

# Reelin provides an inhibitory signal in the migration of gonadotropin-releasing hormone neurons

Anna Cariboni<sup>1,2</sup>, Sonja Rakic<sup>1</sup>, Anastasia Liapi<sup>1</sup>, Roberto Maggi<sup>2</sup>, Andre Goffinet<sup>3</sup> and John G. Parnavelas<sup>1,\*</sup>

<sup>1</sup>Department of Anatomy and Developmental Biology, University College London, London WC1E 6BT, UK

<sup>2</sup>Department of Endocrinology, Centre of Excellence on Neurodegenerative Diseases, University of Milan, 20133 Milan, Italy

<sup>3</sup>Developmental Neurobiology Unit, University of Louvain Medical School, B1200 Brussels, Belgium

\*Author for correspondence (e-mail: j.parnavelas@ucl.ac.uk)

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## Summary

Gonadotropin-releasing hormone (GnRH) neurons, a small number of cells scattered in the hypothalamic region of the basal forebrain, play an important role in reproductive function. These cells originate in the olfactory placode and migrate into the basal forebrain in late embryonic life. Here, we show that reelin, which is expressed along the route of the migrating cells, has an inhibitory role in guiding GnRH neurons to the basal forebrain. Only a small (approximately 5%) subpopulation of these neurons expresses one of the reelin receptors (ApoER2/Lrp8), and all GnRH neurons appear to lack the intracellular adaptor protein Dab1, suggesting that the function of reelin is not mediated by the conventional signal transduction pathway. The importance of reelin in the establishment of GnRH

neurons in the hypothalamus was confirmed by our finding that the brains of developing and adult *reeler* mice of both sexes contained a markedly reduced number of these neuroendocrine neurons. Furthermore, the testes of adult males showed dilation of seminiferous tubules and reduction in their density when compared with controls. Mutants lacking the reelin receptors ApoER2 and Vldlr, and *scrambler* mice lacking Dab1, showed a normal complement of GnRH neurons in the hypothalamus, confirming that the effect of reelin in their migration is independent of Dab1.

Key words: GnRH neurons, Migration, Reelin

## Introduction

A small number of cells known as the gonadotropin-releasing hormone (GnRH) neurons are scattered in the basal forebrain from the medial septal/preoptic region to the posterior hypothalamus. They form the final pathway for the central regulation of fertility by projecting to the median eminence where they secrete the decapeptide GnRH into the pituitary portal vessels to induce the release of gonadotropins from the pituitary gland into the general circulation (Merchenthaler et al., 1984; Barry et al., 1985).

GnRH neurons originate in the nasal compartment at the level of the medial olfactory placode where they are identified by the production of GnRH mRNA or the peptide early in embryonic life (E10–11 in mouse; E12–13 in rat) (Schwanzel-Fukuda and Pfaff, 1989; Wray et al., 1989a; Wray et al., 1998b; Tobet et al., 2001). They migrate in association with olfactory/vomeronasal nerves (VNN) until they pass the cribriform plate. The fascicles of VNN split at this level, with the majority entering the main and accessory olfactory bulbs; the remaining axons, the so-called caudal VNN (cVNN), take a caudal and ventral turn and enter the basal forebrain (Schwartz et al., 2004). These pioneer axons can be labelled with the intermediate filament marker peripherin (Wray et al., 1994). Migrating GnRH neurons follow the cVNN into the basal forebrain where they detach from the guiding fibres and find their positions in the hypothalamic region by the time of

birth in rodents. Only a small number of these neurons proceeds dorsally and enter the cerebral cortex (Yoshida et al., 1995). In the human, failure of GnRH neurons to migrate normally results in reproductive dysfunction and delayed or absent pubertal maturation. Kallmann's syndrome (KS) is a genetic developmental disorder characterized by anosmia and hypogonadotropic hypogonadism (Seminara et al., 1998). In the X-linked form of the disease, the VNN and GnRH neurons fail to cross the cribriform plate and remain clustered in this area (Schwanzel-Fukuda et al., 1989).

What are the molecular mechanisms that guide GnRH neurons during their long and tortuous journey from the nasal compartment to the forebrain? A number of molecules have been shown to affect their migration (reviewed by Wray, 2001; MacColl et al., 2002; Pimpinelli and Maggi, 2004; Wierman et al., 2004). Amongst them are: adhesion molecules (NCAM and its polysialylated form PSA-NCAM, peripherin, TAG-1 and nasal embryonic LHRH factor), secreted molecules (GABA, netrin-1, HGF), the transcription factor Ebf2 (Corradi et al., 2003), and the gene product responsible for the X-linked KS, anosmin-1 (Cariboni et al., 2004). Most of these molecules affect the migration of GnRH neurons indirectly by altering the underlying migratory pathway and nearly all appear to act at the early stages of their migration within the nasal compartment. The cues that instruct GnRH neurons to avoid

the olfactory bulb and migrate caudally to the basal forebrain remain largely unknown.

Reelin is an extracellular protein extensively studied for its function in neuronal migration and lamination in the cerebral and cerebellar cortices (D'Arcangelo et al., 1995; Ogawa et al., 1995; D'Arcangelo and Curran, 1998). It has also been found to have important roles in a number of developmental events in the hippocampus (Del Rio et al., 1997; Niu et al., 2004; Zhao et al., 2004) and other areas of the CNS (Lambert de Rouvroit and Goffinet, 1998), and has been implicated in the migration of autonomic neurons in the spinal cord (Yip et al., 2000). *Reelin* is highly expressed in the olfactory system, including the olfactory bulb, vomeronasal organ and VNN (Ikeda and Terashima, 1997; Alcantara et al., 1998; Teillon et al., 2003), and investigators have recently queried its role in this system. Here, we tested the hypothesis that reelin acts as a guidance signal for the migration of GnRH neurons and their disposition in the forebrain. Using immortalized GnRH neurons as well as in vitro and in vivo experiments in rodents, we found that reelin does affect the migratory activity and distribution of GnRH-releasing neurons. Analysis of embryonic and postnatal *reeler* mouse brains of both sexes showed a reduction in GnRH neurons in the hypothalamus, providing an explanation for the observed reduced fertility in these animals (Caviness et al., 1972; Green, 1989). Finally, examination of animals mutants for *ApoER2/Vldlr* and *Dab1*, the components of reelin signalling, showed a normal complement of GnRH neurons, suggesting that this pathway is not involved in mediating the effects of reelin in their migration to the hypothalamus.

## Materials and methods

### Animals

All animal procedures were performed in accordance with institutional guidelines.

Brains of embryos, removed from pregnant Sprague Dawley albino rats at different stages during the last week of gestation (E1, day vaginal plug was found), were used for the preparation of slice cultures and for immunofluorescence experiments. *Reeler*, *scrambler* and double *Vldlr/ApoER2* mutant brains (provided by A.G.) were fixed by intracardiac perfusion with 4% paraformaldehyde in PBS and postfixed in the same fixative solution for 24 hours. *Reeler* mutant mice (Orleans allele) were bred by crossing homozygous mutant animals on a mixed, predominantly CD1 background (Goffinet, 1984; D'Arcangelo et al., 1997). *Scrambler* mutants with a spontaneous inactivation of *Dab1* (Sheldon et al., 1997; Ware et al., 1997) were also maintained by crossing homozygous animals on a predominant CD1 background. The *Vldlr/ApoER2* mutant mice were previously generated by targeting in ES cells (Trommsdorff et al., 1999) and were bred on mixed 129 and CD1 backgrounds. The testes of adult (3 months) *reeler* and wild-type animals were fixed by intracardiac perfusion with Bouin's fluid and postfixed in the same solution for 24 hours.

### Immunohistochemistry

Embryonic or adult fixed brains were embedded in 3.5% agarose and sectioned at 60–80  $\mu\text{m}$  with a Vibroslice (Campden Instruments, UK). Sections were blocked with 5% normal goat serum (Gibco, NY, USA) in PBS + 0.3% Triton X-100 for 30 minutes, and incubated in primary antibody (see list below) diluted in PBS + 0.3% Triton X-100 for 36 hours. After washes in PBS, sections were incubated with Alexa FITC- or TRITC-conjugated isotype-specific secondary antibodies at a dilution of 1:400 for 2 hours at room temperature, washed and mounted with Citifluor anti-fading solution (Agar, Essex, UK).

Preparations was then examined with a confocal microscope (Leica). Images were reconstructed using MetaMorph imaging software (Universal Imaging, West Chester, PA, USA).

For immunoperoxidase experiments, sections were incubated with an anti-rabbit biotinylated secondary antibody and processed using an ABC kit (Vector Laboratories, Burlingame, CA) with 3,3'-diaminobenzidine (0.015%; Sigma) as a chromogen. Sections were then washed in PBS, mounted, dehydrated and coverslips placed on top. Images were taken using black and white camera (Quantix Photometrics, Princeton Instruments, UK) and edited using Adobe Photoshop.

The primary antibodies used were: anti-GnRH (rabbit polyclonal, 1:400 for immunofluorescence and 1:4000 for immunoperoxidase; Immunostar Inc., Wisconsin), anti-peripherin (rabbit polyclonal, 1:4000, Chemicon Int., Temecula), anti-reelin (mouse monoclonal, clone G10, 1:500), anti-ApoER2 (mouse monoclonal, clone 2H8, 1:50), anti-Dab1 (mouse monoclonal, clone L2, 1:500), anti- $\alpha$ 3 integrin (mouse monoclonal, clone MAB1952Z, 1:500; Chemicon Int.), anti-EGFP (rabbit polyclonal, 1:400; Molecular Probes, Eugene, OR) and anti-beta III tubulin (mouse monoclonal, 1:500; Sigma, UK).

### Histology of testes

The testes of *reeler* and wild-type mice were embedded in paraffin wax, and serially sectioned at 10  $\mu\text{m}$ . Sections were deparaffinized, dehydrated and stained with Haematoxylin and Eosin. The number of seminiferous tubules was assessed by counting three random 1 mm<sup>2</sup> fields of testes derived from two *reeler* and two wild-type animals.

### Preparation of slice cultures and application of fluorescent tracers

Pregnant Sprague Dawley albino rats at E18 ( $n=3$ ) were killed by cervical dislocation. The fetuses were rapidly removed and placed in Gey's balanced salt solution medium supplemented with glucose (6.5 mg/ml) at 4°C. Brain slices were prepared as described previously (Nadarajah et al., 2002). Briefly, brains embedded in 3% low-melting point agarose (Sigma) were sectioned in the sagittal plane in ice-cold oxygenated artificial cerebrospinal fluid (ACSF), pH 7.4, at 300  $\mu\text{m}$  using a Vibroslice (Campden Instruments). Slices, proceeding from lateral to medial forebrain, were mounted onto porous nitrocellulose filters (0.45  $\mu\text{m}$ ; Millipore, London, UK) and transferred to 24-well culture plates.

To label the population of neurons arising or passing through the olfactory bulb, tungsten particles coated with a fluorescent tracer, 4-chloromethyl benzoyl amino tetramethyl rhodamine (CMTMR; Molecular Probes) were applied adjacent to the olfactory bulb (Fig. 2A) using micropipettes as described by Alifragis et al. (Alifragis et al., 2002). Following application of particles, slices were placed in an incubator for 48 hours in culture medium containing DMEM (Sigma), 5% N-2 (Gibco), 100  $\mu\text{M}$  heat-inactivated foetal bovine serum (Gibco), 1  $\times$   $\mu\text{M}$  L-glutamine, 2.4 g/l D-glucose and penicillin/streptomycin (1:1000, Sigma). For the reelin-blocking experiments, monoclonal anti-reelin antibody (clone CR-50; kindly provided by Kazunori Nakajima, Keio University, Japan) was added to the culture medium every 6 hours at a dilution of 1:100 (Ogawa et al., 1995; Miyata et al., 1997; Nakajima et al., 1997). After incubation, sections were fixed in 4% paraformaldehyde and mounted on slides with Citifluor. Images of CMTMR-labelled neurons were examined using a confocal microscope (Leica) and edited using Photoshop imaging software.

### Cell lines

GN11 cells, generously given to R.M. by S. Radovick (University of Chicago, Chicago, IL, USA), and COS-7 cells (American Type Culture Collection, ATCC, Manassas, VA, USA) were grown as a monolayer at 37°C in a humidified CO<sub>2</sub> incubator in Dulbecco's MEM containing 1 mM sodium pyruvate, 100 mg/ml streptomycin, 100 U/ml penicillin and 10 mg/l of phenol red (Biochrom KG, Berlin,

Germany) and supplemented with 10% foetal bovine serum (FBS; Gibco, Grand Island, NY, USA). The medium was replaced at 2-day intervals. Subconfluent cells were harvested by trypsinization and cultured in 57 cm<sup>2</sup> dishes (at a density of 1×10<sup>5</sup> and 2.5×10<sup>5</sup> for GN11 and COS-7 cells, respectively). Cells within six passages were used in all experiments. A stable line of GN11 cells expressing EGFP (GN11-EGFP) was obtained by G418 selection following transfection with the pEGFP-N<sub>2</sub> plasmid (ClonTech, La Jolla, CA, USA) (Cariboni et al., 2004).

### Collagen gel co-cultures

Cell aggregates of GN-EGFP cells were prepared by the 'hanging drop' technique as described previously (Kennedy et al., 1994). Rat tail collagen solution was prepared as described by Guthrie and Lumsden (Guthrie and Lumsden, 1994). Collagen gels were generated by adding 10% (v/v) of 10× concentrated DMEM (without phenol red) and 0.8 M sodium bicarbonate to an aliquot of collagen stock solution. Pairs of olfactory bulbs were dissected from E18 rats brains and cut into pieces (explants) of 100-300 μm in diameter; explants and GN11-EGFP cell aggregates were subsequently plated on four-well dishes coated with poly-L-lysine and overlaid with 80 μl of collagen solution. Following polymerization (30 minutes), the preparation was covered with 500 μl of serum-free medium containing B-27 supplement (Gibco) and cultured in a humidified, 5% CO<sub>2</sub>, 37°C incubator. After 48 hours in vitro, olfactory bulb explants and GN11 cell aggregates were fixed in 4% paraformaldehyde and immunostained with a monoclonal anti-beta III tubulin antibody (Sigma) and a polyclonal anti-EGFP antibody, respectively. Cell displacement away from the aggregate was studied by estimating the difference in the percentage of migrating cells present in the proximal (P) and distal (D) quadrants as illustrated in Fig. 2B.

For the reelin-blocking experiments, 1 μl of CR-50 antibody (100 μg/ml, diluted in culture medium) was added 3 times a day during the 48 hours incubation period.

This analysis was performed blindly with the aid of the Metamorph image analysis system (Universal Imaging Corporation). Statistical analysis was performed using the Prism4 program (GraphPad Software Inc., San Diego, CA, USA).

### Production of reelin-enriched conditioned media

Full-length *reelin* cDNA plasmid (pCRI) was kindly provided by Gabriella D'Arcangelo (Houston, TX, USA) and Tom Curran (Memphis, TN, USA); pCDNA3 plasmid was chosen as a control. For transfection, COS-7 cells (at 80% confluence) were grown in culture plates in complete culture medium for 24 hours and incubated for 3 hours with the selected expression vector (1 μg/ml) in the presence of Lipofectamine-2000 (Life Technologies, MD, USA) according to the manufacturer's instructions. The expression of the different constructs was verified by immunofluorescence. Conditioned media from pCRI (reelin-CM) or pCDNA<sub>3</sub> (control-CM) transfected COS-7 cells were obtained as described by Hack et al. (Hack et al., 2002). Briefly, transfected COS-7 cells were left in culture for 48 hours in complete medium; this was then replaced with serum-free medium and the cells incubated for 24 hours. Cell supernatant was centrifuged at 3000 g for 5 minutes and immediately used for microchemotaxis assays. Secretion of reelin was confirmed by immunohistochemistry and western blot analysis (data not shown).

### Chemomigration assays

In order to quantify the migratory activity of GnRH neurons in the presence of reelin, we performed chemomigration assays on GN11 cells (Maggi et al., 2000) using a 48-well Boyden's chamber according to the manufacturer's instructions (Neuroprobe, Cabin John, MD, USA). Briefly, subconfluent cells were suspended (10<sup>5</sup> cells/50 μl) in serum-free medium and placed in the open-bottom wells of the upper compartment of the chamber. These wells were separated from facing wells of the lower compartment by a polyvinylpyrrolidone-free

polycarbonate porous membrane (8 μm pores) precoated with gelatin (0.2 mg/ml in PBS). To measure chemotaxis (the directed migration of cells towards a concentration gradient of chemotactic factors), control-CM and reelin-CM were placed into the lower compartment of the chamber. Haptotaxis (the directed movement up to a gradient of substratum-bound chemoattractant) was measured by coating the lower surface of the gelatin treated-porous membrane with control- or reelin-CM at 4°C for 24 hours.

The chamber was kept in an incubator at 37°C for 3 hours, which is the minimum time required to attain significant migratory activity of GN11 neurons (Pimpinelli and Maggi, 2004). The cells migrated through the pores during incubation, and adhered to the underside of the membrane. They were subsequently fixed and stained using the Diff-Quick stain kit (Biomap, Milan, Italy) and mounted onto glass slides. For quantitative analysis, the membranes were observed using an Olympus light microscope with a 20× objective. Three random fields of stained cells were counted for each well, and the mean number of migrating cells/mm<sup>2</sup> for each experimental condition was calculated. Statistical analysis was performed using the Prism4 program.

### RT-PCR

Total RNA was isolated by extraction with TRIzol (Life Technologies, Inc.).

Single-strand cDNA was synthesized by AMV reverse transcriptase (Promega) and random hexamers (Promega). PCR was carried out using 0.5 μl cDNA and the appropriate oligonucleotides (0.6-6 μM) in 25 μl reaction mix using Taq DNA polymerase (Qiagen) and the following conditions: 35 cycles of denaturing for 60 seconds at 94°C, annealing for 60 seconds at 60°C and extension for 120 seconds at 72°C. In order to verify quality and quantity of RNA retrotranscribed into cDNA, GAPDH amplification was carried out under the same conditions (data not shown). PCR products were analysed by electrophoresis on a 2% agarose gel, and bands visualized under UV illumination following ethidium bromide staining. The primers used are listed in Table 1.

## Results

Previous studies have demonstrated the abundant presence of reelin at various levels of rodent olfactory system (Ikeda and Terashima, 1997; Alcantara et al., 1998; Teillon et al., 2003). Specifically, strong reelin expression has been detected in the olfactory epithelium, main and accessory olfactory bulb, vomeronasal organ and VNN. We have confirmed these observations using reelin immunohistochemistry at various stages of embryonic development. We found strong reelin immunoreactivity in the olfactory bulb and in the region where GnRH neurons penetrate into the brain (Fig. 1). Double-labelling experiments indicated that GnRH neurons were reelin negative. In order to test whether this extracellular protein affects their migration, we first conducted a series of in vitro experiments on immortalized GnRH neurons and in embryonic brain slices.

### Reelin affects the migration of GnRH neurons

In vivo studies of GnRH neurons are hindered by their small number and widespread distribution in the basal forebrain (Merchenthaler et al., 1984). Furthermore, attempts to maintain a pure population in culture have been unsuccessful (Terasawa et al., 1993; Kusano et al., 1995). However, a number of cell lines have been obtained by genetically targeted tumourigenesis of GnRH neurons in mice (Mellon et al., 1990; Radovick et al., 1991). These cells express neuronal markers

**Table 1. Sequences (5'-3'), amplicon length and accession numbers of primers used in this study**

| Primer                       | Sequence              | Position in the transcript | Accession no.             |
|------------------------------|-----------------------|----------------------------|---------------------------|
| <i>ApoER2</i> F              | TGGCGCTGTTGCTGTTGCTG  | 187-481 (313 bp)           | NM_053073                 |
| <i>ApoER2</i> R              | CACGTGGCCTTCGATTCGT   |                            |                           |
| <i>ApoER2</i> F <sup>n</sup> | AGTCAAGGAGTGTGAAGAGG  | 263-444 (201 bp)           |                           |
| <i>ApoER2</i> R <sup>n</sup> | TCCTCCTCGCCGTCGCACTT  |                            |                           |
| <i>Vldlr</i> F               | ACTGCAAGCGGGAAGAAAGCC | 254-486 (253 bp)           | NM_013703                 |
| <i>Vldlr</i> R               | GGCACTGTCAGGGCTTCAT   |                            |                           |
| <i>Vldlr</i> F <sup>n</sup>  | GTTTCAGTGCACAAATGGCG  | 292-477 (186 bp)           |                           |
| <i>Vldlr</i> R <sup>n</sup>  | TCTTCGCAATCAGGATCCCC  |                            |                           |
| <i>Dab1</i> F                | GAAAGACTCCAGGAAGA     | 311-384 (79 bp)            | NM_Y08381, Y08380, Y08379 |
|                              |                       | 217,271,555 isoforms       |                           |
| <i>Dab1</i> R                | GGACCCCTTCGCCTTAAAC   |                            |                           |
| <i>Dab1</i> F <sup>n</sup>   | AGGAAGAAAGGTCAGGATCG  | 321-380 (60 bp)            |                           |
|                              |                       | 217,271,555 isoforms       |                           |
| <i>Dab1</i> R <sup>n</sup>   | CCCTTCGCCTTAAACCTCT   |                            |                           |

F, forward; R, reverse; <sup>n</sup>nested PCR.

and retain many of the features of GnRH-secreting neurons (Liposits et al., 1991; Wetsel, 1995; Gore and Roberts, 1997). One of these lines, GN11, shows a strong chemomigratory response in vitro and is thought to represent a good model to study the molecular mechanisms of GnRH neuronal migration (Maggi et al., 2000; Giacobini et al., 2002; Pimpinelli et al., 2003). We used GN11 cells to study the effects of reelin on the migratory activity of GnRH neurons.

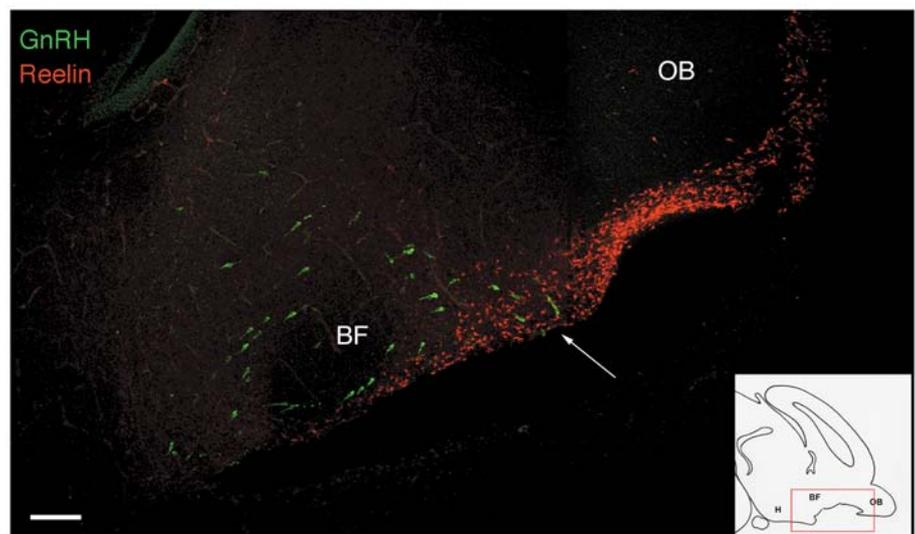
We first performed co-culture experiments in three-dimensional collagen gels containing rat E18 olfactory bulb explants, as a source of reelin (Hack et al., 2002), and aggregates of GN11-EGFP cells. The co-cultures were maintained for 48 hours and subsequently fixed, stained and analysed with a confocal microscope (Fig. 2A). The percentages of cells that had moved away from the aggregate were estimated for the proximal and distal quadrants as illustrated in Fig. 2B. Analysis of 24 samples showed that roughly twice as many GN11-EGFP cells had moved into the distant quadrant, away from the olfactory bulb explant, than in the proximal quadrant (Fig. 2C). However, when this experiment was performed in the presence of reelin-blocking antibody (CR-50), no difference in the percentage of GN11-EGFP neurons migrating away from the aggregate was observed (Fig. 2D,E).

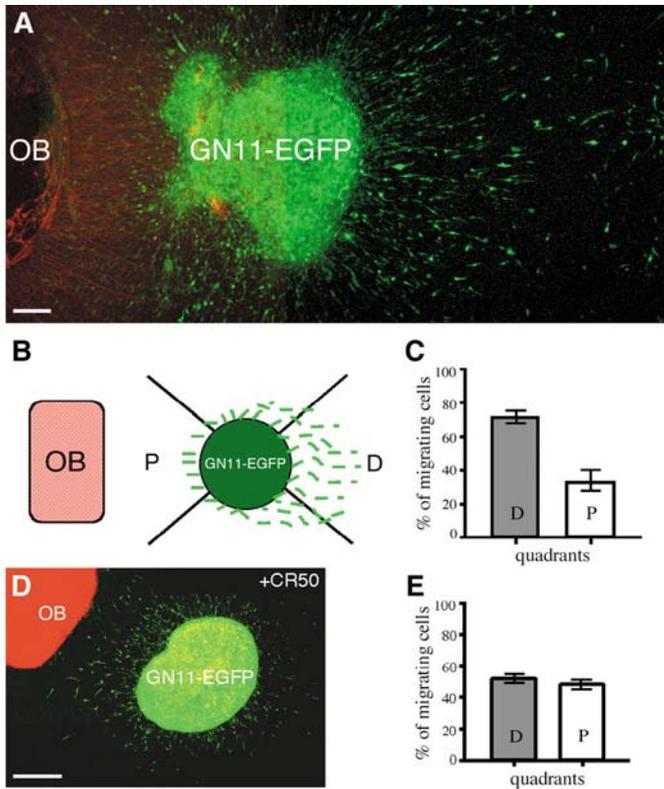
To confirm the repulsive activity of reelin in the migration of GN11 cells, we performed chemomigration assays using a 48-well Boyden's chamber, a method that provides a sensitive and quantitative

measure of cellular responses to specific chemotropic signals. In these experiments, the effects of reelin on GN11 cells were analysed in terms of their chemotactic and haptotactic responses (see Materials and methods). The chemotactic response of GN11 cells was no different to a gradient of soluble reelin, presented by placing the extracellular protein-enriched conditioned media (CM) into the wells of the lower compartment of the chamber, than to control-CM (Fig. 3A). However, when reelin-enriched CM was adsorbed to the gelatin-coated lower surface of the porous membrane, the haptotactic response of GN11 cells was significantly reduced compared to control-CM (Fig. 3B). These results indicate that reelin exerts an inhibitory effect on the migration of immortalized GnRH neurons, but only when it is bound to the substratum.

In order to substantiate the observed effects of reelin on the migration of GnRH neurons, we employed slice cultures of E18 rat brains. In sagittal slices, crystals of the fluorescent tracer CMTMR were placed at the point where GnRH neurons enter the brain (Fig. 4A) and neuronal migration was followed into the basal forebrain. After 48 hours, we observed a stream of labelled cells in the hypothalamic region and only a small number of neurons in the cerebral cortex (Fig. 4B,E). However,

**Fig. 1.** Expression of reelin during GnRH neuronal migration into the forebrain. GnRH and reelin immunoreactivities in a sagittal section through an E18 rat brain (boxed area in schematic drawing). GnRH immunoreactive cells (green) enter the forebrain (arrow) and migrate caudally to the basal forebrain. Reelin immunoreactivity (red) is present in the olfactory bulb and adjacent region, an area that GnRH neurons tend to avoid. OB, olfactory bulb; BF, basal forebrain; H, hypothalamus. Scale bar: 250  $\mu$ m.



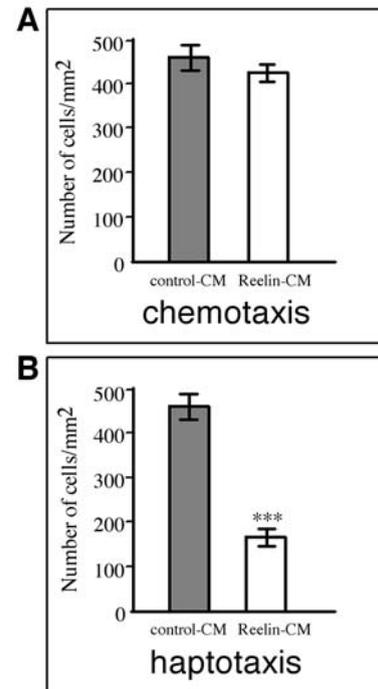


**Fig. 2.** Migration of GN11 cells is inhibited by reelin-rich olfactory bulb explants in three-dimensional collagen matrix. Olfactory bulb explants (OB) from E18 rats were cultured with GN11-EGFP cells in collagen gels. (A) GN11-EGFP cells (green) migrated away from the OB (red, axonal staining). (B) The zone surrounding the cell aggregates was divided into quadrants, thus defining a proximal (P) and a distal (D) quadrant in relation to the OB. (C) Migrating cells were counted in 24 co-cultures and expressed as a percentage of cells  $\pm$  s.e.m. in each of the two quadrants out of the total number of cells in both quadrants. We found that  $66.54 \pm 1.40\%$  of cells migrated into D and  $33.46 \pm 1.40\%$  into P. Scale bar: 250  $\mu$ m. (D,E) Addition of CR-50 antibody resulted in roughly equal proportions of GN11-EGFP cells in P ( $53.53 \pm 2.60\%$ ) and D ( $46.47 \pm 2.60\%$ ;  $n=12$ ). Scale bars: 250  $\mu$ m.

when slices were incubated in the presence of CR-50, an antibody known to block the effects of reelin (Ogawa et al., 1995; Zhao et al., 2004), the majority of CMTMR-labelled cells were found in the cortex rather than the basal forebrain (Fig. 4C,E). These experiments suggested a role for reelin in directing migration and eventual fate of neurons entering the forebrain from the nasal compartment.

### GnRH neurons and the reelin signalling pathway

It is widely thought that reception of the reelin signal requires the presence of at least one of two receptors of the lipoprotein receptor family, namely ApoER2 (also known as Lrp8) and Vldlr (Hiesberger et al., 1999; Trommsdorf et al., 1999). The signal is then transduced by tyrosine phosphorylation of the intracellular adaptor Dab1 (Howell et al., 1997; Sheldon et al., 1997; Ware et al., 1997). Thus, we investigated the presence of reelin receptors both on immortalized GnRH cells and in sections of basal forebrain during the period of migration of GnRH neurons. Using RT-PCR experiments, we found



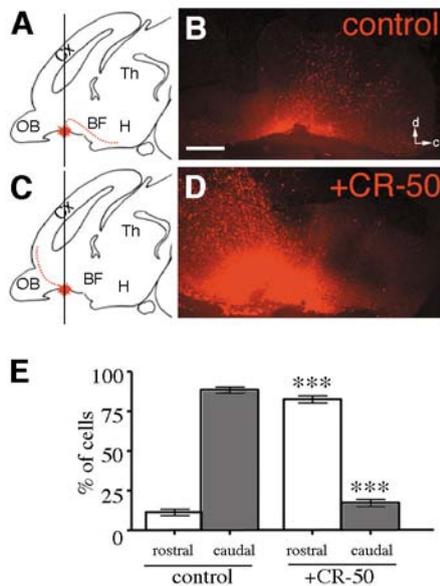
**Fig. 3.** Reelin inhibits the migration of GN11 cells in Boyden's chamber assay. (A) In a chemotactic assay, reelin-CM did not alter the migration of GN11 cells compared to control-CM. However, the haptotactic response of these cells was significantly inhibited by the presence of reelin-CM ( $***P < 0.001$ ; Student's *t*-test). On average,  $469.1 \pm 49.76$  cells/mm<sup>2</sup> migrated in the presence of control-CM while  $183.5 \pm 30.90$  cells/mm<sup>2</sup> migrated in the presence of reelin-CM. Means  $\pm$  s.e.m. obtained from 12 independent experiments.

transcripts of expected molecular sizes for ApoER2 and Vldlr in mRNA from immortalized GnRH neurons (Fig. 5A,B). However, RT-PCR analysis using specific primers that recognize all three known forms of Dab1 mRNA showed no expression of the intracellular adaptor by GN11 cells (Fig. 5A,B).

We then carried out double immunofluorescence experiments on fixed E18 rat brain sections in order to confirm the expression of reelin receptors on migrating GnRH neurons. Using a monoclonal anti-ApoER2 and a polyclonal anti-GnRH antibody, we found that a small percentage (approximately 5%) of GnRH neurons were positive for ApoER2 (Fig. 5C,E). It should be noted that there was a recent report by Perez-Garcia et al. (Perez-Garcia et al., 2004) of the presence of this lipoprotein receptor, but not Vldlr in the olfactory bulb region. However, none of these neuroendocrine neurons were immunoreactive for the adaptor protein Dab1 (Fig. 5F,H). These immunohistochemical studies indicated that, although a subpopulation of migrating GnRH neurons express ApoER2, they lack the intracellular adaptor protein Dab1.

### GnRH neurons in *reeler*, *scrambler* and reelin receptor mutant mice

Reelin deficiency results in the *reeler* phenotype. Characteristics of the mutant include abnormal lamination of cerebral, cerebellar and hippocampal cortices and neuronal

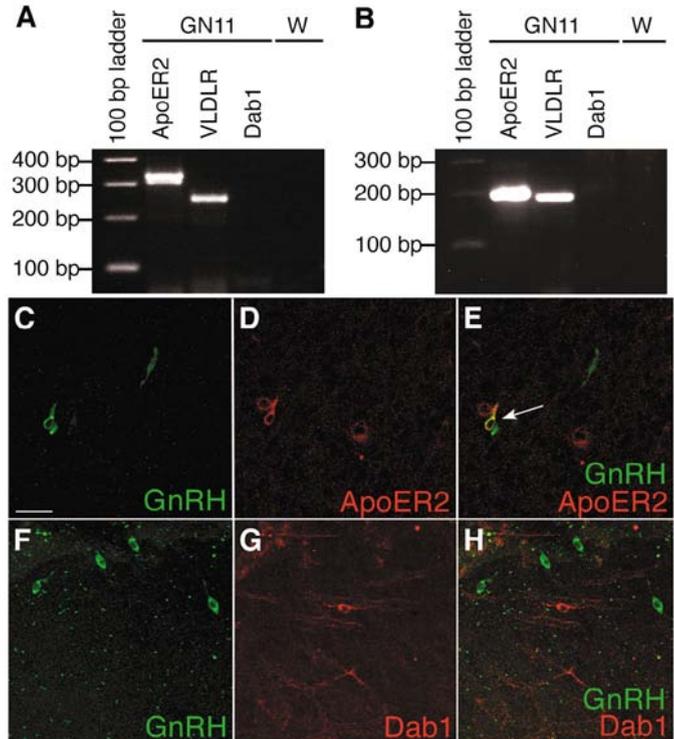


**Fig. 4.** Reelin-blocking antibody alters the direction of migration of basal forebrain neurons. (A,C) Drawings of sagittal sections through E18 rat brains showing placements of fluorescent CMTMR particles at the junction of the olfactory bulb region and the basal forebrain as well as the migratory routes (red dotted lines) taken by labelled cells in control (A) and CR-50-treated slices (C). Vertical lines separate the parts of the sections rostral to the dye placement from those located caudally. (B,D) Streams of labelled cells moving away from the site of CMTMR placement. Note that in the treated slice, a mass of fluorescent cells is directed rostrally. (E) Counts of labelled cells in control and CR-50-treated slices indicated that in the former, cells moved caudal to CMTMR placement but, in the latter, cells took a rostral course ( $n=12$  slices for each case;  $***P<0.001$ ; Student's  $t$ -test). OB, olfactory bulb; Cx, cerebral cortex; Th, thalamus; BF, basal forebrain; H, hypothalamus. Scale bar: 500  $\mu\text{m}$ .

ectopias in other brain areas (Lambert de Rouvroit and Goffinet, 1998). In mice, inactivating mutations of *Dab1* (*scrambler* mice), and double mutations of *ApoER2* and *Vldlr* generate *reeler*-like phenotypes (Tissir and Goffinet, 2003). To confirm the importance of reelin in the migration of GnRH neurons, we examined brains of developing and adult *reeler* mice, as well as of *scrambler* and double lipoprotein receptor (*ApoER2* and *Vldlr*) mutants at the adult stage.

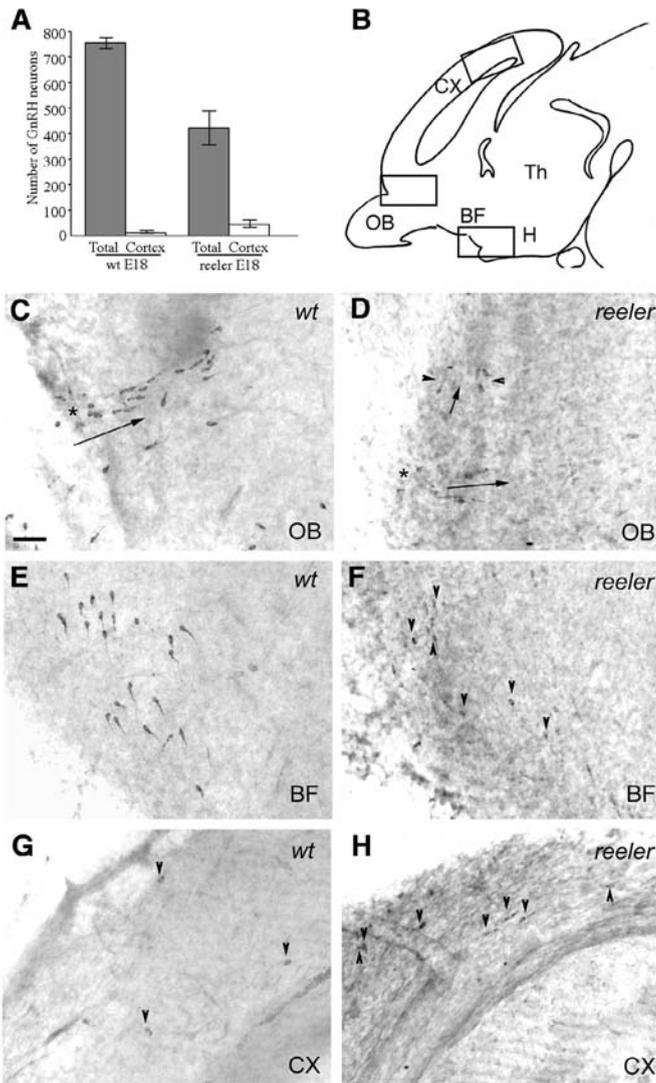
Staining of serial sections from three E18 *reeler* and wild-type brains with an antibody against GnRH showed a significant reduction in the total number of GnRH neurons in the mutants. In this analysis, all labelled GnRH cells were counted in all contiguous sections taken through entire brains, including olfactory bulbs (Fig. 6A,B,E,F). Furthermore, there was a significant increase in the number of labelled cells in the cortex compared to controls (Fig. 6A-D,G,H). However, sections from the same animals stained with an antibody against peripherin in order to visualize the cVNN associated with migrating GnRH neurons, showed that, in agreement with a recent report (Teillon et al., 2003), there was no anomaly in the pattern of cVNN fibres (data not shown).

Analysis of serial sections taken through entire brains of postnatal day (P) seven *reeler* mice (two males and one female) also revealed a marked reduction of GnRH immunoreactive



**Fig. 5.** Expression of reelin signalling molecules in GN11- and GnRH neurons. (A) RT-PCR analysis, using GN11 cell mRNA, showed the presence of *ApoER2* and *Vldlr* transcripts (313 bp and 253 bp respectively), but absence of *Dab1* (predicted amplicon 79 bp). These results were confirmed by nested PCR experiments (B), yielding the predicted 201 bp and 186 bp products for *ApoER2* and *Vldlr*, but not the expected band of 60 bp for *Dab1*. (C-E) Double immunohistochemistry experiments showed the localization of ApoER2 (red, D and E) in a small proportion (arrow in E) of GnRH neurons (green, C and E). (F-H) Similar experiments failed to identify *Dab1* (red, G and H) in GnRH neurons (green, F and H).  $n=3$  brains. Scale bar: 50  $\mu\text{m}$ .

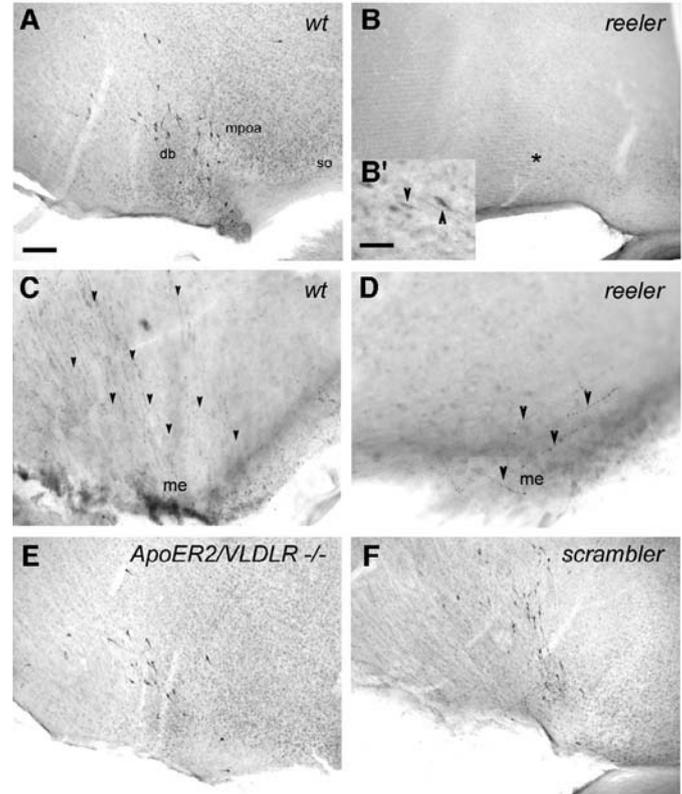
neurons in the hypothalamus compared to wild-type controls (*reeler*  $304\pm24.6$ ; wt  $528\pm23.3$ ;  $n=3$ ;  $P<0.01$ ; Student's  $t$ -test), with very few labelled cells present in the cortex at this stage. By the adult stage, *reeler* brains contained a paucity of faintly labelled GnRH neurons scattered in the hypothalamic region (Fig. 7A,B,B'). In the *reeler* median eminence, where the long processes of GnRH neurons normally converge to access the pituitary portal vessels, labelled processes were very few compared to the profuse array of such processes observed in the wild-type animals (Fig. 7C,D). This picture was consistent for all six (3 males and 3 females) adult *reeler* brains examined. To investigate whether the reduction in the number of GnRH neurons is reflected in alterations in the gonads, we analysed the testes of adult male *reeler* and wild-type mice ( $n=2$  for each type). There was no gross difference in the weight, volume or appearance of testes between the wild-type and *reeler* mice. However, histological examination revealed a significant reduction in the density of seminiferous tubules (mean count/ $\text{mm}^2 \pm \text{s.d.}$ ; wt  $21.33\pm5.84$ ; *reeler*  $11.00\pm2.28$ ;  $P<0.01$ ; Student's  $t$ -test) and dilation of these tubules in *reeler* animals. Spermatogenesis appeared complete in the seminiferous



**Fig. 6.** Reduction in number and aberrant position of GnRH neurons in E18 *reeler* mice. (A) Cell counts revealed a significant reduction in the total number of GnRH neurons in the brains of *reeler* mice (*reeler*,  $417.0 \pm 31.75$ ; wt,  $748.0 \pm 4.04$ ;  $n=3$  for each genotype;  $*P < 0.05$ ; Student's *t*-test), but a relative increase in the number of these cells in the cortex compared to wild type (wt) animals (*reeler*  $41.11 \pm 9.77$ ; wt,  $9.793 \pm 3.97$ ;  $*P < 0.05$ ; Student's *t*-test). (B) Drawing of a sagittal section depicting the areas (boxes) selected for analysis. (C–H) Images of immunohistochemically stained GnRH neurons in OB, BF and Cx of wild-type and *reeler* brains. Streams of neurons entering the brain (asterisk in C and D) are directed towards the basal forebrain (large arrows in C and D indicate direction of stream) in both groups of animals, but *reeler* show an additional stream directed towards the cortex (small arrow in D). Note the overall reduction in GnRH neurons in the BF of *reeler* mice (arrowheads in F) and the increase of these neurons in the cortex (arrowheads in H). OB, junction of olfactory bulb and forebrain; BF, basal forebrain; H, hypothalamus; Cx, cortex. Scale bar: 50  $\mu\text{m}$ .

tubules of both groups of animals and Sertoli cells were present in all samples. In addition, there was no hyperplasia of the Leydig cells, but an apparent decrease in the interstitial tissue in *reeler* testes (data not shown).

Unlike *reeler* mice, young adult *ApoER2* and *Vldlr* double



**Fig. 7.** GnRH neuron distribution in the hypothalamus of adult *reeler*, *scrambler* and *ApoER2/Vldlr* mutant mice. (A,B) Sagittal sections taken from adult wild-type and *reeler* animals, respectively (A) Typical distribution of GnRH neurons in the hypothalamus. (B) In the *reeler* mouse there are very few, faintly stained GnRH neurons. At this magnification, GnRH neurons are hardly visible in *reeler*. The inset B', taken from the area indicated by an asterisk in B, illustrates a few of these neurons at a higher magnification. (C) A profuse array of GnRH processes (arrowheads) streaming to the median eminence (me) region from an adult wild-type animal. In *reeler* mice (D), only very few such processes (arrowheads) converge to the median eminence. (E,F) Sections taken from the brains of *ApoER2/Vldlr* mutants (E) and *scrambler* mice (F) illustrate that the density and appearance of GnRH neurons are similar to those in wild-type animals. Scale bars: 100  $\mu\text{m}$  (A,B,E,F); 50  $\mu\text{m}$  (B',C,D). mpoa, medial preoptic area; db, diagonal band of Broca; me, median eminence; so, supraoptic nucleus.

knockouts as well as *scrambler* mice had a normal complement of GnRH neurons in the hypothalamic region (Fig. 7E,F). These observations, taken together with the finding that GnRH neurons lack the intracellular adaptor Dab1, suggest that these migrating cells do not respond to reelin through the conventional reelin signalling pathway.

Cell surface adhesion  $\alpha 3\beta 1$  receptors have also been found to bind to reelin (Dulabon et al., 2000). We sought to investigate whether this integrin is involved in the reception of the reelin signal by GnRH neurons. However, we failed to detect  $\alpha 3\beta 1$  integrin in GnRH neurons by double immunohistochemistry and, furthermore, incubation with a blocking anti- $\alpha 3$  integrin antibody (Niu et al., 2004) did not alter the migration of GN11 cells away from olfactory bulb

explants in collagen gels (data not shown). Thus, it appears that  $\alpha 3\beta 1$  is not involved in the reception of reelin in this system.

## Discussion

Our results demonstrate that reelin plays a role in guiding the migration of GnRH neurons in the developing basal forebrain. However, this function does not appear to be mediated by the conventional reelin signalling pathway that includes two receptors, ApoER2 and Vldlr, and the intracellular adaptor protein Dab1. First, we demonstrated the repulsive activity of reelin on the migration of GnRH immortalized neurons, both in three-dimensional collagen gels and in chemomigration assays. Second, we observed that the migratory path of neurons moving from the olfactory region to the basal forebrain was altered in slices of developing brain when the activity of reelin was blocked with CR-50 antibody. Third, we found that the action of reelin on the migration of GnRH neurons is independent of Dab1. Finally, we confirmed the importance of reelin in the establishment of GnRH neurons in the hypothalamus: in brains of developing and adult *reeler* mice, we found a reduction in the number of these neuroendocrine neurons that might explain the known reduced fertility of *reeler* mice (Caviness et al., 1972; Goffinet, 1984; Green, 1989). However, mutants lacking receptors, *ApoER2* and *Vldlr*, and *scrambler* mice lacking *Dab1* showed a normal complement of GnRH neurons in the hypothalamus, indicating that the activity of reelin in this developing neuroendocrine system is not mediated by the conventional signal transduction pathway.

### Reelin and GnRH neuronal migration

Reelin, an extracellular protein, is present in the nervous system of all vertebrates and its expression is widespread. Numerous studies have focused on its role in the developing brain (reviewed by Curran and D'Arcangelo, 1998; Rice and Curran, 2001; Tissir and Goffinet, 2003), particularly in providing positional cues to radially migrating neurons in the cerebral cortex (D'Arcangelo and Curran, 1998). Reelin is highly expressed in the olfactory system and especially in the main (mitral cell layer) and accessory olfactory bulbs (Alcantara et al., 1998; Teillon et al., 2004). In this area, migrating GnRH neurons appear to lose their association with the olfactory and VNN fibres, change their course and move caudally along the cVNN to enter the basal forebrain. Furthermore, recent reports have documented the expression of ApoER2 in the olfactory bulb region (Perez-Garcia et al., 2004). These observations prompted us to hypothesize that reelin acts as a repellent during the migration of GnRH neurons, guiding them away from the olfactory bulb and into the basal forebrain.

Functional studies of GnRH-secreting neurons have been facilitated by the availability of two cell lines of immortalized mouse GnRH-secreting neurons: GT1 cells [which includes GT-1, -3 and -7 subclones (Mellon et al., 1990)] and GN cells [which includes GN10, GN11 and NLT subclones (Radovick et al., 1991)]. These cell lines may be considered as postmigratory and migratory GnRH neurons, respectively. In particular, GN11 cells retain features of GnRH-secreting neurons, including strong chemomigratory activity in vitro (Maggi et al., 2000) and, as such, have been used to investigate

mechanisms involved in GnRH neuron migration (Giacobini et al., 2002; Cariboni et al., 2004).

Using collagen gels and microchemotaxis assays, we found that reelin inhibits the migration of GN11 cells. In the collagen gel assay, we used embryonic olfactory bulb explants as a source of reelin and observed that the migration of immortalized GnRH neurons is inhibited. However, when the action of reelin was blocked by CR-50 antibody, no differences were observed in GN11-EGFP cell displacement from the aggregate, supporting the hypothesis for a role of this protein in the migration of GnRH neurons.

More direct evidence was provided by the chemomigration assays that showed the repulsive activity of substrate-bound reelin on GN11 cells. The results of both migration assays are in accordance with the purported nature of reelin as a secreted protein bound to extracellular matrix represented here by the collagen gel matrix or by the gelatin-coated surface of the porous membrane in the Boyden's chamber. Notably, the inhibitory effect on GN11 cells was observed in the haptotaxis but not the chemotaxis assays, pointing to the requirement of a physical substrate for the action of reelin and not to the presence of a soluble gradient of concentration.

The results of these migration assays are consistent with our observations in cultured embryonic rat brain slices. When the activity of reelin is blocked with CR-50 antibody in these slices, the migratory path of the majority of cells labelled with CMTMR at their point of entry into the brain is diverted to the cerebral cortex instead of the hypothalamic region. It is known that CR-50 is directed against an N-terminal epitope, interferes with the aggregation of reelin, and blocks its function in vitro and in vivo (Ogawa et al., 1995; Miyata et al., 1997; Nakajima et al., 1997; D'Arcangelo et al., 1999; Kubo et al., 2002). Although it was not technically feasible to double label cells for CMTMR and GnRH, it may be assumed that at least some of the cells labelled with the fluorescent tracer were indeed neurosecretory neurons. The fate of these neurons in the cortex, similar to the small number of GnRH neurons that enter the cortical anlage in normal animals, is presently unknown.

Reception of the classical reelin signal requires the presence of at least one of two lipoprotein receptors, ApoER2 and Vldlr, as well as the intracellular adaptor protein Dab1. Using RT-PCR, we found that GN11 cells express both receptor mRNAs, but in immunohistochemically stained sections of embryonic brains, a small proportion of GnRH neurons express only ApoER2. This may be due to the presence of the receptor in one of the different subpopulations of GnRH neurons (Tobet et al., 1996) or, alternatively, ApoER2 may be transiently expressed during migration. Furthermore, we observed that both GN11 cells and GnRH neurons lacked Dab1. These observations indicate that the repulsive activity of reelin on migrating GnRH neurons is independent of Dab1 and unlikely to be mediated by the conventional reelin signalling pathway. Other molecules have been suggested to bind reelin. Among them, the protocadherin CNR1 has been proposed to act as a co-receptor for reelin (Senzaki et al., 1999), but this proposal has recently been disproved (Jossin et al., 2004). Reelin has been found to inhibit radial neuronal migration by binding to  $\alpha 3\beta 1$  integrin receptors (Anton et al., 1999; Dulabon et al., 2000; Schmid et al., 2005). However, our results of migration assays using GN11 cells together with immunohistochemical

observations of brain sections suggest that this integrin is not involved in the reception of the reelin signal by GnRH neurons.

### Mutants of the reelin signalling pathway

Here we demonstrated that the inhibitory activity of reelin is important for guiding the migration of the GnRH neurons to the basal forebrain. Our observations in developing and adult *reeler* mice confirmed the importance of reelin in the development of these neuroendocrine neurons in the hypothalamus. We first noted a defect in their migration during embryonic life, with a significant increase in cells misrouted to the cerebral cortex instead of the hypothalamus. This resulted in a marked reduction of cell number in postnatal animals, with adults showing only sparse and faintly stained GnRH-secreting neurons in the hypothalamus and few processes projecting to the median eminence. This reduction in hypothalamic GnRH neurons is likely the cause of the consistently observed delayed pubertal maturation and low fertility of *reeler* mice, which was thought to be due to their ataxic behaviour (Caviness et al., 1972; Goffinet, 1984; Green, 1989). However, our histological analysis of adult *reeler* testes showed a clear reduction in the density of seminiferous tubules, but no hyperplasia of Leydig cells. The lack of hyperplasia of Leydig cells is indicative of hypogonadotropic hypogonadism rather than an intrinsic defect of the testes (Mason et al., 1986; Corradi et al., 2003).

Our in vitro experiments suggest that the repulsive activity of reelin on the migration of GnRH neurons is not mediated by the classical reelin signalling pathway. The presence of a normal complement of GnRH-secreting neurons in the hypothalamus of mutants lacking both lipoprotein receptors and in *scrambler* mice lacking *Dab1* is consistent with the in vitro findings. Although we are at present unable to identify a mechanism that mediates the action of reelin in this neuroendocrine system, it is worth noting that effects of reelin, independent of the conventional reelin signalling pathway, have recently been described (Rossel et al., 2005) in the developing mouse hindbrain. These authors identified a ventral reelin-dependent migration that is independent of the receptors, ApoER2 and Vldlr.

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