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The adhesion molecule TAG-1 is required for proper migration of the superficial migratory stream in the medulla but not of cortical interneurons

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Abstract

The neural cell adhesion molecule TAG-1 has been implicated in the tangential migration of neurons of the caudal medulla and of cortical interneurons. In the former case, protein is expressed by the neurons as they migrate, and blocking its function results in altered and reduced migration in vitro. In the latter case, protein is expressed, in part, by the pathway the interneurons use to reach the cortex, and in vitro experiments propose a role for TAG-1 in this system, as well. However, the in vivo requirement of TAG-1 in these migrations has not been investigated. In this report, we analyze the developmental phenotype of TAG-1-deficient animals in these two migratory systems. We show that mutant mice have smaller lateral reticular nuclei as a result of increased cell death in the superficial migratory stream of the caudal medulla. On the other hand, the absence of TAG-1 does not affect the number, morphology, timing and routes of GABAergic interneurons that migrate from the ganglionic eminences to the cortex. Therefore, TAG-1 function is required for the survival of the neurons of some precerebellar nuclei, while it is not required for cortical interneuron migration in vivo.

Keywords: Adhesion; Migration; Cell death; TAG-1/axonin-1; IgSF; Cell adhesion molecules; Precerebellar nuclei; GABAergic interneurons

Introduction

TAG-1 (Cntn-2) is a neuronal adhesion molecule of the immunoglobulin superfamily (IgSF) expressed in the developing and adult nervous system with a dynamic and complex pattern (Dodd et al., 1988; Wolfer et al., 1994; Denaxa et al., 2003). This GPI-linked glycoprotein promotes adhesion and neurite outgrowth of sensory neurons as shown by in vitro or ex vivo assays (Furley et al., 1990; Stoeckli et al., 1991; Tsiotra et al., 1996; Pavlou et al., 2002). In chick embryos, it has been shown to influence, along with other members of the IgSF, the guidance of commissural axons towards the spinal cord midline, the floor plate (Stoeckli and Landmesser, 1995; Stoeckli et al., 1997; Fitzli et al., 2000).

We have shown that TAG-1 is involved in tangential migrations in two developing structures in the mouse CNS, the caudal medulla and the neocortex. The protein is expressed by the migrating cells of the superficial migratory stream (SMS) at the caudal medulla giving rise to precerebellar nuclei such as the lateral reticular and external cuneate nuclei (LRN/ECN; Fig. 1A, Kyriakopoulou et al., 2002). TAG-1 also marks the corticofugal fibers and influences the migration of GABAergic interneurons derived from the medial ganglionic eminence (MGE) to the neocortex (Fig. 1B; Denaxa et al., 2001). In both the medulla and the cortex, blocking TAG-1 function ex vivo results in reduced migration. In the adult, protein is produced by myelinating glia of the central and peripheral nervous system and is clustered in the area of the node of Ranvier in myelinated fibers (Traka et al.,

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2002). TAG-1 is thought to act as a scaffold, together with the axonal protein Caspr2, to keep *Shaker*-type voltage-gated K^+ (Kv) channels clustered under the myelin sheath at the juxtaparanodes (Traka et al., 2003; Poliak et al., 2003).

TAG-1-deficient (*Tag-1*$^{-/-}$) mice have been described to be viable and fertile with no gross abnormality in their nervous system except for an upregulation of A1 adenosine receptors in the hippocampus (Fukamauchi et al., 2001). The mice show increased sensitivity to epileptogenic stimuli. In addition, TAG-1 is required in vivo for the clustering of Kv and Caspr2 around the area of the node of Ranvier (Traka et al., 2003). The in vivo requirement of TAG-1 function during development, however, where its role has been demonstrated by ex vivo or in vitro migration assays has not been established. The purpose of the study described here is to analyze the phenotype of the *Tag-1*$^{-/-}$ mice during development in terms of the migratory behavior of the cells of the superficial migratory stream in the caudal medulla and of the GABAergic neurons in the neocortex. We show that TAG-1 is necessary for the proper migration and development of some precerebellar nuclei. On the other hand, it is not required for the migration of GABAergic interneurons to the cortex, where other factors may compensate for its absence in vivo.

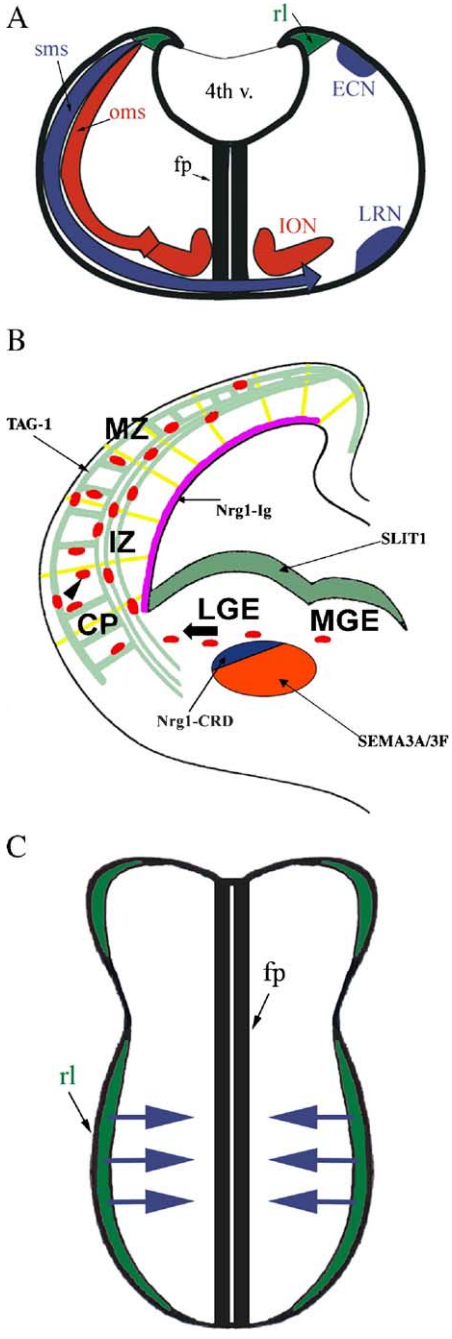


Fig. 1. Schematic representation of the migratory routes in the developing cortex and hindbrain. (A) Diagram of a transverse section from E13.5 hindbrain at the level of the 4th ventricle (4th v). The blue and red arrows indicate the superficial (SMS) and olivary migratory (OMS) routes respectively. The cells of the OMS stop before crossing the midline and form the inferior olive nucleus (ION), but the cells of the SMS pass beneath the floor plate (FP) and form the lateral reticular (LRN) and external cuneate nucleus (ECN) at the contralateral side of their origin (rl: rhombic lip). (B) Diagram of a coronal section from E14.5 telencephalon. The arrow in bold indicates the tangential migration of GABA expressing neurons from the subpallium to the pallium and the arrowhead their radial migration in the cortical plate. Cortical interneurons migrate away from the medial and lateral ganglionic eminence (MGE and LGE respectively), driven by the repulsive activities of Slit1 and Sema 3A/3F, in the permissive corridor of non-secreted forms of neuregulin (Nrg1-CRD). In the cortex, a proportion of them migrate in close association with tangentially oriented TAG-1 expressing fibers (green) in the marginal (MZ) and intermediate zone (IZ). A proportion of them is also attracted by the secreted isoforms of neuregulin (Nrg1-Ig). They reach their final positions in the cortical plate (CP) by using the radially arranged TAG-1-immunopositive efferent axons or radial glial fibers (yellow). (C) Diagram of a hindbrain explant dissected from E13.5 embryos. The blue arrows indicate the superficial migratory stream.

Materials and methods

Animals

Tag-1$^{-/-}$ mice were produced as described (Fukamauchi et al., 2001) and kept as heterozygous breeding pairs. The day of the vaginal plug detection was considered as embryonic day 0.5 (E0.5). We used E11.5–17.5 embryos, postnatal day (P) 0 and adult (3 months old) mice. All genotypes were confirmed by PCR (Fukamauchi et al., 2001). There was no difference between wild type and heterozygous animals in any of the assays that were performed, either in the cortex or in caudal medulla. Therefore, we show comparisons between heterozygous and homozygous mutants. The housing and animal procedures used were in agreement with the European Union policy.

Tissue preparation

Pregnant females were sacrificed by cervical dislocation. Embryos were collected in PBS and then fixed in 4% paraformaldehyde in PBS (4% PFA) at 4°C or dissected to prepare explants for culture. Postnatal and adult mice were anesthesized with Avertin (0.425 mg/g, i.p.) and fixed with transcardial perfusion of cold (4°C) 4% PFA. The fixed brains were cryoprotected with 30% sucrose in PBS, embedded in 15% sucrose, 7.5% gelatin in PBS and cut in the cryostat in 14-μm-thick sections (Denaxa et al., 2001; Kyriakopoulou et al., 2002). Some brains were dehydrated with ethanol, embedded in paraffin and cut in 10-μm sections. Some cryostat and paraffin sections were processed for histological staining with cresyl violet or haematoxylin–eosin (H/E).

In situ hybridization

In situ hybridization experiments were performed on whole mount embryos, on 200-μm vibratome sections and on 14-μm cryostat sections as described previously (Denaxa et al., 2001; Kyriakopoulou et al., 2002). The

following cDNAs were used as antisense probes: dlx2 (J.L.R. Rubenstein, UCSF), lhx6 (V. Pachnis, NIMR-MRC, Mill Hill), gad67 (F. Guillemot, NIMR-MRC, Mill Hill), Rig-1/Robo3 (A. Chedotal, Univ. Paris 6), Barhl1 (M. Xiang, Univ. of N. Jersey, R.W.Johnson Medical School).

Immunohistochemistry

Immunohistochemical experiments were performed as described previously (Denaxa et al., 2001; Kyriakopoulou et al., 2002). The following antibodies were used: mouse monoclonal abs: anti-PSA-NCAM (5A5; Developmental Studies Hybridoma Bank, Iowa City, IA), anti-parvalbumin (Chemicon, Temecula, CA), anti-calbindin (Sigma, St. Louis, MO); rabbit polyclonal abs: anti-GABA (Sigma), anti-L1 (kindly provided by Dr. Rathjen), anti-GAD (Chemicon), anti-calretinin (Swant, Switzerland), anti-Pax6 (BAbCo, Richmond, CA). Secondary antibodies used: biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA), anti-rabbit or anti-mouse IgG FITC or Cy3 (Jackson ImmunoResearch, West Grove, PA), anti-mouse IgM Alexa Fluor 488 or 568, anti-mouse or anti-rabbit IgG Alexa Fluor 488 (Molecular Probes, Eugene, OR).

Carbocyanine dye tracing

Corticofugal fibers were visualized by DiAsp tracing in the cortex, according to Molnár and Cordery (1999). Unilateral DiI injections in the cerebellum were done as follows: a small crystal of DiI was applied on one side of E17.5 cerebella of heterozygous and homozygote mutant mice fixed in 4% PFA. The brains were kept at 37°C for 4 weeks in the dark, embedded in 4% low melting agarose and cut into 200-μm vibratome sections. DiI placements in the rhombic lip of hindbrain explants and subsequent culturing of mutant and heterozygous animals were performed as described in Kyriakopoulou et al. (2002).

TUNEL assay

For the detection of cell death in the region of the caudal hindbrain, we used the In Situ Cell Death Detection kit, TMR red (Roche Diagnostics, Nutley, NJ), to perform TUNEL assays. The hindbrain region from E12.5–E14.5 embryos were dissected and fixed in 4% PFA for 1 h, washed in PBT (PBS, 0.1% Triton X-100) and dehydrated in 70% EtOH for 30 min. After rehydration, the tissues were incubated for 1 h in a solution of 6% H_2O_2 in methanol, washed in PBT, digested in 20 μg/ml proteinase K for 5 min and postfixed in 4% PFA for 30 min. After permeabilization (2 min on ice in 0.1% sodium citrate, 0.1% Triton X-100), we followed the labeling reaction with the TUNEL reaction mixture and analyzed the results in the confocal microscope. Transverse vibratome sections of E13.5 caudal medulla were processed for TUNEL and immunohistochemistry for Pax-6. We performed TUNEL assays in coronal cryostat sections of heterozygous and mutant E14.5 cortices according to the protocol suggested by the manufacturer.

5-Bromo-2-deoxy-uridine (BrdU) labeling and immunocytochemistry

Pregnant female mice were injected with a solution of BrdU (2 mg/ml in 0.9% NaCl, 20 μg/g body weight) at different developmental stages (E12.5–E14.5) to label the mitotic neurons of the embryos on these ages. The newborn pups (at postnatal day 0) were sacrificed, and the brains were cut in 150-μm-thick vibratome sections. For the detection of the incorporated BrdU, we followed previously published protocols (Kyriakopoulou et al., 2002; de Diego et al., 2002) for immunofluorescence detection.

Explant cultures

Explants of the neural tube containing the hindbrain and cerebellum were dissected from E12.5 embryos and cultured in an "open book" configuration as described previously (Kyriakopoulou et al., 2002) and illustrated in Fig. 1C. Organotypic slice cultures of E13.5–E14.5 mouse telencephalon, DiI labeling or GABA immunohistochemistry of migrating neurons and culture in the presence of Fab fragments of anti-TAG-1 and control antibodies, were performed as described previously (Denaxa et al., 2001).

Microscopy

Immunofluorescent or DiI injected sections and explants were viewed, and images were generated in a Leica TCS-NT Laser Scanning Confocal microscope. Other images were photographed in a Leica MZ12 microscope equipped with a Leica MPS60 camera (Germany) and a PowerShot G6 Canon digital camera.

Quantification and statistical analysis

Quantification of the width of the SMS

The number of the cell bodies which are found beneath the floor plate at the level of the ventral midline was measured in 10-μm-thick transverse sections of E13.5 embryos.

Quantification of the size of adult lateral reticular nucleus (LRN)

We used the Scion Image Program in order to calculate the volume and the cell density of the LRN nucleus in serial 10-μm-thick transverse sections of adult hindbrain.

Quantification of GABAergic interneurons in the cortex

Coronal sections of adult mutant and control mice ($n = 3$ for each genotype) hybridized with the GAD67 probe were used. Cells were counted on images acquired in a Hamamatsu Chilled CCD camera (C5985) attached to a Zeiss Axioskop. Three sections per animal were counted at bregma −1.40 to −2.06, while sections at more rostral or caudal levels were also observed. Retrosplenial, motor, somatosensory and perirhinal areas of the cortex were counted. Quantification of the three major GABAergic interneuron subtypes in the cortex (parvalbumin-, calretinin-, calbindin-immunolabeled cells) was performed as above except that three bregmata were chosen (+1.00, −0.70, −1.94), and the number of cells counted per section was divided by the area in mm^2 (Scion Image Program) that each section occupies.

Quantification of GABAergic cells in cortical slices

Pixel density measurements of GABA-labeled cells in cortical slices were performed as described previously (Denaxa et al., 2001) with Scion Image Program.

Quantification of TUNEL-positive cells in the superficial migratory stream (SMS) of the hindbrain

We quantified TUNEL-positive cells (red fluorescence) by measuring the area of the fluorescent pixels in the SMS with the Scion Image Program.

Quantification of the BrdU-labeled cells at LRN nucleus of P0 mice

We detected the incorporated BrdU by immunofluorescence, and we used the Scion Image Program to calculate the ratio of fluorescent pixel density to the total measured area.

In all cases, we used the single factor ANOVA test to analyze the results.

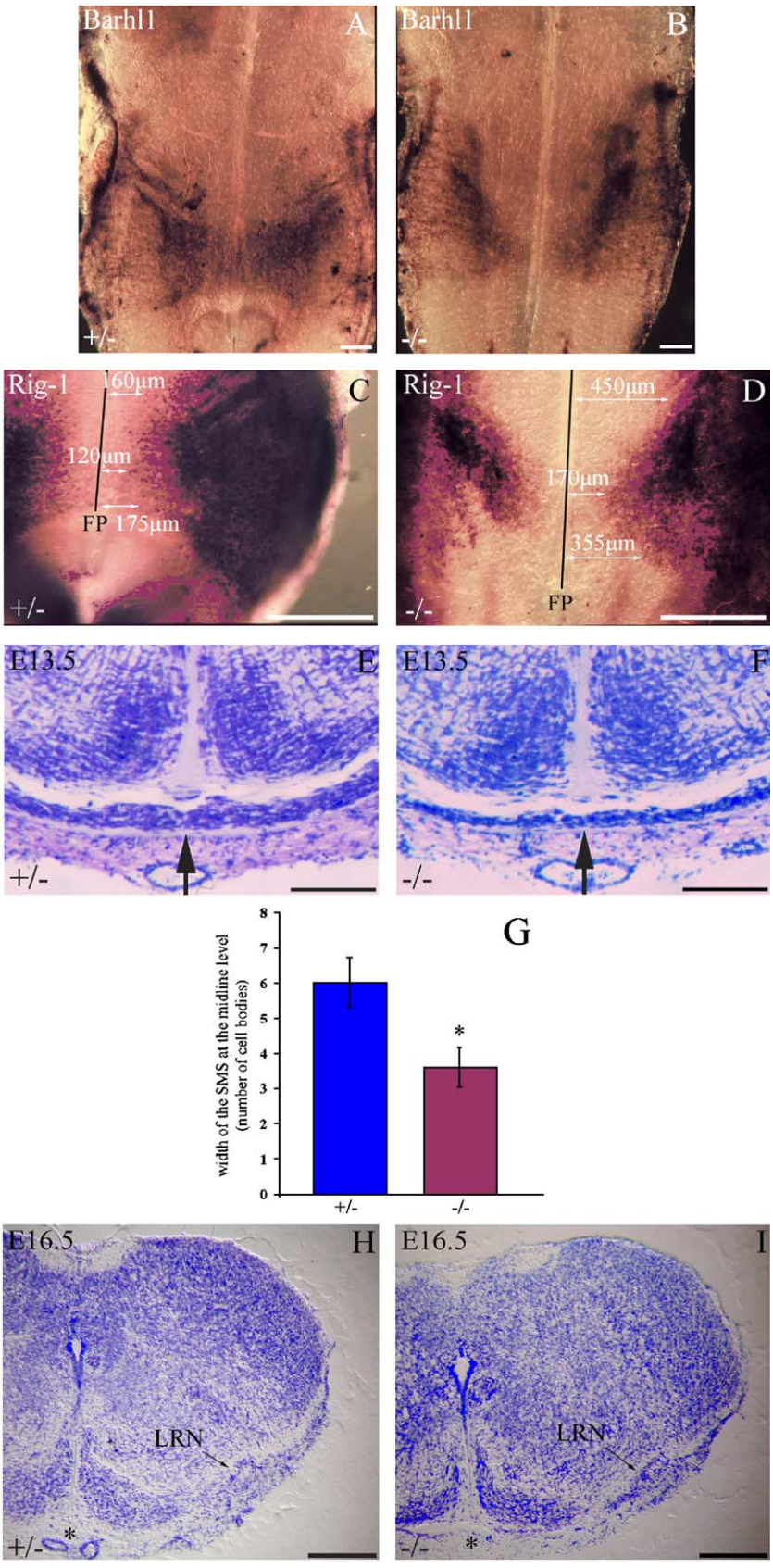
Results

The superficial migratory stream is affected in Tag-1$^{-/-}$ mice

The birthdates and trajectories of the SMS cells migrating in the caudal hindbrain have been described initially by ^{3}H-thymidine and tracing studies (Altman and Bayer, 1987; Bourrat and Sotelo, 1990); These cells express TAG-1 only during the period of their migration from E12.5 to E15.5 (Wolfer et al., 1994; Kyriakopoulou et al., 2002). We have shown that in vitro blocking of TAG-1 function causes alterations in the migration of these neurons (Kyriakopoulou et al., 2002). In order to investigate the effect of the loss of TAG-1 in vivo, we examined the phenotype of *Tag-1*$^{-/-}$ mice during development.

We performed in situ hybridization experiments in toto on E13.5 animals, since the majority of SMS neurons are

migrating at this age, using two markers of the SMS, the homeobox transcription factor Barhl1 (Bulfone et al., 2000; Li et al., 2004) and the Slit receptor Rig-1/Robo3 (Marillat et al., 2004). Figs. 2A–D show that mutant mice exhibit fewer Barhl1 and Rig1-positive cells than controls. Furthermore, the SMS was thicker in the Tag-1$^{+/-}$ animals than in Tag-1$^{-/-}$



mice as observed on transverse sections at the level of the caudal medulla stained with cresyl violet (arrows in Figs. 2E–F). To quantify the reduction of the thickness of the SMS, we measured the number of cell bodies beneath the floor plate at the level of the midline. As illustrated in Fig. 2G, this reduction in the thickness of the SMS was statistically significant ($n = 7$ for each genotype).

We have examined later stages (up to E16.5) of mutant and control brains to test the possibility that there may be a delay in the SMS migration in mutant brains. No such evidence was found since by E16.5 the migration was complete in both *Tag-1*$^{+/-}$ and *Tag-1*$^{-/-}$ animals (Figs. 2H–I).

In order to investigate the migratory behavior of the wild type and mutant SMS cells in vitro, we prepared explants from the hindbrain at the beginning of the migration period (E12.5) as detailed in de Diego et al. (2002) and Kyriakopoulou et al. (2002). We placed DiI crystals in the rhombic lip of control and mutant explants at the beginning of the culture period (E12.5) and looked at the morphology of migrating cells as well as the rate of the migration during defined time periods in vitro. After 24 h in vitro, cells in explants from both genotypes (*Tag*$^{+/-}$ $n = 3$, *Tag*$^{-/-}$ $n = 4$) exhibit similar morphology, namely, they were unipolar and extended long leading processes directed towards the midline, as described in Kyriakopoulou et al. (2002) in the case of the wild type (Figs. 3A–D). After 40 h in culture, cells that have migrated further ($n = 8$ for each genotype) and their leading processes have crossed the midline looked still similar in both genotypes (Figs. 3E–F). After 45–50 h in vitro, cells bodies have reached and cross the midline in both mutant and heterozygous mice ($n = 8$ for each genotype; data not shown). Finally, after 65 h in culture, the majority of the DiI-labeled cells have reached the contralateral side of the explant in both mutant and heterozygous mice (*Tag*$^{+/-}$ $n = 7$, *Tag*$^{-/-}$ $n = 2$; data not shown). Thus, the absence of TAG-1 does not affect the morphology or behavior of cells that continue to migrate in the explants.

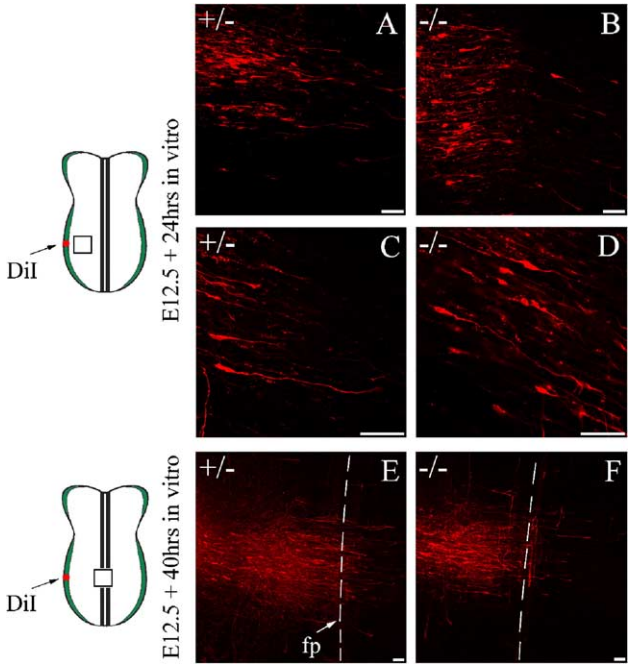


Fig. 3. Morphology of migrating SMS cells. Confocal images of whole mount explants injected with DiI at the rhombic lip at E12.5. (A–D) DiI-labeled cells with unipolar shape and long leading processes migrate towards the midline 24 h after the beginning of the culture. The morphology of the cells is the same in both *Tag-1*$^{+/-}$ (A, C) and *Tag-1*$^{-/-}$ animals (B, D). (E–F) After 40 h in vitro, some leading processes have reached and crossed the midline (dotted line) in both *Tag-1*$^{+/-}$ (E) and *Tag-1*$^{-/-}$ animals (F). Some labeled processes seen close to the floor plate perpendicular to the direction of migration are likely to be axons located deeper than the leading processes. The images illustrate the region boxed on the diagram on the left. Scale bar: 50 μm.

The mature lateral reticular nucleus (LRN) is smaller in Tag-1$^{-/-}$ *mice*

The cells of the SMS form the LRN and the ECN when they reach their final positions. To investigate whether the reduction in the number of SMS cells in *Tag-1*$^{-/-}$ mice resulted in alterations of the adult LRN, we compared the size of this nucleus in *Tag-1*$^{+/-}$ and *Tag-1*$^{-/-}$ adult mice, as it was more accessible than the ECN to quantification, due to its morphology and position in the hindbrain. The LRN was formed in both heterozygous and deficient animals (Figs. 4A–B). To investigate whether the size of the LRN was reduced, we calculated the volume of the adult nucleus from serial sections at the level of caudal hindbrain, and we found that there was a statistically significant reduction in the volume of the LRN in *Tag-1*$^{-/-}$ animals (Fig. 4C; $n = 5$ for each genotype). We also observed that there was a small reduction in the area of the LRN, but the greatest effect was found in the length of the nucleus which is smaller in the deficient mice about 20% (data not shown). However, the cell density of the LRN was not affected by the absence of TAG-1, as illustrated in Fig. 4D.

In order to detect the possibility of ectopic LRN cells, we performed in situ hybridization with *Barhl1* in transverse sections of the medulla at P0. The LRN was visualized, but no evidence of ectopic *Barhl1* signal was found (Figs. 4E–F).

We next performed unilateral DiI injections in the cerebella of E17.5 embryos in order to test whether the mutant LRN extend their mossy fibers normally. Such injections result in ipsilateral labeling of LRN neurons and contralateral labeling of inferior olivary neurons (data not shown). We observed the

Fig. 2. The number of migrating cells of the superficial stream is reduced in the TAG-1-deficient mice. (A–D) Whole mount in situ hybridization with Barhl1 (A, B) and Rig-1 (C, D) of *Tag-1*$^{+/-}$ (A, C) and *Tag-1*$^{-/-}$ mice (B, D). The labeled migrating cells of SMS are less in *Tag-1*$^{-/-}$ in both cases. The arrows in panels C and D indicate the distance of Rig-1-positive cells from the midline. (E–F) Transverse sections of E13.5 embryos at the level of superficial migration, stained with cresyl violet. In *Tag-1*$^{-/-}$ mice (F), the number of the migrating cells beneath the floor plate (arrow) is fewer than in *Tag-1*$^{+/-}$ (E) littermates. (G) Quantification of the reduction of the number of migrating cells in the superficial stream with single-factor ANOVA analysis. Values represent the number of the migrating cell bodies at the level of the ventral midline of *Tag-1*$^{+/-}$ (blue) and *Tag-1*$^{-/-}$ (red) animals. Error bars represent the SD, *P value <0.01. (H–I) Transverse sections of E16.5 embryos at the level of superficial migration, stained with cresyl violet. The SMS cells have reached their final destination and formed the LRN nucleus. No migrating cells were observed at the level of the midline (asterisk). Scale bars: (A–D) 500 μm; (E–F) 250 μm; (H–I) 250 μm.

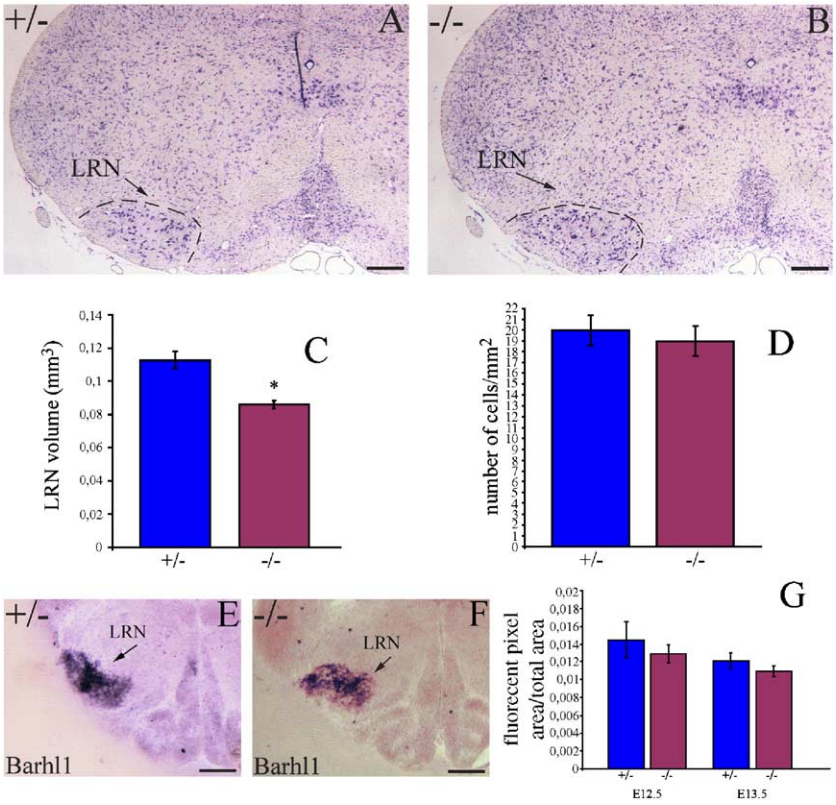


Fig. 4. The mature LRN is smaller in TAG-1-deficient mice. (A–B) Transverse sections of adult medulla stained with cresyl violet. The LRN is formed in both *Tag-1*$^{+/-}$ and *Tag-1*$^{-/-}$ animals (the dotted line surrounds the LRN nucleus). (C) Quantification of the reduction of the volume of LRN in adult *Tag-1*$^{-/-}$ mice. Values represent the volume in mm^3 of the LRN nucleus in *Tag-1*$^{+/-}$ (blue) and *Tag-1*$^{-/-}$ (red) mice. Error bars represent the SD, **P* value <0.01. (D) Quantification of cell density in the adult LRN of Tag$^{+/-}$ and Tag$^{-/-}$ mice. (E–F) Barlh1 in situ hybridization on transverse sections of medullas of *Tag-1*$^{+/-}$ (D) and *Tag-1*$^{-/-}$ (E) P0 mice. The LRN is visible, and no ectopic expression is detected. (G) Quantification of the proliferating cells of the SMS at E12.5 and E13.5. Values represent the ratio of the fluorescent pixel area/total pixel area in both *Tag-1*$^{+/-}$ (blue) and *Tag-1*$^{-/-}$ (red) mice. Error bars represent the SD. Scale bars: (A, B) 500 μm, (E–F) 500 μm.

same pattern of retrograde tracing in control and mutant medullas; thus, the absence of TAG-1 does not affect mossy fiber projections from the LRN to the cerebellum.

All of the results described above point to a reduction of the number of the SMS cells which reach their final positions in TAG-1-deficient mice. We performed BrdU injections at E12.5–E14.5 in vivo to check whether the proliferation of SMS cells is affected. Our results do not show any statistically significant reduction in the proliferation of heterozygous or mutant SMS cells at E12.5 and E13.5 (Fig. 4G). At E14.5, there were no more mitotic cells of SMS to be labeled. We observe a small, not statistically significant reduction in the numbers of proliferating cells in the mutants which we believe is due to cell death that has occurred during migration (see paragraph below and Fig. 5).

To investigate the fate of the cells that do not manage to migrate in the mutant environment, we examined the alternative possibility that these cells may die perhaps due to their inability to migrate properly. We performed TUNEL assays in transverse sections of hindbrains dissected at E13.5, when the migration is taking place, in combination with immunohistochemistry for the transcription factor Pax6, another marker of the migrating LRN cells (Engelkamp et al., 1999) as shown in Figs. 5A–B. We noticed that the increased cell death was localized mainly in pax6-positive cells in *Tag-1*$^{-/-}$ mice (Fig. 5B) but not in heterozygous littermates (Fig. 5A). Measurements of the TUNEL signal in Pax6-positive cells at E13.5 indicate that 35% of these as compared to 5% of control cells, die during migration (Fig. 5C; $n = 5$ for each genotype).

We also performed TUNEL assays in whole mount E12.5–E14.5 hindbrains and detected increased signal in the mutants throughout the trajectory of the migrating cells at E13.5 (Figs. 5D–E) and E14.5. Increased, statistically significant TUNEL signal is only detected while cells migrate (E13.5–E14.5), and it is not observed before migration starts (E12.5; Fig. 5F).

Taken together, our results indicate that a significant proportion of the cells of the superficial stream die during migration, thus explaining the reduced size of the LRN in the mutant adult animals.

The migration of interneurons and the distribution of corticofugal axons are not affected in Tag-1$^{-/-}$ *mice*

We have previously shown that blocking TAG-1 function in vitro results in a marked decrease of MGE-derived GABAergic cells in the cortex indicating that TAG-1, expressed by the corticofugal fibers, is involved in the tangential migration of neurons from the MGE to the cortex (Denaxa et al., 2001). To

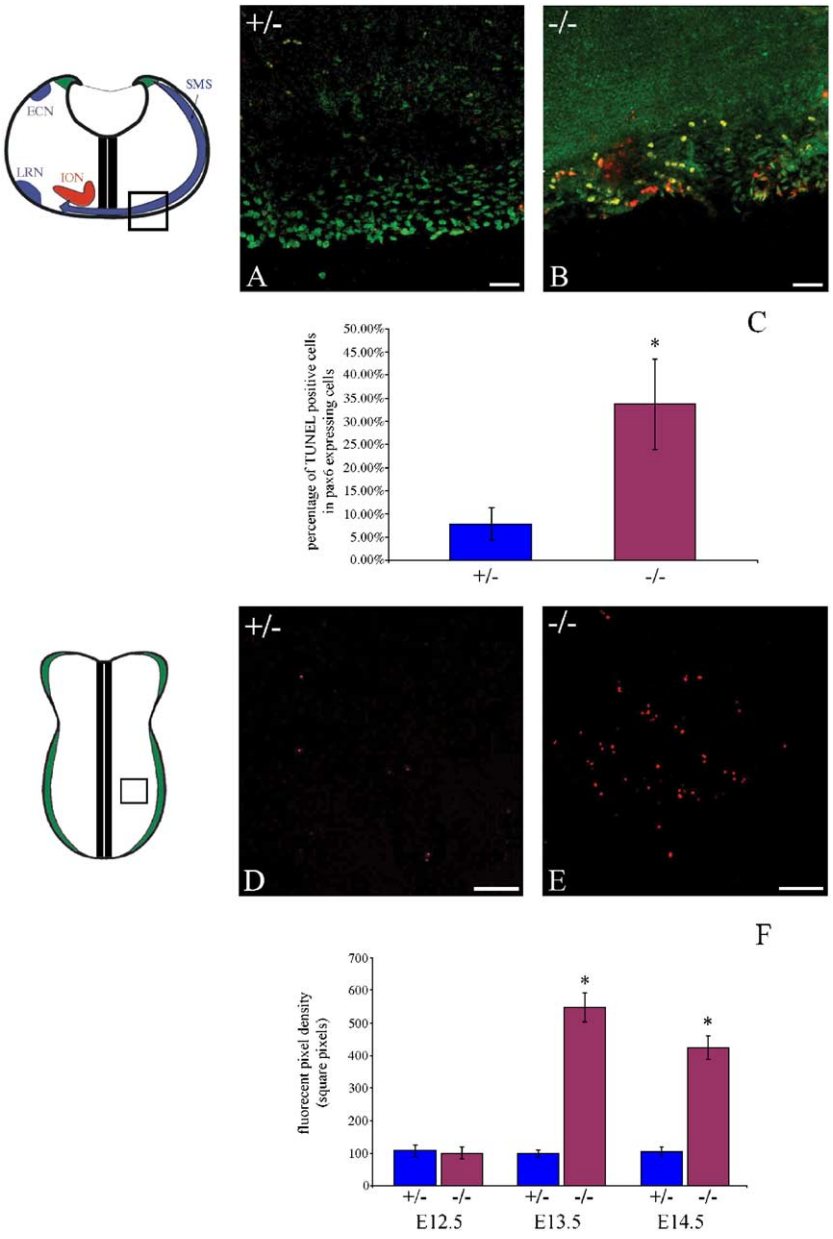
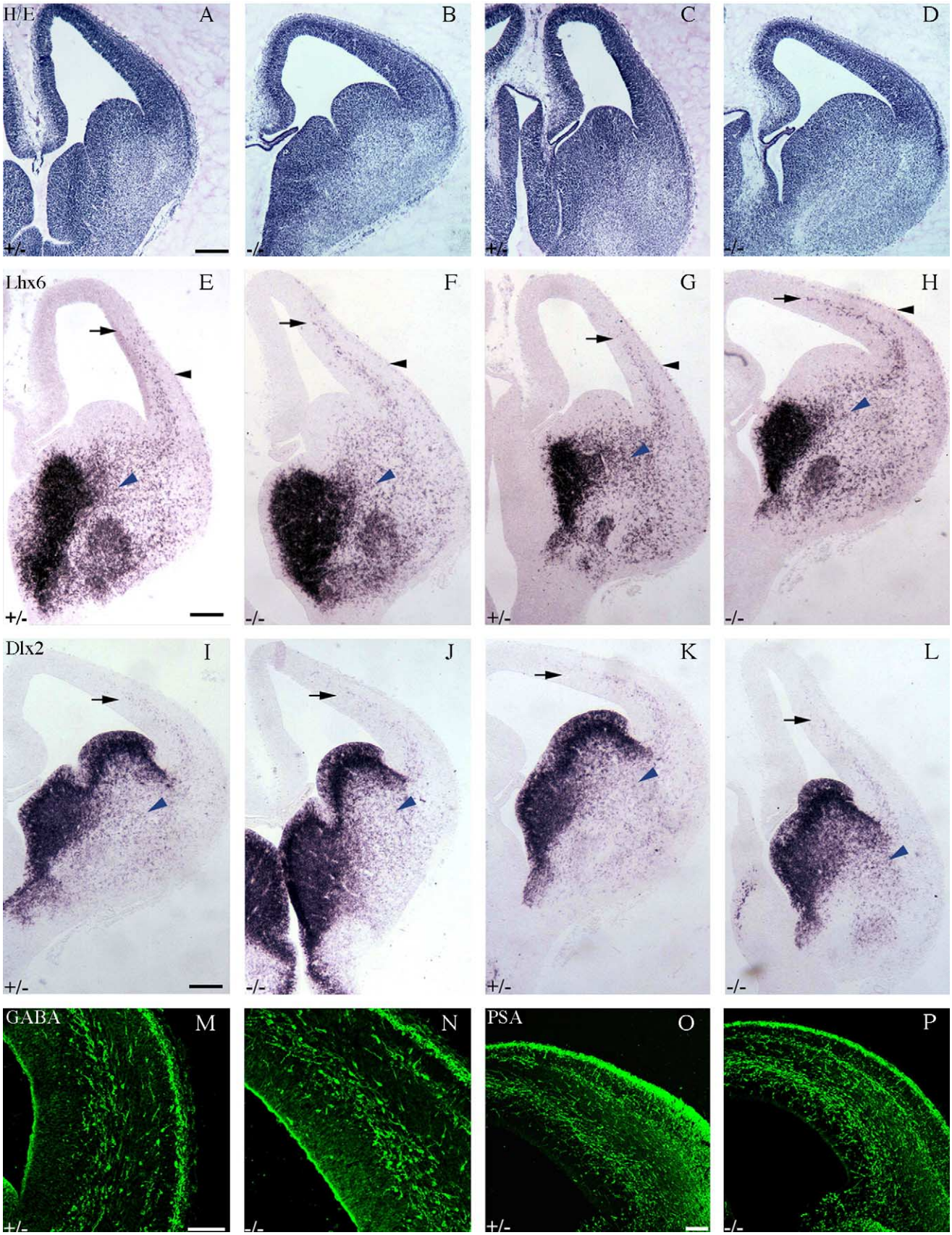


Fig. 5. Increased cell death in the SMS of TAG-1 mutants. (A–B) Immunohistochemistry for pax6 (green) and TUNEL assay (red) on transverse sections of E13.5 embryos. Increased cell death is detected in the pax6-expressing cells of the SMS of *Tag-1*$^{-/-}$ mice (compare panels A, B, the yellow color indicates co-localization of red and green). (C) Quantification of cell death in the SMS at transverse sections of the hindbrain. Values represent the percentage of TUNEL-positive cells of pax6-expressing cells. Error bars represent the SD, **P* value <0.01. (D–E) Cell death is increased in the SMS of *Tag-1*$^{-/-}$ mice (E) compared with the *Tag-1*$^{+/-}$ controls (D) as revealed by TUNEL. The images illustrate the region boxed on the diagram on the left. (F) Quantification of cell death in the SMS of whole mount hindbrains. Values represent the fluorescent pixel area (TUNEL-positive cells) of *Tag-1*$^{+/-}$ (blue) and *Tag-1*$^{-/-}$ (red) mice at E12.5–E14.5. Error bars represent the SD, **P* value <0.01. Scale bars: (A–E) 50 μm.

address the requirement of the TAG-1 protein in the guidance of cells tangentially migrating from the subpallium to the cortex in vivo, we studied the phenotype of *Tag-1*$^{-/-}$ mice during cortical development.

Corticofugal axons are thought to be among the substrates interneurons use to migrate to the cortex (Metin and Godement, 1996; Denaxa et al., 2001; McManus et al., 2004; Morante-Oria et al., 2003). They selectively express TAG-1 (Denaxa et al., 2001) in the proximal part of their pathway, up to the level of the corticostriatal boundary (Jones et al., 2002). We examined whether the ability of corticofugal axons to navigate through the cortex to the internal capsule of the basal forebrain was affected in *Tag-1*$^{-/-}$ brains by tracing corticofugal axons in fixed brains. DiAsp crystal placement into the dorsal cortex of E13.5 or E14.5 control (*Tag-1*$^{+/-}$) or *Tag-1*$^{-/-}$ mice labeled corticofugal axons extending through the intermediate zone of the developing pallium to reach the internal capsule. We observed no differences in the trajectories of the DiAsp axons, between control and *Tag-1*$^{-/-}$ brains (Supplementary Figs. S1A, $n = 4$; B, $n = 5$). L1, a known marker of thalamocortical axons, has been reported to be also present in corticothalamic axons at E14.5 (Jones et al., 2002; Wiencken-Barger et al.,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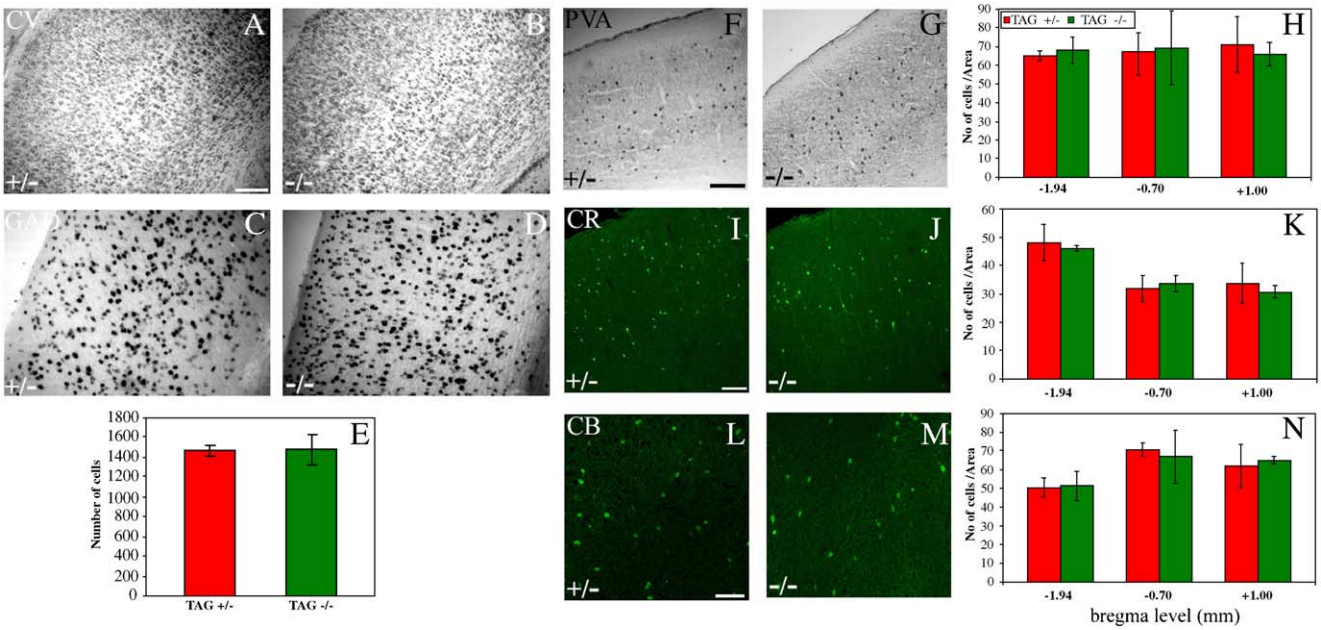

Fig. 7. Normal numbers and distribution of different classes of GABAergic interneurons in the adult cortex of Tag-1$^{-/-}$ mice. (A–D) Cresyl violet staining (A, B) and in situ hybridization for Gad67 (C, D) on coronal sections of the somatosensory cortex of Tag-1$^{+/-}$ and Tag-1$^{-/-}$ adult mice. (E) Number of Gad67-positive cells counted throughout the cortex at bregmata levels −1.40 to −2.06. Error bars represent the SD; one-way ANOVA was performed with genotype as the independent factor. $F_{1,4} = 0.008$, $P = 0.931$ (SPSS v.10.0.1, SPSS Inc.). (F–K) Coronal sections of adult cortex of heterozygous and homozygous mice stained for parvalbumin (F–H), calretinin (I–K) and calbindin (L–N). Number of PVA-positive (H), calretinin-positive (K) and calbindin-positive (N) cells counted divided by the area of the section is shown at bregmata levels −1.94, −0.70, +1.00. No statistically significant differences are observed by single factor ANOVA. The *P* values are for PVA, 0.70, 0.93 and 0.75; for calretinin, 0.77, 0.31, 0.71; for calbindin, 0.93, 0.84, 0.83. Scale bars: (A–G) 150 μm; (H–I) 100 μm; (J–K) 150 μm.

2004). We performed immunohistochemistry for L1 without observing any differences in the trajectories of L1 immunopositive axons between *Tag-1*$^{+/-}$ and *Tag-1*$^{-/-}$ sections (Supplementary Figs. S1C–D).

The gross morphology of the developing forebrain was examined by H/E staining. Comparison of cortical sections between *Tag-1*$^{+/-}$ and *Tag-1*$^{-/-}$ mice, at different rostrocaudal levels and at different embryonic ages (E12.5–E16.5), revealed no gross histological defects (Figs. 6A–D). Additionally, we performed TUNEL assays at E14.5 without observing differences between control and mutant tissue (Supplementary Figs. S2A–B, $n = 2$).

We then examined the distribution of tangentially migrating interneurons in the embryonic cortex, as revealed by the expression of *Lhx6* and *Dlx2*, two transcription factors expressed by the MGE-derived migrating cells (Lavdas et al., 1999; Anderson et al., 1997). Comparison of their expression within the cortex of *Tag-1*$^{+/-}$ and *Tag-1*$^{-/-}$ mice, at embryonic ages from E12.5 to E16.5 and different rostrocaudal levels, showed no obvious differences in the number or laminar distribution of the *Lhx6* and *Dlx2* expressing cells (Figs. 6E–L).

We further assayed *Tag-1*$^{+/-}$ and *Tag-1*$^{-/-}$ animals with classical markers of cortical interneurons such as GABA and GAD without observing any measurable change (Figs. 6M, N and data not shown). In addition to these two markers, we have used immunohistochemistry for PSA-NCAM, the polysialic form of the neural adhesion molecule NCAM, which is present on tangentially migrating cells in the cortex (Seki and Arai, 1991). The ages examined were E12.5 to E16.5, encompassing the period of GABAergic migration to the cortex. We show that at early developmental stages, PSA-NCAM is expressed by most embryonic GABA interneurons during their migration to the cortex (Supplementary Figs. S3A–D). Therefore, PSA-NCAM can be used as an additional independent marker for cortical interneurons. No changes in the distribution of PSA-NCAM-expressing cells within the cortex of *Tag-1*$^{+/-}$ and *Tag-1*$^{-/-}$ mice were observed (Figs. 6O, P).

No gross morphological defects were observed in cresyl violet-stained coronal sections through the cortices of adult *Tag-1*$^{+/-}$ and *Tag-1*$^{-/-}$ mice (Figs. 7A–B). Consistent with the lack of interneuron migration defects observed in *Tag-1*$^{+/-}$ and *Tag-1*$^{-/-}$ animals during development, analysis of GAD67 expression in cortical sections of adult mice at different rostrocaudal levels did not reveal statistically significant differences between *Tag-1*$^{+/-}$ and *Tag-1*$^{-/-}$ animals (Figs. 7C–E; $n = 3$ for each genotype). Nevertheless, we examined the distribution and numbers of the three major interneuron

Fig. 6. Normal distribution of cortical interneuron markers in the absence of TAG-1. (A–D) Eosin–haematoxylin staining in cryostat sections of E14.5 *Tag-1*$^{+/-}$ (A, C) and *Tag-1*$^{-/-}$ (B, D) mouse brains, in different rostrocaudal levels. (E–L) In situ hybridization for *Lhx6* (E–H) and *Dlx2* (I, L) in coronal sections of E13.5 *Tag-1*$^{+/-}$ and *Tag-1*$^{-/-}$ brains, in different rostrocaudal levels. Black arrows indicate the stream of migrating interneurons, which express both transcription factors, blue arrowheads point to the subpallium. (M–P) Immunohistochemistry for GABA (M, N) and PSA-NCAM (O, P) in coronal sections of E13.5 or E14.5 *Tag-1*$^{+/-}$ (M and O, respectively) and *Tag-1*$^{-/-}$ (N and P respectively) mouse brains. No differences are observed in the number or the migratory routes of cortical interneurons. Scale bars: (A–L) 500 μm; (M, N) 160 μm; (O–P) 100 μm.

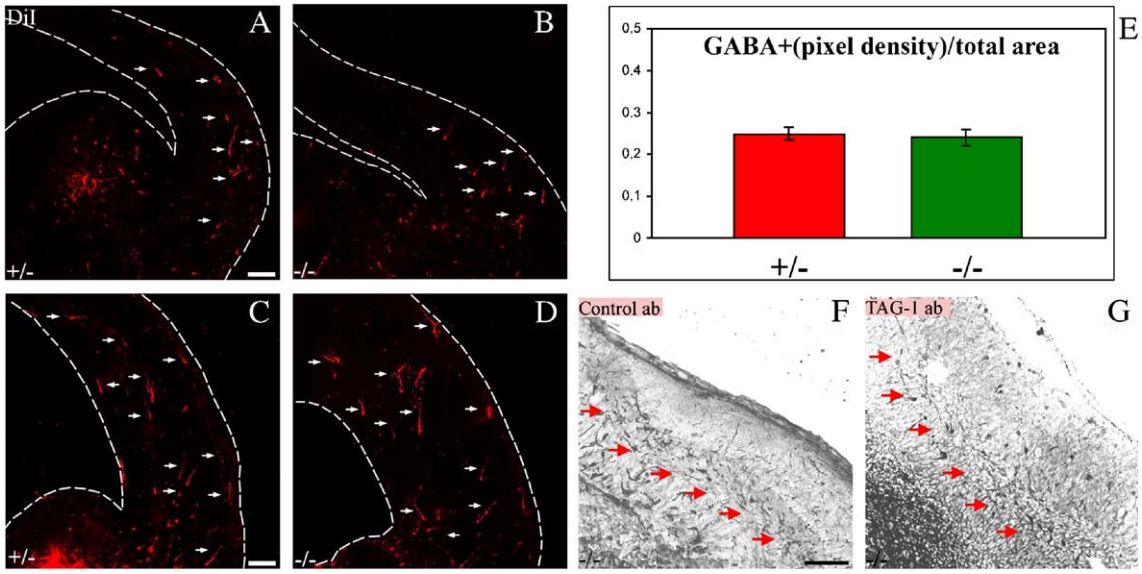

Fig. 8. DiI labeling in cortical slice cultures from Tag-$1^{+/-}$ and Tag-$1^{-/-}$ mouse brains reveals no differences in the morphology or migratory routes of cortical interneurons. (A–D) DiI tracing in cortical slice cultures from E13.5 (A, B) or E14.5 (C, D) mouse brains. In both cases, slices were cultured for 2 days. No alterations in the migration of MGE derived interneurons are observed between *Tag-1*$^{+/-}$ (A, C) and *Tag-1*$^{-/-}$ (B, D) mice. The broken line demarcates the ventricle and the edge of the slice culture, and the arrows show migrating neurons. (E) Quantification of the migration of GABA-labeled neurons in slice cultures of *Tag-1*$^{+/-}$ and *Tag-1*$^{-/-}$ brains. Values represent the ratio of the area where pixel densities were measured to the total area measured (i.e., all of neocortex). Value 1.00 would represent the entire length of the neocortex. Error bars represent the SD; $P > 0.05$, one-factor ANOVA. Values were collected from independent slice cultures where one embryo per culture is used. (F–G) Cortical slices from E13.5 *Tag-1*$^{-/-}$ mouse brains, cultured in the presence of control Fab (F) or TAG-1 Fab antibodies (G). No differences are revealed by immunohistochemistry for GABA. Red arrows indicate the stream of migrating interneurons. Scale bars: (A–D) 100 μm; (F–G) 160 μm.

subtypes, the parvalbumin (PVA; Figs. 7F–H)-, calretinin (CR; Figs. 7I–K)- and calbindin (CB; Figs. 7L–N)-positive neurons in control and mutant adult brains. No differences were seen in the distribution or numbers of these interneuron populations in the various cortical layers at different rostrocaudal levels.

We finally examined the mode of tangentially migrating neurons in slice cultures using DiI labeling for cells derived from the ganglionic eminences of E13.5 or E14.5 *Tag-1*$^{+/-}$ and *Tag-1*$^{-/-}$ mice. DiI-labeled cells were observed in the cortex in both *Tag-1*$^{+/-}$ and *Tag-1*$^{-/-}$ slices (Figs. 8A–D). Furthermore, analysis of density measurements of GABA-expressing cells in cortical slice cultures from E14.5 *Tag-1*$^{+/-}$ and *Tag-1*$^{-/-}$ mice revealed no statistically significant difference between the two genotypes (Fig. 8E; $n = 5$ for each genotype). We have also cultured cortical slices from *Tag-1*$^{-/-}$ mice in the presence of Fab fragments of anti-TAG-1 or control antibodies as described in Denaxa et al. (2001). No decrease is observed in GABAergic cells that have migrated to the cortex in the mutant animals that received control Fab or Fab anti-TAG-1 antibodies (Figs. 8F–G), thus further validating the specificity of the TAG-1 blocking reagents used in our in vitro study (Denaxa et al., 2001).

Taken together, the results described above indicate that TAG-1 is not required in vivo for the tangential migration of embryonic GABAergic interneurons from the subpallium to the cortex.

Discussion

TAG-1, a neural GPI-CAM of the IgSF, has been shown to play a role in adhesion, neurite outgrowth and migration in the embryo and in the maintenance of the molecular organization of myelinated fibers in the adult (reviewed in Karagogeos, 2003). We have shown that TAG-1 is involved in the tangential migration of the superficial migratory stream in the caudal medulla and the migration of GABAergic interneurons from the GE to the neocortex by using ex vivo assays (Kyriakopoulou et al., 2002; Denaxa et al., 2001). The in vivo requirement of TAG-1 in these two processes has been tested by analyzing the phenotype of *Tag-1*$^{-/-}$ mice and is presented in this report.

In the caudal medulla, a significant decrease in the cells of the SMS is observed in *Tag-1*$^{-/-}$ embryos due to increased cell death. The volume of the resulting LRN is smaller in mutant animals, but the neuron density of the nucleus is unaffected. In contrast, in the developing telencephalon, the GABAergic interneurons populate the cortex in apparently normal numbers and distribution in the absence of TAG-1.

The cells of the SMS migrate with two different modes of migration. At the beginning, they migrate by following each other in groups, while closer to the midline, they follow preexisting axons. TAG-1 is expressed by the migrating cells during both phases of migration (Kyriakopoulou et al., 2002). In the absence of TAG-1, a significant number of the neurons of the superficial migratory stream die in situ, indicating that TAG-1 is required by this population for its proper migration, in agreement to our previous ex vivo experiments (Kyriakopoulou et al., 2002). The morphology of migrating cells is similar in the *Tag-1*$^{+/-}$ and *Tag-1*$^{-/-}$ explants, pointing to the fact that SMS cells in the *Tag-1*$^{-/-}$ mice are not defective per se and are able to migrate.

The behavior and rate of migration of mutant cells were not different from the control ones in that they have reached the midline floor plate at the same time (40–45 h), while no errors in trajectories or aberrant pathways were observed. This phenotype is different from phenotypes described in mice deficient in axon guidance/migration factors, where nuclei are not formed, or their trajectories are aberrant, or even the final positioning of the neurons is affected. *Dcc* and *netrin 1*-deficient animals lack pontine nuclei, and their IO nuclei are severely atrophic (Serafini et al., 1996; Keino-Masu et al., 1996; Bloch-Gallego et al., 1999). In Slit receptor *Rig-1/Robo3*-deficient mice, precerebellar nuclei do not develop normally as the neurons and axons fail to cross the midline (Marillat et al., 2004). *Barhl1*$^{-/-}$ mice have smaller precerebellar nuclei and pronounced cerebellar defects (Li et al., 2004). This transcription factor controls expression of NT-3 and appears to be involved in the fine positioning of neurons within all five target precerebellar nuclei (Li et al., 2004).

The cell death observed in the medulla of the TAG-1 mutants suggests that TAG-1 may exert a "survival" factor effect. This would indicate a novel function for IgSF proteins. TAG-1 may help "concentrate" such a signal to the migrating neurons. Another possibility may be that loss of TAG-1 function may cause LRN neurons to become unresponsive to local survival factors. These two possibilities are not mutually exclusive.

Why is the requirement for TAG-1 in the SMS only partial? Our previous data (de Diego et al., 2002) actually point to the fact that additional factors may play a role in this migration. These neurons are partly responsive to floor plate-derived signals and in particular netrin/Dcc signaling (de Diego et al., 2002). However, the LRN forms normally in netrin-1 mutants, thus netrin is not required in vivo, in contrast to TAG-1 (Bloch-Gallego et al., 1999; this work). Other adhesion molecules are expressed by the migrating cells (i.e., PSA-NCAM) or by axons at the environment of the migration (i.e., L1), thus, we cannot exclude the possibility that these molecules compensate partially the loss of TAG-1 from this system.

The LRN is receiving inputs from the spinal cord, motor areas of the neocortex and from vestibular nuclei and is projecting primarily to the spinocerebellum. Thus, it is implicated in the control of motor activity and nociception. A defect in LRN, if not compensated by other neuronal systems, may be expected to cause alterations in locomotor activity. In addition, the phenotype of adult *Tag-1*$^{-/-}$ myelinated fibers may lead to motor deficits. Preliminary data testing the behavior of the *Tag-1*$^{-/-}$ mice in several motor-related tests point to such deficiencies (our unpublished data). Experiments in progress are designed to elucidate the full spectrum of behavioral deficits of *Tag-1*$^{-/-}$ mice.

In contrast to the phenotype present in the caudal medulla, TAG-1 is not necessary for the tangential migration of cortical interneurons in vivo. TAG-1 in the cortex is not expressed by the migrating cells themselves but by corticofugal fibers; these have been proposed to be one of the substrates that interneurons use to migrate (Denaxa et al., 2001; McManus et al., 2004; Morante-Oria et al., 2003). In addition, it is known that TAG-1, like most GPI-neural CAMs, is released from the cell surface (Karagogeos et al., 1991). In fact, these released isoforms, at least in the case of TAG-1/axonin-1 and F3/contactin, are functional since they promote neurite outgrowth (Durbec et al., 1992; our unpublished data). Thus, they may also act as short distance permissive cues or by modulating the activity of other environmental signals, which are found in the cortex. It has become clear from recent work that the corticofugal axons and their surrounding "microenvironment" cannot be the only path the interneurons follow since some of them migrate in the lower intermediate and subventricular zones (which are poor in these axons). Thus, GABAergic interneurons are subjected to additional attractive/permissive factors present in the cortex (Wichterle et al., 2001, 2003; Marin et al., 2003; Tanaka et al., 2003; see also below).

An impressive multiplicity of cues have been shown to influence different steps of migration in/from the subpallium. These include diffusible molecules (slit/robo, class 3 semaphorins, HGF/SF, NT4/5) and extracellular substrate-related proteins (TAG-1, neuregulin-1) (Zhu et al., 1999; Marin et al., 2001; Hamasaki et al., 2001; Powell et al., 2001, 2003; Denaxa et al., 2001; Polleux et al., 2002; Tamamaki et al., 2003; Flames et al., 2004). However, the phenotypes of mice deficient for guidance factors point to the fact that a lot of them are dispensable in vivo, implicating the presence of in vivo compensatory mechanisms. Semaphorin/neuropilin signaling is necessary for sorting striatal and cortical interneurons (Marin et al., 2001), while neuregulin-1/ErbB4 has been recently shown to have an in vivo role as a permissive substrate as well as a chemoattractant in cortical interneuron migration (Flames et al., 2004). On the other hand, analysis of double deficient mice for Slit1 and Slit2 or mice simultaneously lacking Slit1, Slit2 and netrin1 showed that these factors are not required for controlling the tangential migration of interneurons to the cortex (Marin et al., 2003), as has been previously suggested by ex vivo or in vitro experiments (Zhu et al., 1999).

The apparent difference in the results from ex vivo assays and the analysis of mutant mice that is often observed may be related to the fact in the former type of assay, one is examining the abrupt blocking of the function of a particular molecule in a short developmental time window. In analyzing phenotypes of mutant mice instead, one is looking at the effect of the absence of a particular protein throughout development; in the latter case, the organism may adjust by modulating the activity of compensatory molecules. In addition, in ex vivo assays, the putative influence of efferent and afferent fibers as well as other cues, for instance, intrinsic cortical connections, is missing, thus making the function of a single molecule limiting, therefore, detectable.

Most of neural CAMs are expressed with a very dynamic pattern during development in a number of neuronal subpopulations. The employment of in vitro assays pointed to roles in adhesion, neurite outgrowth regulation, fasciculation and plasticity in many neuronal populations. However, the range of phenotypes of mice deficient in these proteins varies and is often evident in one type of neuronal population at one particular time point during development or in the adult

(Cremer et al., 1994; Dahme et al., 1997; Chazal et al., 2000; Moré et al., 2001; Weiner et al., 2004). The avian homolog of TAG-1, axonin-1 has been implicated in the guidance of commissural spinal axons towards the floor plate along with NrCAM (Stoeckli and Landmesser, 1995; Stoeckli et al., 1997; Fitzli et al., 2000). The phenotype of TAG-1 mutant mice, however (Yeomans et al., FENS Forum, 2000, abstract 125.23) reported no abnormalities in the pathfinding of spinal commissural axons.

In summary, we show here that, during development, TAG-1 is necessary in vivo for the survival of precerebellar neurons during their migration in the medulla while being dispensable for interneuron migration to the cortex. Its absence results in fewer migrating neurons in the medulla generating smaller LRN nuclei in the adult. Therefore, our previous in vitro results are in agreement with the present in vivo observations indicating that the function of TAG-1 is required in some tangential migrations.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2005.09.021.

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