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Developmental regulation of *EVF-1*, a novel non-coding RNA transcribed upstream of the mouse Dlx6 gene

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Abstract

We previously reported that sonic hedgehog (Shh) induces the differentiation of rat ventral forebrain neurons expressing a novel marker, *EVF-1* [Development 125 (1998) 5079]. In this report, we show that *EVF-1* is a novel, developmentally regulated, non-coding RNA, with no homology to other known non-coding RNA sequences. Sequence analysis, in vitro translation, and comparison of the rat and mouse *EVF-1* sequences suggest that *EVF-1* contains no protein coding regions. Chromosomal location indicates that *EVF-1* maps adjacent to the Dlx6 gene on mouse chromosome 6. RNA in situ hybridization of the embryonic rat forebrain shows that *EVF-1* is expressed by immature neurons in the subventricular zone and its expression decreases during forebrain development. Whole mount in situ hybridization shows that *EVF-1* is expressed at high levels in the branchial arches, ventral forebrain, olfactory bulb, and limbs. *EVF-1* expression is linked to Shh and the Dlx family of proteins, genes with a demonstrated importance to ventral forebrain and craniofacial development.

Keywords: Non-coding RNA; Sonic hedgehog; Telencephalon; Forebrain development; Ventral forebrain; Dlx genes; Homeodomain; Nuclear RNA; Differentiation; Immature neurons

1. Results and discussion

Proper forebrain development depends on the interaction of multiple genes. Sonic hedgehog (Shh), one of the key secreted signaling proteins in the embryo, is essential for ventralizing the mouse neural tube (Chiang et al., 1996, reviewed by Ingham and McMahon (2001)). In the rodent ventral forebrain, Shh induces the expression of Dlx2 (Kohtz et al., 1998; Gaiano et al., 1999; Kohtz et al., 2001), a member of the Dlx family of homeodomain-containing proteins originally reported by Porteus et al. (1991). The co-ablation of *Dlx1* and *Dlx2* results in the loss of specific populations of ventral forebrain neurons, as well as the loss of *Dlx5* and most *Dlx6* expression in the subventricular zone of the ventral telencephalon (Anderson et al., 1997). In mice lacking both *Dlx5* and *Dlx6*, craniofacial abnormalities are observed (Robledo et al., 2002). Although obvious ventral forebrain defects are not observed in mice lacking *Dlx5* and *Dlx6* (Robledo et al., 2002), it is not known whether specific populations of ventral forebrain neurons are affected. This is a possibility because mice lacking both *Dlx2* and *Dlx1* show a loss of specific populations of neurons without obvious ventral forebrain defects (Anderson et al., 1997). Taken together, these studies suggest that patterning and differentiation in the ventral forebrain and branchial arches depends on a cascade initiated by the Shh protein that ultimately involves members of the Dlx gene family.

The mechanisms involved in the activation of the Dlx genes by Shh remain poorly understood. However, one step in this cascade has been elucidated through elegant experiments reported by Zerucha et al. (2000). This report showed that transcriptional activation of *Dlx5* and *-6* occurs by the direct action of the Dlx2 and Dlx1 proteins on a conserved enhancer located within the *Dlx5/6* intergenic region. In an effort to further investigate mechanisms involved in the activation of ventral forebrain-specific genes by the Shh protein, we performed a screen for novel cDNAs

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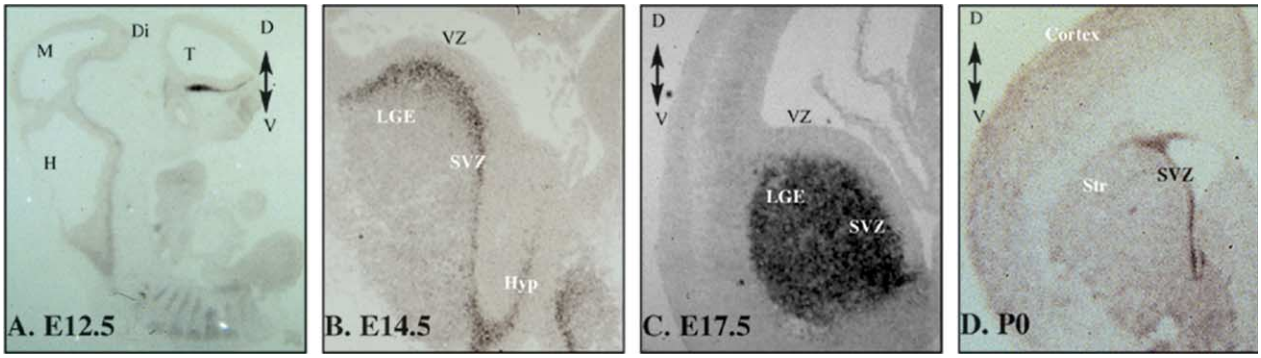

Fig. 1. *EVF-1* expression is restricted to differentiating ventral forebrain neurons at different stages of rat telencephalic development. In situ hybridization of rat embryonic sections using an anti-sense *EVF-1* digoxigenin labeled probe and visualization with alkaline phosphatase conjugated anti-digoxigenin antibodies. (E, embryonic day, P, post-birth) A. saggital section through the whole embryo. The purple stripe of cells mark the first appearance of *EVF-1* expressing cells in the subventricular zone. T, telencephalon, Di diencephalon, M, midbrain, H, hindbrain, D. (B–D) Coronal sections through the telencephalon. (B,C) Section through the lateral ganglionic eminence, the embryonic structure that will give rise to the striatum, D. *EVF-1* positive cells (purple) mark what is remaining of the subventricular zone, the ventricular zone is gone at this stage. D, dorsal, V ventral, LGE, lateral ganglionic eminence, SVZ, subventricular zone, VZ, ventricular zone, Hyp, hypothalamus.

specific for the ventral telencephalon using the differential display method (Liang and Pardee, 1995). Differential mRNA profiles from rat embryonic day 12 (E12) dorsal and ventral telencephalon were analyzed, and eight different genes were found to be differentially expressed by RT-PCR and Northern analysis (data not shown). In situ hybridization revealed that one of these genes, *EVF-1* (embryonic ventral forebrain) exhibits an expression pattern very similar to the *Dlx* genes, which encode homeobox containing transcription factors known to be critical to the patterning and migration of ventral forebrain neurons in the developing embryo.

Using E11 rat neural explants and RT-PCR, we previously showed that the Shh protein induces the expression of *EVF-1* (Kohtz et al., 1998). In addition, in situ hybridization analysis showed that *EVF-1* is restricted to the ventral forebrain, overlapping and adjacent to Shh and the Dlx homeodomain proteins (Kohtz et al., 1998). In order to further investigate *EVF-1* expression in the developing forebrain, we performed in situ hybridization analysis of *EVF-1* for four different times in the developing rat forebrain. Fig. 1A shows that *EVF-1* is expressed in the subventricular zone when it first appears (E12.5). As development proceeds, *EVF-1* is expressed in more differentiated cells in the post-mitotic layer (E14.5–E17.5). By birth (P0), the ventricular zone is absent, and *EVF-1* expression is limited to the cells that remain in the subventricular zone. These data show that *EVF-1* remains ventrally restricted in the developing forebrain. However, the relationship of *EVF-1* expression and rapidly dividing cells changes from E12.5 to P0 in the developing forebrain.

In order to determine the relationship between the rapidly dividing cells in the ventricular zone, subventricular zone, and *EVF-1*, BrdU labeling studies were performed. In vivo injection of BrdU labels rapidly dividing cells by becoming incorporated into DNA. Cells that have incorporated BrdU into their DNA can be visualized by staining with an anti-BrdU antibody. A one-hour pulse given to a pregnant rat labels rapidly dividing cells in the ventricular zone. Fig. 2 shows the relative expression of *EVF-1* with dividing cells in the ventricular zone that incorporate BrdU. Adjacent sections of *EVF-1* and anti-BrdU were used, confirming that *EVF-1* is expressed by cells in the subventricular zone, which is immediately adjacent to the ventricular zone.

In order to further characterize the type of cells that express *EVF-1*, we asked whether *EVF-1* was being expressed by neurons. Antibodies to neuron-specific tubulin (III) are commonly used as a marker for immature neurons (TUJ1, Caccamo et al., 1989). We used the monoclonal antibody TUJ1 as a neuron-specific marker, and performed double anti-TUJ1–*EVF-1* in situ hybridization to determine if *EVF-1* cells express TUJ1. Fig. 3 shows co-localization of *EVF-1* and TUJ1. These data indicate that *EVF-1* is expressed by immature neurons upon migration out of the ventricular zone into the subventricular zone during ventral

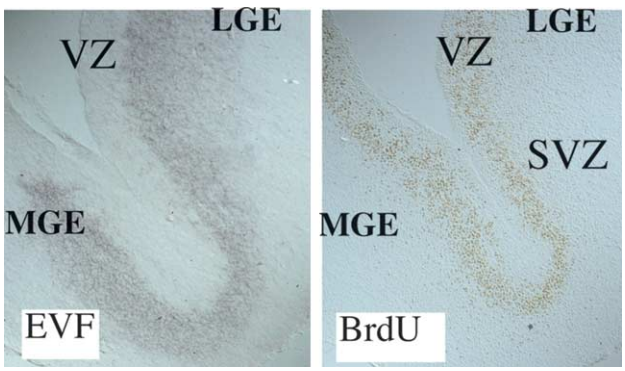

Fig. 2. *EVF-1* is expressed in the subventricular zone. E13.5 pregnant rats were injected once with BrdU intraperitoneally, and embryos were sacrificed 1 h later. Coronal, adjacent sections of E13.5 embryonic telencephalon were processed for *EVF-1* in situ hybridization (left), or anti-BrdU staining (monoclonal anti-BrdU, Becton Dickinson). *EVF-1* is visualized with alkaline phosphatase substrate (purple), BrdU is visualized with a peroxidase substrate (brown). VZ, ventricular zone, SVZ, subventricular zone, LGE, lateral ganglionic eminence, MGE, medial ganglionic eminence.

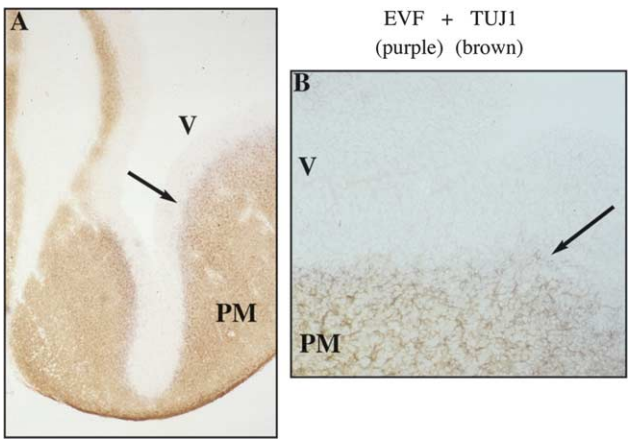

Fig. 3. *EVF-1* is expressed by immature neurons as they migrate out of the ventricular zone to the subventricular zone. Double RNA in situ hybridization using anti-sense *EVF-1* probe (purple) and immunohistochemistry using anti-TUJ1 (brown) define the relationship between *EVF-1* and immature neurons in a coronal section of rat E13.5 ventral forebrain (A) low magnification (B) high magnification. V, ventricular zone, PM, post-mitotic layer.

telencephalic differentiation. Fig. 4 is a whole mount in situ hybridization of rat E12 embryos showing the expression of *EVF-1*. Expression of *EVF-1* is seen predominantly in the branchial arches, ventral forebrain, olfactory bulb and limb buds. This expression pattern of *EVF-1* is similar to that previously reported for *Dlx5* and *6* (Liu et al., 1997; Eisenstat et al., 1999). In addition, the *EVF-1* expression pattern is more similar to *Dlx5/6* than *Dlx1/2*, in that it is absent from ventricular zone cells (Kohtz et al., 1998).

One clue as to how the *EVF-1* ncRNA may function derives from its subcellular localization. In situ hybridization analysis of *EVF-1* in subventricular cells suggested that *EVF-1* is located in the cytoplasm and has a diffuse staining pattern (Kohtz et al., 1998, and this report).

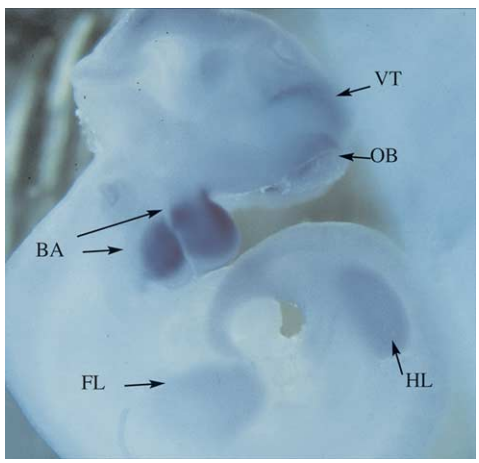

Fig. 4. *EVF-1* exhibits a restricted pattern of expression in the whole embryo. Whole mount in situ hybridization of rat E12.5 embryo using *EVF-1* anti-sense RNA as a probe. BA, branchial arches, VT, ventral telencephalon, OB, olfactory bulb, FL, forelimb, HL, hindlimb.

However, permeabilization of rat E14.5 LGE sections with 1% Triton X-100 reveals that *EVF-1* RNA is localized in dense areas that appear to be nuclei, in cells as they exit the ventricular and subventricular zone (Fig. 5B, arrows). Co-staining with DAPI, a nuclear stain, shows that the *EVF-1* anti-sense probe intensely stains the nucleus (Fig. 5D,E).

A 400 bp PCR fragment was identified using the differential display method (Liang and Pardee, 1995) and comparing the profiles from dorsal and ventral telencephalon. Screening of a rat embryonic brain, oligo dT primed library with the 400 bp PCR fragment originally isolated by differential display resulted in the isolation of a 2737 bp cDNA. Sequencing of the rat *EVF-1* cDNA failed to reveal any significant open reading frames (greater than 200 base pairs) or homology with other cDNAs or non-coding RNAs in the database. In order to rule out the possibility of sequencing errors, sequencing of single-stranded DNA was performed on both strands. Although conservation of the mouse and rat *EVF-1*s is present, the longest putative coding region (beginning at 1670 in the rat sequence) is predicted to encode a 63 amino acid rat protein or a 45 amino acid mouse protein (Fig. 6A). This region is located in the least conserved stretch (80%) between the rat and the mouse sequences. The highest region of conservation is in the 5′ and (94%). These observations support the idea that the *EVF-1* RNA is non-coding. In addition to sequence comparisons, the *EVF-1* cDNA was in vitro translated to test whether any proteins could be translated from in vitro transcribed *EVF-1* RNA. No in vitro translated products could be detected, whereas cDNAs encoding the Dlx2 protein generated a 40 kD protein (data not shown). In order to determine if the 2737 bp is a full-length *EVF-1* cDNA, 5′RACE was performed (data not shown). Results from the 5′RACE confirmed that the *EVF-1* cDNA obtained in the library screen is a full-length transcript. Together with the sequencing data, cross-species conservation analysis, and the inability to translate proteins from *EVF-1* RNAs support the hypothesis that *EVF-1* is a non-coding RNA.

Using information from the mouse genome sequencing database, the genomic organization of the *EVF-1* gene was determined. Fig. 6 shows a schematic representation of this region. The *EVF-1* ncRNA is encoded by two exons separated by a large 37.5 kb intron. The *EVF-1* exon1 is 4 kb upstream of the Dlx 6 gene and 8 kb distant from the conserved enhancer ii, identified by Zerucha et al. (2000). This is a region of synteny between the mouse chromosome 6 and human chromosome 7, supporting the idea that functionally important genes are coded in this region.

Of the ncRNAs (reviewed by Kelley and Kuroda (2000), Eddy (2001), Erdmann et al. (2001) and Eddy (2002)), isolated to date, Xist (reviewed by Plath et al. (2002)) and H19 (reviewed by Tilghman (1999), Hurst and Smith (1999), Sasaki et al. (2000) and Arney (2003)) are the most similar to the *EVF-1* ncRNA, in that both are

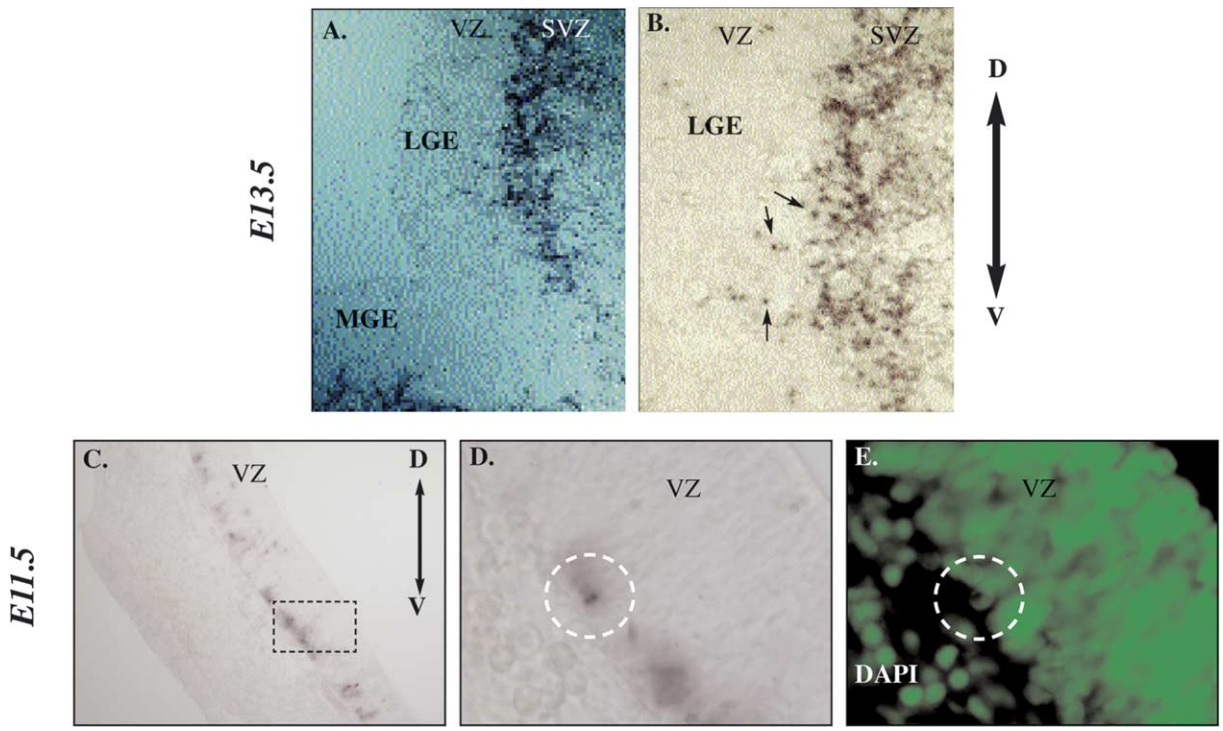

Fig. 5. Triton permeabilization reveals the nuclear expression of *EVF-1* RNA. In situ hybridization of coronal sections and anti-sense *EVF-1* probe. A and B. Rat E13.5 coronal section showing the ventral telencephalon (A) low magnification (B) high magnification. The arrows indicate regions of intense *EVF-1* staining. (C–E) Rat E11.5 coronal section showing the ventral telencephalon (C) low magnification (D,E) high magnification. (C,D) In situ hybridization using anti-sense *EVF-1* probe. (E) DAPI nuclear staining. VZ, ventricular zone, SVZ, subventricular zone, arrows in (B) point to dense nuclear staining, LGE, lateral ganglionic eminence, MGE, medial ganglionic eminence, D, dorsal, V, ventral.

polyadenylated non-coding RNAs. Additional similarities between H19 and *EVF-1* include the following: both were isolated in a differential expression screen, are developmentally regulated and spliced, and similar in size (H19 = 2.3 kb, *EVF-1* = 2.7 kb). Both H19 and *EVF-1* were found to contain no open reading frames by cross-species sequence conservation analysis and testing by in vitro translation. Characteristics that distinguish *EVF-1*

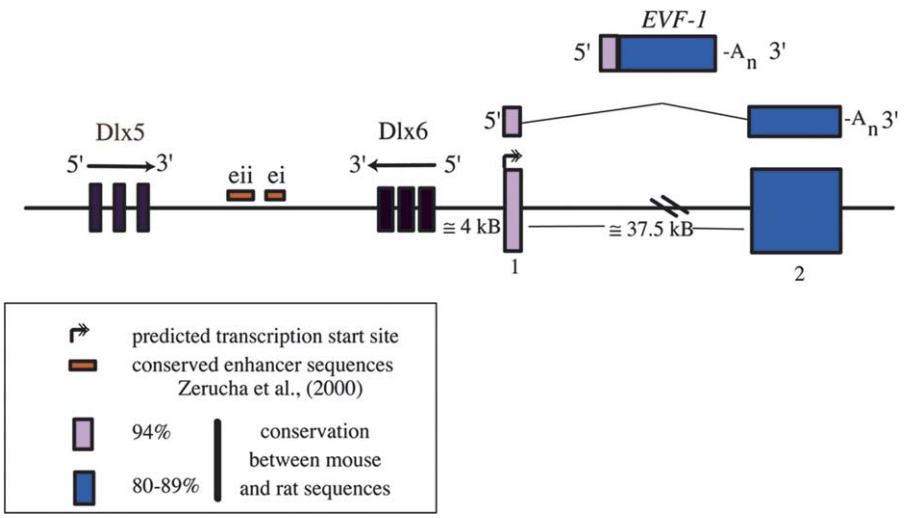

Fig. 6. Mouse EVF-1 genomic organization. The mouse EVF-1 gene consists of two exons. The genomic structure is based on the mouse genome sequence available from the Genbank database, the rat and mouse cDNAs previously sequenced in our laboratory, mouse ESTs available in the Genbank database. *Note*. These regions are not drawn to scale. The full length rat *EVF-1* cDNA sequence is available on Genbank (accession number AY518691).

and H19 are the proximity of *EVF-1* to the Dlx6 coding region (this paper) and its induction by Shh in the forebrain (Kohtz et al., 1998).

2. Methods

2.1. Differential display

Total RNA from the lateral ganglionic eminence (LGE) and cortex (Cor) was isolated from rat embryonic day 13.5 and day 17.5 tissue using the method described by (Chomczynski and Sacchi, 1987), and treated with DNASE. Differential display was performed according to Liang and Pardee (1995) using a combination of five arbitrary and two anchored primers on the following four RNAs: E13.5 LGE, E17.5 LGE, E13.5 Cor and E17.5 Cor. The primers were as follows:

1. Anchored 1: 5′ T_{12} GG 3′
2. Anchored 2: 5′ T_{12} GC 3′
3. Arbitrary 1: 5′ GCT TGA GTC C 3′
4. Arbitrary 2: 5′ GCA CAG CGA A 3′
5. Arbitrary 3: 5′ GCC AGC TTG T 3′
6. Arbitrary 4: 5′ AGG TGA CCG T 3′
7. Arbitrary 5: 5′ GAA CGT CAG G 3′

Only bands that were significantly different between LGE and Cor at both E13.5 and E17.5 were isolated and further characterized. Eight bands were subcloned and sequenced; the band corresponding to *EVF-1* was 400 bp long, and based on its expression pattern (see below) was chosen for further study. The 400 bp *EVF-1* fragment was used to screen a rat E15 brain library (J. Boulter, Salk Institute for Biological Studies), resulting in the identification of a 2.7 kb cDNA containing a 400 bp identity with the original differential display PCR fragment.

2.2. In situ hybridization

Section in situ hybridization was performed using digoxigenin-labeled anti-sense RNA probe against *EVF-1* according to Schaeren-Wiemers and Gerfin-Moser (1993). Whole mount in situ hybridization was performed according to Wilkinson (1992). The *EVF-1* transcript was made from a *Bam* HI linearized 2.7 kb cDNA template, transcribed with T7 polymerase. Triton permeabilized in situ hybridizations were performed by modification of the procedure described by Schaeren-Wiemers and Gerfin-Moser (1993). Modifications are listed as follows: (1) Embryonic tissue was fixed in 4% paraformaldehyde at 4 °C overnight, transferred to 30% sucrose overnight, embedded in OCT, and quick frozen in the cryostat (−30 °C). (2) Ten-micrometer cryostat sections were air dried, fixed on slides in 4% paraformaldehyde for 10 min at room temperature, rinsed in PBS 3× (10 mM sodium phosphate pH 7.4, 150 mM NaCl), treated with proteinase K (1 μg/ml) for 5 min, refixed with 4% paraformaldehyde for 10 min at room temperature, and washed 4 × 5 min in PBS. (3) Sections were permeabilized for 1 h at room temperature in PBS containing 1% Triton X-100, followed by pre-hybridization, hybridization, and color development as described by Schaeren-Wiemers and Gerfin-Moser (1993). For nuclear localization with *EVF-1* in situ/TUJ1 (Caccamo et al., 1989) antibody double labeling, anti-TUJ1 antibody was co-incubated with the anti-digoxigenin antibody, followed by incubation with peroxidase conjugated anti-mouse antibody, alkaline phosphatase substrate (BCIP/NBT) development, and then peroxidase substrate development (DAB).

2.3. BrDu incorporation

Timed pregnant E13.5 rats were injected with BrdU (50 mg/kg) (stock solution = 20 mg/ml BrdU, 2 mg/ml 5-fluoro-deoxyuridine in 0.007N NaOH, Sigma) every 1 h before the time of sacrifice. Animals were then sacrificed and transcardially perfused with 4% paraformaldhyde in PBS. Brains were post-fixed overnight in fixation solution containing 30% sucrose. Brains were washed three times in PBS, quick frozen in isopentane at −40 °C and embedded in OCT compound for cryostat sectioning. Brains were then sectioned at 10 μm and mounted on gelatin-coated glass slides. Sections were DNASE treated and then incubated with mouse anti-BrDu antibody (Becton Dickinson), followed by peroxidase conjugated anti-mouse, and visualization with DAB.

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