

Differential Regulation of Connexin 26 and 43 in Murine Neocortical Precursors

K. S. Bittman and J. J. LoTurco

Department of Physiology and Neurobiology, University of Connecticut, Storrs, CT 06268, USA

Proliferating cells of the developing murine neocortex couple together into clusters during neurogenesis. Previously, we have shown that these clusters contain neural precursors in all phases of the cell cycle except M phase, and that they extend a nestin-expressing process from the cluster to the pial surface. In addition, coupling within neocortical cell clusters is a dynamic process related to the cell cycle, with maximal coupling in S/G2 phase, uncoupling in M phase and then recoupling during G1 and S phases of the cell cycle. In the present study, we use immunohistochemistry to demonstrate that cycling neocortical cells as well as radial glial cells express the gap junction proteins connexin 26 and connexin 43. Furthermore, we demonstrate that biocytin labeled clusters extend processes to the pial surface that express the glial cell antigen RC2. Lastly, by combining bromodeoxyuridine and connexin immunohistochemistry on acutely dissociated neocortical cells, we show that the percentage of cycling cells immunoreactive to connexin 26 and connexin 43 changes through the cell cycle. These results indicate that radial glial cells as well as neural precursors couple into clusters, and suggest that through differential regulation of connexins, neocortical precursors may compartmentalize as they progress through the cell cycle.

Introduction

Neurons of the adult mammalian neocortex are generated through a series cellular divisions within the ventricular zone (VZ), a sheet of mitotically active epithelial cells lining the lateral ventricles. Two main cell types populate the ventricular zone: neural precursors and radial glial cells (Levitt, 1981). Dye-transfer studies have demonstrated that during neocortical neurogenesis undifferentiated cells within the VZ couple into clusters via gap junction channels (LoTurco and Kriegstein, 1991; Bittman *et al.*, 1997). These clusters extend a single nestin-expressing process to the pial surface, suggesting that radial glial cells as well as neural precursors may be a part of clusters of coupled VZ cells (Bittman *et al.*, 1997). Both populations of VZ cells are mitotically active (Boulder, 1970; Levitt *et al.*, 1981; Misson *et al.*, 1988a,b), and disrupting gap junction communication reduces the percentage of VZ cells that enter S phase (Bittman *et al.*, 1997), suggesting that gap junction communication increases the probability of VZ cells entering the cell cycle. Moreover, the cells forming VZ clusters couple and uncouple throughout the cell cycle. Precursors uncouple in M phase and recouple during S and G2 phases. In late neurogenesis, when a majority of neocortical progenitors exits the cell cycle (Takahashi *et al.*, 1996), recoupling is delayed until the end of the next phase of DNA synthesis (S phase). The process of coupling and uncoupling may regulate the entry or exit of cells from the cell cycle. One possible mechanism that may regulate cell coupling and uncoupling is differential expression of gap junction proteins through the cell cycle.

Thirteen rodent gap junction protein subunits (connexins)

have been identified, and each hemichannel (connexon) consists of six subunits (Bennet *et al.*, 1991; Dermietzel, 1996). Functional gap junction channels form among cells expressing complimentary hemichannels. In the developing rat neocortex, connexins of molecular weight 43 (Cx43) and 26 (Cx26) kDa have been identified and the expression of these connexins changes during neurogenesis of the rat neocortex. Cx43 and Cx26 expression levels are moderate early in neurogenesis, increase and then diminish as neurogenesis proceeds to completion (Nadarajah *et al.*, 1997). In addition, differentiated neurons express Cx26 while glial cells express Cx43 (Dermietzel *et al.*, 1989; Nadarajah *et al.*, 1997). These observations indicate the presence of connexin proteins among a variety of cell types in developing mammalian neocortex, but do not clearly indicate whether or not they are expressed by proliferating neocortical cells, by radial glial cells, or whether or not Cx43 and Cx26 co-localize to the same cells.

In order to further understand the function of cell coupling and the role of connexin proteins during neurogenesis we have combined the techniques of intracellular biocytin labeling, 5-bromo-2'-deoxyuridine (BrdU) pulse labeling, and immunohistochemistry. Our results indicate that radial glial cells express both Cx26 and Cx43, and can couple into VZ clusters. In addition, we show that Cx43 and Cx26 are co-localized within some, but not all VZ cells. We have also determined that Cx26 expression is regulated during the cell cycle and that the number of precursors expressing Cx26 increases from S to early G1 phase and remains moderately high throughout the rest of the cell cycle. In contrast, the number of precursors expressing Cx43 is highest in S/G2 phase and diminishes during the remainder of the cell cycle. These experiments demonstrate that radial glial cells as well as undifferentiated neuroepithelial cells can couple to clusters of VZ cells, that radial glial cells express Cx26 and Cx43, and that the dynamic process of coupling and uncoupling may be the result of regulated connexin expression through the cell cycle of proliferating neocortical cells. We suggest that Cx26 and Cx43 may mediate a dynamic interaction between cells in the VZ of mouse neocortex.

Materials and Methods

CD1 mice (Charles River) were mated between 5.00 and 6.00 a.m. and checked for vaginal plugs at 9.00 a.m. the same morning. Presence of a vaginal plug indicated the first day of conception (E0). Fourteen, sixteen and seventeen (E14, E16 and E17) day old mouse embryos were used for this study. Timed pregnant CD1 mice were injected with 80 mg BrdU/kg body wt diluted in 0.9% NaCl with 0.007 N NaOH. Injections were done between 9.00 and 10.00 a.m. Animals survived 2, 4 or 8 h prior to being killed. Embryos were dissected from the mother and placed in cold Hank's balanced salt solution (HBSS). Brains were removed and processed for either dissociated cell culture or cryostat sectioning.

For dissociation and plating of cells, the cortex was dissected from the rest of the brain and dissociated in warm minimal essential media (MEM) with 30 mM glucose, 1% L-glutamine (Fluka), 0.2% gentomycin (Fluka) by gently titrating with a fire-polished glass pasteur pipette for several minutes. 1×10^6 cells in 25–50 μ l of volume were plated into wells on poly-D-lysine (100 μ g/ml) coated slides. Slides were placed in a 37°C humidified incubator with 5% CO₂ and 95% O₂ for 1.5 h. Media was aspirated from each well and cells were fixed with chilled 2% paraformaldehyde in 0.1 M phosphate buffer in the refrigerator for 30 min. Fixative was removed and the slides were rinsed thoroughly in phosphate-buffered saline (PBS). For storage purposes, some slides were cryoprotected with 30% sucrose in PBS in the refrigerator for 30 min. These slides were then frozen at –20°C and stored until use.

Immunocytochemical double-labeling for BrdU and either Cx26 or Cx43 was performed on dissociated cells. Slides were rinsed in PBS and placed in 65°C PBS for 10 min. Following several rinses with PBS, slides were then placed in 0.1 mg/ml pepsin (Sigma) diluted in 0.1 N HCl for 1.5–3 min. After one rinse in PBS, slides were placed in 2 N HCl warmed to 37°C for 10 min. Slides were thoroughly rinsed in PBS and then blocked in 5% normal goat serum (NGS) in PBS with 0.1% bovine serum albumin (BSA) for 30 min. Slides were removed from the blocking serum and placed directly in a mixture of primary antibodies [rabbit anti-Cx26 (Zymed) 1:100 and mouse anti-BrdU (Novacastra) 1:200] diluted in PBS with 1% NGS and 0.01% BSA for 1 h. After several rinses in PBS, slides were incubated in a mixture of goat anti-rabbit fluorescein isothiocyanate (FITC) (1:100 Jackson) and goat anti-mouse Texas Red (1:100 Jackson) in PBS with 1% NGS and 0.01% BSA for 1 h. Slides were rinsed well in PBS and coverslipped under Fluoromount-G (Fisher).

For cryostat sections, whole cortices not used for dissociated cell plating were fixed in 2% paraformaldehyde in 0.1 M phosphate buffer in the refrigerator for 30 min. Fixative was next removed and the brains were cryoprotected with 15% sucrose in PBS at 4°C until the brains sunk. Sucrose was removed and the brains were placed in a 1:1 mixture of 15% sucrose/PBS and OCT (Sigma) for 2 h to overnight at 4°C. Brains were rapidly frozen with liquid nitrogen fumes, sliced at 10 μ m on a cryostatic microtome and collected onto gelatin-coated slides.

Tissue sections and dissociated cells (prepared as described above) were immunofluorescently double labeled for Cx26 and the glial cell marker RC2 or Cx43 and RC2. Some tissue sections were immunofluorescently labeled for Cx26 or Cx43 expression only. Slides were rinsed in PBS and then placed in 5% NGS with 0.1% BSA in PBS for 30 min. Slides were transferred directly to a mixture of polyclonal anti-Cx26 or anti-Cx43 (1:100) and monoclonal anti-RC2 (1:5, Developmental Hybridoma Bank) in PBS supplemented with 1% NGS, 0.01% BSA and 0.1% Triton-X100 for 45 min. After several rinses in PBS, slides were incubated in a mixture of goat anti-rabbit FITC (1:100) and goat anti-mouse Texas Red (1:100) in PBS with 1% NGS, 0.01% BSA and 0.1% Triton-X100 for 45 min. Sections were rinsed in PBS and coverslipped under Fluoromount-G with 1 mg/ml phenylenediamine as an anti-fade agent.

VZ imprints were immunofluorescently double-labeled for Cx26 and Cx43. Briefly, neocortical tissue from the dorsomedial telencephalon of an E17 mouse was laid ventricular side down on the surface of a 35 mm² coverslip coated with 100 mg/ml poly-D-lysine. A non-coated rectangular coverslip was then laid on top of the tissue and excess HBSS was aspirated from between the two slides. After 15 s, the top coverslip was removed and the circular coverslip was placed in a 24-well plate. Chilled 2% paraformaldehyde in 0.1 M phosphate buffer was added and the imprint was incubated at 4°C for 20 min. The coverslip was rinsed thoroughly in PBS and then incubated in 5% NGS, 1% dry milk and 0.1% Triton-X100 in PBS for 1 h. Coverslips were then placed directly in a mixture of polyclonal anti-Cx26 (Zymed 1:100) and monoclonal anti-Cx43 (Zymed 1:500) in PBS with 1% NGS, 1% dry milk and 0.1% Triton-X100 for 1 h. Coverslips were rinsed in PBS and then placed in secondary goat anti-rabbit Texas Red (1:100 Jackson) and goat anti-mouse FITC (1:100 Jackson) in PBS with 1% NGS, 1% dry milk and 0.1% Triton-X100 for 1 h. Cells were next rinsed in PBS, allowed to partially dry, and were coverslipped under anti-fade reagent (Molecular Probes).

In order to test the specificity of the gap junction antibodies, immunohistochemistry was performed on tissue known to express exclusively Cx26 or Cx43. Adult mouse heart and brain were dissected

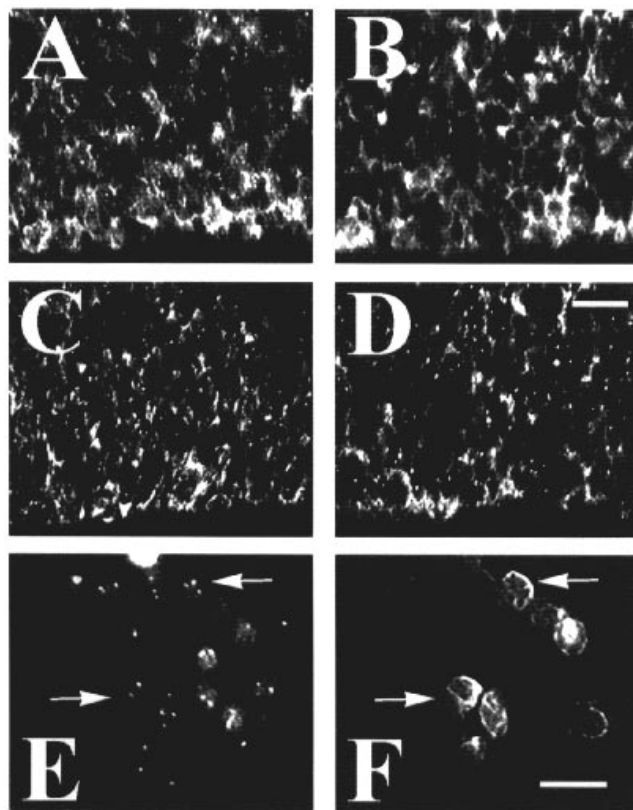


Figure 1. Both Cx26 and Cx43 are expressed in the mouse VZ. (A) Cx26 immunoreactivity in the VZ of E14 mouse neocortex; punctate, plaque-like immunofluorescence is seen along the basal border and intermediate zone. (B) Cx43 immunostaining throughout the VZ of E14 mouse neocortex. (C) Cx26 immunoreactivity in the VZ of E17 mouse neocortex. (D) Cx43 immunolabeling in the VZ of E17 mouse neocortex. (E) Cx26 expression in an imprint of E14 mouse VZ cells. (F) Cx43 expression in the same VZ cells as (E) stained with a monoclonal anti-Cx43 antibody. Arrows indicate VZ cells expressing both Cx26 and Cx43. Scale bars: (A–D) = 20 μ m; (E,F) = 20 μ m.

and rapidly frozen in chilled isopentane. Sections (10–12 μ m) were sliced on a cryostatic microtome and collected onto gelatin-coated slides. The slides were immersed in chilled 2% paraformaldehyde for 20 min, rinsed in PBS and stained for the gap junction proteins Cx26 and Cx43 as described above for tissue sections of embryonic mouse brain.

Sections were visualized with either a Nikon Optiphot-2 microscope with an episcopic-fluorescence attachment (EFD-3, Nikon) or a Zeiss LSM 410 confocal microscope with an argon–krypton laser. Images from the Nikon Optiphot-2 were captured with a cooled CCD video camera (Photometrics Imagepoint) using standard filters and NIH Image 1.59 software. All software was run on a Power PC Macintosh computer. BrdU-immunoreactive cells were visualized using a rhodamine filter set. FITC labeling was observed using an excitation filter of 465–495 nm, and a barrier filter at 515–555 nm. Confocal images were post-processed using Adobe PhotoShop 3.5.1 software. Quantification was performed by counting the percentage of connexin-labeled cells among 50 BrdU-positive cells. A positively labeled connexin-expressing cell exhibited punctate fluorescent labeling, or plaques, seen on or within the cell (Dermietzel *et al.*, 1989). We considered a cell positively labeled by connexin antibodies if two or more plaques were visible. In addition, we also quantified the percentage of BrdU-labeled cells labeled with three or more connexin plaques. These cells were considered to be high expressors of connexin protein. Plaques were similar in appearance; however, in some cells, several plaques combined to form larger plaques. In some cases most or all of a cell would be stained. These cells were considered to contain more than four connexin plaques. Statistical analysis was performed using SuperAnova software.

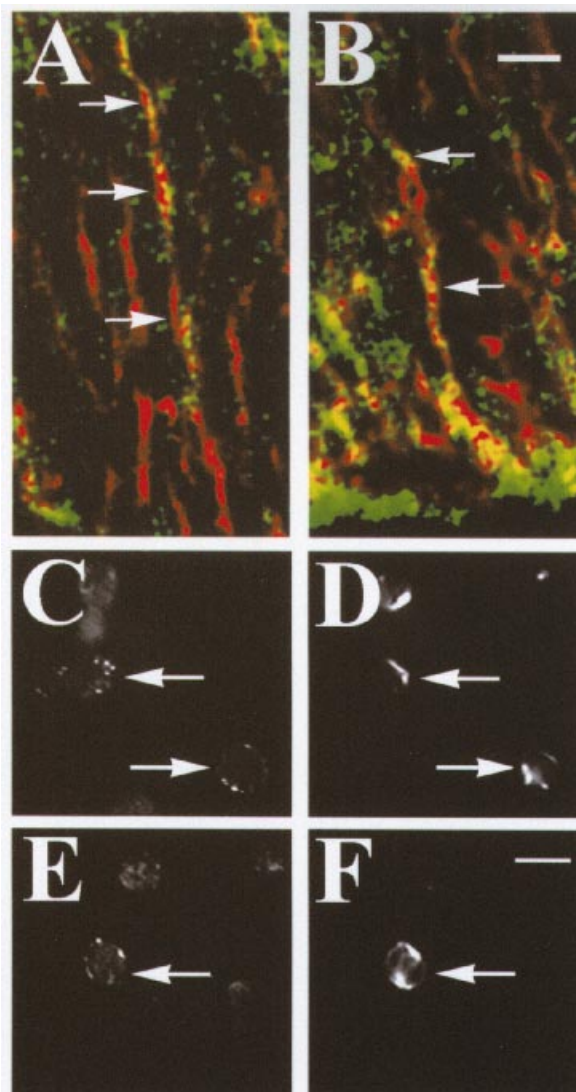


Figure 2. Cx43 and Cx26 are expressed by radial glial cells. (A) Confocal double immunofluorescence of Cx26 (green) and RC2 (red) in E14 mouse neocortex illustrates double labeling (yellow) along an RC2-positive fiber extending from the VZ through the intermediate zone (arrows). (B) Confocal microscopy of Cx43 (green) and RC2 (red) double labeling (yellow) along a fiber (arrows) from E17 mouse VZ and IZ. (C) Cx43 immunolabeling in acutely dissociated E17 mouse neocortical cells. (D) From the same field as (C), the radial glial marker RC2 labels a small percentage of acutely dissociated E17 mouse neocortical cells. RC2-labeled cells were also immunoreactive to Cx43 antibodies (arrows). (E) Cx26 immunostaining among acutely dissociated E17 mouse neocortical cells. (F) From the same field as (E), RC2-labeled cells were also stained with Cx26 antibodies (arrow). Scale bars: (A,B) = 20 μ m; (C–F) = 10 μ m.

Results

Cx26 and Cx43 are Expressed in the Mouse VZ

Previous research has examined the expression of Cx26 and Cx43 throughout neurogenesis in the rat neocortex; however, no reports of connexin expression in the developing mouse neocortex exist. In addition, no studies have examined co-localization of Cx26 and Cx43 in developing neocortex. We used immunohistochemistry on tissue sections of E14 and E17 mouse neocortex to demonstrate that both proteins are present in the mouse neocortex throughout the period of mid to late neurogenesis (E13–E17) (Fig. 1A,B). Expression of both connexins is greatest at the apical portion of the VZ. To determine

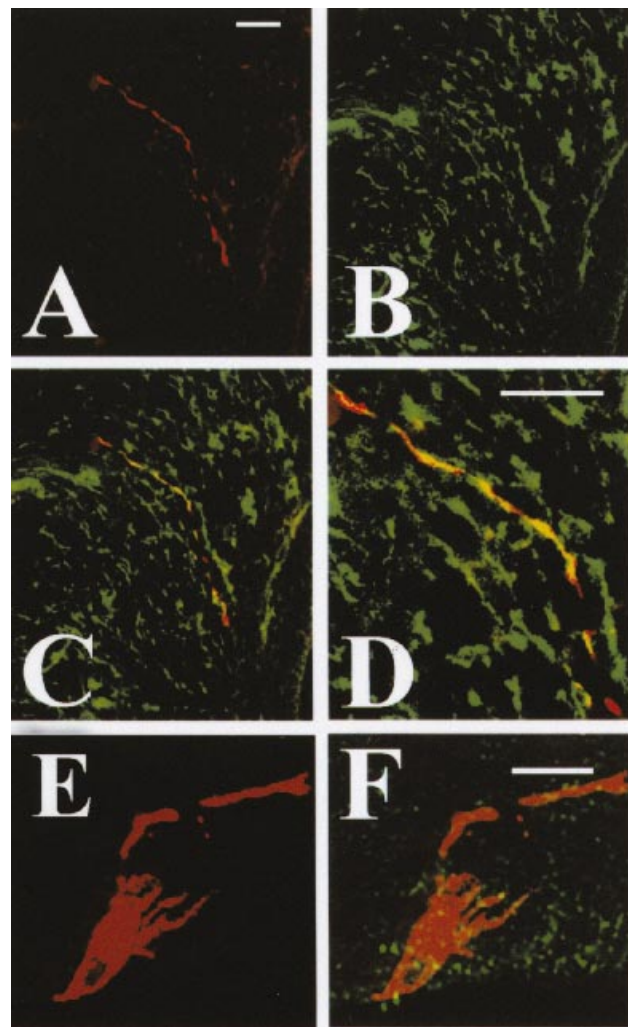


Figure 3. Radial glial cells couple to clusters of VZ cells. (A) Confocal image of a fiber from an E16 VZ cluster labeled by an intracellular injection of biocytin labeled cluster is shown in (E). The fiber extends through the entire intermediate zone. (B) Confocal image of RC2 immunoreactivity from the same tissue as (A). (C) Confocal double image of biocytin and RC2 staining. Where the two labels co-localize, the overlay is yellow. (D) Higher magnification image of biocytin and RC2 double labeling. Once again, where the two labels co-localize the overlay is yellow. (E) The biocytin-labeled cluster in the VZ which extends the fiber shown in (A)–(D). (F) Overlay of the biocytin-labeled cluster in (E) and RC2 immunolabeling in the VZ. Scale bars: (A)–(C) = 40 μ m; (D)–(F) = 20 μ m.

whether Cx26 and Cx43 are co-localized within the same VZ cells we used double immunohistochemistry on imprints of E17 mouse ventricular zone cells with a rabbit anti-Cx26 polyclonal antibody (Zymed) and a mouse anti-Cx43 monoclonal antibody (Zymed): 39.81% (84/211) of VZ cells expressed Cx26 and 39.33% of VZ cells expressed Cx43 (83/211). Moreover, Cx26 and Cx43 were co-localized in 16.11% (34/211) of total VZ cells, and 20.36% of gap junction expressing VZ cells (34/167) (Fig. 1C,D). Cells within the ventricular zone, therefore, can express Cx26 and Cx43 either alone, or in combination.

Radial Glial Cells Express Cx26 and Cx43

The VZ of the developing mouse neocortex is composed of two major cell types: radial glial cells and neural precursors (Levitt *et al.*, 1981). In order to determine if radial glial cells express Cx26 and/or Cx43 we used double-immunofluorescent confocal microscopy with the radial glial cell marker RC2 (Misson *et al.*,

1988a) and antibodies raised against either Cx26 or Cx43. Our results demonstrate that in tissue sections of E14 mouse neocortex, radial glial cell fibers in the VZ and intermediate zone express both Cx26 and Cx43. Punctate connexin immunostaining can be found along RC2-expressing fibers as they exit the VZ *en route* to the pial surface (Fig. 2A,B). In addition, we performed double-immunofluorescence on a population of acutely dissociated neocortical cells from E14 or E17 mouse neocortex (Fig. 2C-F). At E14, 78% (78/100) of RC2-expressing radial glial cells also express Cx26 and 76% (76/100) of radial glial cells express Cx43 (Fig. 2C-F). At E17, 58% of RC2-expressing cells were positively labeled with Cx26 (59/101) while 74% of the RC2-expressing cells stained positively for Cx43 (79/106). These results demonstrate that a substantial portion of the radial glial cell population can express Cx26 or Cx43.

RC2-positive Fibers Extend from VZ Clusters

Since RC2-labeled radial glial cells express Cx26 and Cx43, we wanted to determine if RC2 cells are coupled to VZ clusters. VZ cells were injected with biocytin and were then labeled fluorescently for both biocytin and the radial glial cell antigen RC2 (Misson *et al.*, 1988a). Labels were simultaneously visualized using a confocal laser scanning microscope (Fig. 3). In E14 and E16 mouse neocortex, clusters of coupled cells labeled by biocytin frequently projected a single fiber to the pial surface. As these fibers wound through the intermediate zone and cortical plate they often bifurcated into multiple branches, each extending to the pial surface. These fibers also label with RC2 antibodies (10/14), indicating that radial glial cells can be a part of VZ clusters (Fig. 3). Since our RC2 staining poorly labeled the ventricular zone itself, and did not label cell bodies or processes within the VZ, we were unable to clearly co-localize individual cell bodies in VZ clusters with RC2 staining; however, all fibers in the intermediate zone and cortical plate that doubly stained with RC2 and biocytin extended from clusters and not individual cells in the VZ. Consistent with these results, we have shown previously that processes extending from clusters also express Rat401 (Bittman *et al.*, 1997).

Cx26 and Cx43 Expression in Neocortical Precursor Cells

In order to determine if Cx26 and Cx43 are expressed by neocortical precursors, we developed an immunohistochemical double-labeling protocol using acutely dissociated prenatal mouse neocortical cells. The S phase marker BrdU was injected into timed pregnant mice (E14 or E17) to label actively cycling neocortical cells. Dissociated cells were prepared as described and double-stained for connexin (either Cx26 or Cx43) and BrdU immunofluorescence (Fig. 4). The results demonstrated that acutely dissociated neocortical cells exhibited punctate immunofluorescence, or plaques of connexin immunoreactivity similar to the punctate staining seen in tissue (Fig. 4B,D). With both antibodies, from one to nine connexin plaques were clearly discernible (Fig. 4B,D). We then quantified the percentage of BrdU-stained cells double-labeled with connexin antibodies. In order to be certain that the data did not result from background staining, we also quantified the percentage of BrdU-labeled cells labeled with three or more connexin-immunoreactive plaques, reasoning that it would be unlikely for background staining to be present at this high level. The percentage of BrdU-positive cells immunoreactive to Cx26 was significantly higher than Cx43 for

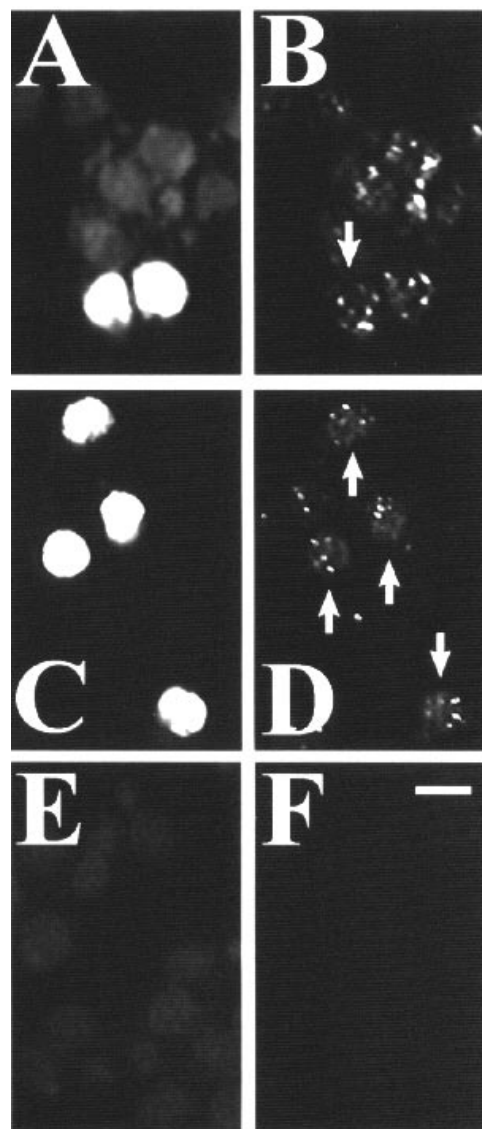


Figure 4. Cx26 and Cx43 are expressed in acutely dissociated cycling progenitor cells. (A,C) BrdU immunolabeling of a population of acutely dissociated neocortical cells. (B) Cx26-immunoreactive cells from acutely dissociated cells of mouse neocortex. Arrows indicate BrdU-labeled cells from the same field in (A). (D) From the same field as (C), Cx43 immunoreactivity of acutely dissociated neocortical cells. Arrows indicate double-labeled cells. (E,F) The same field under rhodamine (E) and FITC (F) filters in which omission of both primaries results in no non-specific staining by the secondary antibodies. Scale bar = 20 μ m.

each measurement at both E14 and E17 (Fig. 5). At E14, a mean of 68.87% of BrdU-positive cells was immunoreactive to Cx26 while an average of 59.44% was immunoreactive to Cx43 (Tukey, $P < 0.05$, $n = 18-23$, Fig. 5). A mean of 28.61% of BrdU-labeled cells expressed three or more Cx26-immunoreactive plaques, while a mean of 15.33% expressed three or more Cx43 plaques (Tukey, $P < 0.01$, $n = 18-23$, Fig. 5). At E17, the difference in the mean percentages of BrdU-labeled cells expressing connexin protein was 70.48% for Cx26 and 63.78% for Cx43 (Tukey, $P < 0.01$, $n = 18-21$, Fig. 5). An average of 30.76% of BrdU-labeled cells express three or more Cx26-immunoreactive plaques, while a mean of 18.56% express three or more Cx43-immunoreactive plaques (Tukey, $P < 0.01$, $n = 18-21$, Fig. 5). Overall, the percentage of BrdU-positive cells

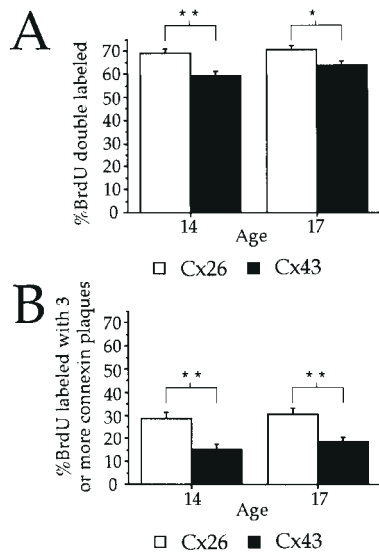


Figure 5. The percentage of cycling VZ precursors expressing connexin protein does not change through neurogenesis. (A) Bar graphs of the percentage of BrdU-labeled cells double-labeled by connexin antibodies in acutely dissociated E14 and E17 neocortex. (B) Bar graphs of the percentage of BrdU-labeled cells also labeled with three or more connexin plaques in E14 and E17 acutely dissociated neocortical cells.

immunoreactive to Cx26 was higher than Cx43 for each measurement (double-labeled and labeled with three or more connexin plaques) at both E14 and E17 (ANOVA $P < 0.01$, $n = 36-44$, Fig. 5). The percentage of BrdU-labeled cells expressing Cx26 did not change between E14 and E17. Similarly, the percentage of BrdU-labeled cells expressing Cx43 did not change between E14 and E17. These results demonstrate that both connexins are found among cycling cells, a greater percentage of cycling cells express Cx26 than Cx43, and the number of cycling cells expressing connexin protein does not change through mid to late neurogenesis.

The Percentage of Cycling Cells Expressing Connexins Changes Differentially Through the Cell Cycle

Previously, we demonstrated that cell coupling and uncoupling was related to the cell cycle. In order to evaluate connexin expression through the cell cycle, 14 and 17 day old pregnant mice were injected with BrdU and allowed to survive 2, 4 or 8 h, thereby labeling cells in S/G2, G2/M/G1 or late G1 phases of the cell cycle (Takahashi *et al.*, 1993). Acutely dissociated neocortical cells were prepared from the embryos and double-stained immunocytochemically for BrdU and either Cx26 or Cx43. We then quantified the percentage of BrdU-stained cells double-labeled with connexin antibodies and labeled with three or more connexin plaques as described above.

Between S and early G1 phases, the percentage of BrdU-labeled cells expressing Cx26 increases, while the percentage expressing Cx43 decreases. At E14, the percentage of BrdU cells double-labeled with Cx26 increases significantly between the 2 and 4 h survival times, from a mean of 60.86% to a mean of 78.25% (Tukey $P < 0.01$, $n = 6-8$ per group, Fig. 6). Similarly, the percentage of BrdU-labeled cells stained with three or more Cx26 plaques rises significantly from a mean of 17.71% at 2 h to a mean of 39.25% by 4 h (Tukey $P < 0.01$, $n = 6-8$ per group; Fig. 6). During late neurogenesis (E17), there is a significant increase in the percentage of BrdU-positive cells double-labeled with

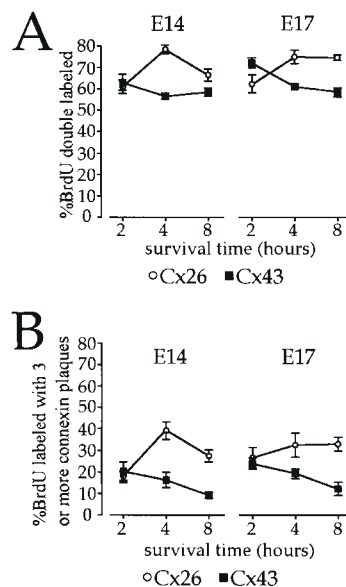


Figure 6. The percentage of BrdU-labeled cells also labeled with either connexin (Cx26 or Cx43) changes differentially through the cell cycle. (A) At E14, the percentage of BrdU-labeled cells expressing Cx26 increases between 2 and 4 h after the BrdU injection (Tukey, $P < 0.01$, $n = 6-8$ per group). At E17, the percentage of BrdU-labeled cells immunoreactive to Cx43 decreases between 2 and 4 h after the BrdU injection (Tukey, $P < 0.01$, $n = 6$). There is also a significant interaction between connexin and survival period at E14 and E17 (ANOVA, $P < 0.01$, $n = 12-16$ per group). (B) At E14, there is an increase in the percentage of BrdU-labeled cells expressing three or more Cx26-immunoreactive plaques between 2 and 4 h after the BrdU injection (Tukey, $P < 0.01$, $n = 6-8$ per group). At E17, the percentage of BrdU-positive cells labeled with three or more Cx43-immunoreactive plaques decreases between 2 and 8 h after the BrdU injection (Tukey, $P < 0.01$, $n = 6$ per group).

Cx26 antibodies between 2 and 4 h (Tukey $P < 0.05$, $n = 8$ per group). In contrast to Cx26, the percentage of BrdU-labeled neocortical cells also labeled with Cx43 decreases between the 2 and 8 h survival times (Fig. 6). During late cortical neurogenesis (E17) the percentage of BrdU/Cx43 double-labeled cells decreases significantly from a mean of 72% at 2 h to a mean of 58.33% by 8 h (Tukey $P < 0.01$, $n = 6$). The percentage of BrdU-labeled cells labeled with three or more Cx43 plaques also decreased significantly from 24% after 2 h to 9.33% by 8 h (Tukey $P < 0.01$, $n = 6$ per group, Fig. 6). Lastly, there is a significant interaction between the type of connexin and time after BrdU injection at both E14 and E17 (ANOVA $P < 0.01$, $n = 6-8$ per group, Fig. 6). This interaction was significant for both measurements at both E14 and E17. This interaction demonstrates that the percentage of cycling neocortical cells immunolabeled with Cx43 or Cx26 changes differentially through the cell cycle in both early and late neocortical neurogenesis. The percentage of cells expressing Cx26 increases between S and G1, while the percentage expressing Cx43 decreases between S and G1.

Cx26 and Cx43 Antibodies are Specific

In order to demonstrate the specificity of the Cx26 and Cx43 antibodies used for immunohistochemistry in this study, we tested the antibodies on tissue known to express exclusively Cx26 or Cx43. Cx26 is highly expressed by liver cells, while Cx43 is highly expressed by heart cells (reviewed by Bennet *et al.*, 1991). We used the polyclonal antibodies from this study to stain both tissue types from an adult mouse. Cx26 immunoreactivity was robust in adult mouse liver and absent in adult mouse heart. Conversely, Cx43 immunostaining was evident in

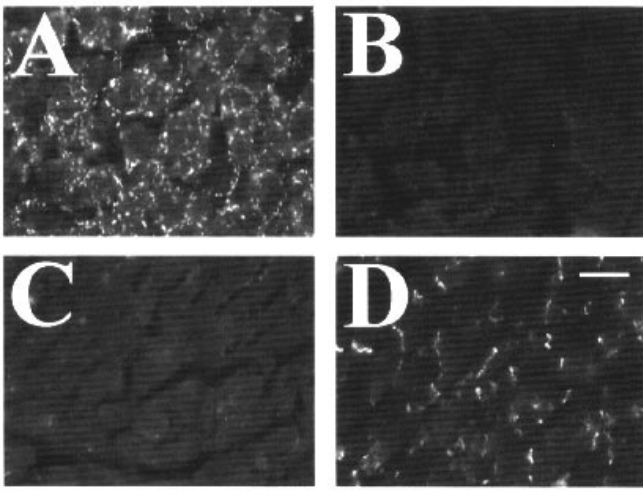


Figure 7. Anti-connexin antibodies do not cross-react. (A,B) Cx26 staining in adult mouse liver (A) and heart (B). Cx26 immunoreactivity is abundant in liver and absent in the heart. (C,D) Cx43 immunostaining in liver (C) and heart (D). Cx43 immunoreactivity is present in adult mouse heart cells, but not liver cells. Scale bar = 20 μ m.

heart tissue, but not in liver (Fig. 7). In addition, the monoclonal anti-Cx43 antibody (Zymed) used in this study (Fig. 1E) recognizes a 43 kDa band from an isolated E17 mouse neocortical protein preparation (data not shown). The Cx26 and Cx43 antibodies used in these experiments do not cross-react and appear specific to the antigens they were raised against.

Discussion

Neurons of the adult mammalian neocortex are generated through a series of cellular divisions through a discrete period in embryogenesis. In the mouse, neocortical neurogenesis occurs between gestational days 11 and 17 with a majority of neurons generated during the last two days (Takahashi *et al.*, 1996). During this period, dividing cells within the VZ couple together into clusters (LoTurco and Kriegstein, 1991). Coupling of neocortical precursor cells into clusters is a dynamic process related to the cell cycle (Bittman *et al.*, 1997). Coupling is maximal by the time cells enter early G1 phase. Cells then uncouple and recouple during S phase. In the present study we demonstrate that cycling neocortical cells and radial glial cells express Cx26 and Cx43, and that both undifferentiated neuroepithelial cells and radial glial cells are found among clusters of coupled VZ cells. By combining BrdU and connexin immunohistochemistry we demonstrate that Cx26 and Cx43 are differentially expressed through the cell cycle. The percentage of cycling neocortical cells expressing Cx26 increases from S to early G1 phase of the cell cycle and the percentage of cycling neocortical cells expressing Cx43 decreases from S to G1 phase of the cell cycle. We postulate that through differential expression of these connexin proteins, VZ cells couple and uncouple through the cell cycle. This dynamic process may compartmentalize cells expressing homologous connexins and could therefore synchronize populations of cycling VZ cells (Cai *et al.*, 1997a,b). In addition, the presence of radial glial cells among clusters of coupled neural precursors may be related to radial units (Rakic, 1988).

Glial Cells Couple into VZ Clusters

Radial glial cells of developing mammalian VZ are used as guides by young neurons migrating from the VZ to the cortical plate

(Rakic, 1988; Gasser and Hatten, 1990; Hatten, 1990). Since radial glial cells of the VZ extend processes to the pial surface (Misson *et al.*, 1988a,b; Takahashi *et al.*, 1990), these processes may relay information from outside the VZ to coupled neural precursors within the VZ. Recently, Owens and Kriegstein (1998) have shown fluctuations in intracellular calcium in VZ cells clusters, and pharmacological agents known to block synaptic transmission do not disrupt these fluctuations. However, pharmacologically reducing intracellular stores of calcium abolishes calcium propagation among VZ clusters (Owens and Kriegstein, 1998). Cell coupling between radial glia and neuronal precursors may provide a mechanism for relaying and restricting cell signaling to a local population of progenitors in the VZ.

Cx26 and Cell Coupling

Regulated expression of gap junction proteins through the cell cycle has been reported in a variety of species and tissue types. For example, in regenerating rat tracheal epithelia, Gordon *et al.* (1982) have shown that there is an increase in the number of gap junctions between S and G2 phases of the cell cycle. Further, Lee *et al.* (1992) have demonstrated that Cx26 expression increases in late S and early G2 phase in synchronously cycling human mammary epithelial cells. Our results demonstrate that the percentage of cycling neocortical cells immunoreactive to Cx26 increases significantly from S to early G1 phase of the cell cycle during the middle of neocortical neurogenesis (Fig. 6). In addition, in our previous work we have shown that mouse VZ cells are maximally coupled by early G1 phase of the cell cycle during both early and late neurogenesis (Bittman *et al.*, 1997). As a result of these observations, we suggest that an increase in the percentage of neural precursors expressing Cx26 from S to early G1 phase of the cell cycle may promote cell coupling during the middle of neurogenesis.

Cx43 and Cell Coupling and Uncoupling

No previous studies have indicated any change in Cx43 expression through the cell cycle. In fact, Tomasetto *et al.* (1993) found Cx43 expression levels to remain constant throughout the cell cycle of synchronously cycling mammary epithelial cells. Here we describe a decrease in the number of cycling neocortical cells immunoreactive to Cx43 from S to G1 phase of the cell cycle. The decrease in the percentage of cycling cells immunoreactive to Cx43 through G1 phase of the cell cycle correlates with cell uncoupling in G1 phase of the cell cycle (Bittman *et al.*, 1997). This decrease is most prominent during later neurogenesis when a majority of cells are becoming post-mitotic (Caviness *et al.*, 1995; Takahashi *et al.*, 1996). The changes in Cx43 expression through the cell cycle during late cortical neurogenesis may be related to cell differentiation.

The functional significance of a decrease in the percentage of cycling cells immunoreactive to Cx43 through the cell cycle is unclear. However, in human neuronal cell lines and immortalized mouse hippocampal cells, Cx43 levels decrease as neuronal differentiation occurs. Differentiation has been correlated with reduced cell coupling (Rozental and Spray, 1996; Bani-Yaghoob *et al.*, 1997; Rozental *et al.*, 1998). Additionally, astrocyte growth is significantly reduced in primary culture of astrocytes lacking the Cx43 gene (Naus *et al.*, 1992). Also, bFGF increases Cx43 expression in neocortical precursors and increases the number of cells that enter S phase (Nadarajah *et al.*, 1998). Subsequently, it may be during G1 phase of the cell cycle that proliferating cells become responsive to new signals that

initiate the process of differentiation. Taken together, these observations suggest that decreased Cx43 expression during G1 phase of the cell cycle may increase the probability of uncoupling from a cluster and initiate differentiation of some VZ cells.

Interaction Between Cx26 and Cx43

Our results demonstrate that cycling precursor cells as well as radial glial cells express both Cx26 and Cx43. In addition, VZ cells can express a combination of Cx26 and Cx43 or either protein alone. Pharmacological blockade of gap junction channels reduces uptake of BrdU in the VZ of cortical explants (Bittman *et al.*, 1997), suggesting that gap junction communication is necessary for cells to continue progressing through the cell cycle. A wide variety of chemical agents are capable of blocking gap junction channels, but very few disrupt specific connexin hemichannels. Consequently, it remains uncertain whether Cx26 and Cx43 contribute equally to VZ cell coupling and proliferation. Since cycling cells and radial glial cells express both Cx26 and Cx43, it may be possible that an interaction between the two connexins contributes to synchronizing the activities of cells locally within the VZ (Cai *et al.*, 1997a,b). The actual effect of cell coupling may vary depending on the connexin expressed, phase of the cell cycle and period of neurogenesis.

Cx43 hemichannels are not capable of forming dye-permeable channels when co-cultured with cells expressing Cx26 hemichannels (Tomasetto *et al.*, 1993). The lack of Cx26 and Cx43 hemichannel coupling could serve to compartmentalize populations of VZ cells in different phases of the cell cycle. If Cx26 and Cx43 hemichannels cannot combine, then cells expressing the same connexins would be more likely to couple together into clusters. As cells progress through S phase of the cell cycle, the percentage of cells expressing Cx26 increases and the likelihood of Cx26/Cx26 channel formation rises. As a result, VZ cell coupling would increase. During early S phase of the cell cycle, the percentage of cells expressing Cx43 is similar to the percentage expressing Cx26. Subsequently, coupling at this time would depend on whether neighboring cells express the same connexin protein. In addition, since a high percentage of radial glial cells express both Cx26 and Cx43, cells in all phases could interact directly with radial glial cells. Through differential connexin expression, a dynamic relationship between radial glial cells and neural precursors may be established within a developing radial unit.

Notes

This work was supported by the Ester A. and Joseph Klingenstein Foundation and the Human Frontiers Science Program and by Public Health Service Grant MH56524.

Address correspondence to Joseph J. LoTurco, Department of Physiology and Neurobiology, University of Connecticut U-156, 3107 Horsebarn Hill Road, Storrs, CT 06268-4156, USA. Email: LoTurco@oracle.pnb.uconn.edu.

References

Bani-Yaghoob M, Bechberger J, Naus C (1997) Reduction of connexin43 expression and dye-coupling during neuronal differentiation of human NTera2/clone D1 cells. *J Neurosci Res* 49:19-31.

Bennet M, Barrio L, Bargiello T, Spray D, Hertzberg E, Saez J (1991) Gap junctions: new tools, new answers, new questions. *Neuron* 6:305-320.

Bittman K, Owens D, Kriegstein A, LoTurco J (1997) Cell coupling and uncoupling in the ventricular zone of developing neocortex. *J Neurosci* 17: .

Boulder C (1970) Embryonic vertebrate central nervous system: revised terminology. *Anat Rec* 166:257-261.

Cai L, Hayes N, Nowakowski R (1997a) Local homogeneity of cell cycle length in developing mouse cortex. *J Neurosci* 17: 2079-2087.

Cai L, Hayes N, Nowakowski R (1997b) Synchrony of clonal cell proliferation and contiguity of clonally related cells: production of mosaicism in the ventricular zone of developing mouse neocortex. *J Neurosci* 17: 2088-2100.

Caviness V, Takahashi T, Nowakowski R (1995) Numbers, time and neocortical neurogenesis: a general developmental and evolutionary model. *Trends Neurosci* 18:379-383.

Dermietzel R (1996) Molecular diversity and plasticity of gap junctions in the nervous system. In: *Gap junctions in the nervous system* (Dermietzel R, Spray D, eds), pp. 13-38. New York: Chapman and Hall.

Dermietzel R, Traub O, Hwang T, Beyer E, Bennett M, Spray D (1989) Differential expression of three gap junction proteins in developing and mature brain tissues. *Proc Natl Acad Sci USA* 86:10148-10152.

Gasser U, Hatten M (1990) Central nervous system neurons migrate on astroglial fibers from heterotypic brain regions *in vitro*. *Proc Natl Acad Sci USA* 87:4543-4547.

Gordon R, Lane B, Marin M (1982) Regeneration of rat tracheal epithelium: changes in gap junctions during specific phases of the cell cycle. *Exp Lung Res* 3:47-56.

Hatten M (1990) Riding the glial monorail: a common mechanism for glial-guided neuronal migration. *Trends Neurosci* 13:179-184.

Kandler K, Katz, I (1998) Coordination of neuronal activity in developing visual cortex by gap junction-mediated biochemical communication. *J Neurosci* 18:1419-1427.

Lee S, Tomasetto C, Paul D, Keyomarsi K, Sager R (1992) Transcriptional downregulation of gap-junction proteins blocks junctional communication in human mammary tumor cell lines. *J Cell Biol* 118:1213-1221.

Levitt P, Cooper M, Rakic P (1981) Coexistence of neuronal and glial precursor cells in the cerebral ventricular zone of the fetal monkey: an ultrastructural immunoperoxidase analysis. *J Neurosci* 1:27-39.

LoTurco J, Kriegstein A (1991) Clusters of coupled neuroblasts in embryonic neocortex. *Science* 252:563-566.

LoTurco J, Owens D, Heat M, Davis M, Kriegstein A (1995) GABA and glutamate depolarize cortical progenitor cells and inhibit DNA synthesis. *Neuron* 15:1287-1298.

Misson J, Edwards M, Yamamoto M, Caviness V (1988a) Identification of radial glial cells within the developing murine central nervous system: studies based upon a new immunohistochemical marker. *Dev Brain Res* 44:95-108.

Misson J-P, Edwards M, Yamamoto M, Caviness V (1988b) Mitotic cycling of radial glial cells of the fetal murine cerebral wall: a combined autographic and immunohistochemical study. *Dev Brain Res* 38:183-190.

Nadarajah B, Jones A, Evans W, Parnavelas J (1997) Differential expression of connexins during neocortical development and neuronal circuit formation. *J Neurosci* 17:3096-3111.

Nadarajah B, Makarenkova H, Becker D, Evans W and Parnavelas J (1998) Basic FGF increases communication between cells in the developing cortex. *J Neurosci* 18:7881-7890.

Naus C, Zhu D, Todd S, Kidder G (1992) Characteristics of C6 glioma cells overexpressing a gap junction protein. *Cell Mol Neurobiol* 12:163-175.

Nedergaard M (1994) Direct signaling from astrocytes to neurons in cultures of mammalian brain cells. *Science* 263:1768-1771.

Owens D, Kriegstein, A (1998) Patterns of intracellular calcium fluctuation in precursor cells of the neocortical ventricular zone. *J Neurosci* 18:5374-5388.

Rakic P (1988) Specification of cerebral cortical areas. *Science* 241:170-176.

Rozental R, Spray D (1996) Temporal expression of gap junctions during neuronal ontogeny. In: *Gap junctions in the nervous system* (Spray D, Dermietzel R, eds), pp. 261-278. New York: Chapman and Hall.

Rozental R, Morales M, Mehler M, Urban M, Kremer M, Dermietzel R, Kessler J, Spray D (1998) Changes in properties of gap junctions during neuronal differentiation of hippocampal progenitor cells. *J Neurosci* 18:1753-1762.

Spray D (1996) Physiological properties of gap junction channels in the nervous system. In: *Gap junctions in the nervous system* (Spray D, Dermietzel R, eds), pp. 39-60. Austin, TX: Chapman and Hall.

Takahashi T, Misson J, Caviness V (1990) Glial process elongation and branching in the developing murine neocortex: a qualitative and quantitative immunohistochemical analysis. *J Comp Neurol* 302:15-28.

Takahashi T, Nowakowski R, Caviness V (1993) Cell cycle parameters and patterns of nuclear movement in the neocortical proliferative zone of the fetal mouse. *J Neurosci* 13:820-833.

Takahashi T, Nowakowski R, Caviness V (1996) The leaving or Q fraction of the murine cerebral proliferative epithelium: a general model of neocortical neurogenesis. *J Neurosci* 16:6183-6196.

Tomasetto C, Neveu M, Daley J, Horan P, Sager R (1993) Specificity of gap junction communication among human mammary cells and connexin transfectants in culture. *J Cell Biol* 122:157-166.