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Expression of the Prader-Willi gene Necdin during mouse nervous system development correlates with neuronal differentiation and p75NTR expression

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Abstract

The expression pattern of Necdin, a gene involved in the etiology of Prader-Willi syndrome and a member of the MAGE family of genes, is described during mouse nervous system development. Using RNA in situ hybridization, immunohistochemical staining, and colocalization with neuronal differentiation markers, we found that Necdin RNA and protein are expressed within post-mitotic neurons at all stages studied. From E10 to E12, Necdin is detected in all developing neurons, in both central and peripheral nervous system, with the highest expression levels in the diencephalon and the hindbrain. After E13, Necdin is expressed in specific structures of the nervous system, in particular the hypothalamus, the thalamus, and the pons, suggesting a specific developmental role therein. In addition, Necdin expression is also detected in non-neural tissues, such as the somites, the developing limb buds, the first branchial arches, the tong, and the axial muscles. Recently, Necdin and other MAGE proteins were found to interact in vitro with the intracellular domain of the p75NTR neurotrophin receptor, but this interaction has not been validated in vivo. We report here that the spatial and temporal expression of p75NTR is included in Necdin expression domain. These results are in agreement with Necdin proposed role on cell cycle arrest, inhibition of apoptosis and facilitation of neuronal differentiation in vitro, and with hypothalamic cellular deficiencies reported in mice with abrogation of the Necdin gene. Furthermore, they are also consistent with the putative role of Necdin in signaling events promoted by p75NTR during mouse nervous system development.

Keywords: Brain development; In situ hybridization; Protein immunostaining; Necdin; p75NTR; Prader-Willi; Mouse; Gene; Protein expression pattern

1. Results and discussion

The gene Necdin, a member of the MAGE (Melanoma Antigen Gene Expression) family, whose function is unknown, is involved in the etiology of a multigenic neurodevelopmental disorder, the Prader-Willi syndrome (PWS). Studies in vitro have suggested that Necdin may be a neuron-specific growth suppressor that facilitate cell cycle exit and neuronal differentiation and inhibit apoptosis (reviewed by Yoshikawa, 2000). Mice with abrogation of the Necdin gene exhibit a decrease in the number of oxytocin (OT) and luteinizing hormone-releasing hormone (LH-RH)-producing neurons in the hypothalamus, and other features reminiscent of the PWS phenotype (Muscatelli et al., 2000). Recently, Necdin and other MAGE proteins, MAGE-D1 (also named NRAGE or Dlxin-1) and MAGE-H1, were found to interact in vitro with the intracellular domain of the p75NTR neurotrophin receptor (Tcherpakov et al., 2002; Salehi et al., 2000). Necdin expression pattern has not been studied in detail, and several questions remain unanswered. First, it is unclear if Necdin is a pan neuronal developmental marker (Aizawa et al., 1992), or if its expression during nervous system development is limited to discrete areas as reported in the adult (Muscatelli et al., 2000). Second, Necdin mRNA and protein expression have not been compared, and it is unknown if they exhibit distinct patterns as recently reported for MAGE-D1 (Kendall et al.,

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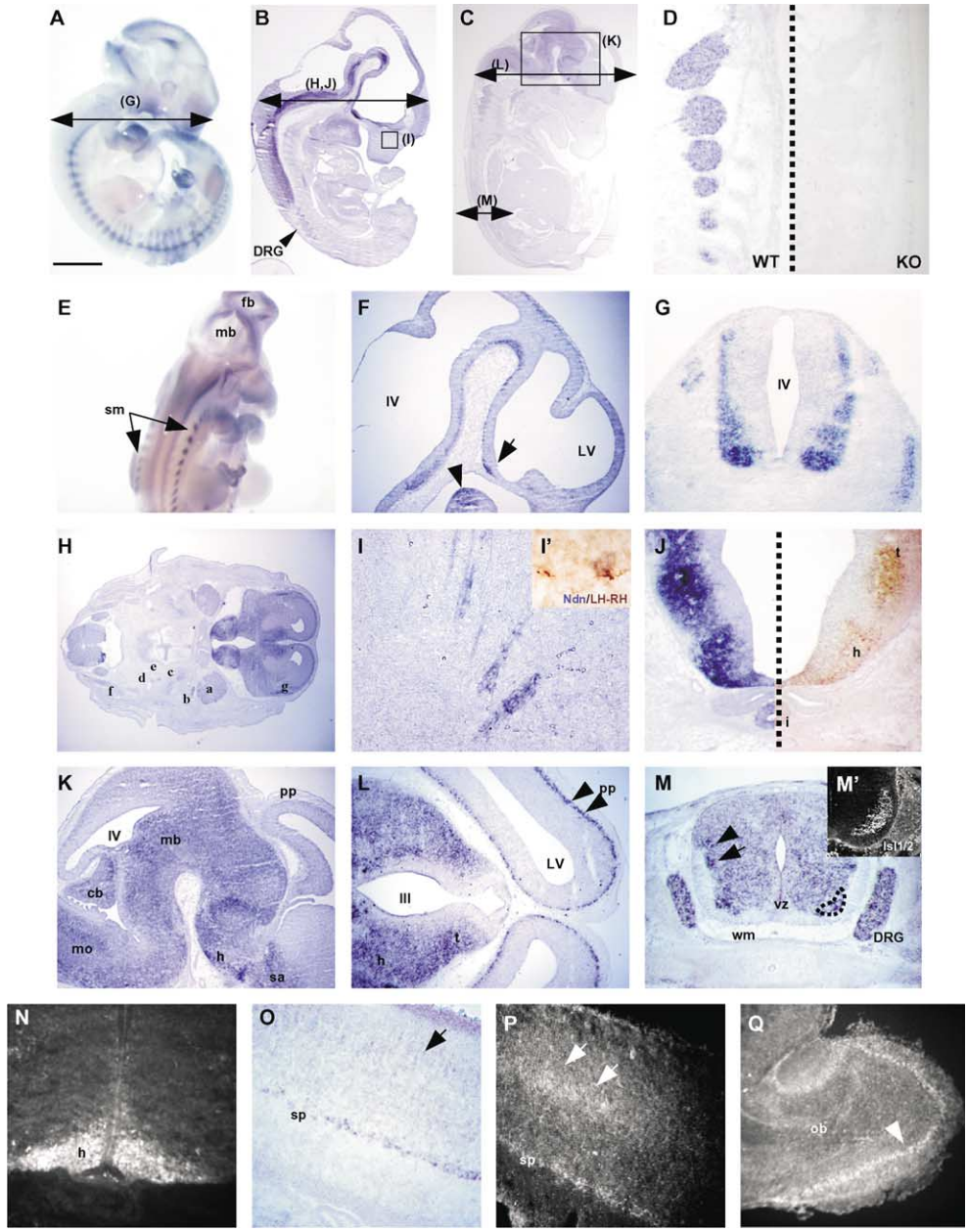

Fig. 1. Expression pattern of Necdin mRNA and protein during mouse nervous system development. (a) Necdin in situ hybridization and immunostaining on whole-mount mouse embryos at E10 (A,E), and on tissue sections at E10 (F,G), E12 (B,H–J), E13 (C,D,K–M). Dorsal is up, except H,L. (A) Side view showing Necdin mRNA expression in the neural tube at E10. Note also non-neural expression in the mandibular component of the first branchial arch, hindlimb and forelimb, and in the somites. Level of section in G is indicated. (B) Sagittal section of a 12-day old mouse embryo reveals wide expression of Necdin in the neural tube (mostly ventral part), the dorsal root ganglion (DRG) and the tong. Levels of sections in H and J are indicated. (C) Sagittal section at E13 showing mRNA signal in the diencephalon, along the ventral part of the neural tube as well as in the dorsal telencephalon and the septal area. Levels of sections in L and M are indicated. (D) Close-up view of the DRG (sagittal section at E13 from wild type mice on the left and from Necdin-deficient mice on the right) demonstrating the specificity of the Necdin riboprobe used in the in situ hybridization studies. (E) Dorso-lateral view of embryo in A, displaying expression in the neural tube, in particular in the midbrain (mb) and in the forebrain (fb), and also on the somitic series (sm). (F) Rostral part of a 10-day old mouse embryo showing mRNA expression in the most external cell layer of the neural tube (earliest born neurons), in particular in the hypothalamic primordium (arrow). Note also expression in the first branchial arch (arrowhead). (G) Transverse section though the hindbrain showing Necdin mRNA expression in the marginal zone. (H) Illustration of Necdin mRNA expression pattern in cranial ganglia. Note the labeling in the trigeminal (*Vth*, *a*), facial (*VIIth*, *b*), vestibulocochlear (*VIIIth*, *c*), vagus (*Xth*, *d*), cranial accessory (*XIth*, *e*) ganglia. Necdin is also expressed in axial muscles (f), and in the ganglionic eminence of the telencephalon (the primordium of the corpus striatum, g). (I) Close-up view of the nasal septum of embryo in B showing Necdin mRNA expression in a cord of migrating LH-RH neurons. Cellular colocalization of LH-RH peptide (brown) and Necdin mRNA (purple) is shown in I′. (J) Transverse serial sections through the diencephalon: Necdin expression domain (mRNA on the left and protein on the right) lines the third ventricle, in the ventral thalamus (t), the anterior hypothalamus (h) and the neural component of the pituitary (infundibulum, i). (K) Magnification of the rostral part of embryo in C, showing expression in the hindbrain (medulla oblongata, mo), the marginal zone of the cerebellum primordium (cb), the midbrain tegmentum (mb), the diencephalon, and the telencephalic neuroepithelium (pp). (L) Transverse section showing expression in the pp and in the diencephalon. (M) Transverse section with Necdin expression in the DRG and in the ventral

2002). Third, Necdin is described as a marker of post-mitotic cells (Aizawa et al., 1992; Niinobe et al., 2000; Muscatelli et al., 2000), but the relationship between Necdin expression and neuronal differentiation has not been investigated using specific differentiation markers. Fourth, it is unknown if Necdin and p75NTR have overlapping expression patterns and could thus potentially interact in vivo. In this report, we definitively establish Necdin mRNA expression pattern during mouse nervous system development. At several stages, the in situ hybridization pattern is compared with Necdin protein immunostaining. We also establish the relation between Necdin expression and neuronal differentiation, focusing on the early stages of neurogenesis, and using several neuronal differentiation cell markers on serial adjacent sections. In addition, we compare Necdin and p75NTR expression in embryonic nervous system.

Necdin mRNA expression was first detected at E10 throughout the nascent mantle layer of the neural tube (Fig. 1A,E–G). Necdin is expressed in particular in the hypothalamic primordium in newly formed neurons at the pial surface (Fig. 1F) and labeled with the TuJ1 antibody (Fig. 2A,B), the first marker of post-mitotic immature neurons in the diencephalon (Mastick and Andrews, 2001). By E12 and thereafter, Necdin transcript is expressed in all the ventral parts of the neural tube, in marginal areas where differentiating neurons reside (Fig. 1B,C,H–M). Strong signal is detected in the diencephalon (thalamus, zona limitans intrathalamica, hypothalamus, see Fig. 1C,J–L), in post-mitotic areas labeled with MAP2 antibody (Fig. 2C,D), but also in the hindbrain, and in differentiated cranial ganglia (Fig. 1B,C,H,K). Necdin-deficient mice have a decreased number of OT and LH-RH hypothalamic neurons (Muscatelli et al., 2000). OT production is barely detectable before birth and therefore, could not be used as a reliable marker, but we found that Necdin is expressed in the sensory nasal (olfactory) epithelium, in LH-RH neurons migrating to the hypothalamus (Fig. 1B,I,I′), and could thus play a role in LH-RH neurons development. After E12, Necdin mRNA expression extend dorsally in the post-mitotic outer layer of all neuroepithelia of the central nervous system, such as the lateral and third ventricle and the cerebellum primordium (Fig. 1C,H,K,L). In the spinal cord (see at E13, Fig. 1M), Necdin is detected in the ventral horn, in developing post-mitotic motor neuron precursors expressing Islet1/2, in a small population of dorsal interneurons, and in more dispersed cells of the alar plate interneurons (Fig. 1M,M′). In the peripheral nervous system, Necdin expression is observed in dorsal root ganglia (DRG) (Fig. 1B,D,H,M) and sympathetic ganglia (not shown). Necdin expression is also detected in non-neural tissues, such as the somites and the developing limb buds (Fig. 1A,E), the first branchial arches (Fig. 1F), the tong (Fig. 1B), and the axial muscles (Fig. 1H), all of which contain post-mitotic cells.

To confirm that Necdin in neural tissues is expressed in neurons but not in glial cells, double labeling experiments were performed with anti-Necdin antibody, neuronal-specific TuJ1 antibody, and glial-specific GFAP antibody. At all developmental stages studied (E10, E12, E17, PO, P2, adult), Necdin protein expression, within the nervous system, was found to be restricted to neurons (see Fig. 2B′ at E10). In addition, in all tissue sections examined at these stages, no difference was found between Necdin protein immunostaining and the pattern of Necdin detected transcripts (see Fig. 1J, O,P). No conclusion could be raised about Necdin sub cellular localization in vivo because the antibody used in this study predominantly detects Necdin in the cytoplasm (Niinobe et al., 2000). We further investigated the link between Necdin expression and neuronal differentiation in the neuroepithelium of the telencephalon. From E11 to E13.5, Necdin mRNA and protein are expressed in the preplate (pp), a subpial layer of cells containing the earliest post-mitotic neurons in the developing neocortex, and then in the early cortical plate (cp) labeled with TuJ1 and neurofilament (NF) antibodies (Figs. 1H,K,L and 2E–H). At all stages, no signal is detected in the proliferating ventricular zone (vz) and the intermediate zone (iz) of the neuroepithelium (Fig. 2E–H). From E15 and thereafter, Necdin is expressed in the subplate (sp), a transitory structure at the base of the developing cp, in the mid-cortical layers (at P0 and P2, see Fig. 1O and P, respectively), and more faintly in the marginal zone.

Abundant Necdin expression persisted throughout gestation. At P0–P2, Necdin is broadly expressed in the diencephalon (see hypothalamus at E17, Fig. 1N), the hindbrain (not shown) and in specific layers of the neocortex (see above and Fig. 1O and P). Staining also persist in specific neuronal populations in the olfactory bulb (Fig. 1Q), the cerebellum, the hippocampus, and other specific neural structures (data not shown). By P7, after the peak of cell death in the sp, Necdin expression declines in the cortex. Last, as previously reported (Aizawa et al., 1992; Muscatelli et al., 2000), Necdin expression in adult mouse nervous

horn of the spinal cord, in developing post-mitotic motor neuron precursors (dashed line) expressing Islet1/2 (M′), and at low level, in a small population of dorsal interneurons (arrow), and in more dispersed cells of the alar plate (arrowhead). No mRNA is detected in the ventricular zone (vz) and in the white matter (wm). Other abbreviations: sa, septal area; III, third ventricle; IV, fourth ventricle; LV, lateral ventricle. Scale bar (shown in A), 1.5 mm (A,B), 2.7 mm (C), 150 μm (D,M,M′), 1.2 mm (E), 450 μm (F), 375 μm (G), 1 mm (H), 80 μm (I), 35 μm (I′), 160 μm (J), 800 μm (K), 300 μm (L). (b) Necdin in situ hybridization and immunostaining on tissue sections at E17 (N), P0 (O), and P2 (P,Q). Dorsal is up. (N) Necdin protein staining in the suprachiasmatic nucleus of the hypothalamus at E17. Sagittal sections showing Necdin expression in the subplate (sp) and mid-cortical layers (arrows) of the dorsal cortex: (O) Necdin mRNA at P0. (P) Necdin protein at P2. (Q) Sagittal section at P2 with Necdin protein expression (arrowhead) in the olfactory bulb (ob). Scale bar (shown in A), 260 μm (N), 200 μm (O,P), 600 μm (Q).

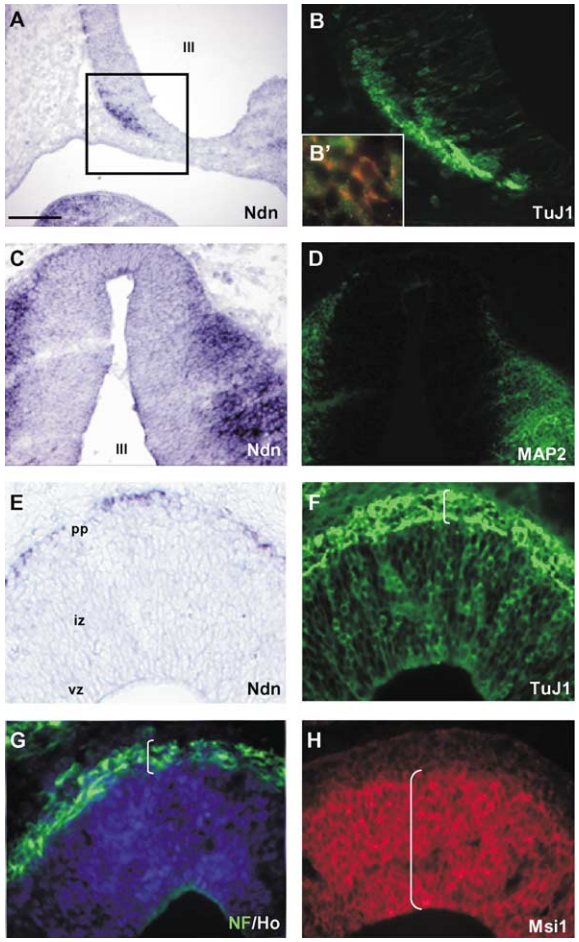

Fig. 2. Necdin is expressed in post-mitotic neurons during embryonic development. Comparison of Necdin (Ndn) transcript (A,C,E) and protein (B′) expression with immunostaining with neuronal differentiation markers TuJ1, anti-microtubule associated protein 2 (MAP2), anti-Neurofilament (NF), and anti-mouse Musashi1 (Msi1) antibodies, on serial adjacent sections. TuJ1 detects neuronal progenitors cells undergoing terminal division. Late differentiation markers, MAP2 and NF, are primarily expressed in postmigratory more mature neurons. Msi1 is a marker of neural progenitor cells. (A,B) Sagittal view at E10 showing Necdin expression in the presumptive hypothalamus, in newly formed neurons at the pial surface of the third ventricle, labeled with TuJ1. (B′) Merged images of Necdin protein (red) and Tuj1 (green) showing their coexpression in the diencephalic neuroepithelium. (C,D) Transverse section through the diencephalon at E12, showing Necdin expression in the dorsal hypothalamus and MAP2 immunoreactivity in the corresponding area. (E–H) Magnification of the dorsal telencephalic neuroepithelium at E13. Pial surface is up. Necdin is detected in post-mitotic neurons in the preplate (pp) near the pial surface of the neuroepithelium (E), in the external part of the zone labeled with early neuronal differentiation marker TuJ1 (F) and late differentiation marker NF (G), but is absent from the ventricular zone (vz) and the intermediate zone (iz) area labeled with Msi1 (H). Abbreviation: Ho, Hoechst dye. Scale bar (shown in A), 150 μm (A), 75 μm (B), 18 μm (B′), 100 μm (C,D), 50 μm (E–H).

system is limited to discrete areas, in particular in the hypothalamus, the thalamus, and the hindbrain (data not shown).

These results suggest that during mouse nervous system development, Necdin protein expression is restricted to neurons and has a similar distribution pattern to the mRNA. Necdin begin to be expressed at E10, around the time the first diencephalic neurons become post-mitotic. From E10 to E12, in both central and peripheral nervous system, Necdin expression correlates with the initial formation of all post-mitotic neurons, labeled with TuJ1 and MAP2. Studies conducted in vitro have suggested that Necdin may be implicated in cell cycle arrest, cell differentiation and survival (reviewed by Yoshikawa, 2000; Tcherpakov et al., 2002). The current results support the hypothesis that Necdin could also facilitate cell cycle exit of most developing neurons in vivo. After E13, Necdin expression remains high in both embryonic and adult thalamus, hypothalamus and pons, but diminish in other post-mitotic structures such as the neocortex (Fig. 2E–H). In addition, Necdin expression is not limited to nervous system cells. Taken together, these results indicate that Necdin is not a pan neuronal marker as previously described.

To determine if Necdin and p75NTR could be functional partners in vivo, we compared the expression of the two genes during mouse nervous system development, using specific riboprobes and antibodies. We found that p75NTR expression is included in Necdin expression domain (see details of expression in Fig. 3A–F, and cellular colocalization in Fig. 3G and H). Both genes are also expressed in the thalamus, in the inner nuclear layer of the retina, which gives rise to the retinal ganglion cell layer, in the preoptic and septal area of the basal telencephalon, in the diagonal band of Broca, in the striatum, in sympathetic ganglia, and in muscle components (data not shown). In the telencephalic neuroepithelium, from E13 to E18, p75NTR expression in the pp, and then in the sp (McQuillen et al., 2002) is similar to Necdin expression described in this study (see Fig. 1L,O,P). In post-natal animals, both genes are expressed in the olfactory bulb, in the piriform cortex, and in the cerebellum. In very few cases, such as in the cerebellum, p75NTR expression is more extended than Necdin domain (not shown). Although we could not detect p75NTR in developing hypothalamic nuclei, p75NTR expression have been reported in adult hypothalamus as well as in LHRH producing neurons (Berg-von der Emde et al., 1995). These results indicate that Necdin and p75NTR spatial and temporal expression partially overlap within post-mitotic neuronal populations. This is consistent with a putative role of Necdin in signaling events promoted by p75NTR and neurotrophins during development. In particular, Necdin may accelerate differentiation in response to nerve growth factor (Tcherpakov et al., 2002). Interestingly, both p75NTR and Necdin-deficient mice exhibit improved spatial learning (Muscatelli et al., 2000; Greferath et al., 2000), but it is unknown if they have similar neuronal histological abnormalities.

2. Methods

Specificity of Necdin mRNA and protein detection were controlled on tissues from Necdin-deficient animals.

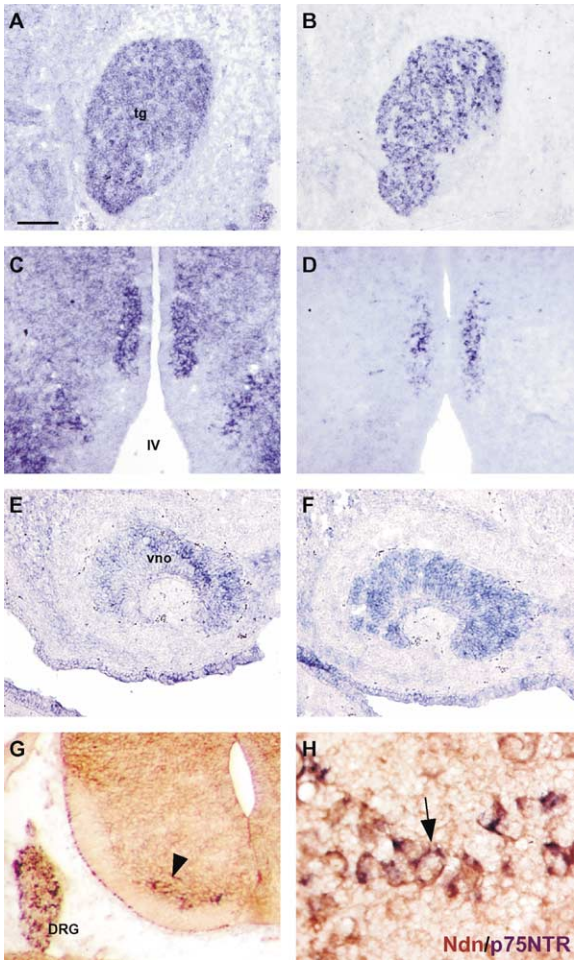

Fig. 3. Comparison of Necdin and p75NTR expression pattern during development. Comparison of Necdin (A,C,E) and p75NTR (B,D,F) mRNA expression on adjacent transverse sections at E12.5. p75NTR expression is partly included in Necdin expression domain. For example, in: (A,B) trigeminal ganglia (tg), (C,D) raphe nucleus in the pons, and (E,F) vomeronasal organ (vno, a chemoreceptive structure situated at the base of the nasal septum). Simultaneous detection on transverse sections of Necdin protein (brown) and p75NTR mRNA (purple): (G) At E13 in developing motor neuron precursors of the ventral horn of the spinal cord (arrow) and in DRG. (H) High magnification showing cellular coexpression (arrowhead) in the pons at P2. Scale bar (shown in A), 150 μm (A–F), 70 μm (G), 18 μm (H).

The probe used in this study recognizes the 3′-UTR (from nucleotide 1174 after the ATG to nucleotide 1466) of the Necdin gene that is unrelated to other MAGE encoding genes. In situ hybridization was carried out on 12 μm paraformaldehyde-fixed cryosections with antisense probes transcribed with T7 RNA polymerase, and the digoxigenin label detected using anti-digoxigenin Fabs coupled to alkaline phosphatase and NBT/BCIP (Roche). No signal was detected in the sense control riboprobes. For immunohistochemistry, primary antibodies were: NC243 rabbit anti-mouse Necdin (Niinobe et al., 2000) that predominantly detects Necdin in the cytoplasm when using conventional immunohistochemical analysis, rat anti-mouse Musashi1 (kind gift of Dr Hideyuki Okano, Osaka University), 9651 rabbit anti-mouse p75NTR receptor (kind gift of Dr Moses Chao, New York University), LR1 rabbit anti-LH-RH (kind gift of Dr Robert Benoit, Montreal General Hospital), rabbit anti-Neurofilament M (Chemicon), TuJ1 (mouse monoclonal to neuron-specific class III β-tubulin, Berkeley Antibody), anti-MAP2 (mouse monoclonal HM-2 to rat brain microtubule associated protein 2, Sigma), anti-GFAP (mouse monoclonal to glial fibrillary acidic protein, Sigma), and anti-Islet (mouse monoclonal 2D6 and 4D5, Developmental studies Hybridoma Bank of Iowa University). Sections were examined on a Zeiss Axioplan 2 equiped with a CARV module.

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