Migratory Response of Interneurons to Different Regions of the Developing Neocortex

The interactions between migrating interneurons and their environment that lead to stereotypic migration pathways remain largely undefined. We have used time-lapse imaging to record the migratory responses of labeled interneurons to different regions of the migratory pathway in organotypic slice cultures. We tested the hypothesis that the length of the migratory pathway is not equally permissive for interneuron migration, with separate zones of inhibition and attraction. Three different experimental approaches were used to address this issue, including explant cocultures, cortical overlay cultures, and rotation of cortical slices. The results clearly identify the lateral region to be an attractive substrate for interneuron entry at embryonic day 12.5, whereas the medial region at this stage contains a zone of inhibition. This property of the medial neocortex is temporally regulated with switching from inhibition to attraction within 24 h. We suggest that this temporal regulation may provide a mechanism for gating the entry of interneurons into the hippocampus while ensuring that cortical interneurons are properly confined within the neocortical wall. In this manner, interneurons arising from common precursors and sharing common migratory pathways are able to populate different pallial structures.

Keywords: GAD67, interneuron, migration, neocortex

Introduction

During development, neurons can arise from a progenitor source that is far removed from where the differentiated neuron becomes integrated into specific neuronal circuits (see review, Marin and Rubenstein 2001; Sotelo 2004). In the neocortex, 2 modes of cell migration are present: glial-guided and glial-independent radial migration of projection neurons and the tangential migration of γ-aminobutyric acidergic (GABAergic) interneurons (Kriegstein and Noctor 2004). The projection neurons are generated from the germinial ventricular zone and migrate radially along the ventricular–pial axis, in a well-described “inside-out” manner, to form the laminated neocortex. In contrast, interneurons originate from a source of progenitor cells in the ganglionic eminences and migrate as postmitotic neurons into the neocortex, hippocampus, and olfactory bulb (Anderson and others 1997, 1999; Lavdas and others 1999; Pleasure and others 2000; Wichterle and others 2002; Nery and others 2002).

From the onset, postmitotic interneurons are guided by a set of extrinsic factors present in the surrounding environment. For example, Slit-1/2 and semaphorin3A, expressed in the striatum, prevent the ventral migration of neurons from the medial ganglionic eminence (MGE) into the striatum (Marin and others 2001, 2003; Tamamaki, Fujimori, and others 2003). Structures developing medioventral to the MGE, that is, hypothalamus, thalamus, or septum, appear to be nonpermissive for cell migration (Wichterle and others 2003); thus, the direction of migrating neurons is restricted to the dorsal regions leading to the neocortex.

Recent work has verified that both repulsive and attractive cues act in a combinatorial manner to shape interneuron migration. Neuregulin 1-cystein-rich domain, brain-derived neurotrophic factor; neurotrophin-4, glial cell line-derived neurotrophic factor; and hepatocyte growth factor have been shown to stimulate migration across the corticostriatal boundary (Powell and others 2001; Polleux and others 2002; Flames and others 2004; Pozas and Ibanez 2005). Upon entering the neocortex, interneurons migrate from the lateral to medial position, and the question arises as to what factors influence this process. Not all interneurons that enter the neocortical domain are destined to stay within the cortical wall. GABAergic neurons are known to traverse the cortical wall before arriving into the hippocampal anlage (Pleasure and others 2000; Polleux and others 2002). These studies suggest that interneurons destined for the neocortex and hippocampus arise from a common germinal zone (MGE), and this raises the issue of how they are able to find their separate targets.

In this study, we sought to determine whether or not the entire length of the cortical substratum is equally permissive for interneurons leaving the corticostriatal boundary at embryonic day 12.5 (E12.5). To address this, we have used time-lapse imaging to monitor migrating interneurons present in short-term organotypic slice cultures. These cultures were generated from mice carrying a green fluorescent protein (GFP) targeted into the glutamic acid decarboxylase (GAD67) locus (Tamamaki, Yanagawa, and others 2003). E12.5 to E13.5 mouse embryos were isolated from timed matings of heterozygous GAD67+/−/gfp males and C57BL/6j females (E0.5, morning of vaginal plug detection). Time-mated pregnant mice were anesthetized and
GAD67\textsuperscript{+/+} embryos identified by the intensity of forebrain fluorescence under epifluorescent illumination. Coronal slices were prepared from embryonic forebrain as previously described (Noctor and others 2001). Slices were selected from the anterior half of the cerebral hemispheres at the plane where MGE and lateral ganglionic eminence (LGE) could be separately identified and cultured individually on slice culture inserts. Dissected slices were manipulated using microscalpel blades and tungsten needles and incubated for 3–4 h before the first images (0-h time point) of interneuron migration were collected.

**Confocal Time-Lapse Imaging**

Interneurons positive for GFP were imaged on an inverted Zeiss Axiovert 200-LSM 5-Pa confocal microscope using 488-nm excitation and 505- to 530-nm emission filters. All images were captured using a \( \times 10 \) objective set at the sectional plane that contained the majority of migrating cells (optical slice \(<10 \mu m\)). Higher magnification was achieved using a water immersion \( \times 40 \) objective, and projection images were compiled from Z-stacks generated to include all visible processes. Images were acquired at 24-h intervals, for up to 72 h, to allow visualization of the migratory population of interneurons into the neocortex, and transmitted light images were taken at each time point as a point of reference. Time-lapse imaging was performed using minimum laser exposure to prevent photodamage and bleaching, and between time points, slices were kept in a humidified incubator at 37°C, 5% CO\(_2\). Montages of brightfield and fluorescent images were assembled and aligned using Adobe Photoshop.

**Results**

We have utilized the GAD67-GFP (Δneo) mouse to evaluate factors that affect migration of interneurons throughout the neocortex (Tamamaki, Yanagawa, and others 2003). This reporter line had been previously used to characterize the generation of cortical interneurons and has proven to be a reliable marker in the analysis of all subpopulations of interneurons (Tamamaki, Yanagawa, and others 2003). To examine how the initial migratory front of interneurons respond to the neocortex, we have cultured slices of E12.5 telencephalon and monitored how interneurons respond differentially to regions along the mediolateral axis.

**Interneuron Migration Is Inhibited by the Medial Neocortex**

To determine whether the entire extent of the neocortex was permissive to interneuron migration, we utilized a coculture system of GAD67\textsuperscript{+/+} and wild-type tissue. Previous experiments in our laboratory have shown that interneurons can traverse the ventricular zone of the neocortex and enter into the cortical plate (Q. Sang, personal communication). By explanting an E12.5 GAD67\textsuperscript{+/+} MGE into the ventricle of an E12.5 wild-type slice, it was possible to visualize in real time the degree of interneuron migration along the entire mediolateral axis (Fig. 1A,B). Cultures were monitored every 6–12 h, and after 48 h, there was a clear disparity in the extent of migration along the mediolateral axis (Fig. 1C). Few interneurons entered into the medial neocortex (Fig. 1D), whereas many interneurons were found more laterally, suggesting that they have migrated in the opposite direction with their leading processes pointing in the reverse direction (Fig. 1E). Interneurons positioned in the ventrolateral region migrated into the lateral neocortex in large numbers (Fig. 1F). To delineate the spatial changes of receptivity to interneuron migration, smaller fragments of GAD67\textsuperscript{+/+} MGE were cocultured at various positions along the mediolateral axis to monitor migration of individual interneurons (data not shown). These experiments showed a similar effect with the lateral neocortex being a more permissive environment than the medial neocortex for E12.5 interneurons.

Although the juxtapositioning of MGE explants in the ventricle is artifactual, it validated the hypothesis that different

**Figure 1.** Inhibition of interneuron migration by the medial neocortex. (A) Schematic illustration of the brain slice coculture showing explant of GAD67\textsuperscript{+/+} MGE in the lateral ventricle at E12.5. (B) Brightfield–fluorescent image at 0 h showing explant containing GFP-positive interneurons placed adjacent to the ventricular zone (dashed line). (C) After 24 h in culture, interneurons show various levels of migration throughout the neocortex. Higher magnification of boxed regions show (D) few interneurons enter into the medial neocortex, (E) interneurons that enter more laterally migrate in the reverse direction (arrow), and (F) bidirectional migration of interneurons into the lateral neocortex. Scale bar: (B, C), 100 μm; (D, E, F), 20 μm.
cortical substrates along the mediolateral axis have different receptivity to the influx of interneurons. To test this further, overlay cultures were performed where a slice of GAD67+/gfp E12.5 telencephalon was cultured with an overlying piece of neocortex obtained from a wild-type littermate. Coronal slices of the wild-type neocortex were cut to the same thickness, and a strip of neocortex was placed over the GAD67 slice. Although this may effectively double the number of cells when the slices merge during the culture period, it does not have a deleterious effect on the survival or viability of migrating interneurons (Fig. 2A-C). Telencephalon slices were selected from the anterior half of the cerebral hemispheres at the plane where the MGE and LGE can be discriminated from each other, thus alleviating the risk of caudal ganglionic eminence tissue contamination from which interneurons undertake a separate migratory route (Xu and others 2004; Yozu and others 2005). When the overlay was placed in the same orientation, interneurons migrate through the corticostriatal boundary, and the migratory front moves into the neocortex in the direction of the medial neocortex after 24 h (Fig. 2A-C). In contrast, if the mediolateral axis of the overlay is rotated 180° so that the medial neocortex is now positioned over the lateral migratory front, migrating interneurons avoid regions of the brain slice that would normally have been permissive and are now forced into the surrounding domains (Fig. 2D-F). The size of this nonpermissive region (~200 μm) correlates with the width of an E12.5 neocortex. The consequence of this is that large clusters of interneurons are observed in some sections of the neocortex, whereas other regions are interneuron sparse (Fig. 2F). Interneuron migration can be abolished completely if both the mediolateral and ventricular–pial axes are rotated in the overlay (Fig. 2G-I). In this instance, very few migrating cells enter the neocortex. These experiments indicate that the migration of GAD67-positive interneurons into the neocortex is a consequence of factors, or positioning cues, that exist along both axes of the neocortex.

Confronting the migratory front of interneurons with different cortical regions along the mediolateral axis also tested this inhibitory effect. To achieve this, small or large regions of the neocortex were removed and the cut edges juxtaposed to allow potential migration of interneurons across the joined boundaries. Single cuts in the slice had no effect on interneuron migration.}

Figure 2. Migration of interneurons is perturbed by overlay cultures placed in the reverse axial orientation. (A, D, G) Schematic illustrations of neocortex overlay paradigm used to assess the effect of cortical regions on the leading interneuron population. Overlays were placed in the same, reversed M-L axis, or reversed M-L and V-P axis, respectively. (B) Brightfield–fluorescent image showing E12.5 GFP-positive interneurons and position of control overlay (dashed line) at 0 h. (C) After 24 h in culture, interneurons tangentially migrate into the neocortex and are unaffected by the overlay cortical slice. (E) Image showing position of reverse M-L overlay (dashed line) at 0 h. (F) Interneurons migrate toward medial neocortex, but after 24 h in culture, the trajectory is altered to avoid a region of the slice where medial overlay was placed (dashed circle). (H) Image showing position of reverse M-L and V-P overlay (dashed line). (I) Interneuron migration is abolished after 24 h in culture (dashed circle). L, lateral region of the neocortex; M, medial region of the neocortex; P, pial edge of neocortex; V, ventricular zone of neocortex. Scale bar: (B, C, E, F, H, I), 100 μm.
migration (data not shown), and removal of small areas of intervening neocortex showed no effect (Fig. 3A–C). When larger slices of intervening neocortex were removed, interneurons did not transverse the cut site, changed their normal trajectory, and migrated in the reverse direction (Fig. 3D–F). This confirms that the migratory front of interneurons at E12.5 is adverse to factors present in the medial neocortex.

**Temporal Regulation of Neocortical Inhibition**

The question of an attractive gradient being expressed in the neocortex has been previously tested. Marin and others (2003) showed that at E13.5, migrating interneurons preferentially migrate toward the medial neocortex when both medial and lateral cortical regions were placed equidistant from a source of migrating interneurons. This suggests that not only does the medial neocortex at E13.5 constitute a highly permissive substrate for migration but also it contains chemoattractive activity that influences the behavior of tangentially migrating neurons.

The above contrasts with findings obtained with E12.5 medial neocortex; however, the possibility remains that there is an E12.5–E13.5 switch in the medial neocortex from inhibition to attraction. To test this, we examined the capacity of E13.5 medial neocortex to support interneuron migration when the neocortex was rotated 180° on the mediolateral axis (Fig. 4A,D,G). The response of interneurons to this manipulation was monitored at various time points to capture when migration first occurred across the cut surface. In E12.5 control manipulations (where a strip of neocortex is removed and replaced in the same orientation), interneurons migrate liberally, although some retardation was observed at the cut site after 24 h in culture (Fig. 4B,C). In contrast, the rotation of the mediolateral axis, without changing the ventricular-pial axis, showed no migration of interneurons medial to the corticostriatal boundary after 24 h. Nevertheless, by 40 h in culture, an alternative migratory stream was sometimes observed where interneurons migrated into the more permissive flipped lateral edge. This highlights that the neurons maintain the capacity to migrate and are therefore restricted by factors present in the medial neocortex (Fig. 4E,F). Interestingly, when the neocortex is rotated at E13.5, interneurons transversed the cut site and actively migrated into the neocortex within 8 h of culture (Fig. 4G). Higher magnification of this region revealed interneurons with large leading processes and branching to indicate active migration (Fig. 4H). Thus, it would appear that at various developmental stages, interneurons can be either repelled or attracted to the medial neocortex, and this may be a major component in controlling tangential migration of interneurons into the hippocampus.

**Discussion**

Mechanisms underlying the tangential migration of cortical interneurons are important, given the essential role that these neurons play in cortical function and the number of human neurological disorders that arise due to abnormal migration (Ross and Walsh 2001). The molecular events in neuronal migration are multifaceted, and the focus of this study has been on extrinsic factors that alter migratory dynamics throughout the developing neocortex. It has been proposed that an increasing gradient of attractive cues, from medial to lateral neocortex, provides the impetus for migrating interneurons (Marin and Rubenstein 2001), and a number of attractive cues have been suggested to provide the necessary guidance systems (Powell and others 2001; Polleux and others 2002; Flames and others 2004; Pozas and Ibanez 2005). To date, there has been no discussion on inhibitory zones present within the dorsal cortical

![Figure 3](image-url)
region that might constrain the spatial and temporal limits of interneuron migration. The present study clearly indicates inhibitory activity within the medial neocortex that is temporally transient and opens up the possibility that it might act as a gatekeeper to control the influx of interneurons into the developing hippocampal anlage.

**Inhibitory Activity in the Medial Neocortex**

Using time-lapse recording of interneuron migration in brain slices, the current study provides evidence to show that not all areas of the mediolateral neocortex are equally permissive and that the affinity of interneurons for the medial neocortex is temporally regulated to control the timing of neuronal targeting between separate brain regions. The focus of recent papers has been on understanding how different subtypes of interneurons are generated and how these diverse populations are organized within the neocortex (Monyer and Markram 2004; Xu and others 2004). We have chosen to visualize all interneurons by using GAD67 to mark the diverse subtypes of tangentially migrating neurons (Tamamaki, Yanagawa, and others 2003). Even though other methods of labeling result in the analysis of smaller subpopulations or subtypes of neurons, we were interested in assessing the global effects on the first populations present at E12.5 to cross the corticostriatal boundary. The constituents of these early-born populations remain to be determined and may comprise only deeper layer interneurons (Valcanis and Tan 2003) or may contain multiple interneuron subtypes that arise from heterogeneous progenitors in the ganglionic eminences (Xu and others 2004).

It is not unprecedented to propose that the medial and lateral neocortex differ as a permissive substratum for interneuron migration. Specification or arealization of the neocortex is established by key transcription factors that are differentially expressed early in development (Stoykova and Gruss 1994; Gulisano and others 1996). Of these, Emx2 and Pax6 are probable candidates for controlling inhibitory activity in the medial neocortex as they are expressed in either a low rostrolateral–high caudomedial or high rostromedial–low caudolateral gradient, respectively (Bishop and others 2002). Analysis of Emx2 null mice has shown that this transcription factor is upstream of a number of key axon guidance molecules such as ephrin-A5 and Eph-A7 (Bishop and others 2002). Although the Eph–ephrin signaling pathway has not been implicated in interneuron migration, this family of proteins is a potential candidate for

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**Figure 4.** Temporal regulation of inhibitory activity in the medial neocortex. (A, D, G) Schematic illustrations of slice culture manipulations used to analyze the migration of interneurons at the lateral cortical position. (B) A brightfield–fluorescent image showing a fragment of E12.5 neocortex that was excised and replaced in the same orientation (arrows) before interneurons had entered into the cortical wall. (C) After 24 h in culture, interneurons migrate over the juxtaposed edge (arrow) and migrate toward the medial neocortex. (E) Area of E12.5 neocortex removed and replaced in the reverse M–L orientation (arrows) at 0 h. (F) Cultures were extended to 40 h to account for delayed migration, but no interneurons were observed to enter the flipped medial neocortex (arrow). An alternate migratory stream was detected in the flipped lateral neocortex (asterisk). (H) Image shows an E13.5 neocortex flipped on the M–L axis. (I) Higher magnification of boxed region shows active migration of interneurons across the cut site (dashed line) and into the flipped medial neocortex. L, lateral region of the neocortex; M, medial region of the neocortex. Scale bar: (B, C, E, F, H), 100 μm; (I), 20 μm.
the inhibitory activity present in the medial neocortex, and further investigations of these molecules would be informative.

**Temporal Regulation of Interneuron Migration**

Previous studies have shown that interneurons destined for the hippocampus and neocortex are generated in the MGE and share the same tangential migratory routes in the cortical wall (Anderson and others 1999; Pleasure and others 2000). Given that both populations share a common developmental pathway, there are a number of hypotheses that can account for the temporal regulation at the medial neocortex. First, if the initial population of interneurons to enter the neocortex is destined for the hippocampus, then temporal regulation may be required to allow the hippocampal primordium to reach a certain stage of maturity before the GABAergic neurons can be accepted. Second, if the initial population of interneurons to enter the neocortex is destined to remain in the cortical plate, then inhibitory cues present in the medial neocortex are necessary to restrict forward migration into the hippocampus. This would mean that a second migratory wave of interneurons would bypass the initial wave of cortical interneurons and migrate into the hippocampus. The results of the present study are consistent with both hypotheses, with the demonstration that the medial neocortex is inhibitory to the passage of interneurons at E12.5, but this changes to a permissive activity at E13.5 when interneurons are able to enter the medial neocortex. This notion is consistent with birthdating studies that suggest that hippocampal interneurons in mice peak at E12.5 (Soriano and others 1986), and we reason that they would arrive at the medial neocortex at least 24 h later. The mechanism of suspended migration, as suggested by the first hypothesis, is not uncommon in the development of the vertebrate nervous system. In the dorsal root ganglia, semaphorin 3A and chondroitin sulfate proteoglycans are known to be molecular gatekeepers to repel axon entry into the spinal cord at the dorsal root entry zone (Masuda and Shiga 2005). In the hindgut, neurons from the sacral neural crest are known to observe suspended migration in the extramural ganglia before entering the hindgut mesenchyme a few days later (Kapur 2000).

In conclusion, this study touches on the issue of how interneurons that are generated in the MGE are able to properly find their targets in different regions of the dorsal and medial pallium. The identity and function of the adult interneurons in the hippocampus and neocortex are nonequivalent, although their developmental strategies appear to be similar (Pleasure and others 2000). In the case of striatal interneurons, the absence of neuropilin-1—semaphorin 3A interaction appears to be necessary and sufficient to ensure that they do not migrate into the neocortex (Marin and others 2001). Based on the current results, we propose that a similar chemoinhibitory—chemoattractive interaction may also be present at the medial neocortex to regulate the proper segregation of hippocampal and neocortical interneurons.

**Notes**

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**References**


