriptional control of the rat *olfactory marker protein* gene (*Olf1*) (Kudrycki et al., 1993; Wang and Reed, 1993). Three other rodent genes showing high similarity to *Ebf* (renamed *Ebf1*), *Ebf2* (also known as *Mmot1* or *O/E-3*), *Ebf3* (also known as *O/E-2*) and *O/E-4*, were subsequently identified (Garel et al., 1997; Malgaretti et al., 1997; Wang et al., 1997; Wang et al., 2002). In other species, *Ebf* orthologs have been found in *C. elegans*, *Drosophila*, *Xenopus* and zebrafish (Crozatier et al., 1996; Bally-Cuif et al., 1998; Dubois et al., 1998; Prasad et al., 1998; Pozzoli et al., 2001). All Ebf proteins share an atypical non-basic helix-loop-helix motif (HLH), with an additional type 2 helix and a novel zinc coordination motif. This latter motif is involved in DNA

binding, which also requires homodimerisation or heterodimerisation with other family members mediated by the HLH structure (Hagman et al., 1995). A detailed analysis performed in the mouse revealed that *Ebf1*, *Ebf2* and *Ebf3* are expressed along the entire rostro-caudal axis of the developing CNS, with overlapping patterns, except in the forebrain where each of them is restricted to specific regions (Garel et al., 1997; Wang et al., 1997). Expression is observed in differentiating neurones, *Ebf2* being restricted to early post-mitotic neurones whereas *Ebf1* and *Ebf3* expression is maintained in more differentiated cells. These data suggest a general role for Ebf genes in neuronal differentiation. In accordance with this, overexpression of a dominant-negative mutant of *Xenopus Ebf2*, *Xcoe2/XEbf2*, prevents primary neurone differentiation in *Xenopus* embryos (Dubois et al., 1998). Both *XEbf2* and *XEbf3* were shown to have neurogenic activity in this system, but were proposed to be involved in different steps of the neurogenic cascade. Whereas *XEbf2* acts at an early stage during the commitment process, downstream to *XNgn1* but upstream of *XNeuroD*, *XEbf3*, which is expressed later, functions downstream to *XNeuroD*, in neuronal differentiation (Dubois et al., 1998; Pozzoli et al., 2001). In the mouse, analysis of *Ebf1*$^{-/-}$ mutants revealed neuronal differentiation defects in CNS regions where *Ebf1* is the sole family member to be expressed. Hence, in the striatum, post-mitotic neurones that leave the subventricular zone (SVZ) en route to the mantle layer appear unable to downregulate genes normally restricted to the SVZ, or to activate some mantle-specific genes (Garel et al., 1999). In the hindbrain, facial branchiomotor neurones show an abnormal migratory pathway, presumably as a result of incorrect interpretation of environmental guiding cues (Garel et al., 2000). A migration defect was also observed in gonadotropin-releasing hormone-synthesizing neurones in *Ebf2*$^{-/-}$ mice (Corradi et al., 2003). These latter animals also show peripheral nerve defects, although it was not established whether this is of central and/or peripheral origin. Altogether these studies confirm that the Ebf genes play important and different roles in neurogenesis, and suggest that significant redundancy exists between them.

Although work performed in *Xenopus* has provided information on the position of Ebf genes in the neurogenic cascade, it was restricted to primary neurogenesis in the ectoderm, which significantly differs from neurogenesis in higher vertebrates, in which neurones are continuously generated in the proliferative neuroepithelium and migrate to the pial surface when they differentiate. Furthermore, in gene targeting experiments in the mouse, compensation between the different Ebf genes is likely to have prevented the unravelling of important aspects of their function. This prompted us to undertake a study of Ebf gene function in neurogenesis by electroporation of the chick embryo neural tube, where we can use both gain- and loss-of-function approaches in a higher vertebrate. We provide evidence that Ebf genes play general and essential functions in the neurogenic process in higher vertebrates, acting downstream to proneural genes. They are necessary for initiation of both migration toward the mantle layer and neuronal differentiation, but are not required for cell cycle exit, and thus allow the uncoupling of these two aspects of neurogenesis.

Materials and methods

Plasmid constructs and chick cDNAs cloning

All expression constructs were derived from plasmid pAdRSV-Sp (Giudicelli et al., 2003). For mouse *Ebf1*, a 1.8 kb DNA fragment containing the entire coding sequence was obtained by PCR amplification from plasmid pEBF17 (Hagman et al., 1993) using the following primers: 5′-TTTCATGCTAGCGATCCAGGAAAGCAT-CC-3′ and 5′-AAATTCCTTAAGGCAATTCTTTCACATGG-3′. The dominant-negative *Ebf* construct (Δ*Ebf*), lacking 321 bp corresponding to the 107 N-terminal amino acids, was obtained by subcloning a *Bam*HI-*Afl*II fragment from the former construct. For chick *Ngn2* and *NeuroM*, we used 700 bp *Dra*III-*Pvu*II, and 1.6 kb *Eco*RI, DNA fragments from plasmids *c-ngn-2* (Perez et al., 1999) and pBSK-*NeuroM* (Roztocil et al., 1997), respectively. For chick *NSCL1*, we used a 400 bp DNA fragment obtained by PCR amplification from the cDNA (Li et al., 1999), using the following primers: 5′-ATCCGTCTAGACATGCTCAACTCGGAGCA-3′ and 5′-AGACAATCGATGGGCGGCTCAGACATCCA-3′. *N-cadherin* and *CRABPI* cDNA probes were obtained by RT-PCR from 5-day-old whole chick embryo total RNA, using the following primers:

N-cadherin, 5′-TGGTAACTGTTGTCAAGCCCA-3′ and 5′-GGGTCTACAGCAGTGATGTTA-3′; and

CRABPI, 5′-ATGCCTAACTTCGCCCGCACCT-3′ and 5′-GCTCATCATTAGCTAATTCTCGAGT-3′.

PCR-amplified DNA fragments and cloning junctions were checked by sequencing. cDNA clones corresponding to chick *Ebf1* and *Ebf3* DNA binding domains were obtained essentially as described (Garel et al., 1997), using 5-day-old whole chick embryo total RNA. Degenerated oligonucleotide primers corresponding to the conserved amino acid sequences AHFEKQP and DNMFVHNN that flank the *Drosophila collier* and mouse Ebf1 DNA binding domains were as described previously (Garel et al., 1997). GenBank Accession numbers for chick *Ebf1* and *Ebf3* clones are AY270034 and AY270035, respectively.

In ovo electroporation

Electroporations were carried out as described (Itasaki et al., 1999; Giudicelli et al., 2001). Briefly, fertilised eggs were incubated at 37°C to the indicated embryonic stages. DNA in 10 mM Tris (pH 8.0) was injected into the neural tube at the following concentrations: Δ*Ebf*, 2 μg/μl; reporter plasmids [pAdRSVβgal (Le Gal La Salle et al., 1993) and pEGFP-N1 (Clontech)], 0.5 μg/μl; other constructs, 1.5 μg/μl. The total DNA concentration in each electroporation experiment was kept constant by adding vector plasmid DNA. Embryos were electroporated at hindbrain or thoracic spinal cord levels. Electroporations were performed with a BTX820 electroporator (Quantum) with the following parameters: hindbrain level, 4 pulses of 25 V and 40 milliseconds at a frequency of 1 Hz; spinal cord level, 6 pulses of 25 V and 50 milliseconds at a frequency of 1 Hz. To evaluate the efficiency of electroporation, a GFP expression vector (pEGFP-N1) was systematically co-transfected in embryos to be processed for in situ hybridisation or peroxidase-revealed immunohistochemistry, and only efficiently electroporated embryos were analysed. Following electroporation, the eggs were incubated for 10 to 40 hours, as indicated. The embryos were harvested in phosphate buffered saline (PBS), fixed in paraformaldehyde (PFA, 4% in PBS) for 3 hours for immunohistochemistry, or for more than 6 hours for in situ hybridisation, and dehydrated in a methanol series. For β-galactosidase detection, the embryos were fixed for 20 minutes and stained with X-gal as previously described (Schneider-Maunoury et al., 1993).

In situ hybridisation

In situ hybridisation with digoxigenin-labelled riboprobes on whole-mount embryos or vibratome sections was performed as described (Wilkinson and Nieto, 1993) and double in situ hybridisation was as

described previously (Giudicelli et al., 2001). In this latter case one of the probes was labelled with fluorescein-UTP. Digoxigenin and fluorescein were detected sequentially with alkaline phosphatase-coupled antibodies (Roche). NBT/BCIP (purple) staining was carried out first, typically on digoxigenin-labelled probes. After removal of the antibody, INT/BCIP (orange, red) staining was performed on fluorescein-labelled probes. After whole-mount in situ hybridisation, embryos were flat-mounted or microtome sectioned. For vibratome section (50 μm thick), embryos were embedded in 4% agarose or albumin/gelatine. The chick probes were as follows: *cEbf1*, *cEbf3*, *N-cadherin* and *CRABPI* (this work); *R-cadherin* (Inuzuka et al., 1991a); *Cash1* (Jasoni et al., 1994); *Ngn1* and *Ngn2* (Perez et al., 1999); *Islet1*, *Islet2* and *Lim1* (Tsuchida et al., 1994).

Cell proliferation and cell apoptosis analyses

Cell proliferation was evaluated by bromodeoxyuridine (BrdU) incorporation. BrdU, 15% (w/v) in PBS, was injected into the lumen of the neural tube and the embryos were harvested 2 hours later. BrdU immunodetection was performed on sections treated with 2 N HCl, 0.5% Triton X-100 in PBS for 30 minutes at 37°C, after blocking and before incubation with primary antibodies. Apoptosis was detected by fluorescein labelling of DNA strand breaks (TUNEL, Roche) on 50 μm-thick vibratome sections.

Immunohistochemistry

Immunohistochemistry was normally revealed by fluorescence, except for the detection of neurofilaments in flat-mounted hindbrains. In this latter case, we used a biotinylated hamster antibody directed against mouse IgG (Vector, 1:400) and streptavidin-horse radish peroxidase (Amersham, 1:500), to detect the anti-neurofilament antibody. Peroxidase activity was revealed with diaminobenzidine (Sigma), in the presence of nickel ammonium to enhance the staining. For immunofluorescence, 50 μm-thick vibratome sections were prepared from embryos embedded in 4% agarose, and blocked in PBS containing 0.25% Triton X-100 and 5% donkey serum. Primary antibodies were incubated in the same solution overnight at 4°C. Incubations with the secondary antibodies were at room temperature for 2 hours. All washes were performed with PBS containing 0.25% Triton X-100. Sections were mounted in Vecta Shield (Vector). Immunofluorescence pictures were acquired on a Leica TCS 4D confocal microscope and assembled with Adobe Photoshop. Antibodies and dilutions were as follows: neurofilaments, mouse monoclonal 3A10 (1:20, Developmental Studies Hybridoma Bank (DSHB)); β-tubulin-type III (Tuj1), mouse monoclonal and rabbit polyclonal (1:500, Babco); BrdU, mouse monoclonal (1:100, Becton Dickinson); β-galactosidase, rabbit polyclonal (1:700 Cappel); Islet 1/2, mouse monoclonal 39.4D5 (1:100, DSHB); HA, rat monoclonal (1:400, Roche); Flag, rabbit polyclonal (1:200, Sigma); FITC-, Cy3- and Cy5-conjugated secondary antibodies (1:200-1:800, Jackson Immuno Research).

Results

Identification and expression of chick Ebf genes

Before performing functional analyses of Ebf genes in the chick embryo, it was important to identify chick Ebf family members and to study their expression pattern in the developing neural tube. We followed a strategy that previously led us to the identification of mouse *Ebf2* and *Ebf3* (Garel et al., 1997). Degenerated oligonucleotide primers derived from highly conserved regions of the DNA binding domains of mouse *Ebf1* and the *Drosophila Ebf* homologue *collier* (*knot* – FlyBase) were used for RT-PCR amplification of RNA from five-day-old whole chick embryos (see Materials and methods). Two different amplified sequences were identified as belonging to putative Ebf family members (GenBank Accession Numbers AY270034 and AY270035). Nucleotide and amino acid sequence comparisons strongly suggested that they correspond to the chick orthologs of mouse *Ebf1* and *Ebf3*, respectively. Chick Ebf1 presented 87% and 99% identity with mouse Ebf1 at the nucleotide and amino acid levels, respectively (data not shown). Chick Ebf3 showed 86% and 100% identity with mouse Ebf3 at the nucleotide and amino acid levels, respectively (data not shown).

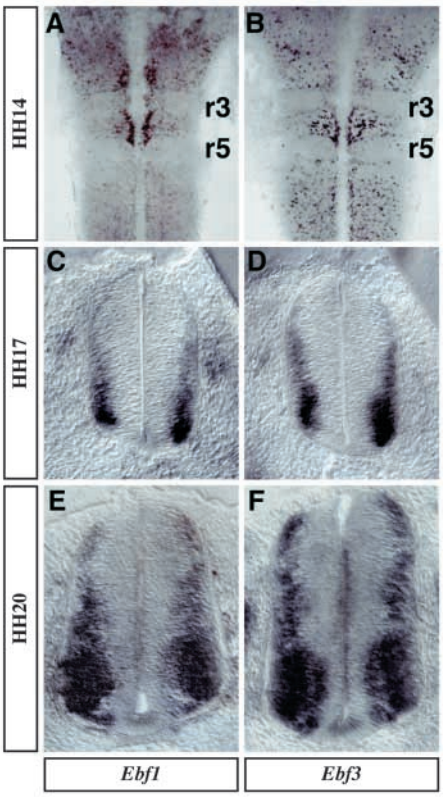

Fig. 1. Expression of *Ebf1* and *Ebf3* in the chick neural tube. In situ hybridisation was performed as indicated (stages and probes). (A,B) Flat mounts of the hindbrain region showing the generalised expression of *Ebf1* and *Ebf3*, with the exclusion of rhombomeres 3 and 5, coincident with neurogenesis at stage HH14. (C-F) Transverse sections through the spinal cord at stages HH17 and HH20 showing high expression of *Ebf1* and *Ebf3* in the mantle layer. r, rhombomere.

In situ hybridisation analysis of the expression of chick *Ebf1* and *Ebf3* in the developing neural tube revealed an activation coincidental with the onset of neurogenesis (Fig. 1). This was particularly obvious in the hindbrain, where neurogenesis is known to occur first in even-numbered rhombomeres (r), and then in odd-numbered ones. A similar dynamic pattern of activation was seen for *Ebf1* and *Ebf3*, which were activated throughout the hindbrain except in r3 and r5 at stages HH13-HH14 (Fig. 1A,B); expression in these latter rhombomeres occurred only from stage HH15 (data not shown). Sections through the spinal cord revealed that *Ebf1* and *Ebf3* expression was mainly associated with the mantle layer, and was likely to correspond to differentiating post-mitotic neurones (Fig. 1C-F). These expression patterns suggest that Ebf gene activation may constitute a general feature of neuronal differentiation, in this part of the CNS at least. In addition, they are very similar

to those observed for the murine orthologs (Garel et al., 1997; Wang et al., 1997). The conservation of gene sequences and expression profiles suggest that Ebf genes perform similar functions in mammals and birds.

Ngn2 and NeuroM promote Ebf gene expression

Since Ebf gene expression is induced during neurogenesis, we investigated whether genes known to promote this latter process might also cause extended Ebf gene expression. The *atonal*-related genes *Ngn1* and *Ngn2* are normally activated in proliferating cells in the ventricular zone, and are involved in the initiation of neurogenesis (Ma et al., 1996; Ma et al., 1999; Perez et al., 1999). To test a possible epistatic link between proneural and Ebf genes, we misexpressed *Ngn2* in the chick neural tube by in ovo electroporation. The *Ngn2* coding sequence was cloned into plasmid pAdRSV-Sp, placing it under the control of the Rous sarcoma virus long terminal repeat promoter, enhanced by the human type 5 adenovirus terminal repeat. This combination of regulatory elements leads to efficient transcription of genes inserted downstream (Giudicelli et al., 2003). Electroporation of the *Ngn2* expression plasmid in one half of the neural tube at stage HH9-HH10 led to strong activation of *Ebf1* and to weaker, but significant, enhancement of *Ebf3* expression, as detected by in situ hybridisation (Fig. 2A,D).

We then investigated whether genes possibly acting more downstream in the neurogenic cascade could also lead to Ebf gene activation. The genes *NeuroM* and *NSCL1* are transiently expressed in early post-mitotic neurones starting their migration to the mantle layer (Begley et al., 1992; Roztocil et al., 1997). As in the case of *Ngn2*, both genes were ectopically expressed in the neural tube by electroporation using the pAdRSV-Sp expression plasmid, and the expression of *Ebf1* and *Ebf3* was monitored by in situ hybridisation. The effect of *NeuroM* misexpression was very similar to that of *Ngn2*, leading to the activation of both Ebf genes (Fig. 2B,E). By contrast, *NSCL-1* ectopic expression did not affect the Ebf gene expression pattern (Fig. 2C,F).

These experiments indicate that Ngn2 and NeuroM can promote Ebf gene expression during neuronal differentiation. They suggest that proneural and early differentiation genes lie upstream of the Ebf genes in the cascade of events controlling neurogenesis.

Ebf1 misexpression promotes neuronal differentiation

To directly assess Ebf gene function in neurogenesis, we first performed gain-of-function experiments by using in ovo electroporation in the chick neural tube with the pAdRSV-Sp expression vector. We chose to misexpress the mouse *Ebf1* gene because the complete coding sequence was available and this gene appears to be very similar to the chick ortholog, both in terms of sequence and expression pattern. In the first series of experiments, stage HH15 embryos were co-electroporated with *Ebf1* and *lacZ* expression plasmids to identify the electroporated cells [it has been shown that under these conditions, most electroporated cells receive both plasmids, (Dubreuil et al., 2000)]. As a control, some embryos were electroporated with the *lacZ* expression plasmid alone. In the control experiments, analysis of the *lacZ*-positive cells, performed by X-gal staining, indicated that, as expected, 20 hours after electroporation most cells presented a morphology and a localisation of neuroepithelial progenitors (Fig. 3A). This was still the case for the majority of the electroporated cells at 40 hours after electroporation, but at that time some X-gal-positive cells were restricted to the mantle layer and had presumably undergone differentiation (Fig. 3B). Analysis of the distribution of X-gal-positive cells in neural tubes co-electroporated with the *Ebf1* expression plasmid revealed a very different situation: whereas at 20 hours after electroporation most of the cells presented a neuroepithelial morphology (Fig. 3C), like in the control, at 40 hours essentially all of the *lacZ*-positive cells were localised in the mantle layer (Fig. 3F). A time course analysis indicated that the migration of *Ebf1*-electroporated cells was largely engaged at 24 hours and was almost completed at 30 hours (Fig. 3D,E). Therefore ectopic expression of *Ebf1* in neuroepithelial progenitors appears to result in the early onset of cell migration toward the mantle layer.

As neuroepithelial progenitors normally exit the cell cycle before engaging in migration towards the mantle layer, we investigated whether the early migrating *Ebf1*-expressing cells could still be proliferating. To track the *Ebf1*-electroporated cells in this case, we modified the expression vector to introduce an HA epitope at the N-terminal end of the protein. We then verified that this modification did not affect the activity of the protein in co-electroporation experiments with *lacZ* (data not shown). To analyse proliferation, chick neural tubes were electroporated with either the *HA-Ebf1* expression construct or a control construct, *Krox20m-HA* (see below), incubated for 18 or 28 hours, and then subjected to a 2-hour BrdU incorporation pulse, which labels cells in S-phase. The embryos were then processed for

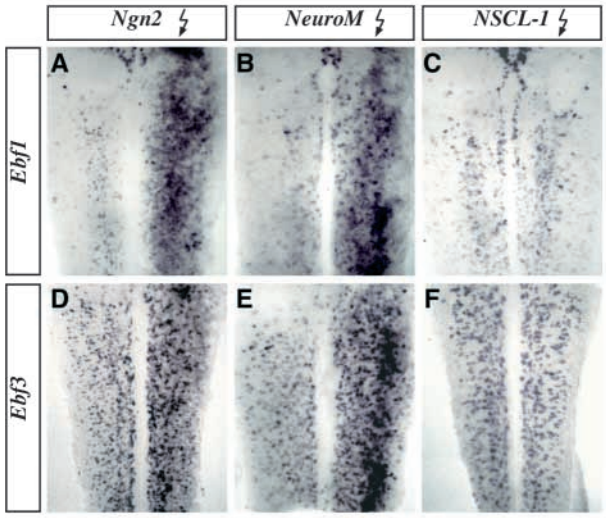

Fig. 2. Induction of Ebf gene expression by Ngn2 and NeuroM. Stage HH10 chick embryos were co-electroporated at the level of the anterior spinal cord with the GFP expression vector and *Ngn2* (A,D), *NeuroM* (B,E) or *NSCL1* (C,F) expression plasmids. Embryos were collected 24 hours later and processed for in situ hybridisation with *Ebf1* (A-C) and *Ebf3* (D-F) probes. The neural tubes were then flat-mounted. *Ebf1* and *Ebf3* were induced by Ngn2 and NeuroM, but not by NSCL1. This conclusion reflects the behaviour of more than 90% of the embryos, from three independent experiments, each involving at least eight embryos per probe. Electroporation was on the right side.

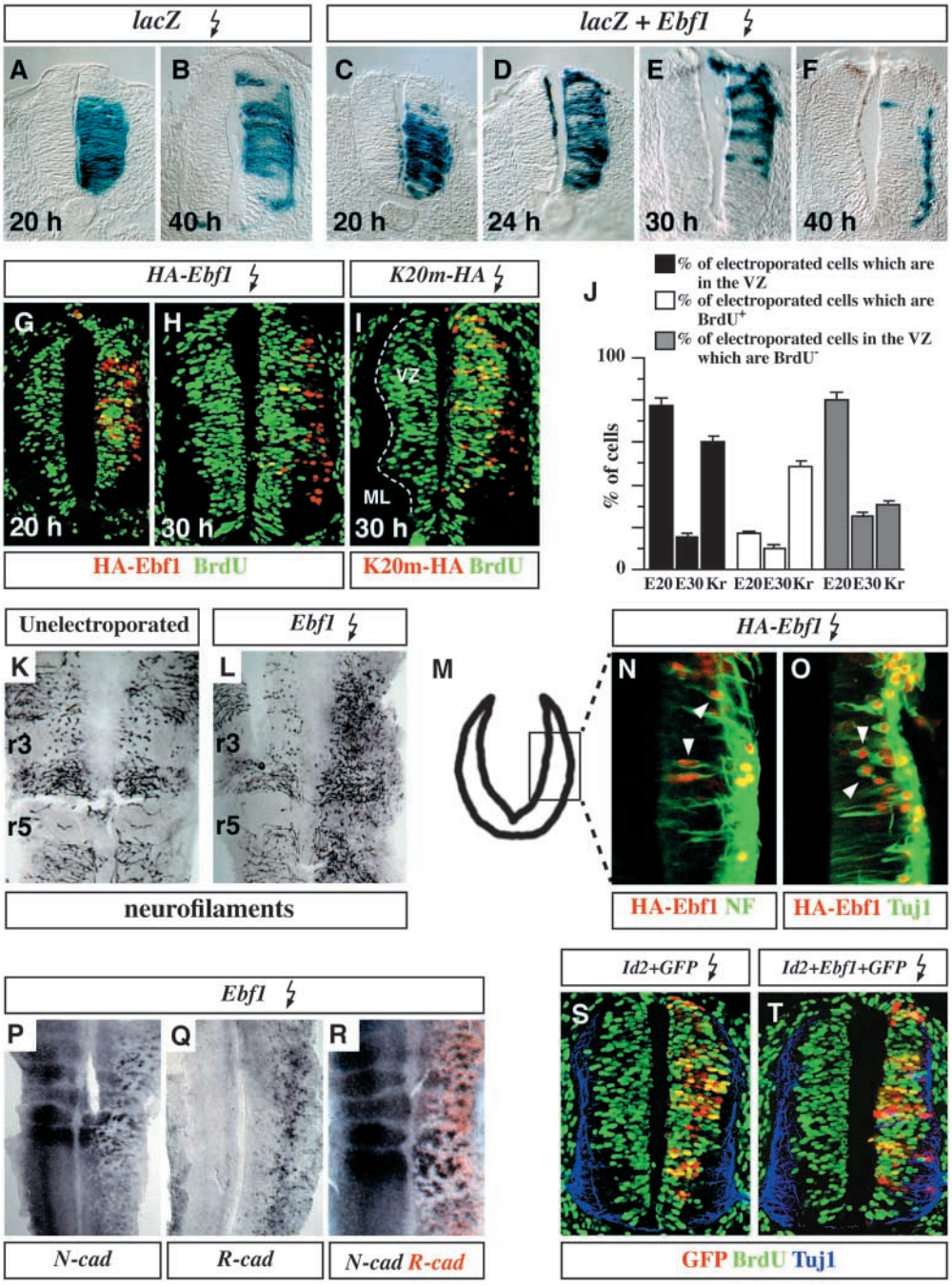

Fig. 3. *Ebf1* misexpression promotes neuronal differentiation. (A-F) Stage HH15 chick embryos were either electroporated with a *lacZ* expression plasmid, or co-electroporated with *lacZ* and *Ebf1* expression plasmids, incubated for the indicated periods, processed for X-gal staining and transversally sectioned. Each pattern shown reflects the situation in more than 90% of the embryos, from eight independent experiments, each involving six embryos per condition. (G-I) Stage HH15 embryos were electroporated with HA-tagged *Ebf1* or R409W mutant *Krox20* (*K20m-HA*, encoding an inactive transcription factor) and then subjected to 2-hour BrdU pulse-labelling immediately before collection at the indicated time following electroporation. Vibratome sections were then analysed by immunofluorescence with antibodies directed against HA (red) and BrdU (green). The dashed line in I indicates the separation between the mantle layer (ML) and the ventricular zone (VZ). (J) Quantification of the data obtained from experiments presented G-I. The bars represent the percentage of electroporated cells (HA-Ebf1- or Krox20m-HA-positive, red or yellow) that are located in the ventricular zone (black bars), the percentage of electroporated cells that are BrdU-positive (yellow, white bars), and the percentage of electroporated cells within the ventricular zone, which are BrdU-negative (red, grey bars). The cell counts correspond to the analysis of six to nine sections from at least three independently processed embryos for each condition. The data represent mean±s.e.m. (K,L) Flat-mounted hindbrains from embryos that were either not electroporated or co-electroporated with *Ebf1* and GFP expression vectors at stage HH10, then stained for neurofilaments by immunochemistry 24 hours later. (M-O) Stage HH10 embryos were electroporated with HA-tagged *Ebf*, sectioned 24 hours later at the level of r6 and analysed by immunofluorescence with antibodies directed against the HA epitope (red) and neurofilaments (green, N), or the HA epitope and Tuj1 (green, O). The arrowheads point to cells co-expressing the two markers. M shows the part of the sections presented in N,O. Each analysis was performed on three independent series of six embryos. Tuj1 and neurofilaments were detected in approximately 100% and 80% of the HA-Ebf1-positive cells, respectively. (P-R) Flat-mounted hindbrains from embryos co-electroporated with *Ebf1* and GFP expression vectors at stage HH10, and processed 24 hours later for in situ hybridisation with *N-cadherin* (*N-cad*) and *R-cadherin* (*R-cad*) probes. In R, double in situ hybridisation was performed (*N-cad*, purple; *R-cad*, red). In K,L,P-R the patterns shown were observed in more than 90% of the embryos, from four independent experiments, each involving at least six embryos per condition. (S,T) Stage HH15 embryos were co-electroporated with GFP and chicken *Id2* (S), or GFP, *Id2* and *Ebf1* expression vectors (T). They were then subjected to 2-hour BrdU pulse-labelling immediately before collection, 30 hours after electroporation. Vibratome sections were then analysed by immunofluorescence with antibodies directed against BrdU (green), GFP (red) and Tuj1 (blue). In S and T, 60±5% and 55±3% of transfected cells were BrdU$^+$, respectively, and 98±0.6% and 88±4% were located in the VZ, respectively. The data represent mean±s.e.m. and correspond to the analysis of seven sections from three independently processed embryos for each condition. Electroporation was on the right side. Electroporated constructs are indicated at the top of each panel and immunolabelling or in situ hybridisation probes at the bottom. h, hours.

immunofluorescence with antibodies directed against HA and BrdU. Analysis of neural tube transverse sections from embryos incubated for 30 hours after electroporation confirmed the previous results, i.e. most HA-Ebf1-positive nuclei were located in the mantle layer (Fig. 3H,J). In addition, it revealed that almost all of these nuclei were BrdU-negative (Fig. 3H), which suggests that the electroporated cells had left the cell cycle. When the embryos were recovered 20 hours after electroporation, more than half of the HA-Ebf1-positive nuclei were still within the ventricular zone (Fig. 3G,J). Nevertheless, the majority of these ventricular nuclei were BrdU-negative (Fig. 3J). The control construct, *Krox20m-HA*, was designed to produce a mutant derivative of another transcription factor, Krox20. This mutant, R409W, does not bind to DNA and has no transcriptional activity (Warner et al., 1999; Giudicelli et al., 2001); it is therefore expected to have no effect on neurogenesis. Indeed, 30 hours after electroporation, most Krox20m-HA-positive nuclei were still within the ventricular zone (Fig. 3I,J), in agreement with the *lacZ* electroporation experiments (data not shown). Furthermore, most of these ventricular nuclei were BrdU-positive (Fig. 3J), indicating that the corresponding cells were proliferating. In conclusion, these experiments indicate that ectopic expression of *Ebf1* in neuroepithelial progenitors leads to early onset of both exit from the cell cycle and migration toward the mantle layer.

Finally, we investigated whether forced *Ebf1* expression would result in modifications in the expression pattern of neuronal and neuroepithelial markers. As indicated above, at around stages HH13-HH14 the hindbrain is an interesting region in which to analyse neuronal differentiation because, in contrast to even-numbered rhombomeres, odd-numbered rhombomeres are still largely devoid of differentiated neurones, as indicated by neurofilament labelling (Fig. 3K). Analysis of neurofilament expression in *Ebf1*-electroporated hindbrain revealed both an increased number of differentiated neurones in even-numbered rhombomeres and an early onset of neuronal differentiation in odd-numbered ones (Fig. 3L). Furthermore, double-labelling for HA-Ebf1 and neurofilaments or another neurone-specific marker, neuronal class III β-tubulin (Tuj1), indicated that most Ebf1-positive cells also expressed these markers (Fig. 3M-O). An important aspect of the neuronal differentiation programme is the shift in the expression pattern of adhesion molecules that is associated with neuronal migration: for example, N-cadherin is expressed in neuroepithelial progenitors, whereas R-cadherin expression is activated later during neurogenesis and has been suggested to be associated with post-mitotic cells (Inuzuka et al., 1991b; Redies and Takeichi, 1996). We found that *Ebf1* ectopic expression led to the repression of N-cadherin expression and the activation of R-cadherin expression (Fig. 3P-R). Therefore, Ebf1 promotes downregulation of a progenitor-associated adhesion molecule and expression of a neurone-associated one. This may play a role in the early onset of the migration of the electroporated cells towards the mantle layer. In conclusion, these data indicate that ectopic expression of *Ebf1* in neuroepithelial progenitors promotes neuronal differentiation, as indicated by modifications in the expression of several markers.

Ebf1-mediated neurogenesis involves activation of bHLH genes

As *Ebf1* misexpression promotes neurogenesis in a similar way to proneural genes, we wondered whether this outcome might be reached through activation of the proneural genes. We

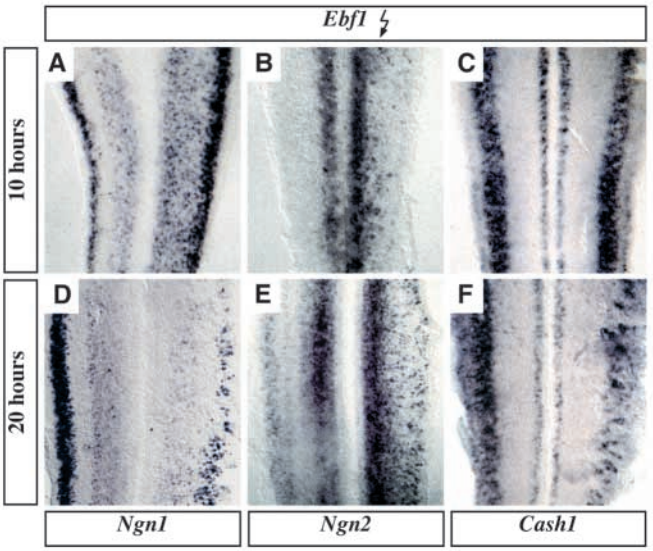

Fig. 4. Transient activation of proneural genes following *Ebf1* misexpression. Stage HH10-HH12 chick embryos were co-electroporated on the right side with *Ebf1* and GFP expression plasmids, collected 10 hours (A-C) or 20 hours (D-F) later, and processed for in situ hybridisation with the indicated probes. The pictures show flat mounts of the anterior region of the spinal cord. The cases shown are representative of more than 90% of the embryos, from three independent experiments, each involving at least eight embryos per probe.

therefore tested the effect of *Ebf1* misexpression on *Ngn1* and *Ngn2*, as well as on the *achaete-scute*-related *Cash1* gene. Ten hours after electroporation, expression of both *Ngn1* and *Ngn2* was induced on the experimental side (Fig. 4A,B). By contrast, *Cash1* expression was not affected (Fig. 4C). At later stages, consistent with the neuronal differentiation induced by *Ebf1*, we observed a general downregulation of the proneural genes. In the case of both *Ngn1* and *Cash1*, this was already visible at 20 hours after electroporation, whereas the decrease in *Ngn2* expression was only observed after 30 hours (Fig. 4D-F, and data not shown). These data indicate that Ebf1 can promote the expression of some, but not all, proneural genes, which suggests the existence of specific, positive regulatory loops linking these genes.

Activation of *Ngn1* and *Ngn2* provides a possible means for induction of neurogenesis by Ebf1. But is proneural, and more generally bHLH, gene function actually mediating aspects of Ebf1 neurogenic activity? To address this issue we used an Id inhibitor of bHLH proteins (Norton, 2000) to antagonise their activity. As expected, electroporation of a chicken Id2 (Martinsen and Bronner-Fraser, 1998; Dubreuil at al., 2002) expression vector prevented migration of neuroepithelial cells towards the mantle layer and neuronal differentiation (Fig. 3S, compare with 3I). Co-electroporation of the *Ebf1* and *Id2* expression vectors led to only a minority of the electroporated cells migrating into the mantle layer and expressing the neuronal marker Tuj1 (Fig. 3T). This is in sharp contrast with the electroporation of *Ebf1* alone, which drives most of the cells into the mantle layer (Fig. 3H,J). Therefore, we can conclude that induction of neurogenesis by Ebf1 relies for a large part on bHLH (including proneural) protein activity. In addition, the fact that no cycling cells were observed among

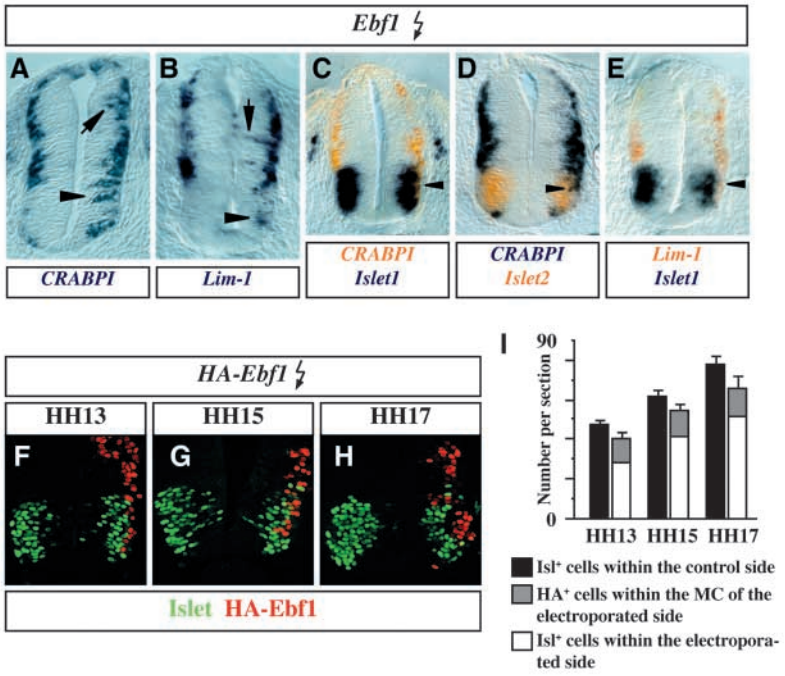

Fig. 5. *Ebf1* misexpression affects neuronal subtype specification. (A-E) Stage HH15 chick embryos were co-electroporated with *Ebf1* and GFP expression vectors, incubated for 30 hours, sectioned and processed for in situ hybridisation with *CRABPI* (A) and *Lim1* (B) probes, or for double in situ hybridisation with *CRAPBI* and *Islet1* (C), *CRABPI* and *Islet2* (D), or *Lim1* and *Islet1* (E) probes. In double-labelling experiments, the colour code is shown underneath. Note the presence of cells ectopically expressing *CRABPI* or *Lim1* within the ventricular zone (arrows), and at the level of the motor column on the electroporated side (arrowheads). These latter cells do not express *Islet1* (C,E). The cases shown are representative of more than 80% of the embryos, from six independent experiments, each involving at least six embryos per probe. (F-H) Embryos were electroporated with HA-tagged *Ebf1* at stage HH13 (F), HH15 (G) and HH17 (H), and collected 30 hours later. Vibratome sections were processed for immunofluorescence analysis with antibodies directed against the HA epitope (red) and Islet1/Islet2 (green). (I) Quantification of the data obtained from the experiments presented in F-H. The bars represent the number of Islet-positive cells per section on the control side (green, black bars), the number of HA-Ebf1-positive cells per section within the estimated motor column on the electroporated side (red, grey bars) and the number of Islet-positive cells per section on the electroporated side (green, white bars). The data represent mean±s.e.m. of cell counts and correspond to the analysis of six to nine sections of at least three independently processed embryos for each condition. Only sections containing at least 10 HA-Ebf1-positive cells within the motor column have been taken into account. $P<0.001$ for the differences observed in the number of Islet-positive cells between control and electroporated sides. Electroporation was on the right side. Electroporated constructs are indicated at the top of each panel, and immunolabelling or in situ hybridisation probes at the bottom. MC, motor column.

the co-electroporated cells located in the mantle layer suggests that Ebf1 activity is not sufficient to drive cells into the mantel layer if they have not left the cell cycle.

Ebf1 misexpression alters neuronal subtype

As Ebf1 is able to promote neuronal differentiation, we wondered whether it might also interfere with the specification of neuronal identity. To investigate this possibility, we analysed the effect of *Ebf1* misexpression on neuronal subtype characteristics. At the stage of collection of the embryos (30 hours after electroporation at stage HH15), thoracic spinal cord neurones are normally distributed between *Islet1/Islet2*-positive populations, which correspond to motoneurones located in the ventral region, and *Lim1*- and/or *CRABPI*-positive populations, which correspond to interneurones, mostly located dorsally to the motoneurone pool (Tsuchida et al., 1994; Vaessen et al., 1990), with no co-expression of these two types of markers (Fig. 5A-E,G). Upon *Ebf1* electroporation, we observed an extension of the *CRABPI*- and *Lim1*-positive domains into the motor column (Fig. 5A-E). Furthermore, these markers were also detected in cells still located within the ventricular zone; they are normally restricted to the mantle layer (Fig. 5A,B). Double-labelling experiments indicated that *CRABPI*- or *Lim1*-positive cells located at the level of the motor column were negative for *Islet1* (Fig. 5C,E). Finally, double labelling for electroporated Ebf1 and for Islet1/Islet2 revealed that all electroporated cells were negative for Islet1/Islet2, even though they were located at the level of the motor column (Fig. 5G). Performing the electroporation experiments at different stages of embryo development did not affect this result (Fig. 5F,H). In all cases, the electroporation of *Ebf1* resulted in a reduction of the pool of Islet1/Islet2-positive cells (Fig. 5F-I).

Hence, ectopic expression of *Ebf1* appears to lead to premature activation of interneurone markers and, surprisingly, to repression of motoneurone markers. Double-labelling experiments suggest that progenitor cells normally fated to become motoneurones according to their dorsoventral (DV) localisation are reprogrammed towards an interneurone differentiation pathway. Therefore, under the conditions of these misexpression experiments, Ebf genes can modulate neuronal fate.

A dominant-negative Ebf protein prevents neuronal differentiation

The previous analyses have indicated that Ebf genes can interfere at various levels of the neuronal differentiation pathway. In order to identify their precise role during normal neurogenesis, we attempted to generate a dominant-negative molecule. We introduced a deletion in the *Ebf1* expression vector, resulting in the elimination of the N-terminal 107 amino acids of the wild-type protein. Such a deletion retains the dimerisation domain but has been shown previously to obliterate Ebf1 DNA-binding activity (Hagman et al., 1993). Furthermore, a similar construct derived from the *Xenopus Ebf2* gene was shown to have a dominant-negative activity (Dubois et al., 1998).

To investigate whether the deleted protein, termed ΔEbf, possessed a dominant-negative activity, we performed co-electroporation experiments with varying amounts of the wild-type *Ebf1* expression construct and examined the effect on

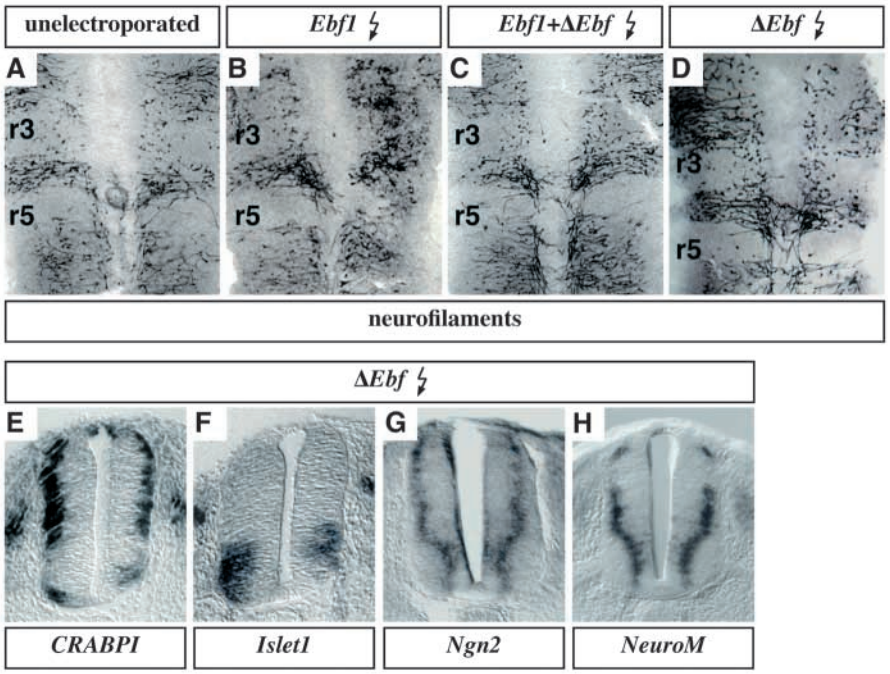

Fig. 6. Δ*Ebf* acts as a dominant-negative mutant and impairs neuronal differentiation. (A-D) Flat-mounted hindbrains from chick embryos that were not electroporated (A) or were co-electroporated at stage HH10 with the indicated constructs (B-D) and the GFP expression vector, then collected 24 hours later and processed for neurofilaments immunochemistry. The examples shown are representative of more than 90% of the embryos, from six independent experiments, each involving at least eight embryos. (E-H) Transverse sections from stage HH15 embryos that were co-electroporated with Δ*Ebf* and GFP expression vectors, collected 30 hours later and processed for whole-mount in situ hybridisation with *CRABPI* (E), *Islet1* (F), *Ngn2* (G) and *NeuroM* (H) probes. The cases shown are representative of more than 80% of the embryos, from four independent experiments, each involving at least eight embryos per probe. Electroporation was on the right side. Note that Δ*Ebf* blocks neurofilament induction promoted by *Ebf1* and reduces the level of late (*CRABPI*, *Islet*), but not early (*Ngn2*, *NeuroM*), neurogenesis markers.

neuronal differentiation at the hindbrain level, as revealed by neurofilament staining (Fig. 6A-D). A concentration of 2 μg/μl of the Δ*Ebf* expression plasmid was able to prevent precocious neuronal differentiation induced by wild-type *Ebf1* at concentrations varying between 1 and 2 μg/μl (Fig. 6B,C, and data not shown). This suggests that ΔEbf antagonises wild-type Ebf1 and, therefore, can act as a dominant-negative molecule. Furthermore, at lower concentrations of the *Ebf1* expression plasmid, or in its absence, Δ*Ebf* led to inhibition of neuronal differentiation (Fig. 5D, and data not shown). This observation suggests that Ebf family members are involved in normal neuronal differentiation, and that ΔEbf can also antagonise endogenous Ebf1 and, presumably, other members of the family owing to heterodimer formation. To better establish this point, we examined the effect of electroporation of Δ*Ebf* on the expression of the interneurone and motoneurone markers, CRABPI and Islet1, respectively. In both cases, we observed a limited but significant decrease in expression on the experimental side (Fig. 6E,F).

As ΔEbf antagonises endogenous Ebf proteins, which themselves can lead to the activation of proneural genes, we wondered whether ΔEbf inhibition of neuronal differentiation might be mediated by repression of proneural or early differentiation genes. We therefore investigated the effect of Δ*Ebf* electroporation on *Ngn2* and *NeuroM* expression. No modification of the patterns of *Ngn2* and *NeuroM* expression was observed (Fig. 6G,H), which suggests that the effect of ΔEbf on neuronal differentiation does not rely on alterations in the expression of genes involved in early neurogenesis.

In conclusion, our data suggest that Ebf activity is necessary for neuronal differentiation, but that it is not required for earlier stages of neurogenesis.

Antagonizing Ebf blocks neuronal differentiation and migration without interfering with cell cycle exit

As expression of Δ*Ebf* prevents expression of neuronal markers, we then investigated whether it could also interfere with neuronal migration and/or cell cycle exit. For this purpose, we performed co-electroporation of the Δ*Ebf* vector with a construct that expresses green fluorescent protein (GFP), in order to trace electroporated cells. To evaluate cell cycle exit, we carried out pulse-labelling with BrdU as described above. Δ*Ebf*-electroporated cells were mostly found within the ventricular zone, which is in contrast to control cells electroporated with the mutant *Krox20* construct, which were distributed between the ventricular zone and the mantle layer, and *Ebf1*-electroporated cells, which were largely confined to the mantle layer (Fig. 7A-D). These data suggest that expression of Δ*Ebf* prevents migration towards the mantle layer. In addition, Δ*Ebf*-electroporated cells did not express the neuronal marker Tuj1 (Fig. 7I), indicating that ΔEbf prevented neuronal differentiation, which is in agreement with the above data (Fig. 6). Analysis of BrdU-positive cells among electroporated cells indicated that they represented similar proportions after electroporation with Δ*Ebf* or with the control construct, whereas, as expected, this proportion was dramatically reduced after electroporation with *Ebf1* (Fig. 7A-D). This indicates that despite blocking neuronal migration and differentiation, ΔEbf did not affect the proportion of cells in S-phase, and presumably of proliferating cells. Accordingly, the proportion of electroporated BrdU-negative cells within the ventricular zone after electroporation with Δ*Ebf* was much higher than in the control, suggesting that cells had exited the cell cycle but stayed within the ventricular zone (Fig. 7D).

As dominant-negative ΔEbf appears to prevent neuronal differentiation/migration without affecting cell cycle exit, we investigated what happens when forcing expression of a proneural gene able to drive both types of processes. For this purpose, we constructed an expression vector for a flagged version of chick Ngn2. As expected, cells electroporated with *Ngn2* were found within the mantle layer, were negative for BrdU incorporation and expressed the neuronal marker Tuj1

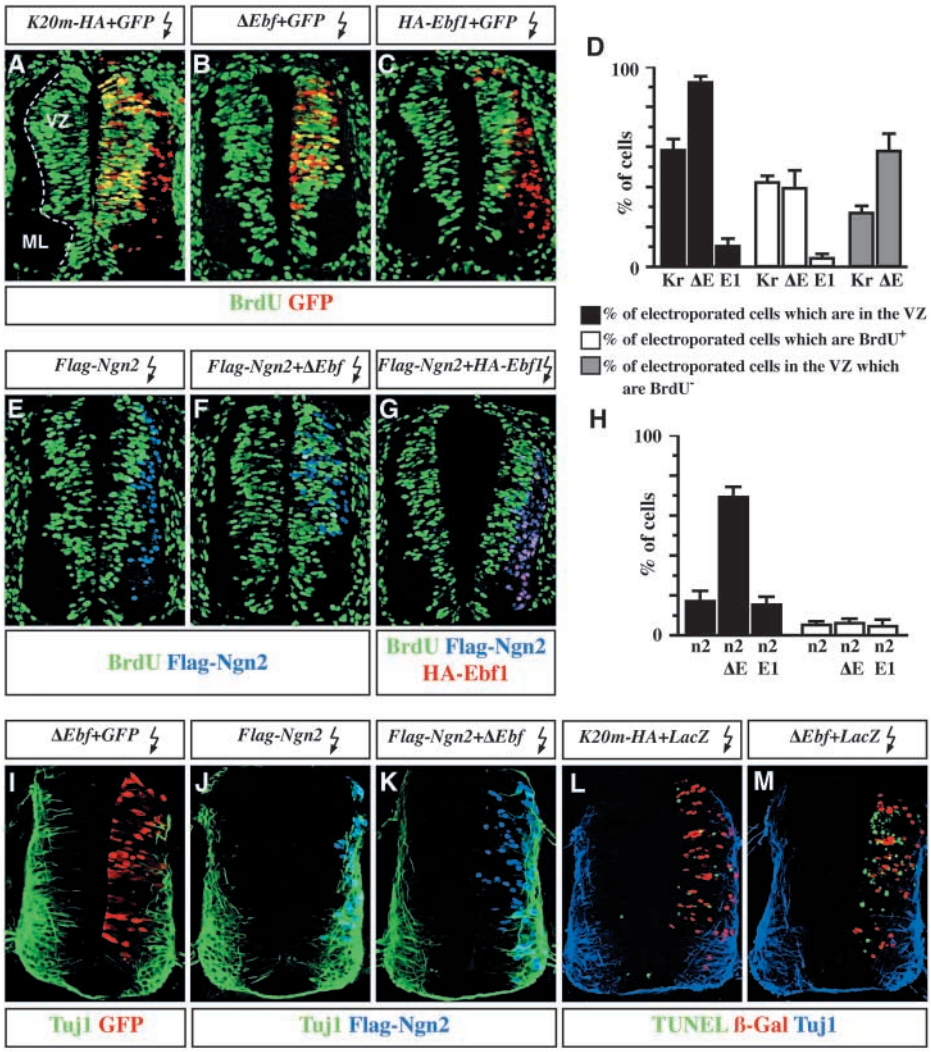

Fig. 7. Δ*Ebf* uncouples cell cycle exit from neuronal differentiation. (A-C) Stage HH15 chick embryos were co-electroporated with *GFP* and mutant *Krox20* (*K20m-HA*, Kr), Δ*Ebf* (Δ*Ebf*, ΔE) or *Ebf1* (*HA-Ebf1*, E1) expressing constructs as indicated, subjected to 2-hour BrdU pulse-labelling 28 hours later, and vibratome sectioned for direct detection of GFP fluorescence (shown in red) and immunofluorescence analysis of incorporated BrdU (green). The mutant Krox20 protein is inactive and was used as a control. The dashed line in A indicates the separation between the mantle layer (ML) and the ventricular zone (VZ). (D) Quantification of the data obtained from the experiments presented in A-C. The bars represent the percentage of electroporated cells (GFP-positive, red or yellow) that are located in the ventricular zone (black bars), the percentage of electroporated cells that are BrdU-positive (yellow, white bars), and the percentage of electroporated cells within the ventricular zone that are BrdU-negative (red, grey bars). (E-G) Stage HH15 chick embryos were electroporated with *Flag-Ngn2* (n2) alone or together with Δ*Ebf* or *Ebf1* expression constructs as indicated, subjected to 2-hour BrdU pulse-labelling 28 hours later, and vibratome sectioned for immunofluorescence analysis of incorporated BrdU (green), Flag (blue) and HA (red) epitopes, marking Ngn2 and Ebf1, respectively. (H) Quantification of the data obtained from the experiments presented in E-G. The bars represent the percentage of electroporated cells that are Ngn2-positive (blue, purple or white), which are located in the ventricular zone (black bars), and the percentage of electroporated cells that are BrdU-positive (white, white bars). In D and H the data represent mean±s.e.m. and correspond to the analysis of six to nine sections from at least three independently processed embryos for each condition. (I-K) Stage HH15 chick embryos were co-electroporated with *GFP* and Δ*Ebf*, *Flag-Ngn2* alone, and *Flag-Ngn2* and Δ*Ebf* expressing constructs as indicated, incubated for 30 hours and then vibratome sectioned for immunofluorescence analysis of Tuj1 (green) and Flag epitope (Blue), or direct detection of GFP fluorescence (red). (L,M) Stage HH15 chick embryos were co-electroporated with *lacZ* and *Krox20m* or Δ*Ebf* expression plasmids, respectively, incubated for 40 hours and then vibratome sectioned for immunofluorescence analysis of Tuj1 (blue), and β-galactosidase (β-gal, red) and TUNEL analysis (green). In sections electroporated to the same extent, as judged by *lacZ* expression, apoptosis is significantly increased (3.5±1-fold) in Δ*Ebf*- versus *Krox20m*-electroporated embryos. More than half of the TUNEL-positive cells in M are also *lacZ*-positive, although only weakly. Cell counts were performed on eight sections from three independently processed embryos. Electroporation was on the right side.

(Fig. 7E,H,J), indicating that they had exited from the cell cycle and differentiated. Co-electroporation of Δ*Ebf* with *Ngn2* largely prevented the migration of electroporated cells to the mantle layer (Fig. 7F,H). In addition, electroporated cells staying in the ventricular zone were prevented from differentiating, as they did not expressed the neuronal marker Tuj1 (Fig. 7K). By contrast, co-electroporation of Δ*Ebf* did not impede cell cycle exit, because none of the *Ngn2*-expressing cells incorporated BrdU (Fig. 7F). Finally, as expected, co-electroporation of *Ebf1* with *Ngn2* led to both cell cycle exit and migration to the mantle layer (Fig. 7G,H). In conclusion, these data indicate that whereas ΔEbf can largely impede migration towards the mantle layer and neuronal differentiation induced by Ngn2, it does not prevent cell cycle exit.

As expression of Δ*Ebf* appears to block neuronal differentiation of cells engaged in the neurogenic pathway, we wondered about the effects of this contentious situation on cell fate and survival at later stages. For this purpose, we analysed cell apoptosis by TUNEL assay, 40 hours after co-electroporation of the neural tube with either the Δ*Ebf* expression construct or the mutant *Krox20* expression plasmid as a control, together with a *lacZ* vector as a tracer. We found that in the control case some apoptosis is observed specifically in the electroporated side (Fig. 7L). This phenomenon might be related to non-specific effects of high levels of expression of the electroporated genes. Cells electroporated with Δ*Ebf* were still largely located within the ventricular zone (89±3%). Electroporation of Δ*Ebf* led to a significant increase of

the number of TUNEL-positive cells (3.5±1-fold) in the electroporated side, as compared with the control case (Fig. 7L,M, note that at least half of the apoptotic cells in the experimental side express *lacZ*, although this is not visible in the figure because of the very low level of β-galactosidase, possibly due to apoptosis). These data suggest that the ΔEbf-mediated block in neuronal differentiation can lead to cell death in at least a subset of the affected cells.

Discussion

In this paper, we have identified and characterized two novel chick genes that are the orthologs of mouse *Ebf1* and *Ebf3*. The very high homology of the amino acid sequences, and the similarity of the expression patterns in post-mitotic neurones along the entire CNS, in mouse and chick indicate that Ebf family members have been highly conserved during higher vertebrate evolution, and are likely to have similar roles in different species. Using gain- and loss-of-function experiments in the chick embryo neural tube, we have carried out a detailed analysis of Ebf gene function during neurogenesis. This allowed precise positioning of these genes along the neurogenic cascade and raised the possibility that they may play multiple roles in this pathway in higher vertebrates. In particular, our data establish that Ebf genes are required for the coupling of neuronal differentiation and migration to the mantle layer with cell cycle exit.

The Ebf genes in the neurogenic cascade

Our conclusions on Ebf gene regulation and function are detailed below, and are based on the following observations.

(1) Analysis of *Ebf1* and *Ebf3* mRNAs in the chick neural tube indicated that their accumulation is coincidental with the onset of neurogenesis and that they are detected within the entire mantle layer (Fig. 1). This is in agreement with the expression pattern of the mouse orthologs (Garel et al., 1997; Wang et al., 1997), and shows that these genes are expressed at a high level in early post-mitotic neurones and that their expression is maintained during neuronal differentiation. Low level, scattered expression has also been observed in the neuroepithelium for mouse *Ebf2* and *Ebf3*, presumably corresponding to cells en route to the mantle layer (Garel et al., 1997; Pattyn et al., 2000).

(2) Forced expression of both *Ngn2*, a proneural gene, and *NeuroM*, an early neuronal differentiation regulator, promoted *Ebf1* and *Ebf3* expression (Fig. 2), indicating that the latter genes are downstream of the former in the neurogenic cascade, consistent with Ebf gene expression pattern.

(3) Expression of a dominant-negative molecule, which presumably antagonizes all Ebf activities, did not affect cell cycle exit, but prevented neuroepithelial precursor migration towards the mantle layer and expression of differentiation markers (Figs 6, 7). Furthermore, the dominant-negative Ebf was also able to prevent neuronal differentiation and migration induced by the forced expression of *Ngn2*, but it did not affect the endogenous expression of this latter gene. Together, these observations suggest that Ebf genes play an essential role in cell engagement into neuronal differentiation and migration towards the mantle layer, coupling these processes to cell cycle exit.

(4) In agreement with a role of Ebf genes in the control of neuronal differentiation and migration, misexpression of *Ebf1* in neuroepithelial progenitors promoted these processes (Fig. 3), which indicates that Ebf genes are both necessary and sufficient. However, surprisingly, forced expression of *Ebf1* also led to exit from the cell cycle. This was correlated with a transient reinforcement of *Ngn1* and *Ngn2* (Fig. 5), and *NeuroM* (data not shown), expression. We could actually demonstrate that induction of the complete neurogenic programme by Ebf1 was largely dependent on bHLH proteins, presumably including proneural gene products, as shown by its inhibition by the bHLH antagonist Id2 (Fig. 3S,T). At this stage, we cannot exclude that forced high level expression of *Ebf1* in neuroepithelial progenitors may lead to non-physiological proneural gene activation, subsequently resulting in the activation of the complete programme. An alternative explanation, involving a second function of Ebf genes, can nevertheless be envisaged and is discussed below.

(5) We have shown that *Ebf1* misexpression also leads to changes in the balance of neuronal subtypes (Fig. 5). This suggests the existence of a third level of intervention of Ebf genes in the neurogenic cascade.

On the basis of these different observations, we propose to position the Ebf genes in the neurogenic cascade in the spinal cord and the hindbrain as indicated in Fig. 8. According to this model Ebf genes are downstream to proneural genes and cell cycle exit, but are absolutely required for neuronal differentiation and migration towards the mantle layer. The details of our conclusions are discussed below.

Uncoupling cell cycle exit from migration and differentiation

This work has revealed that expression of a dominant-negative Ebf gene prevents neuronal differentiation and migration towards the mantle layer without interfering with cell cycle exit. Previous studies performed in the *Xenopus* embryo indicated that blocking cell division by the injection of cell cycle inhibitors did not result in a significant activation of neuronal markers (Hardcastle and Papalopulu, 2000). Together these data indicate that cell cycle exit is not sufficient for induction of neuronal differentiation. In other words, the two types of events, although they are co-induced by proneural genes, are not intrinsically linked, and interfering with Ebf gene function allows them to be uncoupled.

However, uncoupling cell cycle exit from neuronal differentiation and migration may not be without consequences. Our analysis of cell death following expression of dominant-negative *Ebf* indicates that this treatment increases the proportion of apoptotic cells among electroporated cells (Fig. 7), which suggests that the cells might be able to sense this abnormal uncoupling and, consequently, enter into a cell death programme. A related interpretation is that the cells could simply sense the block in differentiation when they are already too far engaged in the commitment process. In several systems, a block in cell differentiation is accompanied by cell death, and this is also the case in the developing striatum of *Ebf1* null mice (Garel et al., 1999).

As Ebf expression is required for both neuronal differentiation and migration towards the mantle layer, a subsequent question is whether Ebf genes independently contribute to each of these two manifestations, or whether differentiation is a consequence of migration or vice-versa. In

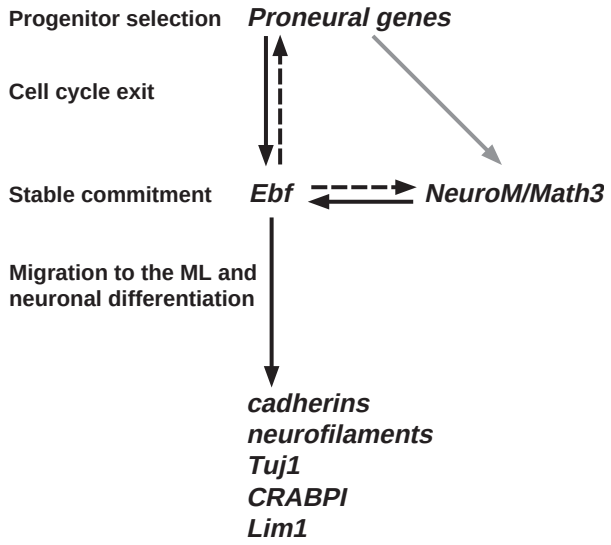

Fig. 8. Schematic representation of epistatic relationships in the CNS neurogenic pathway (see text for detailed explanation). Cellular stages are indicated on the left. Plain arrows indicate established regulatory links between genes. Dashed arrows correspond to links participating in putative positive-feedback loops involved in stable commitment. The grey arrow represents data derived from the work of Perron and collaborators (Perron et al., 1999).

favour of a direct role of Ebf genes in migration, we have observed that *Ebf1* ectopic expression results in a modification of the pattern of expression of adhesion molecules, with repression of N-cadherin and induction of R-cadherin. Such modifications have been proposed to be involved either in the release of undifferentiated progenitors from the ventricular zone and/or in the promotion of their migration towards the mantle layer (Redies and Takeichi, 1996). Furthermore, additional evidence suggests a role of Ebf genes in the control of cellular adhesion, and of cellular and axonal migration (Prasad et al., 1998; Garel et al., 1999; Garel et al., 2000; Corradi et al., 2003). Nevertheless, our work suggests that Ebf genes also control aspects of neuronal differentiation independently from the migration towards the mantle layer. Neuronal markers like *Lim1* and *CRABPI*, which are normally restricted to the mantle layer, are expressed in *Ebf1*-electroporated cells while they are still within the ventricular zone. In addition, in the striatum primordium of *Ebf1* null-mutant embryos, cells in the mantle layer show an aberrant differentiation pattern, being unable to downregulate genes normally restricted to the SVZ, or to activate some mantle-specific genes (Garel et al., 1999). Together these data suggest that the function of Ebf genes is not likely to be restricted either to the promotion of migration towards the mantle layer or to the induction of neuronal differentiation, but rather that these genes directly contribute to both types of processes, and may actually coordinate them.

Additional, putative functions of Ebf genes in neuronal commitment and subtype specification

As indicated above, we do not know whether the transient activation of *Ngn2* and *NeuroM* by Ebf1 (Fig. 4) is physiological, in particular because expression of the dominant-negative *Ebf* does not seem to affect the expression of these genes (Fig. 6). However the establishment of positive-feedback loops, involving proneural, Ebf and possibly other genes, might be involved in the stabilisation of the committed state among selected progenitors (Fig. 8). Another possibility is that Ebf genes might mediate a signal acting after cell cycle exit and ensuring that differentiating cells cannot resume proliferation, as has been proposed for *NeuroD* (Mutoh et al., 1998). If there is redundancy in the establishment or maintenance of these loops, the latter might not be revealed by the loss-of-function mediated by the dominant-negative Ebf. A similar feedback mechanism involving *Xcoe2*/*Ebf2* has been proposed during primary neurogenesis in *Xenopus* (Dubois et al., 1998). This raises the possibility that different aspects of the functions of Ebf genes, and of their relationships with other genes involved in the control of neurogenesis, have been conserved during vertebrate evolution. By contrast, *Xenopus Ebf3* is expressed later than *Xcoe2*, is not involved in maintaining *XNgn1* expression and has been proposed to be required only in late neuronal differentiation, downstream of *XNeuroD* (Pozzoli et al., 2001). Although our experiments did not specifically address the precise level of action of each Ebf gene, the expression data (this work) (Garel et al., 1997; Malgaretti et al., 1997; Wang et al., 1997; Pattyn et al., 2000), together with the absence of a general neuronal phenotype associated with the *Ebf2* null mutation (Corradi et al., 2003), are consistent with an early role of *Ebf1* and *Ebf3*, coincidental and redundant with that of *Ebf2*. Therefore, *Ebf2* and *Ebf3* have functionally diverged in *Xenopus*, whereas they may have conserved similar early neurogenic function in higher vertebrates.

Combinatorial expression of homeobox genes in neural progenitors, established according to their DV location and in response, in particular, to sonic hedgehog signalling, has been shown to play an essential role in neuronal subtype specification in the spinal cord (Jessell, 2000). Proneural genes also have restricted patterns of expression along the DV axis and recently they have been implicated in the specification of neuronal subtype as well (Fode et al., 2000; Gowan et al., 2001). In this study, we have shown that *Ebf1* misexpression in spinal cord progenitors leads to repression of motoneurone markers (Islet1/Islet2) and activation of interneurone markers (Lim1 and CRABPI), at the level of the motor column (Fig. 5). This suggests that progenitors normally fated to become motoneurones according to their DV location are reprogrammed towards an interneurone fate. This was unexpected as Ebf genes are normally expressed in differentiating motoneurones (Fig. 1) (Garel et al., 1997). We can provide two possible, and non-exclusive, explanations for our observations. Firstly, it is possible that high levels or inappropriate timing (leading to premature cell cycle exit in particular) of *Ebf1* expression in neuroepithelial progenitors leads to modifications in the combinatorial expression of genes involved in early DV specification (e.g. homeobox genes, proneural genes) and, consequently, but indirectly, to fate changes. Indeed, we have shown that *Ebf1* misexpression has different effects on the expression of three proneural genes, promoting the expression of *Ngn1* and *Ngn2*, but not of *Cash1* (Fig. 4). Secondly, because the Ebf genes appear as major regulators of neuronal differentiation, it is possible that they directly control the expression of neuronal subtype-specific genes. Indeed, we have shown that *Ebf1* inactivation in the

striatum primordium prevents the activation of *CRABPI* (Garel et al., 1999), and that forced expression of *Ebf1* leads to ectopic activation of *CRABPI* not only in the motor column but also in the ventricular zone (Fig. 5). It is therefore possible that higher than normal levels of Ebf expression in differentiating neurones directly alters the balance between subtype specification genes. Further analyses will be required to precisely delineate the possible function of Ebf genes in this aspect of neuronal differentiation.

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