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Viewing Cell Movements in the Developing Neuroendocrine Brain[1]

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Synopsis. Many studies suggest that migratory guidance cues within the developing brain are diverse across many regions. To better understand the early development and differentiation of select brain regions, an *in vitro* method was developed using selected inbred and transgenic strains of embryonic mice. In particular, organotypic slices are used to test factors that influence the movements of neurons during brain development. Thick 250 μm slices cut on a vibrating microtome are prepared and maintained *in vitro* for 0–3 days. Nissl stain analyses often show a uniform distribution of cells in the regions of interest on the day of plating (embryonic days 12–15). After 3 days *in vitro*, cellular aggregation suggesting nuclear formation or the changing position of cells with a defined phenotype show that reasonably normal cell movements occur in several regions. Movements *in vitro* that mimic changes in vivo suggest that key factors reside locally within the plane of the slices. Video microscopy studies are used to follow the migration of fluorescently labeled cells in brain slices from mice maintained in serum-free media for 1 to 3 days. Transgenic mice with selective promoter driven expression of fluorescent proteins allow us to view specific cell types (*e.g.,* neurons expressing gonadotropin-releasing hormone). The accessibility of an *in vitro* system that provides for relatively normal brain development over key brief windows of time allows for the testing of important mechanisms.

Introduction

There is a growing recognition of disorders of neuronal migration with striking clinical consequences, and an appreciation for the molecular mechanisms that may underlie them (Gleeson and Walsh, 2000). Many studies suggest that migratory guidance cues within the developing brain are diverse across many regions and multiple methods are needed to dissect key molecular mechanisms. *In vitro* slice preparations from mammalian embryos have provided powerful tools for studying the migratory behavior of cells in many regions of the developing nervous system. Experiments have shown behaviors that were not easy to predict a priori, particularly novel orientations and patterns of cell migration (*e.g.,* Andersen *et al.*, 1997*a*, *b*; Nadarajah *et al.*, 2002). The ability to introduce specific reagents *in vitro* has provided for a determination of the role of calcium channels (Komuro and Rakic, 1992) and NMDA receptors (Komuro and Rakic, 1993) on cell migration in the cerebellum. Organotypic slices have long been used to study the developing hypothalamus from the perspective of neurite outgrowth (Toran-Allerand, 1976, 1980) and gene expression (Wray *et al.*, 1989; Thomas *et al.*, 1998), but not for examining cell migration. We adapted *in vitro* organotypic slice procedures to directly examine the movements of cells within parts of the brain associated with neuroendocrine functions; particularly the preoptic area (Tobet *et al.*, 1994; Hendersen *et al.*, 1999) and hypothalamus (Tobet *et al.*, 1999; Dellovade *et al.*, 2001; Davis *et al.*, 2002*a*), and to study the migration of GnRH neurons across the developing mouse nasal septum and into and through the rostral forebrain (Tobet *et al.*, 1996; Bless *et al.*, 2000). The combination of *in vitro* studies with *in vivo* studies allows hypotheses to be tested in a way that ensures cross-validation of results.

The use of GFP to selectively label cell populations provides an additional strong technique for developmental studies of cell migration. Experiments using slice preparations have utilized fluorescent dyes such as DiI (*e.g.,* O'Rourke *et al.*, 1992), Oregon green (Nadarajah *et al.*, 2001; 2002), and others (Alifragis *et al.*, 2002) to label cells *in vitro*. The migration of cells in selected locations can be readily followed, but the chemical identity of the labeled cells is not as easily determined. The discovery of GFP in the jellyfish, *Aequorea victoria*, and its adaptation for transgenic and viral use, allows the targeted expression of a fluorescent marker that can be visualized in live cells (Chalfie *et al.*, 1994; Marshall *et al.*, 1995; Okada *et al.*, 1999). GFP is a 238 amino acid protein that absorbs short wavelength light and emits green light (Chalfie *et al.*, 1994). New mutated forms of GFP that are more intensely fluorescent provide more avenues for its use in vertebrate systems (Moriyoshi *et al.*, 1996; Zernicke-Goetz *et al.*, 1996). By combining GFP with promoters that drive expression in selected cell populations, movements and migration of identified cell populations can be visualized. We are currently utilizing 3 lines of transgenic mice in which specific neuronal promoters are used to drive either GFP (Suter *et al.*, 2000; Stallings *et al.*, 2002) or yellow fluorescent protein expression (YFP; Feng *et al.*, 2000). Cells in the ventromedial nucleus of the hypothalamus (VMH) have been shown to selectively express steroidogenic factor-1 (SF-1; Ikeda *et al.*, 1994; Shinoda *et al.*, 1995; Roselli *et al.*, 1997; Dellovade *et al.*, 2000; Ikeda *et al.*, 2001). The linkage of GFP downstream from a

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promoter that drives selective expression of SF-1 (Stallings *et al.*, 2002) allows us to follow the migration of cells destined for the VMH (Davis *et al.*, 2004). The mouse gonadotropin-releasing hormone (GnRH) gene promoter has been utilized by two groups to drive GFP expression in GnRH neurons (Spergel *et al.*, 1999; Suter *et al.*, 2000), and we have used mice from one of these lines (Suter *et al.*, 2000) to examine GnRH neuron migration directly (Bless *et al.*, 2002). Thy-1 is a cell surface glycoprotein (immunoglobulin superfamily) whose expression in the brain is restricted to neurons, and is spatially and temporally regulated during development leading to expression in the majority of neurons at maturity (Barlow and Huntley, 2000). The Thy-1 promoter has been used to drive YFP expression in a wide variety of neurons in adults (Feng *et al.*, 2000), and we have used the same line of mice at embryonic ages to examine the movement of cells in the preoptic area and hypothalamus.

MATERIALS AND METHODS

Animals

Timed pregnant mice of various strains were obtained from the animal facility at the Shriver Center of the University of Massachusetts Medical School. All mice were maintained in plastic cages with bedding (Sani-Chips, P.J. Murphy Inc. and Carefresh Total Care Bedding, Absorption Corp.) in a 14hr:10hr light: dark cycle (lights on 07:00) with Agway Prolab rat, mouse, hamster 2000 formula and tap water provided *ad lib.* Pregnant mice (day sperm plugs found = Day 0) were fed Lab Diet Mouse Diet (11% protein; PMI Feeds, Inc.). For some experiments, the mitotic marker bromodeoxyuridine (BrdU) is injected on specific days during gestation to determine the positions of neurons born on specific days at subsequent points in development (Miller and Nowakowski, 1988). Pregnant mice are injected intraperitoneally with 25 mg/kg BrdU in 0.05 *M* PBS (pH 7.4). To obtain embryos, pregnant mice were anesthetized with ketamine (80 mg/kg) and xylazine (8 mg/kg) and pups removed one at a time for slice preparations. Neonates on postnatal days 8–21 (P8, P12) were anesthetized using ketamine (80 mg/kg) and xylazine (8 mg/kg). Animal care was in full accordance with institutional guidelines.

In vitro studies: coverslip model

Heads or brains are dissected free from mouse embryos in cold Krebs buffer. After embedding in 8% low melting temperature agarose (Sigma Type VII-A), slices (250 μm thick) were cut using a vibrating microtome (Leica VT1000S) and placed into cold sterile-filtered Krebs buffer containing 0.01 *M* HEPES, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 0.1 mg/ml gentamicin. The plane of section is based on the model system studied and the expected orientation of migration. Thus, sagittal sections are cut to examine the migration of GnRH neurons from the nasal compartment into the brain (Tobet *et al.*, 1996) and coronal sections are cut to examine the differentiation of nuclear groups in the forebrain (Henderson *et al.*, 1999; Tobet *et al.*, 1999). Alternate angles for cutting are used to confirm the predominant orientation of migration. After the final brain from a litter is cut (no longer than 2hr total time), the slices are left in the cold for an additional 15 min and then incubated for 35 min in MEM containing 10% fetal calf serum with 134 units/ml penicillin, 0.13 mg/ml streptomycin, 1.34 mM glutamine, and 0.5% glucose for 35 min at 36°C in an incubator with 5% CO_2. More recent experiments have shown that 10% fetal calf serum is not necessary in this step, and we currently use a medium that is 2% B-27 supplement with other factors kept constant. Per 100 ml, the medium is comprised of 94.3 ml DMEM F12 Phenol Red Free/ 2 ml B-27 supplement/ 1 ml glutamate/ 1.33 ml Pen/Strep/ 248 μl L-glutamine/ 1.1 ml D-glucose). Slices are then washed in the same media and placed on round coverslips that have been precoated (Table 1, adapted from Roberts *et al.*, 1993) with poly-L-lysine and Vitrogen (Cohesion Technology, Inc.). We also have had recent success using 35 mm plastic dishes with preinserted glass bottoms (MatTek Corporation) that we also coat with Vitrogen. Excess media is removed and the sections are put back in the incubator. It is critical that the incubator be highly humidified (*i.e.,* close as possible to 100%). After 1hr, the slices are covered with approximately 50 μl of a Vitrogen solution comprised of 1 ml Vitrogen, 125 μl 10× MEM, 23 μl pen-strep (10,000 units penicillin and 10 mg streptomycin per ml), and 33 μl of 1 *M* sodium carbonate. After another 90 min, serum-free medium is added to the slices (Neurobasal Medium with B27 supplement (GIBCO BRL Laboratories) and supplemented with 134 units/ml penicillin, 0.13 mg/ml streptomycin, 1.34 mM glutamine, and 0.5% glucose. Slices are kept at 36°C in an incubator with 5% CO_2. After specific culture periods, some slices are fixed in 2% acrolein (from Sigma-Aldrich Co.; 90% stock) or 4% methanol-free formaldehdye (from Polysciences Inc.; 10% stock) for 15–30 min at room temperature and stored in 30% sucrose in 0.1 *M* PB for frozen sectioning on a sliding microtome at 50 μm for Nissl stain analyses and immunocytochemistry. Other slices were stored following fixation in 0.1 *M* PB for processing as whole slices in immunocytochemical procedures.

TABLE 1. *Coverslip preparation.*

25 CIR No. 1 coverslips.
8 hours in xylene.
8 hours acetone.
8 hours 100% EtOH.
Dry and then autoclave.
Dip each coverslip in 0.5 mg/ml polylysine (SIGMA, P1274) and dry.
Rinse with distilled water and dry.
Dip in vitrogen solution diluted 1 : 1 with distilled water.
Air dry and store in individual 35 mm petri dishes.
If not used in 14 days recoat starting from polylysine.

Video microscopy and analysis

Slices are removed from the incubator and washed three times with sterile filtered 1X Krebs. The coverslip with the slice to be viewed is taken out of the 35 mm dish and placed in the center of a coverslip holder and locked in to insure viewing directly through the bottom of the coverslip. Krebs buffer or media is then pipetted over the slice and the holder is placed into a heated stage with 5% CO_2, 95% balance air lightly passed over the top, and fresh media provided at the rate of 4 ml/h. Images are collected using a 20X plan achromat phase objective (0.75 n.a.) on a Nikon TE200 inverted microscope with a Dage RC300 video camera. A 4-D script was created for IPLab Spectrum software (Scanalytics, Inc.) that provided for automated shutter control and the collection of 3-D stacks of three images per time point (every 5 min) that provided a clearer image of each field. After video collection, phase images were taken from each slice to record the location of each field. For analysis, each image stack is analyzed as single frame by selecting the brightest pixel value from the stack for each x–y coordinate of the stack. These frames are then compiled into a video sequence. Video sequences are then adjusted for slice movement by matching non-drifting background objects that appear in each frame. Video sequences are analyzed for absolute distance and net distance traveled by each moving cell, the speed and top speed of each moving cell, and the percentage of migratory time of each moving cell. A moving cell is defined as an object that travels at least 1 cell diameter (about 12 μm) over the course of a video sequence. Absolute distance is calculated as the sum of distances traveled from frame to frame. Net distance is calculated as the distance between a cell's starting position (location in 1st frame) and ending position (location in last frame). Speed is calculated by dividing the duration of time that a cell was visible in a video sequence by the absolute distance traveled by that cell.

In vitro studies: Transwell model

Slices are treated identically as for coverslips through cutting, incubation at 4°C, and then at 36°C in media for 35 min. Slices then are gently laid on transwell membranes in 6 well dishes (Fisher, Catalog #07200169) with 2 ml of incubation media. After 2 or 3 days *in vitro*, media is removed and sections fixed as described above.

Immunocytochemistry

To detect specific immunoreactive antigens, sections or slices (at 4°C) are pretreated with 0.1 *M* glycine in 0.05 *M* PBS (pH 7.5; sections from perfusion fixed tissue only) followed by 0.5% sodium borohydride in 0.05 *M* PBS and 5% normal goat serum (NGS) with 0.3% Triton-X 100 (Tx)/PBS and 1% hydrogen peroxide. Washes with PBS separate each step and times are extended for 250 μm thick slices *versus* 50 μm thick sections. The tissue is then incubated with the designated antisera for 2–3 nights (*in vivo* sections) or 6 nights (*in vitro* slices). For secondary antibody processing, tissue was washed with PBS/1% NGS with 0.02% Triton-X100 prior to incubation with the appropriate biotinylated secondary antibody (Vector Laboratories) for 2 hr at room temperature (*in vivo* sections) or overnight at 4°C (*in vitro* slices). After washes in PBS/0.02% Triton-X100 (all at room temperature) tissue is then incubated with Vectastain ABC reagent (Vector Laboratories). Black reaction product is produced in 50 μm tissue sections using 0.25% 3,3′-diaminobenzidine (DAB, freshly dissolved in tris buffered saline and filtered) with 0.2% nickel ammonium sulfate and 0.02% hydrogen peroxide during a 5 min reaction period. Brown reaction product was produced in 250 μm slices using DAB in PBS without nickel. DAB was prepared without hydrogen peroxide for thick slices and hydrogen peroxide was added after a 15 min pre incubation, and reactions were allowed to proceed for 20 min.

All sections or slices are mounted onto gelatin-coated slides. The tissue sections from experiments where DAB is used for visualization are dehydrated and coverslipped using Permount. Fluorescent tissue sections or slices are coverslipped using Vectashield reagent (Vector Laboratories) or using an aqueous mounting media that dries following application (Accurate Chemical and Scientific Corp.).

Results and Discussion

Using our slice model, after 2 or 3 days *in vitro* there is significant evidence of cell rearrangements simply based on Nissl stain analyses. For example, in hypothalamic slices, cell groups that are not evident at the time of embryo harvesting and *in vitro* placement become evident after 2 to 3 days *in vitro* (*e.g.,* Tobet *et al.*, 1999; Henderson *et al.*, 1999; Fig. 1A–E). In each case, the rearrangements result in nuclear formation similar to that found over the comparable period *in vivo*. Formation of apparently normal cell groups *in vitro* in coronal sections from either the preoptic area (Henderson *et al.*, 1999) or the medial basal hypothalamus (Tobet *et al.*, 1999) suggests that key factors reside locally within the plane of the slices. This is notable in light of the fact that there are cell movements that are known to occur in the rostral caudal dimension (*e.g.,* GnRH neurons, see below). Thus for the formation of at least one cell group in the preoptic area and for the VMH there may be minimal contributions from factors located rostral or caudal to the location of the nascent nuclei. This does not imply that there is no contribution from factors that influence tangential migration as there is significant evidence for migration in both regions that is not along the orientation of radial glial fibers, but rather more dorsal-ventral in orientation (Henderson *et al.*, 1999; Dellovade *et al.*, 2001).

An interesting twist on movement relative to the orientation of radial glia fibers arises in the preoptic area where there are dueling orientations of radial glial fibers ventral to the anterior commissure (Tobet *et al.*,

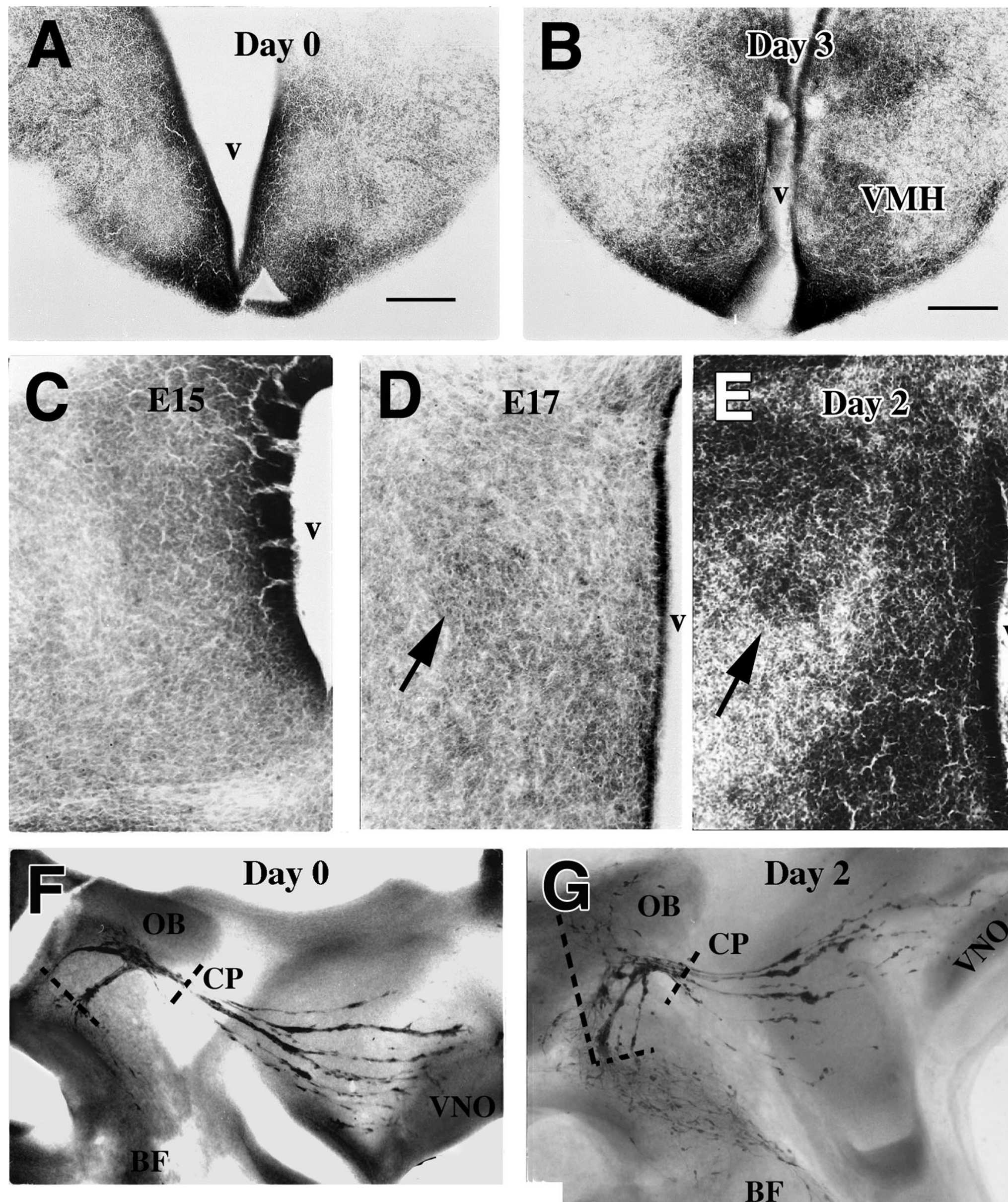

Fig. 1. Digital images show examples of developmental progression and cell rearrangements in slices from different brain regions of embryonic mice. Panel A shows the Nissl-stained cytoarchitecture of a coronal slice cut on embryonic day 15 that includes the region that will form the ventromedial nucleus of the hypothalamus (VMH). Panels B shows a slice that was taken as the same time as the one in A, but left *in vitro* for 3 days prior to processing for Nissl stain revealing the formation of an apparent nuclear grouping similar to the VMH. Panels C and D show the normal development of a cell group in the mouse preoptic area in coronal sections, again using Nissl stain (black arrow). After 2 days *in vitro* following an E15 plating, a similar cell group (black arrow) is discernible in brain slices through the region (Panel E). Panels F and G show the redistribution of cells immunoreactive for GnRH in sagittal slices through embryonic day 13 heads on the day of plating (Panel F) and after 2 days *in vitro* (Panel G). GnRH neurons originate in the vomeronasal organ (VNO) in the nasal compartment and migrate across the cribriform plate (CP) into the basal forebrain (BF). After 2 days *in vitro* GnRH neurons can be seen significantly more in the BF and significantly more spread caudally and ventrally within the BF. Images are adapted from previous publications (Tobet *et al.*, 1999; Henderson *et al.*, 1999; Bless *et al.*, 2000). V = third ventricle; OB = olfactory bulb.

1995; Henderson *et al.*, 1999). One way to visualize the alternate migration of cells along these orthogonal planes is to view the changing positions of cells labeled with the mitotic indicator BrdU. In one experiment, pregnant mice were injected on E15 and slices were placed *in vitro* for 2–3 days. When these slices were processed for BrdU immunocytochemistry, labeled nuclei were noted in progressive lateral and ventral positions in the region of the preoptic area, consistent with migration of cells in both directions along the orientation of radial glial fibers (Fig. 2). Similarly, video microscopy studies showed migration in this same region along both dorsal-ventral and medial-lateral orientations (Henderson *et al.*, 1999). Experiments

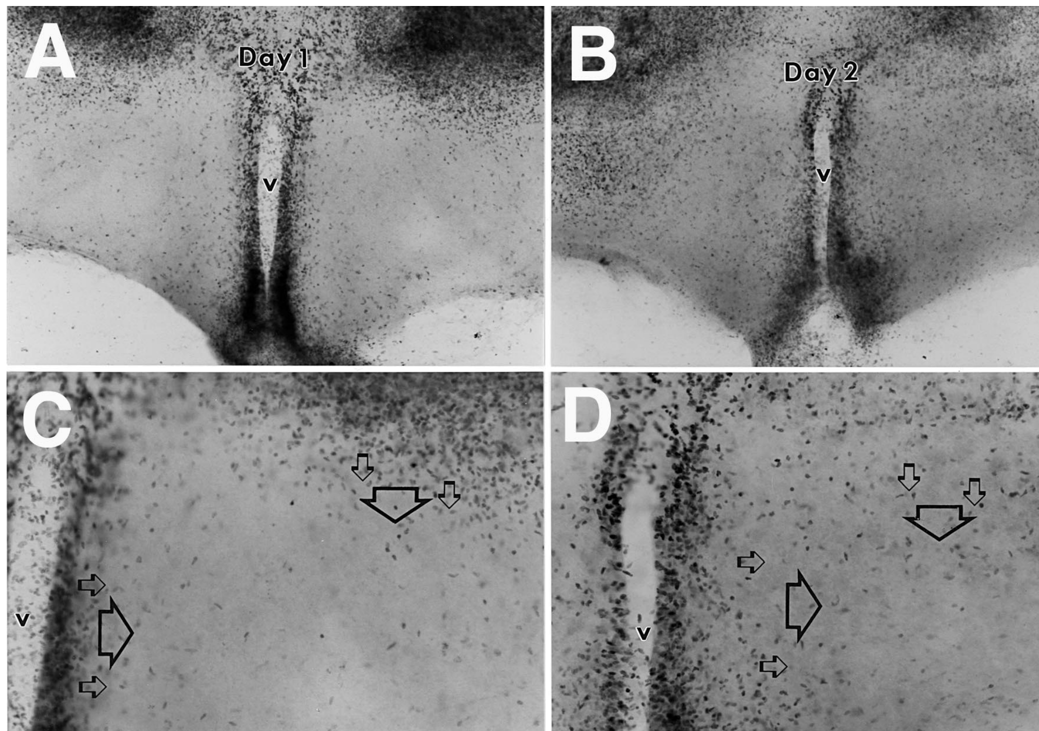

FIG. 2. Digital images show examples of the changing positions of cells labeled *in vivo* with BrdU (E14) one day prior to slicing and plating on E15. The images are taken from slices through the preoptic area that remained *in vitro* for 1 (Panel A and C) or 2 days (Panels B and D). Panels C and D provide higher magnification of the area of interest adjacent to the third ventricle (v) in panels A and B. Over time BrdU immunoreactive cells can be seen in more ventral and more lateral positions (compare the positions of the large and small open arrows in C with those in D) suggesting movements in both orientations in agreement with data from studies utilizing video microscopy in the preoptic area (Henderson *et al.*, 1999).

using retroviral methods in developing chick brain *in vivo* to label clones of cells with a lacZ reporter also suggested that there is significant dorsal ventral migration in the hypothalamus (Arnold-Aldea and Cepko 1996; Golden *et al.*, 1997). While each method has potential methodological caveats, the concordance of the results using the different methods lends promise to the conclusions reached.

Following the changing position of immunocytochemically-identified cells indicates movements within slices maintained *in vitro* over several days. This is particularly true when the total number of cells can be counted and shown to be the same over time. Cells that contain the peptide GnRH are found in stereotyped positions in the nasal compartment and basal forebrain of embryonic mice. If slices are created on either E12 or E13, then after 1 to 3 days *in vitro* GnRH neurons are found in positions within sagittal slices that show the normal pattern of movement over the course of development *in vivo* (Tobet *et al.*, 1996; Bless *et al.*, 2000). Importantly, the number of GnRH neurons detected after 1–3 days *in vitro* remains the same at approximately 400–600 neurons per slice. Interestingly, when this same type of experiment was carried out in rats, only 25% of the GnRH neuronal population could be accounted for *in vitro* after 1, 2 or 3 days (S.A.T., unpublished data). In explants from primate olfactory placodes, GnRH neurons become more detectable after one to three weeks *in vitro* (Terasawa *et al.*, 1993). In explants from E11 mouse olfactory placode, typically only 25% or fewer of the GnRH neuronal population has been accounted for (Fueshko *et al.*, 1998). For GnRH neurons, a problem is that the neurons can only be detected if they make sufficient GnRH to be detected; there are no independent markers. Thus when the cell number changes it is not determined whether the cells die or simply stop synthesizing peptide. This presents an additional problem in that the missing cells may comprise a subset that may have special properties that are not known.

We use video microscopy to follow the migration of fluorescently labeled cells in brain slices from mice maintained in serum-free media for 1 to 3 days. Our early studies utilized DiI to label cells randomly (Tobet *et al.*, 1994; Henderson *et al.*, 1999; Dellovade *et al.*, 2001; Davis *et al.*, 2002) and showed that cell movements in the hypothalamus were of approximately the same rates as those in other brain regions; between 15 and 30 microns/hr (O'Rourke *et al.*, 1992; Komura and Rakic, 1992, 1993; Nadarajah *et al.*, 2001). Our results provide a picture of the different orientations of movement that occur in different portions of the developing hypothalamus and preoptic area. It was a key finding in cortex that cell migration occurs not only radially from the ventricular zone toward the pial surface, but also tangentially (O'Rourke *et al.*, 1992) from the gan-

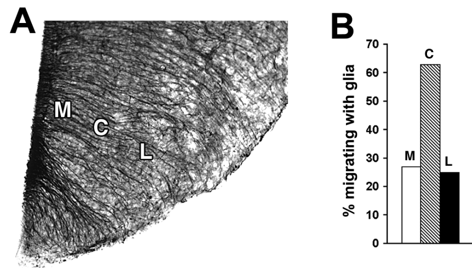

FIG. 3. Digital image (Panel A) shows the pattern of RC2 immunoreactive radial glial processes that span the region of the developing ventromedial nucleus of the hypothalamus (VMH). The movement of DiI labeled cells in this region was analyzed quantitatively for speed and orientation. The rate of movement was similar across the region, but the orientation of motion (Panel B) was significantly aligned with radial glial processes only in the central (C) zone of the developing nucleus and not in the medial (M) or lateral (L) zones. Thus specific cues (perhaps GABAergic; see text) are likely important for determining when cells begin to follow radial glial fibers and when they stop during the development of the VMH. Adapted from Dellovade *et al.*, 2001.

glionic eminence to the cerebral cortex (Anderson *et al.*, 1997*b*; Jimenez *et al.*, 2002). Video microscopy is now revealing potential changes in the behavior or migrating cells such that neurons may switch from tangential migration to ventricle directed migration prior to a radial migration back out to the cortical plate (Nadarajah *et al.*, 2002). In the hypothalamus and preoptic area there is significant tangential migration (Tobet *et al.*, 1994; Henderson *et al.*, 1999; Dellovade *et al.*, 2001; Davis *et al.*, 2002) in addition to the expected radial migration (Altman and Bayer, 1986). As noted above, tangential migration in hypothalamus has been also suggested by retroviral studies in chick brain (Arnold-Aldea and Cepko, 1996; Golden *et al.*, 1997). We are currently taking advantage of animals that are transgenic for GFP under the control of selective promoters (*e.g.,* the promoters for GnRH (Suter *et al.*,2000), SF-1 (Stallings *et al.*, 2002), and Thy-1 (Feng *et al.*, 2000). These mice offer the ability to better identify cells that are viewed compared to dye labeling. Furthermore, by labeling significantly larger populations of cells within each slice, we can view the movements of more cells per field than was previously possible. Our studies in the region of the VMH using DiI showed movements that were predominantly perpendicular to the orientation of glial guides close to the third ventricle, but parallel to the orientation of glial processes in the center of the presumptive VMH (Fig. 3). Due to the low level of labeling with DiI per field of view, this picture of movements took many slices over a substantial timeframe to accumulate the data. Using slices from Thy-1/YFP transgenic mice, a comprehensive image of all of the types of movement have been visible even within a single slices (Fig. 4).

The accessibility of an *in vitro* system that provides for relatively normal brain development over key brief windows of time allows for the significant testing of important mechanisms. Early slice studies in the cerebellum tested the role of calcium channels and

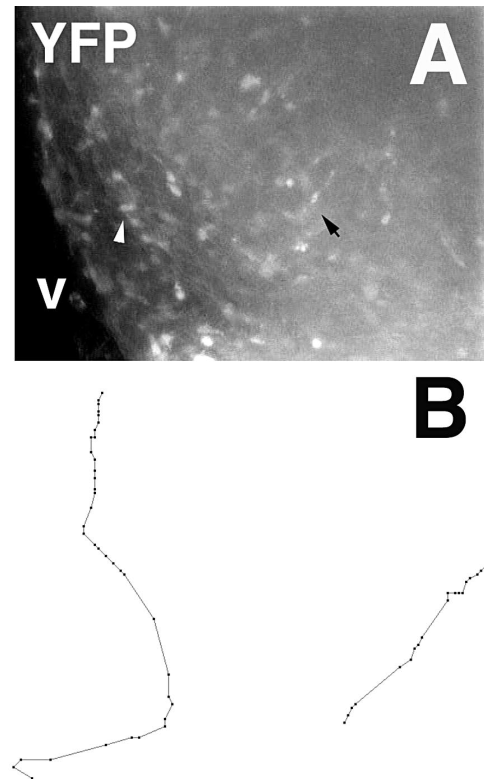

FIG. 4. Digital image (Panel A) shows the fluorescent cell pattern in a slice taken from a thy-1 YFP mouse at E14 and viewed approximately 24hr later. The slice was taken through the region of the developing VMH and the field of view includes the dorsal portion of the developing arcuate nucleus (lower left in panel A) in addition to the medial and central zones of the nascent VMH. The cells indicated by white arrowhead and black arrow are depicted for their movement patterns schematically in panel B. The cell indicated by the white arrowhead is depicted schematically on the left and moved at 21 μm/hr perpendicular to the third ventricle (v) prior to turning to move parallel to the ventricle over the course of 2hr. The cell indicated by the black arrow is depicted schematically on the right and moved at 17 μm/hr parallel to the orientation of radial glial processes in the central region of the nascent VMH. In the schematic images, each dot represents the position of the cell at 5 min intervals.

NMDA receptors (Komura and Rakic, 1992, 1993). We began our studies by determining whether there might be sex differences in cell migration, and by examining the roles of GABA on cell movements in the developing hypothalamus and preoptic area. In the preoptic area we have shown sex differences in cell migration (Henderson *et al.*, 1999), and preliminary data suggests that estradiol may influence the orientation of cell movement in the region (S.A.T., unpublished data). Our studies on the roles of GABA have concentrated on the migration of GnRH neurons from the nasal compartment into the basal forebrain, and on the formation of the VMH. Others have shown a role for GABA in cortical cell migration (Behar *et al.*, 1996, 1998, 2000) acting through both $GABA_A$ and $GABA_B$ receptor mechanisms. It is clear that GABAergic signaling alters cell movements for both GnRH neurons (Bless *et al.*, 2000; Heger *et al.*, 2003) and cells in the

region of the VMH (Dellovade *et al.*, 2001; Davis *et al.*, 2002). While $GABA_B$ receptors may play a significant role in the movement of VMH neurons (inhibiting movement; Davis *et al.*, 2002), it is less likely that they play a role in the movement of GnRH neurons. We found no effects of $GABA_B$ agonist treatments (baclofen) on GnRH neuron positions *in vivo* or *in vitro* (Tobet *et al.*, 2001).

A common feature of cell movements in all brain regions may be the necessity of neurons to detach from fibers that they use for migratory guidance. In the cerebral cortex, reelin has been hypothesized to help signal when migrating cortical neurons release their radial glial guides (Rice and Curran, 2001). For GnRH neurons, GABAergic signaling may help determine the point in the migration when GnRH neurons release their neuronophilic guide fibers that originate with vomeronasal neuroepithelial cells in the peripheral olfactory system. Treatment with the $GABA_A$ receptor antagonist bicuculline caused GnRH neurons to release from peripherin immunoreactive fibers earlier in their migratory path than normal. This caused an abnormal pattern of GnRH neuron system organization in the short term (Bless *et al.*, 2000) and a long-term change in the position of GnRH neuron in the region of the organum vasculosum of the lamina terminalis in adulthood (S.A.T., unpublished data). The functional consequence of this was a significant 3-day delay in puberty as indicated by the day of vaginal opening for animals exposed to bicuculline during gestation. In a complementary experiment, transgenic over-expression of the GABA synthesis enzyme, glutamic acid decarboxylase, in a subset of GnRH neurons led to aberrant GnRH neuronal positions in development, and deficits in reproductive function in adult mice (Heger *et al.*, 2003). In the region of the VMH, $GABA_A$ receptor agonist (muscimol) or antagonist (bicuculline) treatments *in vitro* caused cells to alter their orientation of movement relative to radial glial guides. Thus in 3 very different situations of migratory guidance, neuronophilic (GnRH) or gliophilic for laminar (cerebral cortex) or nuclear organization (VMH), the signals for release from guiding fibers may be critical determinants of final positions.

Much information is needed on the nature of cell movements that could result in the formation of nuclear groups, scattered cells with defined phenotypes, and well-ordered layers. Nuclear groups have irregular boundaries that must be discerned. Layers have regular boundaries. In the hypothalamus, many neuroendocrine cells are scattered across regions without clear boundaries, but with highly stereotyped cell distributions. In each case, the cues that determine final cell positions are critical determinants for producing a nervous system that can then be wired for appropriate connectivity. The use of *in vitro* slices that allow the rearrangement of cells following relatively normal patterns provides a powerful model system for studies that may help determine the cues that shape the developing neuroendocrine brain.

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REFERENCES

Alifragis P., J. G. Parnavelas, and B. Nadarajah. 2002. A novel method of labeling and characterizing migrating neurons in the developing central nervous system. Exp. Neurol. 174:259–265.

Altman, J. and S. A. Bayer. 1986. The development of the rat hypothalamus. Adv. Anat. Embryol. Cell Biol. 100:1–178.

Anderson S. A., M. Qiu, A. Bulfone, D. D. Eisenstat, J. Meneses, R. Pedersen, and J. L. Rubenstein. 1997*a*. Mutations of the homeobox genes Dlx-1 and Dlx-2 disrupt the striatal subventricular zone and differentiation of late born striatal neurons. Neuron 19:27–37.

Anderson, S. A., D. D. Eisenstat, L. Shi, and J. L. Rubenstein. 1997*b*. Interneuron migration from basal forebrain to neocortex: Dependence on Dlx genes. Science 278:474–476.

Arnold-Aldea, S. A. and C. L. Cepko. 1996. Dispersion patterns of clonally related cells during development of the hypothalamus. Dev. Biol. 173:148–161.

Barlow, J. Z. and G. W. Huntley. 2000. Developmentally regulated expression of Thy-1 in structures of the mouse sensory-motor system. J. Comp. Neurol. 421:215–233.

Behar, T. N., Y. X. Li, H. T. Tran, W. Ma, V. Dunlap, C. Scott, and J. L. Barker. 1996. GABA stimulates chemotaxis and chemokinesis of embryonic cortical neurons via calcium-dependent mechanisms. J. Neurosci. 16:1808–1818.

Behar, T. N., A. E. Schaffner, C. A. Scott, C. O'Connell, and J. L. Barker. 1998. Differential response of cortical plate and ventricular zone cells to GABA as a migratory stimulus. J. Neurosci. 18:6378–6387.

Behar, T. N., A. E. Schaffner, C. A. Scott, C. L. Greene, and J. L. Barker. 2000. GABA receptor antagonists modulate postmitotic cell migration in slice cultures of embryonic rat cortex. Cereb. Cortex 10:899–909.

Bless, E. P., A. Westaway, G. A. Schwarting, and S. A. Tobet. 2000. Effects of $GABA_A$ receptor manipulation on migrating gonadotropin-releasing hormone (GnRH) neurons through the entire migratory route *in vivo* and *in vitro*. Endocrinology 141:1254–1262.

Bless, E. P., H. J. Walker, K. W. Yu, S. M. Moenter, G. A. Schwarting, and S. A. Tobet. 2002. Video microscopy of GnRH neurons reveals differences in migration through nasal compartment, cribriform plate and brain. Soc. Neurosci. Abstr.

Chalfie, M., Y. Tu, G. Euskirchen, W. W. Ward and D. C. Prasher. 1994. Green fluorescent protein as a marker for gene expression. Science 263:802–805.

Davis, A. M., T. R. Henion, and S. A. Tobet. 2002. $GABA_B$ receptors and the development of the ventromedial nucleus of the hypothalamus. J. Comp. Neurol. 449:270–280.

Davis, A. M., M. L. Seney, N. R. Stallings, L. Zhao, K. L. Parker, and S. A. Tobet. 2004. Loss of steroidogenic factor 1 after topography in the mouse ventromedial nucleus of the hypothalamus. J. Neurobiol. (In press)

Dellovade, T. L., M. Young, E. P. Ross, R. G. Henderson, K. Caron, K. Parker, and S. A. Tobet. 2000. Disruption of the gene encoding SF-1 alters the distribution of hypothalamic neuronal phenotypes. J. Comp. Neurol. 423:579–589.

Dellovade, T. L., A. M. Davis, C. Ferguson, W. Sieghart, G. E. Homanics, and S. A. Tobet. 2001. GABA influences the development of the ventromedial nucleus of the hypothalamus. J. Neurobiol. 49:264–276.

Feng, G., R. H. Mellor, M. Bernstein, C. Keller-Peck, Q. T. Nguyen,

M. Wallace, J. M. Nerbonne, J. W. Lichtman, and J. R. Sanes. 2000. Imaging neuronal subsets in transgenic mice expressing multiple spectral variants of GFP. Neuron 28:41–51.

Fueshko, S. M., S. Key, and S. Wray. 1998. GABA inhibits migration of luteinizing hormone-releasing hormone neurons in embryonic olfactory explants. J. Neurosci. 18:2560–2569.

Gleeson, J. G., and C. A. Walsh. 2000. Neuronal migration disorders: from genetic diseases to developmental mechanisms. Trends Neurosci. 23:352–359.

Golden, J. A., J. C. Zitz, K. McFadden, and C. L. Cepko. 1997. Cell migration in the developing chick diencephalon. Development 124:3525–3533.

Heger, S., M. Seney, E. Bless, G. A. Schwarting, M. Bilger, A. Mungenast, S. R. Ojeda, and S. A. Tobet. 2003. Overexpression of glutamic acid decarboxylase-67 (GAD-67) in GnRH neurons disrupts migratory fate and female reproductive function in mice. Endocrinology 144:2566–2567.

Henderson, R. G., A. E. Brown, and S. A. Tobet. 1999. Sex differences in cell migration in the preoptic area/anterior hypothalamus of mice. J. Neurobiol. 41:252–266.

Ikeda, Y., W. H. Shuen, H. A. Ingraham, and K. L. Parker. 1994. Developmental expression of mouse steroidogenic factor-1, an essential regulator of the steroid hydrolases. Mol. Endocrinol. 8:654–662.

Ikeda, Y., Y. Takeda, T. Shikayama, T. Mukai, S. Hisano, K. I. Morohashi. 2001. Comparative localization of Dax-1 and Ad4BP/SF-1 during development of the hypothalamic-pituitary-gonadal axis suggests their closely related and distinct functions. Dev. Dyn. 220:363–376.

Jimenez, D., L. M. Lopez-Mascaraque, F. Valverde, and J. A. De Carlos. 2002. Tangential migration in neocortical development. Dev. Biol. 244:155–169.

Komuro, H. and P. Rakic. 1992. Selective role of N-type calcium channels in neuronal migration. Science 257:806–809.

Komuro, H. and P. Rakic. 1993. Modulation of neuronal migration by NMDA receptors. Science 260:95–97.

Marshall, J., R. Molloy, G. W. J. Moss, J. R. Howe, and T. E. Hughes. 1995. The jellyfish green fluorescent protein: A new tool for studying ion channel expression and function. Neuron 14:211–215.

Miller, M. W. and R. Nowakowski. 1988. Use of bromodeoxyuridine-immunohistochemistry to examine the proliferation, migration and time of origin of cells in the central nervous system. Brain. Res. 457:44–52.

Moriyoshi, K., L. J. Richards, C. Akazawa, D. D. M. O'Leary, and S. Nakanishi. 1996. Labeling neural cells using adenoviral gene transfer of membrane-targeted GFP. Neuron 16:255–260.

Nadarajah, B., J. E. Brunstrom, J. Grutzendler, R. O. Wong, and A. L. Pearlman. 2001. Two modes of radial migration in early development of the cerebral cortex. Nat. Neurosci. 4:143–150.

Nadarajah, B., P. Alifragis, R. O. Wong, and J. G. Parnavelas. 2002. Ventricle-directed migration in the developing cerebral cortex. Nat. Neurosci. 5:218–224.

Okada, A., R. Lansford, J. M. Weimann, S. E. Fraser, and S. K. McConnell. 1999. Imaging cells in the developing nervous system with retrovirus expressing modified green fluorescent protein. Exp. Neurol. 156:394–406.

O'Rourke, N., M. E. Dailey, S. J. Smithe, and S. K. McConnell. 1992. Diverse migratory pathways in the developing cerebral cortex. Science 258:299–302.

Rice, D. S. and T. Curran. 2001. Role of the reelin signaling pathway in central nervous system development. Annu. Rev. Neurosci. 24:1005–1039.

Roberts, J. S., N. A. O'Rourke, and S. K. McConnell. 1993. Cell migration in cultured cerebral cortical slices. Dev. Biol. 155: 396–408.

Roselli, C. E., E. Z. Jorgensen, M. W. Doyle, and O. K. Ronnekleiv. 1997. Expression of the orphan receptor steroidogenic factor-1 mRNA in the rat medial basal hypothalamus. Mol. Brain Res. 44:66–72.

Shinoda, K., H. Lei, H. Yoshii, M. Nomura, M. Nagano, H. Shiba, H. Sasaki, Y. Osawa, Y. Ninomiya, O. Niwa, K. I. Morohashi, and E. Li. 1995. Developmental defects of the ventromedial hypothalamic nucleus and pituitary gonadotroph in the *Ftz-F1* disrupted mice. Dev. Dyn. 204:22–29.

Spergel, D. J., U. Kruth, D. F. Hanley, R. Sprengel, and P. H. Seeburg. 1999. GABA- and glutamate-activated channels in green fluorescent protein-tagged gonadotropin-releasing hormone neurons in transgenic mice. J. Neurosci. 19:2037–2050.

Stallings, N. R., N. A. Hanley, G. Majdic, L. Zhao, M. Bakke, and K. L. Parker. 2002. Development of a transgenic green fluorescent protein lineage marker for steroidogenic factor 1. Mol. Endocrinol. 16:2360–2370.

Suter, K. J., W. J. Song, T. L. Sampson, J. P. Wuarin, J. T. Saunders, F. E. Dudek, and S. M. Moenter. 2000. Genetic targeting of green fluorescent protein to gonadotropin-releasing hormone neurons: Characterization of whole-cell electrophysiological properties and morphology. Endocrinology 141:412–419.

Terasawa, E., C. D. Quanbeck, C. A. Schulz, A. J. Burich, L. L. Luchansky, and P. Claude. 1993. A primary cell culture system of luteinizing hormone releasing hormone neurons derived from embryonic olfactory placode in the rhesus monkey. Endocrinology 133:2379–2390.

Thomas, A., D. S. Kim, R. L. Fields, H. Chin, and H. Gainer. 1998. Quantitative analysis of gene expression in organotypic slice-explant cultures by particle-mediated gene transfer. J. Neurosci. Methods 84:181–191.

Tobet, S. A., T. Chickering, I. Kaddis, J. E. Crandall, and G. A. Schwarting. 1994. Can gonadal steroids influence cell position in the developing brain? Horm. Behav. 28:320–327.

Tobet, S. A., R. G. Paredes, T. W. Chickering, and M. J. Baum. 1995. Telencephalic and diencephalic origin of radial glial processes in the developing preoptic area/anterior hypothalamus. J. Neurobiol. 26:75–86.

Tobet, S. A., I. K. Hanna, and G. A. Schwarting. 1996. Migration of neurons containing gonadotropin releasing hormone (GnRH) in slices from embryonic nasal compartment and forebrain. Dev. Brain Res. 97:287–292.

Tobet, S. A., R. G. Henderson, P. J. Whiting, and W. Sieghart. 1999. Special relationship of gamma- aminobutyric acid to the ventromedial nucleus of the hypothalamus during embryonic development. J. Comp. Neurol. 405:88–98.

Tobet, S. A., E. P. Bless, and G. A. Schwarting. 2001. Developmental aspects of the GnRH neuronal system. Mol. Cell Endo. 185: 173–184.

Toran-Allerand, C. D. 1976. Sex steroids and the development of the newborn mouse hypothalamus and preoptic area *in vitro*: Implications for sexual differentiation. Brain Res. 106:407–412.

Toran-Allerand, C. D. 1980. Sex steroids and the development of the newborn mouse hypothalamus and preoptic area *in vitro*. II. Morphological correlates and hormonal specificity. Brain Res. 189:413–427.

Wray, S., R. T. Zoeller, and H. Gainer. 1989. Differential effects of estrogen on luteinizing hormone-releasing hormone gene expression in slice explant cultures prepared from specific rat forebrain regions. Mol Endocrinol 3:1197–1206.

Zernicke-Goetz, M., J. Pines, K. Ryan, K. R. Siemering, J. Haseloff, M. J. Evans, and J. B. Gurdon. 1996. An indelible lineage marker for Xenopus using a mutated green fluorescent protein. Development 122:3719–3724.