

A combination of chain and neurophilic migration involving the adhesion molecule TAG-1 in the caudal medulla

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DOI: [10.1242/dev.129.2.287](https://doi.org/10.1242/dev.129.2.287)

SUMMARY

Neuronal populations destined to form several precerebellar nuclei are generated by the rhombic lip in the caudal hindbrain. These immature neurons gather into the olivary and the superficial migratory streams and migrate tangentially around the hindbrain to reach their final position. We focus on the cells of the superficial stream that migrate ventrally, cross the midline and form the lateral reticular (LRN) and external cuneate (ECN) nuclei. The cells of the superficial stream are preceded by long leading processes; in the dorsal neural tube, they migrate in close apposition to each other and form distinct chains, whereas they disperse and follow Tuj-1 immunoreactive axons on reaching the ventral hindbrain. This suggests that, in the superficial stream, neuronal migration combines both homotypic and heterotypic mechanisms. We also show that the adhesion molecule TAG-1 is expressed by the migrating cells. Blocking TAG-1 function results in alterations in the superficial migration, indicating that TAG-1 is involved in the superficial migration. Other members of the immunoglobulin superfamily and known ligands of TAG-1 are also expressed in the region of the migration but are not involved in the migration. These findings provide evidence that the TAG-1 protein is involved as a contact-dependent signal guiding not only axonal outgrowth but also cell migration.

Key words: Tangential migration, Precerebellar nuclei, LRN, ECN, TAG-1, IgSF, Medulla, Mouse

INTRODUCTION

In the developing nervous system neurons need to migrate, often long distances, to settle into their final positions and form synaptic contacts. The migration of neurons takes place in two directions. Radial migration occurs on the processes of glial cells and has been studied extensively in the cortex and the cerebellum (Rakic, 1985). Tangential migration occurs perpendicular to the glial scaffolding; it is called 'neurophilic' when the cells migrate in close apposition to axonal fascicles and 'chain migration' when the cells migrate in close contact to each other without using a glial or neuronal scaffold (Ono and Kawamura, 1990; Rakic, 1990; Yee et al., 1999). Tangentially migrating neurons are found in the cortex, the cerebellum and in the rhombencephalon (Komuro et al., 2001; Ono and Kawamura, 1989; Ryder and Cepko, 1994; Tamamaki et al., 1997). Some of the cues guiding tangential migration have started being revealed and the picture that is emerging is that the same families of signals involved in the directional guidance of developing growth cones may also guide the translocation of cell bodies (Alcantara et al., 2000; Denaxa et al., 2001; Yee et al., 1999). In the rhombencephalon, tangential migrations underlie the formation of precerebellar nuclei. The neurons that form these nuclei are generated at the level of the rhombic lip in the dorsal neuroepithelium of the caudal hindbrain, and then follow various paths to reach their final positions (Altman and Bayer, 1987a; Altman and Bayer, 1987b; Altman and Bayer, 1987c). The neurons of the external cuneate, lateral reticular, pontine and reticulotegmental nucleus project towards the cerebellum and supply mossy fibers to granule cells. The axons of the inferior olive cells project to the Purkinje cells of the cerebellum, forming the climbing fibers (Rodriguez and Dymecki, 2000; Wang and Zoghbi, 2001; Wingate, 2001). Recent studies indicate that the precursors of the precerebellar mossy fiber neurons appear to derive from a separate progenitor population from the progenitors of the climbing fiber neurons (Rodriguez and Dymecki, 2000).

In the present study, we have focused on the population of precerebellar neurons which are generated in the rhombic lip of rhombomeres 7/8 and are destined to form the lateral reticular nucleus (LRN) and the external cuneate nucleus (ECN). These neurons circumnavigate the medulla oblongata just under the pial surface in a direction perpendicular to the anteroposterior axis (Altman and Bayer, 1987b; Bourrat and Sotelo, 1990; Tan and Le Dourin, 1991). These cells cross the ventral midline and continue dorsally forming the nuclei on the contralateral side. The migration may occur on axon fascicles, suggesting that these tangential fibers serve as guidance substrates for the relocation of neuronal cell bodies (Ono and Kawamura, 1989). We characterize the superficial migration of LRN and ECN neurons in vivo and in vitro. We provide evidence that the neuronal adhesion molecule TAG-1 (Cntn2 – Mouse Genome Informatics) plays a guiding role. TAG-1 is a member of the immunoglobulin superfamily (IgSF) (Furley et al., 1990) and interacts with several

other proteins of the same family (Buchstaller et al., 1996; Buttiglione et al., 1998; Felsenfeld et al., 1994; Fitzli et al., 2000; Grumet, 1997; Lustig et al., 1999; Ono et al., 1994; Rathjen and Schachner, 1984). TAG-1 is transiently expressed by migrating neurons in different regions of the developing nervous system as in granule cells of the cerebellum (Bailly et al., 1996; Stottmann and Rivas, 1998; Yamamoto et al., 1986), and pontine and facial branchiomotor neurons of the hindbrain (Garel et al., 2000; Wolfer et al., 1994; Yee et al., 1999). It is also expressed by migrating neurons in the system under investigation. Blocking the function of the protein in vitro perturbs the migration of precerebellar neurons. The mechanism by which TAG-1 mediates this migration appears not to involve any of its known IgSF interacting partners. Rather, our experiments suggest that homophilic recognition of TAG-1 on the migrating cells may be the mechanism responsible for the superficial migration.

MATERIALS AND METHODS

Animals

CBAxC57Bl/10 mice were used and the day of vaginal plug detection was considered as embryonic day 0.5 (E0.5). Pregnant females were sacrificed with an overdose of ether or chloroform. Embryos were recovered in chilled phosphate-buffered saline (PBS) and were either dissected for explantation or fixed. L1 deficient mice: female hemizygote were crossed with wild-type C57Bl/10 males. Pregnant females resulting from these crosses were sacrificed at E12.5 and each embryo was sex-genotyped and also genotyped by PCR for the normal and the L1-deficient allele (Dahme et al., 1997). In our experiments, we used males that carried the mutant allele of the X-linked L1 gene and wild-type animals.

Immunocytochemistry

Dissected hindbrains or explants were fixed overnight in 4% paraformaldehyde at 4°C. Immunocytochemistry was performed on vibratome sections (100 µm thick) of tissue embedded in agarose 4% in PBS or on cryostat sections (16 µm thick) of sucrose cryoprotected hindbrains or on whole explants. The sections or explants were processed as described previously (Denaxa et al., 2001). Primary antibodies were used as follows: monoclonal antibody (mAb) against TAG-1 (4D7, 1:1000) (Dodd et al., 1988), mAb against class III β - tubulin (Tuj-1, 1:1000, a gift from A. Frankfurter), mAb against PSA- NCAM (5A5, 1:1000) (Dodd et al., 1988), rabbit polyclonal antibody against L1 (1:1000) (Rathjen and Schachner, 1984), rabbit antisera to bacterially expressed F3 (Gennarini et al., 1991). In addition, antibodies against NrCAM were generated in rabbits according to Davis et al. (Davis et al., 1996). These antisera did not reveal any staining by immunohistochemistry in our system, while they detect NrCAM in other rat or mouse neural tissues. Secondary antibodies were

used as follows: biotinylated anti-mouse IgM (1:250, Jackson), FITC- conjugated anti mouse-IgM (1:100, Jackson), FITC-conjugated anti- rabbit (1:250, Amersham) and Cy3-conjugated anti-mouse IgG (1:1000, Jackson). Some sections were processed for histological staining with Cresyl Violet.

In situ hybridization

In situ hybridization experiments were performed on whole-mount explants or on vibratome sections (200 μ m thick) according to the protocol described by Bally-Cuif and Wassef (Bally-Cuif and Wassef, 1994). The TAG-1- and F3-specific probes were produced as described by Denaxa et al. (Denaxa et al., 2001) and Virgintino et al. (Virgintino et al., 1999), respectively. The NrCAM full-length cDNA was used as a template for the antisense and sense probes (Davis et al., 1996). In all cases, no specific signal was observed with the sense probes.

Preparation of the explants

Explants of the neural tube containing the cerebellum and medulla oblongata were dissected from E12.5 brains and freed from meninges in PBS-0.6% glucose or in L15 (Gibco, BRL). They were opened on the dorsal midline in an 'open book' configuration and were cultured on Biopore membranes (Millipore) floating on DMEM/F12 (Gibco) supplemented with 0.6% glucose, 0.02 mM glutamine, 0.0075% sodium bicarbonate, 5 mM Hepes, penicillin (5 U/ml)/streptomycin (5 mg/ml), fetal calf serum (5%) and normal horse serum (5%). Ablation of the rhombic lip was performed on E12.5 hindbrain explants at the beginning of the culture period.

Dil tracing

A small crystal of 1,1'-dioctodecyl-3,3,3',3'-tetramethyl- indocarbocyanine (Dil) was placed at the rhombic lip of E12.5 hindbrain explants. Some of the explants were fixed at E12.5 and other cultured for the appropriate time.

5-bromo-2-deoxy-uridine labeling and immunocytochemistry

Some E12.5 explants were incubated for 30 minutes in culture medium containing BrdU (10 mM) at 37°C, then washed, fixed in 4% paraformaldehyde and processed for in situ hybridization for TAG-1 and BrdU immunocytochemistry.

For the detection of incorporated BrdU, transverse cryostat sections of explants were incubated in 2N HCL for 45 minutes, rinsed in PBS and digested for 5 minutes with 10 mg/ml proteinase K. After rinses with PBS, the sections were postfixed in 4% paraformaldehyde/0.2% glutaraldehyde and incubated with anti BrdU (1/300, Becton Dickinson) in PBS, 0.2% gelatin, 0.5% Triton X-100 at 4°C overnight. After extensive washing with PBS, the sections were incubated for 2 hours at room temperature with biotinylated anti-mouse IgG (1:250, Jackson) and then treated as described above.

Function blocking experiments

Polyclonal antibodies against TAG-1 and Fab fragments were used as described previously in function blocking experiments (Denaxa et al., 2001). Antigen-depleted antibody solutions were produced as follows: CHO cells stably transfected to express TAG-1 on their surface (CHO-TAG-1 cells) (Buttiglione et al., 1998) were grown to confluency in 35 mm petri dishes. Culture medium containing 200-400 $\mu\text{m}/\text{ml}$ antibody against TAG-1 was incubated for 30 minutes with CHO-TAG-1 cells. This operation was repeated 12 times changing to a new 35 mm dish each time. The complete absorption of TAG-1 antibodies from the culture medium was checked by immunocytochemistry on cryostat sections of E13.5 mouse hindbrain. As a control condition, we used non-depleted medium incubated with CHO untransfected cells. In addition, control polyclonal antibodies, Fab fragments from polyclonal antibodies against L1, soluble TAG-1-Fc protein and control MUC18-Fc were used as before (Denaxa et al., 2001; Rathjen and Schachner, 1984; Buttiglione et al., 1998; Lehmann et al., 1989).

For the cleavage of TAG-1 from the surface of the cells we used phosphatidylinositol-specific phospholipase C (PI-PLC, GLYKO) at concentration 100 mU/ml in the culture medium (Masuda et al., 2000). To remove the polysialic acid of NCAM we incubated the explants with 1 $\mu\text{l}/\text{ml}$ of EndoN enzyme according to Hu et al. (Hu et al., 1996). This amount is in a sufficient excess to remove all immunoreactive PSA from the brain for over a week (Ono et al., 1994). The explants dissected at E12.5 were incubated in medium with blocking or control reagents for 2-4 days. Unless otherwise specified, all the reagents were added in the culture medium at the beginning of the culture but PI-PLC was added every 12 hours during the culture period.

Microscopy

Immunofluorescent explants were viewed and images were generated in a Leica TCS-NT Laser Scanning microscope using the 10x, 20x, 40x and 63x objectives. All other samples were viewed in a Zeiss Axioskop microscope and photographs were taken with a 35 mm camera using Fujichrome Sensia I 100ASA film.

RESULTS

TAG-1 is a marker of the superficial migration at the caudal hindbrain

In a screen to identify markers of the superficial migration in the caudal hindbrain, we observed that the adhesion molecule TAG-1 (Wolfer et al., 1994) was expressed in neurons of the superficial migratory stream.

At embryonic day 12.5 (E12.5) on transverse sections of caudal medulla at the level of the

rhombomere 7 and 8 stained with Cresyl Violet (Fig. 1A) there were no superficially migrating cells. At that stage, TAG-1 transcripts were detected at the rhombic lip (asterisks in Fig. 1B) where the cells of the superficial stream are generated. TAG-1 was also expressed in the subventricular zone (s in Fig. 1B) and hypoglossal nucleus (h in Fig. 1B). TAG-1 protein was also widely expressed on superficially located fibers at this level (arrows in Fig. 1C). One day later, at E13.5, cell bodies were detected in the superficial region of the medulla and beneath the floor plate on sections stained with Cresyl Violet (arrowheads in Fig. 1D). This superficial row of cells expressed TAG-1 mRNA (Fig. 1E) and protein (Fig. 1F) and it corresponded to the superficial migratory stream. At E14.5 these cells were still migrating and expressed TAG-1. However, at E15.5, when the migration has been completed (Ono and Kawamura, 1989) and the cells had formed the LRN and ECN, no TAG-1 was detected (data not shown) (Wolfer et al., 1994). TAG-1 was still expressed only by the hypoglossal nucleus. Thus, *in vivo*, TAG-1 is expressed transiently by the cells of the superficial migratory stream at the caudal medulla and is downregulated when the cells are settled in their final positions.

The superficial migration in hindbrain explants *in vitro*

To investigate the mechanisms involved in the superficial migration at the caudal hindbrain we examined whether this migration can resume *in vitro*. We prepared explants containing this region of the medulla by dissecting the hindbrain of embryos at E12.5, cutting at the dorsal midline and placing the explants in an 'open book' configuration with the ventricular side down (Fig. 1G). The explants were cultured *in vitro* for 1 to 5 days (1DIV-5DIV).

It is known that in certain structures of the developing nervous system TAG-1 is expressed by postmitotic cells (Furley et al., 1990). It is also known that the migrating cells of the superficial stream are postmitotic (Altman, 1987b). To investigate whether TAG-1 expression starts after the last mitosis, explants dissected at E12.5 were incubated with BrdU for 30 minutes and then fixed. After TAG-1 *in situ* hybridization and BrdU immunocytochemistry on transverse section of these explants we observed that TAG-1 and BrdU staining were distinct (Fig. 1H). The mitotic cells at this developmental stage were detected at the most lateral side of the rhombic lip (on the left of Fig. 1H) and at the ventricular zone, but TAG-1 was expressed by postmitotic cells that have just started to migrate ventrally (arrow in Fig. 1H).

On explants dissected at E12.5, the cells of the superficial stream were generated at the rhombic lip and migrated towards the floor plate as indicated by the arrows in Fig. 1I. TAG-1 mRNA was detected in whole-mount *in situ* hybridization experiments on explants at E12.5, at the rhombic lip (asterisk at Fig. 1J). TAG-1 was also expressed by the facial branchiomotor neurons (fbm in Fig. 1J) and subventricular zone (Fig. 1J,K). After 1DIV, TAG-1 labeled migrating cells emerging from the rhombic lip on both sides of the explant (between the broken lines in Fig. 1K) and after 2DIV,

the migrating cells have reached and crossed the midline (Fig. 1L). Two different patterns of migration were observed. At the beginning of the migration, when the cells were still located more dorsally (at the lateral parts of the explants), the TAG-1-positive cells formed characteristic stripes as they migrated. By contrast, when the cells reached the ventral part of medulla, near and beneath the floor plate (in the middle of the explant), the TAG-1-positive cells were more individually arranged. In vitro, the migrating cells do not downregulate TAG-1, even if they remain in culture for 5 days (contrary to in vivo conditions, where the migrating cells stop expressing TAG-1 at E15.5 and form the LRN and ECN). It is well established that the expression of many guidance molecules that control the crossing of commissural axons across the midline is downregulated or upregulated upon crossing of the midline (Shirasaki et al., 1998; Stein and Tessier-Lavigne, 2001). Spinal commissural axons express TAG-1 protein on their surface as they project towards the floor plate, but after they crossed the midline TAG-1 is downregulated and another adhesion molecule, L1 is upregulated and expressed by the commissural axons (Dodd et al., 1988).

We set out to investigate whether TAG-1 is still expressed by migrating cells of LRN and ECN after they cross the midline. The symmetric streams of migrating TAG-1 positive neurons from each side of the rhombic lip meet beneath the floor plate at around day E13.5 and then cross the midline, intermingling with each other. In order to be able to follow the expression of TAG-1 in neurons originating from only one side of the rhombencephalon, we performed unilateral ablations of the rhombic lip on explants at E12.5. As illustrated in Fig. 1M, the rhombic lip from one side of the explant was ablated at the beginning of the culture, before the TAG-1 positive cells started their migration. After 1DIV, TAG-1-positive cells emerged only from the intact rhombic lip, suggesting that all the mitotic progenitors have been removed by the ablation. After 2DIV, TAG-1-positive cells were detected mostly on the non-ablated side but a few were also found on the ablated side. These cells had crossed the midline and still expressed TAG-1 mRNA (Fig. 1N) To exclude the possibility that cells of the superficial migration express TAG-1 mRNA but not the protein after crossing the midline, we performed immunocytochemistry in toto on ablated explants after 2DIV. At the normal side of the explant many TAG-1-positive cells, arranged in close contact with each other, were observed (Fig. 1O). At the ablated side, TAG-1-positive cells were also detected (asterisks in Fig. 1P), suggesting that TAG-1 protein was still expressed on the surface of the migrating cells after they have crossed the midline. Thus, the cells of the superficial migratory stream of the caudal hindbrain migrate in vitro while expressing TAG-1 protein even after crossing the midline.

A combination of chain (homotypic) and neurophilic (heterotypic) migration occurs at the posterior superficial migratory stream

It has been reported that the cells of the basilar pontine nucleus, which also express TAG-1, extend a long leading process and migrate circumferentially beneath the pial surface in close contact with each other, without following any neuronal scaffold (Yee et al., 1999). To investigate the morphology of the cells of the posterior superficial migratory stream, we performed Dil injections in the rhombic lip of explants. At E12.5, when the cell bodies are still at the rhombic lip, Dil labeled anterogradely long leading processes of these cells at the most superficial level (Fig. 2A). At deeper levels of the same explants, many labeled axons crossed the midline, reached the contralateral rhombic lip and turned perpendicular towards the anterior part of the hindbrain (Fig. 2B). After 1DIV Dil-labeled cells emerged from the rhombic lip and were directed towards the midline. The migrating cells had a characteristic unipolar morphology, while a long single leading process extended towards the direction of the movement (Fig. 2C). These leading processes could be many hundreds micrometers in length (Fig. 2D). After 2DIV, the migrating cells reached the contralateral part of the explant and they still had the same unipolar shape with long leading processes (Fig. 2E). Thus, the cells of the LRN and ECN extend a long leading process at the superficial level of the caudal medulla, and they follow that towards their final position.

It has been reported that the cells of the superficial stream of the caudal hindbrain migrate on tangentially oriented neuronal fibers (Ono and Kawamura, 1989). We investigated whether TAG-1 was expressed not only by migrating cells but also by these axons. At E12.5 explants, TAG-1 was expressed by tangentially oriented fibers (Fig. 3A) in a direction similar to that of the leading processes. To investigate whether these fibers are axons or long leading processes of cells still located at the rhombic lip, we performed immunocytochemistry for the axonal marker class III β -tubulin (Tuj-1). As illustrated in Fig. 3B, the tangential TAG-1-positive fibers did not express Tuj-1, suggesting that these fibers could be the leading processes. In addition, these fibers are located more superficially as indicated by the overlay in Fig. 3C.

After 1DIV, many TAG-1-positive cells started to emerge from the rhombic lip and migrate in close contact to each other (Fig. 3D). No Tuj-1 staining was detected at the migratory route of these cells (Fig. 3E) and TAG-1-positive cells migrated superficially to the Tuj-1 signal located at the lateral part of the explant (Fig. 3F). After 2DIV, there is a difference in the way of migration at the lateral and medial part of the explants. At the lateral part, the cells migrate without any contact with Tuj-1 positive fibers (Fig. 3G-I), but at the medial part, closer to the midline, they migrate in close contact with axonal fibers, which did not express TAG-1 (Fig. 3J-L).

These results indicate that the cells of the superficial migratory stream follow two different

patterns of migration. Laterally, there is a homotypic interaction between the TAG-1-expressing migrating cells and medially there is a heterophilic interaction between the migrating cells and the pre-existing neuronal axons.

Effects of blocking TAG-1 function on the migrating cells of the superficial stream

TAG-1 is implicated in axon guidance and cell migration in the developing central nervous system (Denaxa et al., 2001; Stoeckli and Landmesser, 1995; Stoeckli et al., 1997). In order to investigate whether TAG-1 is involved in the migration of the cells of the superficial stream, we blocked its function *in vitro*. TAG-1, a GPI-linked molecule, is removed from the surface of cells and axons by cleavage by the enzyme PI-PLC (Karagogeos et al., 1991). We treated explants with PI-PLC and after 2DIV we compared them with non-treated explants. While TAG-1 protein has been removed by PI-PLC (data not shown), TAG-1 mRNA was still present in migrating cells as detected by *in situ* hybridization (Fig. 4A). As shown in Fig. 4A, the superficial migration was perturbed as fewer cells were detected and most of them had stopped in the middle of their way (compare Fig. 4A,B). Additionally, the cells in untreated explants formed rows as they migrated, while in PI-PLC treated explants, cells were more individually arranged. This result indicates that TAG-1 protein and/or other GPI-linked molecules are important for the normal migration of these cells at the caudal hindbrain.

In order to block the function of TAG-1 with more specific reagents, we added polyclonal or Fab fragments of antibodies against TAG-1 in the culture medium of the explants. After 2DIV, we observed that fewer cells of the superficial stream migrated in the presence of Fab fragments (Fig. 4C). On the contrary, in the presence of Fab fragments of control antibodies, the migration of TAG-1-positive cells occurred normally (Fig. 4D).

To confirm the specificity of our blocking reagents and effect, we immunodepleted the polyclonal antiserum to TAG-1 by absorbing it against CHO cells expressing TAG-1 on their surface and control CHO cells (see also Materials and Methods). When we included the immunodepleted medium in explants, the migration occurred normally (Fig. 4F); when the explants were incubated with the medium absorbed against control CHO cells, the migration was affected in the same way as in the two conditions described above (Fig. 4E). From this result we conclude that the blocking effects observed were due to the presence of the polyclonal antibodies against TAG-1 in the culture medium.

Finally, we incubated explants with soluble TAG-1 protein (TAG-1-Fc) (Buttiglione et al., 1998; Denaxa et al., 2001; Stoeckli and Landmesser, 1995; Stoeckli et al., 1997). TAG-1 molecules can bind to each other homophilically (Felsenfeld et al., 1994; Tsiotra et al., 1996), therefore an excess of soluble TAG-1 in the culture medium would compete away the binding of TAG-1 expressed on

the surface of migrating cells with its endogenous homophilic and heterophilic binding partners. The excess of soluble TAG-1 protein in the medium can also interact with TAG-1 receptor on the explant. After 2DIV, we observed alterations in the migration of the TAG-1 positive cells, since the migrating cells were fewer and they did not form stripes as they migrated (Fig. 4G). In control experiments where we used the MUC18-Fc protein, a related immunoglobulin superfamily member, the migration was normal (Fig. 4H).

To investigate the possibility that our blocking reagents cause the death of the TAG-1-positive cells, we prepared explants at E12.5, incubated them with control medium for 2 days and then added the blocking reagents and incubated them for another 2 days. We observed that TAG-1-positive cells migrated normally during the first 2 days of culture and the addition of TAG-1 polyclonal antibody (Fig. 4I) or PI-PLC (Fig. 4J) for the next 2 days did not affect the survival of these cells. Thus, we can conclude that the blocking reagents did not cause the death of TAG-1-positive cells when they were added for the same time period as in blocking experiments. We conclude that we observe fewer TAG-1-positive cells in the blocking experiments because these cells did not migrate properly and either consequently died or remained dorsally in the subventricular zone.

Other adhesion molecules and known ligands of TAG-1 are not implicated at the superficial migration at the caudal hindbrain

In order to identify the binding partner(s) of TAG-1 that contribute to the tangential migration of LRN and ECN neurons, we examined the expression pattern of known TAG-1 partners in the region of the medulla. These proteins are also members of the IgSF and are implicated in neurite outgrowth and/or migration during the development of the nervous system. L1 is an adhesion molecule of the IgSF and one of the partners of TAG-1 (Buchstaller et al., 1996; Dhar Malhotra et al., 1998). By immunocytochemistry on transverse sections of E13.5 caudal hindbrain, L1 protein was detected on some axons at the level of the superficial migration (arrowheads at Fig. 5A). Blocking the function of L1 with Fab fragments of polyclonal antibodies against it (Rathjen and Schachner, 1984) did not cause any alterations in the migration of TAG-1-positive cells (Fig. 5B). In addition, we prepared explants from L1-deficient animals (Dahme et al., 1997) and found that the superficial migration was normal in both null and wild-type littermates (compare Fig. 5C with Fig. 5D). Thus, L1 does not seem to be implicated in this type of migration.

The polysialic form of NCAM (PSA-NCAM) is another molecule that is involved in tangential migrations observed in the olfactory system (Hu et al., 1996; Ono et al., 1994). At the level of superficial migration, PSA-NCAM was expressed by the migrating cells (Fig. 5E). However, when the polysialic acid moiety was removed by the enzyme endoN, the migration of TAG-1-positive cells

occurred normally in explants in vitro (Fig. 5F). All immunoreactive PSA moieties were removed, as shown by lack of signal in treated explants that were stained with monoclonal antibody 5A5 that recognizes PSA (data not shown) (Dodd et al., 1988). Thus, we conclude that the polysialic form of NCAM is not involved in the superficial migration at the caudal hindbrain.

Another IgSF member and partner of TAG-1 is F3 (Buttiglione et al., 1998; Gennarini et al., 1991). F3 was not expressed in the area of the superficial migration (data not shown), when investigated by in situ hybridization and immunocytochemistry, thus rendering the possibility of its involvement unlikely.

The last known partner of TAG-1 that we have tested was NrCAM (Lustig et al., 2001; Lustig et al., 1999). The antisera available did not reveal any staining in the superficial migratory stream, while in situ hybridization experiments did not reveal any signal for NrCAM in the region of the superficial migration at the appropriate stages of development (data not shown), suggesting that this protein is not involved in this process. In addition, sites of NrCAM expression in the developing mouse nervous system as described recently (Lustig et al., 2001) are not correlated with the superficial migration.

DISCUSSION

The developing precerebellar system provides an interesting model for the study of neuronal migration. Immature neurons that eventually will become neurons of the LRN and ECN are born dorsally, migrate ventrally, cross the midline and proceed dorsally to form these two precerebellar nuclei (Altman and Bayer, 1987b; Bourrat and Sotelo, 1990). In other words, they need to circumnavigate the entire length of the brainstem in order to finally settle in positions close to where they were born but on the contralateral side. Earlier morphological studies have suggested that axons can provide a substratum for nonradial neuronal migration in the subpial part of the medulla (Ono and Kawamura, 1989). However, the cellular and molecular components that either initiate or maintain the migration along the entire route remained unknown.

The evidence presented here indicates that the cells of the superficial migratory stream migrate in two different modes of migration. At the beginning they extend a long leading process and migrate in close contact to each other, while closer to the midline they follow pre-existing neuronal fibers as scaffold. The neural adhesion molecule TAG-1, expressed by the migrating cells, is involved in the migration of the neurons of the superficial stream. Blocking the function of TAG-1 either enzymatically or by antibodies or soluble protein, results in alterations of the migration pattern. Furthermore, our evidence suggests a homophilic type of interaction between TAG-1-

positive migrating cells.

Our migration assay in vitro, owing to its open book configuration, maintains the endogenous substrate signals present in the tissue at the time of the migration. Therefore, it allows us to investigate not only the function of long-range signaling molecules, but also molecules involved in contact-mediated guidance. This is in contrast to explant co-culture assays, where the explant containing the origin of cells is faced with a source of putative long-range guidance signals. There is a strong spatiotemporal coincidence of TAG-1 expression and the superficial migration. TAG-1 is expressed transiently by the postmitotic cells of the superficial migratory stream after their last mitosis and it is downregulated, when the cells are settled in their final positions. In the spinal cord, TAG-1 expression by commissural axons is downregulated at the level of the floor plate (Dodd et al., 1988). In our system, the neurons of the superficial stream do not downregulate their TAG-1 expression until they are close to their final position, deep in the parenchyma on the contralateral side of the ventral midline. The observation that some TAG-1-positive neurons migrate contralaterally after unilateral rhombic lip ablation indicates that it is not crossing the floor plate per se that leads to the downregulation of TAG-1 expression.

Fig. 6 presents a model of the superficial migration based on our evidence. At the beginning of their migration, at the most dorsal part of the medulla, cells extend a long leading process and migrate by following each other in a type of chain migration. This type of chain migration is also observed at the pontine migratory stream in the anterior hindbrain (Ono and Kawamura, 1990). Basilar pontine cells extend a long leading process while they migrate and they also express TAG-1 (Yee et al., 1999). Closer to the midline, the cells migrate in contact with neuronal fibers tangentially oriented in a type of neurophilic migration, as proposed also by Ono and Kawamura (Ono and Kawamura, 1989). These two different patterns of migration were also observed with in situ hybridization for TAG-1.

TAG-1 protein has been localized in many fiber systems as well as in migrating neurons in the developing CNS (Dodd et al., 1988; Wolfer et al., 1994; Yamamoto et al., 1986; Yee et al., 1999; Stottmann and Rivas, 1998). Based on its expression pattern on migrating neurons and, more specifically, in contacts between these neurons and the axons they migrate upon, it has been suggested that the protein may be involved in adhesive processes required for migration to take place (Denaxa et al., 2001; Yamamoto et al., 1990). Indeed, we have always observed migrating cells in the superficial stream to move in bundles, in contact to each other. After blocking the function of TAG-1, fewer cells migrate and do so individually. From this result we can conclude that TAG-1 is implicated in the adhesion of each cell to the other, especially at the beginning of their migration, when the cells migrate in close contact to each other. In addition, TAG-1 is expressed only by

postmitotic migrating neurons; therefore blocking its function is not likely to affect the proliferation of these cells before they start to express TAG-1.

The blocking experiments also suggest that there is a homophilic type of interaction between the migrating cells. The use of TAG-1-Fc would result in blocking TAG-1 on the cells via homophilic binding as well as blocking its receptor via heterophilic binding. If the receptor is TAG-1 itself, then blocking with antibodies alone would affect migration to the same extent, which was the case in our experiments. Other partners of TAG-1, such as L1, PSA-NCAM, F3 and NrCAM are most likely not involved in this type of migration. We therefore suggest that homophilic binding between TAG-1 molecules on the migrating neurons is required for the tangential migration in our system. However, we cannot exclude that other unknown binding partners may be involved in this type of migration, especially during the second phase of migration, when the cells follow axonal fibers which do not express TAG-1.

Recently, the TAG-1-deficient mouse phenotype has been published (Fukamauchi et al., 2001). The mice appear normal except for a significant elevation in adenosine A1 receptors in the hippocampus and enhanced seizure susceptibility to convulsant stimuli. However, no analysis of precerebellar neurons and/or their development is mentioned. In light of our in vitro data, it would be interesting to study the generation and migration of these neuronal populations in developing TAG-1- null embryos.

The immature neurons of the superficial migratory stream need to cross the floor plate and proceed dorsally. It has been suggested in the past that the floor plate and in particular, netrin 1 may be the source of guidance signals for precerebellar nuclei (Alcantara et al., 2000; Bloch-Gallego et al., 1999; Bourrat and Sotelo, 1990). Our data does not exclude the action of additional cues that may function at either distinct regions of the migrating pathway or as global cues for attraction to the midline (Shirasaki et al., 1998; Shirasaki et al., 1995). Moreover, we have evidence (de Diego et al., 2002) suggesting that the floor plate exerts an attractive effect on the migrating cells of the superficial stream. This effect is possibly mediated by netrin 1, which is expressed by the floor plate, and DCC, which is expressed by the migrating cells.

In conclusion, we propose, based on the evidence presented here, that the adhesion molecule TAG-1 is involved as a contact- dependent signal guiding the LRN/ECN immature neurons to move in the subpial environment of the medulla. We propose that the protein maintains cell to cell contacts acting in a homophilic manner. Taken together, our experiments provide another example of a molecule playing either a role in axon guidance or in migration depending on the specific neuronal population.

We thank Drs V. Bennett, C. Faivre-Sarrailh, A. Frankfurter, G. Genarrini, S. B. Morton, T. Jessell, F. Rathjen, G. Rougon, D. L. Simmons and I. Talianidis for generous supply of antisera and probes. We are grateful to Dr Melitta Schachner, University of Hamburg, for the L1-deficient mice. We also thank Myrto Denaxa and Maura Strigini for critical reading of the manuscript. This work was supported by the following grants: the Greek Ministry of Education (EPEAEK 1092), IMBB intramural funds, the Greek Secretariat for Research and Technology, the EU BIOTECHNOLOGY grant 980329 (to D. K.) and ERB BIO4CT 960146 (to M. W.) as well as the Greek-French collaborative program PLATON (to D. K. and M. W.).

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Figures

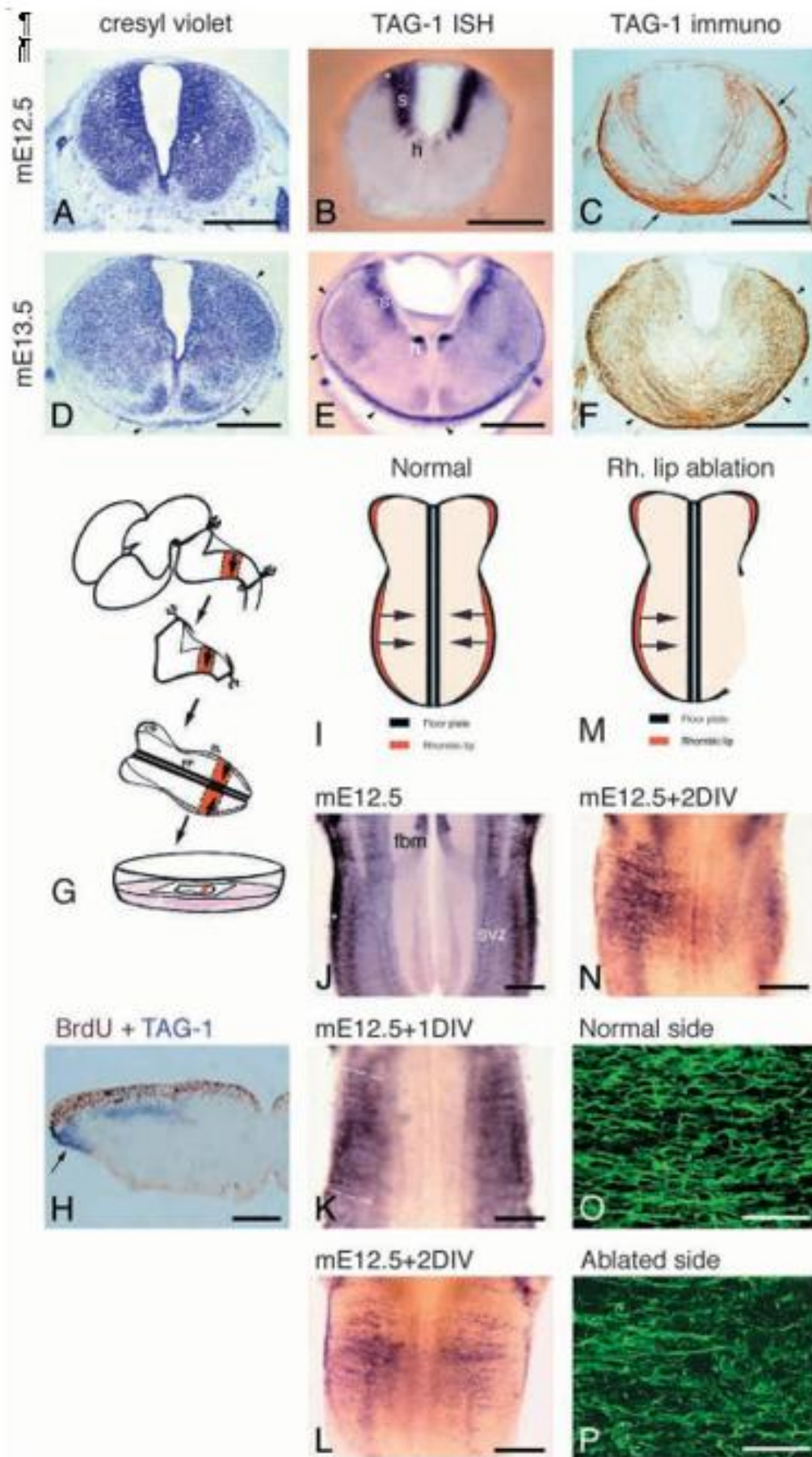


Figure 1. Characterization of the superficial migration in vivo and in vitro. Transverse sections of E12.5 (A- C) and E13.5 (D-F) mouse hindbrain at the level of the superficial migration. Cresyl Violet staining (A,D), in situ hybridization (B,E) and immunocytochemistry (C,F) for TAG-1. At E12.5 the cells of the superficial stream have not started their migration yet (A) but they have just been generated at the rhombic lip (asterisk in B). TAG-1 protein is widely expressed at the superficially located fibers (arrows in C). At E13.5, the migrating TAG-1-positive cells have reached and crossed the midline (arrowheads in D- F). (G) Schematic representation of the dissection procedure. The hindbrains of E12.5 embryos were dissected, cut at the dorsal midline and cultured as an 'open book' configuration on Biopore membranes for the appropriate time (days in vitro, DIV). The region of the migration is marked in red and the arrows indicate the direction of the migration on the explant. (H) Transverse section of an explant taken at E12.5 at the region of the superficial migration. BrdU and TAG-1 staining is distinct. At the region of the rhombic lip mitotic (BrdU positive) cells are detected at the most lateral side and TAG-1 transcripts are expressed only by those postmitotic cells that have started to migrate ventrally (arrow). BrdU-positive cells are also detected in the ventricular zone while TAG-1 signal is found in the subventricular zone. (I) Diagram of a normal explant on E12.5 at the beginning of culture period. Arrows indicate the direction of the migration within the explant. (J-L) Whole-mount in situ hybridization for TAG-1 on explants at E12.5 (J), E12.5 + 1DIV (K) and E12.5 + 2 DIV (L). At E12.5, the cells of the superficial stream have just been generated at the rhombic lip (asterisk in J). One day later they have started to migrate (between the broken lines in K) but have not reached the midline yet, while 2 days later they have crossed the midline (L). The TAG-1-positive cells form characteristic stripes as they migrate. (M-N) Diagram of an explant with a unilateral rhombic lip ablation (M) and whole-mount in situ hybridization for TAG-1 on ablated explant after 2 DIV (N). TAG-1-positive cells have crossed the midline and they still express TAG-1 transcripts. (O,P) Confocal images of whole-mount immunocytochemistry for TAG-1 on ablated explants. TAG-1 protein is expressed by the cells on the normal (left) side of the explant (O), but it is also expressed by the migrating cells after crossing the midline, at the ablated (right) side of the explant (P). Asterisks indicate the TAG-1-positive cell bodies on the ablated side (P). CB; cerebellum; fbm, facial branchiomotor neurons; FP, floor plate; h, hypoglossal nucleus; RL, rhombic lip; SVZ and s, subventricular zone. Scale bars: 500 μ m in A-F,J-N; 200 μ m in H; 50 μ m in O,P.

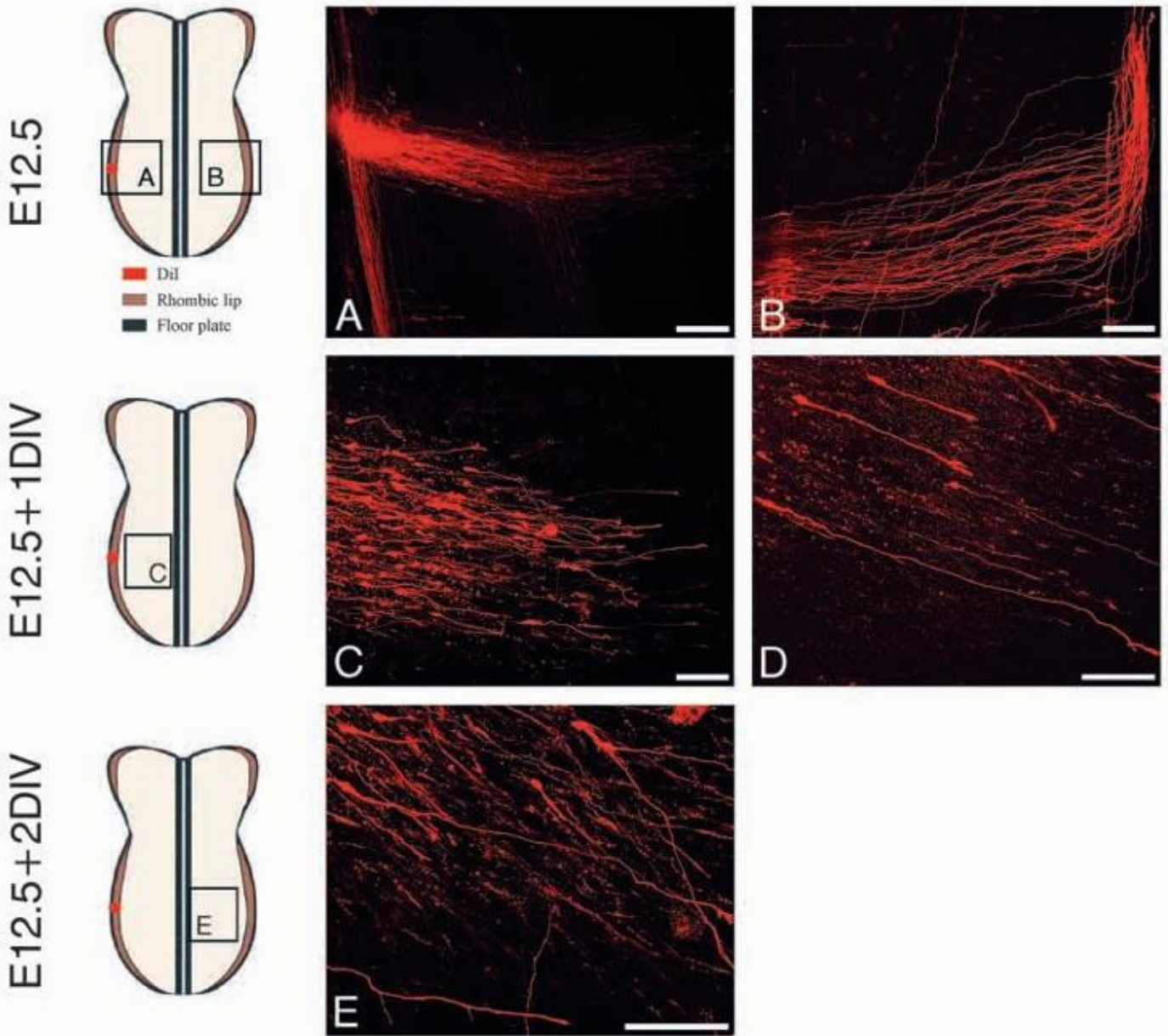


Figure. 2. Migrating cells extend long leading processes towards the direction of the migration. Confocal images of whole mount explants injected with Dil at the rhombic lip. The pictures on the right illustrate the regions boxed on the diagrams. (A,B) Explant injected at E12.5. Close to the pial surface long leading processes, labeled with Dil, extended towards the midline (A). At a deeper level, many labeled axons have crossed the midline and turned anteriorly (B). (C,D) Explant injected at E12.5 and fixed after 1DIV. Dil-labeled cells emerged from the rhombic lip. These migrating cells have a unipolar shape with a long leading process. (E) Explant injected at E12.5 and fixed after 2DIV. The migrating cells have already reached the side contralateral to the injection. Scale bars: 50 μm .

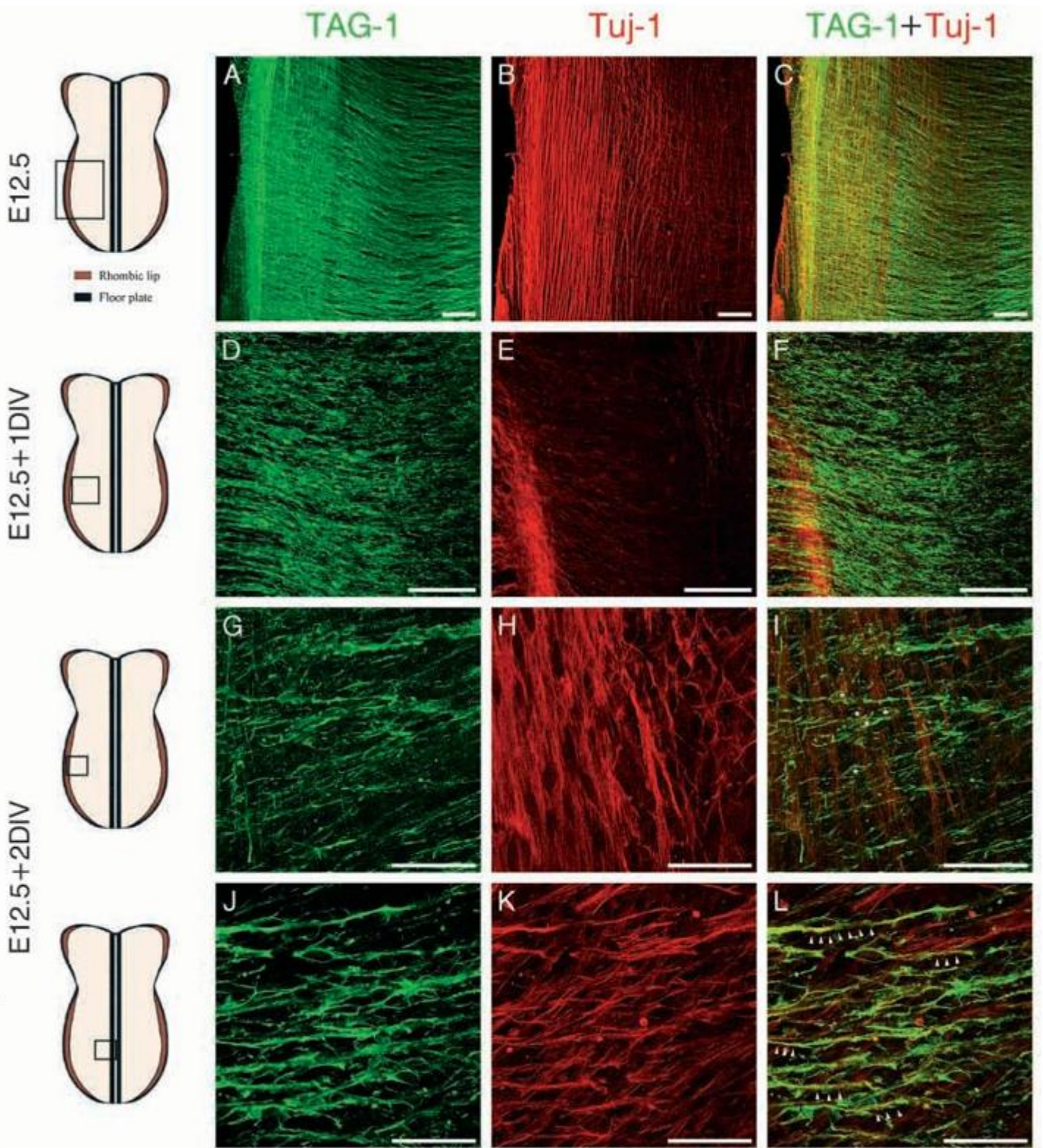


Figure 3. Two different patterns of migration followed by LRN/ECN neurons. Confocal images of whole-mount immunocytochemistry for TAG-1 and Tuj-1 on explants. The pictures on the right illustrate the regions boxed on the diagrams. (A-C) Explants fixed on E12.5. TAG-1 is expressed by superficial transverse fibers (A,C) that are not Tuj-1 positive (B,C). These fibers could coincide with the leading processes of neurons of the superficial migration that are still located at the rhombic lip. (D-F) Explants fixed on E12.5+1DIV. TAG-1 is expressed by the migrating cells emerged by the rhombic lip (D,F). These cells migrate in close contact to each other in an environment void of Tuj-1- positive neuronal fibers (E). The Tuj-1 staining is detected only at the lateral and deeper part of the explant (E,F). (G-L) Explants fixed on E12.5+2DIV. TAG-1-positive cells (G,I) migrating perpendicular to the Tuj-1 positive axonal fibers (H, asterisks in I) are still detected at the lateral part of the explant. Closer to the midline (J-L), TAG-1-positive cells migrate in close contact with neuronal fibers which are Tuj-1 but not TAG-1 positive (arrowheads in L). Scale bars: 50 μm in A-F; 20 μm in G-L.

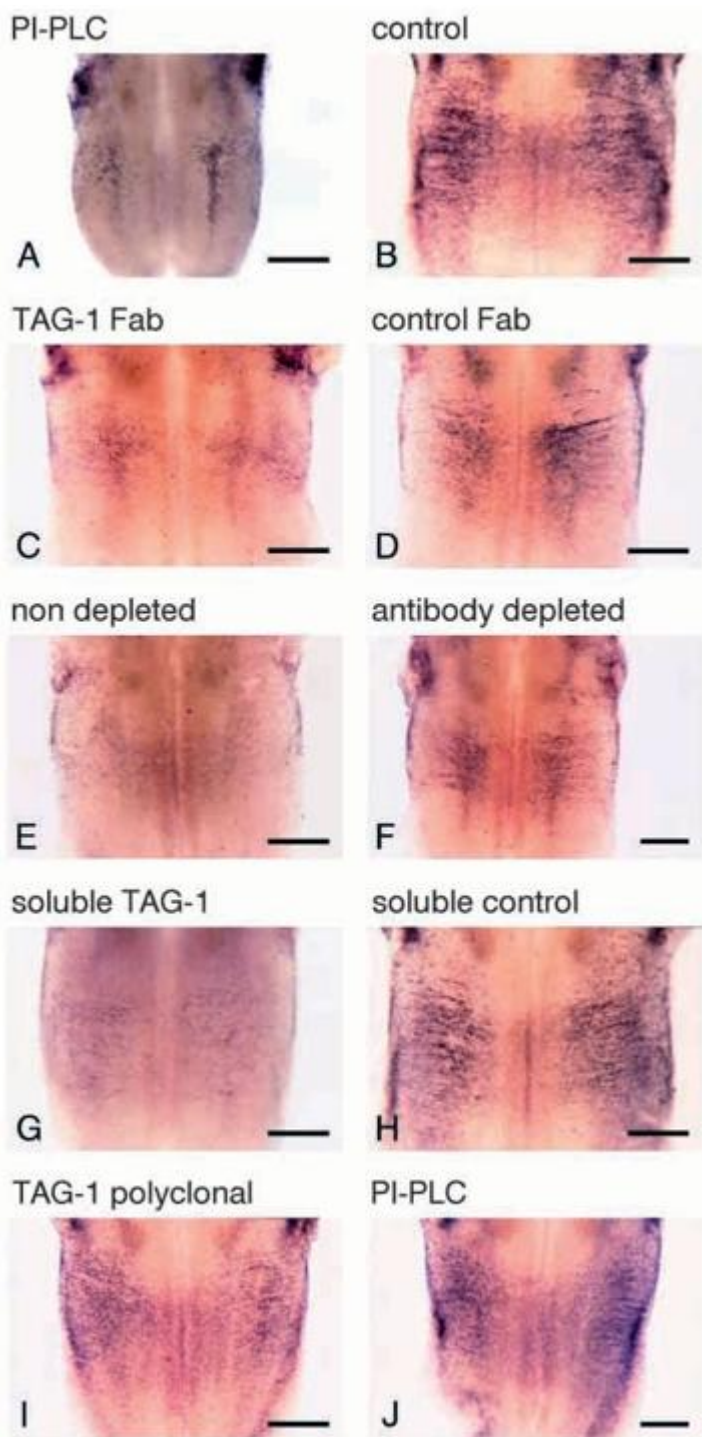
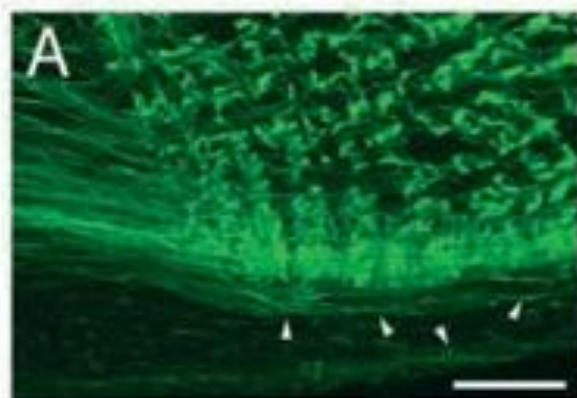
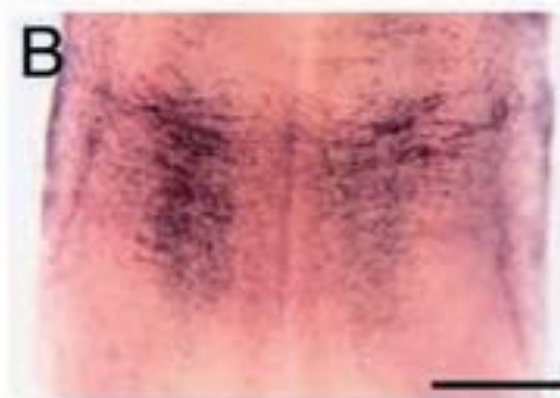


Figure 4. Blocking TAG-1 function in vitro alters the migratory behavior of LRN and ECN cells. Whole-mount in situ hybridization for TAG-1 on E12.5 explants after 2-4 days in culture. (A,C,E,G) Blocking the function of TAG-1 by cutting the GPI link with PI-PLC (A), blocking with Fab fragments of polyclonal antibodies against TAG-1 (C), or incubating with control non-depleted medium (E) or medium with soluble TAG-1-Fc (G). (B,D,F,H) Explants incubated in control medium (B), medium with control Fab fragments (D), in antibody-depleted medium (F) and in medium with control soluble protein MUC18-Fc (H). (I,J) Explants incubated in control medium for 2 days and then for 2 more days in medium with polyclonal antibody against TAG-1 (I) or with PI-PLC (J). The migration of TAG-1-positive cells occurred normally. Scale bars: 500 μ m.

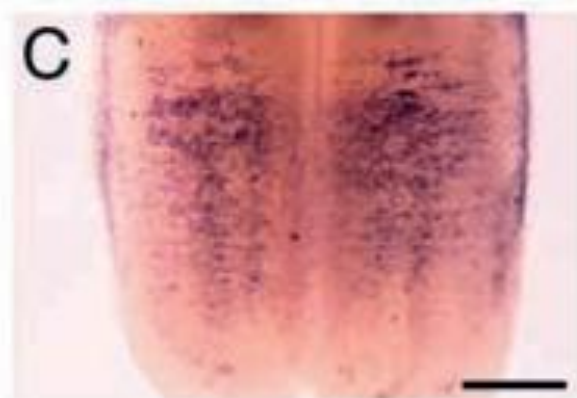
L1 immuno



L1 Fab TAG-1 ISH



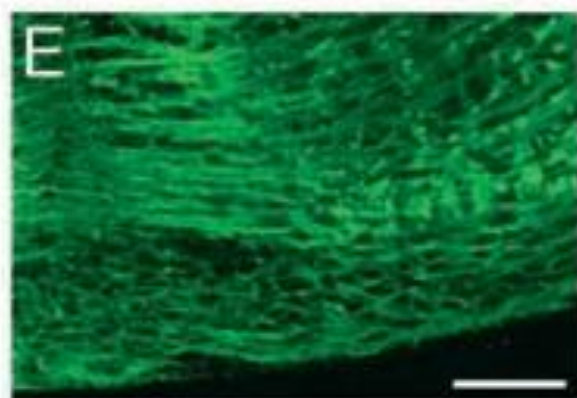
L1 -/- TAG-1 ISH



wt TAG-1 ISH



PSA-NCAM immuno



EndoN TAG-1 ISH

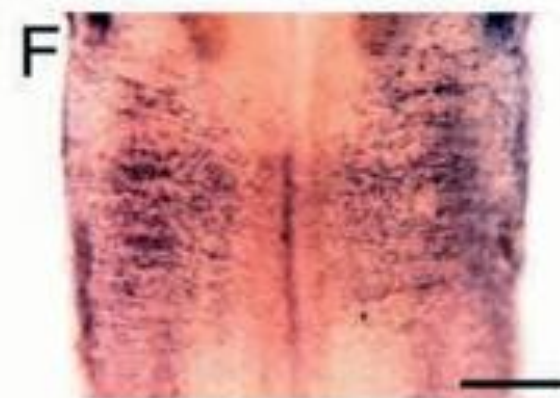


Figure 5. The role of L1 and PSA-NCAM in the superficial migration.

(A) Staining with antibody against L1. Only some axons at the region of the superficial migration are positive for L1 (arrowheads). (B) Whole-mount in situ hybridization for TAG-1 on explants incubated for 2 days in medium with Fab fragments of polyclonal antibody against L1. The trajectories of the migrating cells are normal. (C,D) Whole-mount in situ hybridization for TAG-1 on explants from L1-null (C) and wild-type (D) animals. The migration of TAG-1-positive cells is normal even in L1-deficient animals. (E) Staining with antibody against PSA-NCAM. PSA-NCAM was detected on the cell bodies of the migrating cells. (F) Whole mount in situ hybridization for TAG-1 on explants treated with EndoN during the culture. The TAG-1-positive cells migrated normally when PSA was cut. Scale bars: 50 μm in A,E; 500 μm in B-D,F.

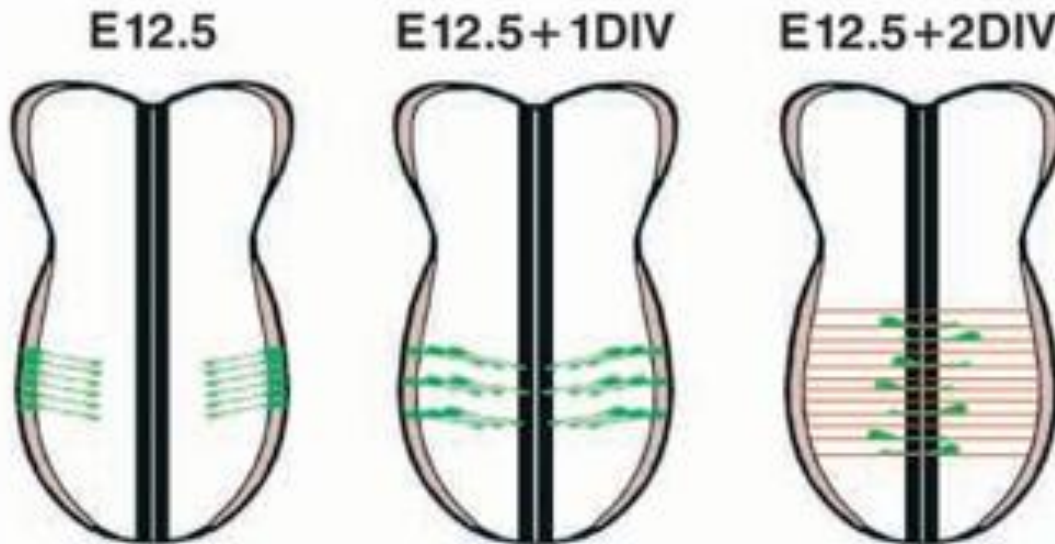


Fig. 6. Superficial migration. Laterally, the cells migrate in contact with each other, extending long leading processes. Medially, close to the floor plate (lines in black), the cells migrate by following pre-existing axonal fibers. Migrating cells and processes are shown in green, while the tangentially oriented axons are indicated in red.