Ethanol Induces Heterotopias in Organotypic Cultures of Rat Cerebral Cortex

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Introduction

During normal migration, neurons with a particular time of origin are distributed within a specific stratum of cortex (e.g. Angevine and Sidman, 1961; Berry and Rogers, 1965; Rakic, 1974; Luskin and Shatz, 1985; Miller, 1985, 1988a). Early-generated neurons migrate to deep cortex and later generated neurons take progressively more superficial positions. This inside-to-outside development of normal cerebral cortex depends upon interactions between young neurons and radial glia. The neuron–glia association is key for establishing the columnar organization that characterizes cerebral cortex.

Neuron–Radial Glia Interactions

The interaction between neurons and radial glia is important for guiding young neurons through the complex matrix of the intermediate zone (IZ) and cortical plate (CP). Breakdown in the neuron–radial glia interaction can result in neurons being distributed in an ectopic site(s). An example of such a disruption occurs in the murine mutant reeler. In the reeler mouse, neuron–glia interactions are obstructed (Pinto-Lord et al., 1982) and cells in the marginal zone (MZ), notably Cajal–Retzius cells, are disorganized (Derer, 1985) and dysfunctional (D’Arcangelo et al., 1995). A small collection of lissencephaly cases in humans are linked to mutations in the reeler gene, characterized by migration defects in the cortex and cerebellum (Hong et al., 2000).

Reeler mice cannot express reelin, a large glycoprotein produced primarily by Cajal–Retzius cells in the MZ and deposited into the extracellular space (D’Arcangelo et al., 1995; Hirotsune et al., 1995; Ogawa et al., 1995; Soda et al., 2003). Reelin binds to at least two receptors, very low density lipoprotein receptor (VLDLR) and apolipoprotein-E receptor type 2 (ApoER2) (Hiesberger et al., 1999; Trommsdorff et al., 1999). Reelin-receptor binding causes the phosphorylation of an adaptor protein Disabled 1 (Dab1). Dab1 is attached to the intracellular domain of VLDLR and ApoER2 (Rice et al., 1998; Howell et al., 1999). Reelin also can bind to integrin α3, integrin β1, and members of the cadherin-related neuronal receptor, all of which are important for neuronal migration (Sezaki et al., 1999; Dulabon et al., 2000).

Though controversial, reelin (i) may act as a chemoattractant, goading the young neurons to migrate into the cortical plate, or (ii) it may serve as a signal for young neurons to stop their migration and to detach from their glial guide (Pearlmman and Sheppard, 1996; Marin-Padilla, 1998; Dulabon et al., 2000; Hack et al., 2002; Nishikawa et al., 2002; Tabata and Nakajima, 2002; Luque et al., 2003). Reelin may also regulate the function and identity of radial glia (Marin and Rubenstein, 2003).

Errors in Neuronal Migration

Small alterations in neuronal migration can produce defects in cortical organization. For example, exposure to moderate amounts of ethanol in vivo can desynchronize the migration process. The initiation of migration can be delayed and the rate of migration retarded (Miller, 1993). The net result is that the columnar organization of cortex can be disrupted so that cells with particular (generational and/or connectional) phenotypes can terminate their migration in ectopic sites (Miller, 1986, 1987, 1988b, 1993, 1997) and form abnormal synaptic relations (Miller, 1987; Al-Rabiai and Miller, 1989). Likely these alterations underlie the mental retardation associated with fetal alcohol exposure. Other neurological disorders, e.g. autism, Asperger’s syndrome, dyslexia and Rett syndrome, exhibit similar alterations in cortical columnar organization and circuitry (Casanova et al., 2002a,b,c, 2003).

Disruptions of the orderly sequence of neuronal migration can produce heterotopic clusters: intra- and supracortical heterotopias. They are common among people with a variety of neurological disorders (e.g. Dunn et al., 1986; Shaw, 1987). These include epilepsy (Blom et al., 1984; Livingston and Aicardi, 1990; Palmini et al., 1991), dyslexia (Galaburda and Kemper, 1979; Galaburda et al., 1985; Humphreys et al., 1990), fetal alcohol syndrome (Clarren and Smith, 1978;
Recent studies suggest that heterotopias form following damage to the pial membrane (PM; also known as pial basal membrane). Mice lacking integrins αv, αq, or β3 display major defects in the cortical PM that lead to the formation of ectopias (Georges-Labouesse et al., 1998; Graus-Porta et al., 2001). During development, damage to the PM caused by physical trauma precedes the appearance of heterotopias (Marin-Padilla, 1996). Loss or dysfunction of proteins that comprise the PM, including laminin (Halfter et al., 2002), perlecan (Costell et al., 1999) and chondroitin sulfate proteoglycans (Blackshear et al., 1997), induce a breakdown of the PM leading to over-migration of neurons and the formation of peripial heterotopias.

Developmental exposure to ethanol can produce errors in migration in rodents (e.g. Miller, 1986, 1992, 1995). Such defects have been identified throughout the nervous system, e.g. neural crest cells (Rovasio and Battiotto, 2002), brainstem neurons (Zhou et al., 2001), and cerebellum (Kornguth et al., 1979; Lewis, 1985; Liesi, 1997). The most thoroughly studied area is the neocortex. Based on their time of origin, neurons migrate to inappropriate sites and the kinetics of neuronal migration is altered (Miller, 1986, 1988c, 1993, 1997; Miller et al., 1990; Komatsu et al., 2001; Siegenthaler and Miller, 2004). A common sequela is the formation of heterotopic cell clusters within cortex and beyond the pial surface (Miller, 1986, 1987, 1988c, 1993; Gressens et al., 1992; Kotkoskie and Norton, 1988; Miller et al., 1990; Komatsu et al., 2001; Sakata-Haga et al., 2002; Mooney and Miller, 2003). Supra-pial heterotopias are also referred to as ‘warts’. We hypothesize that these warts (i) represent neurotoxic effects of ethanol on the termination of reelin expression in the MZ and (ii) result from disruptions in reelin-dependent synaptic integrity.

Materials and Methods

Culture Models

Organotypic cultures were prepared from rat fetuses on gestational day (G) 17. To ensure uniformity, the day on which a sperm-positive plug was first found was designated G1. Pregnant dams were anesthetized (90 mg/kg ketamine and 9 mg/kg xylazine), their uteri were carefully removed and cut into 300 µm thick coronal slices using a McIlwain Tissue Chopper (Mickle Lab. Eng., Gomshall, UK). Tissue was rinsed in ice-cold stock medium [minimum essential medium (GibcoBRL, Grand Island, NY), 24% glucose, 1.0% penicillin/streptomycin and 25 mM potassium chloride] supplemented with 20% fetal bovine serum. Samples were transferred onto a culture filter (PICM0RG50; Millipore, Bedford, MA) in a Petri dish containing fresh supplemented stock medium. After a 3 h incubation (37°C, 6% CO2), the medium was replaced with a serum-free stock medium containing ethanol (0, 200, 400, or 800 mg/dl). This bridges the range of ethanol exposures described in human and rat studies documenting heterotopias (Claren et al., 1978; Kotkoskie and Norton, 1988; Komatsu et al., 2001; Sakata-Haga et al., 2002). Moreover, bioassays show that exposures to ethanol needed to alter neural development in vitro are less than half that needed to replicate the alteration in vitro (e.g. cf. Miller and Nowakowski, 1991; Jacobs and Miller, 2001; and cf. Miller, 1993; Siegenthaler and Miller, 2004). The preparations were maintained in the ethanol-containing medium for 2, 6, 8, 16, 24, or 32 h. Some preparations were incubated for 17 h in a medium containing 0.004% 5-bromo-2-deoxyuridine (BrdU) to label cells passing through the S-phase of the cell cycle.

Unbound primary antibody was removed by washing the sections in mPBS. Tissue was then incubated in biotinylated secondary anti-mouse or anti-rabbit IgG (1:1000; Vector, Burlingame CA) for 1 h. Sections were incubated in avidin–horseradish peroxidase conjugate (Vector). Immunoreactivity was visualized by washing the sections with 3,3’-diaminobenzidine (DAB; Vector) in the presence of hydrogen peroxide. Some secondary antibodies were bound to a fluorescent tag. All immunolabeling steps were performed at room temperature. Finally, sections were counterstained with cresyl violet.

Cultures treated with BrdU were immunolabeled with an anti-BrdU antibody (5+7580; 1:30 in mPBS; BD Biosciences). Sections were treated for immunohistochemistry as described above with the addition of a pretreatment in 2 N HCl prior to application of the anti-BrdU antibody. Some sections were processed for terminal uridylated nick-end labeling (TUNEL, S7100; Intergen) which identifies cells exhibiting fragmented DNA, i.e. apoptotic cells (e.g. cf. Miller, 1992). Briefly, tissue was exposed to terminal deoxynucleotidyl transferase TdT for 1 h at 37°C, rinsed in PBS and then incubated in a peroxidase-conjugated anti-digoxigenin. Immunolabeling was visualized by washing the tissue in a solution of DAB in the presence of hydrogen peroxide (see above). The sections were counterstained with methyl green to assess nuclear appearance.

Results

Normal Cortical Structure

Five main strata were distinguished in the cerebral walls of organotypic slice cultures (Fig. 1). The upper part of the cerebral wall contained the MZ and CP. The MZ was relatively acellular and the CP was cell-dense. The IZ was deep to the CP. It was discriminable by linear arrays of cells. Two deepest zones, the ventricular zone (VZ) and the subventricular zone (SZ) were densely packed with cell bodies. The VZ was a pseudostratified columnar epithelium and the SZ contained more haphazardly arranged cell bodies.

Heterotopias were not evident in control-treated cultures. The MZ was largely devoid of neuronal somata and no cells broached the integrity of the pial surface.
Ethanol-induced Warts

Appearance
Heterotopias were apparent in many ethanol-treated preparations. Within the cerebral wall, heterotopic cells were common in the MZ (Fig. 1; Mooney and Miller, 2003). Often the crowding in the MZ was so great that it was difficult to distinguish the MZ from the CP. That said, these heterotopias did not occupy the entire MZ. Wide expanses of the MZ appeared normal, i.e. as in the untreated slices.

In instances where the pial or ventricular surface was broached, cells were found beyond the normal boundaries in the formation of warts (Fig. 2). Two types of warts were identified: peduncular and appositional. In peduncular warts, the cells formed an enlarged mass that was connected by a stalk to the cerebral wall. In appositional warts, a mass of cells lay directly over a >200 µm wide swath of the cortical surface. Thus, it had the appearance of adhering to the outer surface of the cultured tissue.

Factors Defining Warts
The length of time a slice was treated with ethanol altered the incidence and the morphology of the warts. Warts first appeared in cultures treated with ethanol (400 mg/dl) for 8 h and the frequency of warts increased over time (Fig. 3A). All of the warts detected at 8 h were peduncular warts. With longer exposure to ethanol, the frequency of appositional warts increased.

The presence of warts was concentration-dependent (Fig. 3B). In slices treated with ethanol for 24 h, the incidence of warts was 50% greater in slices treated with 800 mg/dl ethanol than it was for slices treated with 400 mg/dl ethanol. Cultures treated with low concentrations of ethanol (200 mg/dl) did not exhibit warts after 24 h; however, such low concentrations of ethanol eventually can induce warts to form. Small warts were evident in slices treated with only 200 mg/dl ethanol for 72 h (data not shown).

Ethanol concentration altered the morphology of the warts. Following treatment with 800 mg/dl ethanol, the number of slices that had appositional warts was 50% higher than slices treated with 400 mg/dl ethanol. The number of slices showing peduncular warts was the same for slices treated with moderate (400 mg/dl) or high (800 mg/dl) concentrations of ethanol. Interestingly, tissue treated with 800 mg/dl ethanol for 24 h also showed warts on the ventricular surface (Fig. 4).

Composition of the CP and Wart
Few cells in the warts were TUNEL-positive or contained condensed chromatin (Fig. 5). Thus, the majority of cells was viable. No somata in the warts were GFAP-positive. In contrast, virtually all cell bodies in the CP and in the warts were MAP-2-
or NeuN-immunopositive. This suggests that the somata were neuronal. Furthermore, most cell bodies in the superficial CP and in suprapial and ventricular warts, were doublecortin-positive implying that they were actively migrating (Figs 4 and 6). Together, these data argue that most cells in the superficial CP and in the warts were migrating neurons.

Immunoreactivity of calretinin, a calcium binding protein, was localized to cells and neuropil in the MZ, presumably Cajal–Retzius cells. Reelin-positive staining was also evident in the MZ and the superficial CP. The MZ subjacent to a wart exhibited a break in the layer of calretinin immunolabeling. In many preparations, cells in the MZ converged towards this break. Interestingly, calretinin and reelin immunolabeling was seen at the outer margins of the wart, but reelin-positive staining was noticeably absent underneath a wart.

BrDU-positive cells were seen in the proliferative VZ, never in the CP or the wart, indicating that cells in the latter two regions were post-mitotic (Fig. 7).

The developing cortex was rife with nestin- (Fig. 8) and vimentin-immunoreactive elements. Coarse, parallel nestin-positive fibers, presumably bundles of radial glial fibers, were evident through the IZ. These fibers were thinner in the CP. In more superficial CP and in the MZ fibers became more randomly oriented. On occasion, thin fibers could be seen coursing through the MZ and into the suprapial wart. Ventricular warts also contained nestin-positive fibers (data not shown).

Discussion

Ethanol-induced Alterations

Ethanol induces structural malformations in superficial cortex. (i) The density of cells in the MZ is increased. This increased cellularity concurs with previous reports of ectopic cells in the superficial cortex (e.g. Clarren et al., 1978; Sakata-Haga et al., 2002; Mooney and Miller, 2003). These ectopic cells in the MZ are NeuN-positive, i.e. they are neurons. (ii) Clusters of cells, or warts, are evident in the suprapial space. These are similar to heterotopias described in vivo in humans (Clarren et al., 1978) and rodents (Kotkoskie and Norton, 1988; Gressens et al., 1992; Komatsu et al., 2001; Sakata-Haga et al., 2002). (iii) There are abnormalities in the organization of nestin-positive radial glia and calretinin-positive (Cajal–Retzius) neurons.

The changes detected in the slices treated with 400 mg/dl ethanol are similar to those in rats with moderate (−150 mg/dl; Komatsu et al., 2001) or high (<300 mg/dl; Kotkoskie and Norton, 1988) blood ethanol concentrations. This pattern of requiring higher concentrations of ethanol in vitro to match ethanol-induced changes in vivo is evident for specific developmental events. For example, 2½-fold more ethanol is needed in vitro to mimic in vivo changes in cell proliferation (cf. Miller and Nowakowski, 1991; Jacobs and Miller, 2001) and migration (cf. Miller, 1993; Siegenthaler and Miller, 2004).

Effects of Ethanol on Neuronal Migration

The various ethanol-induced changes in the structure of superficial cortex are consistent with data showing that ethanol affects neuronal migration. (i) The infiltrated MZ and warts contain doublecortin-positive cells. Doublecortin is expressed by migrating neurons and, to a lesser extent, by differentiating neurons that have recently completed their migrations (Francis et al., 1999; Gleeson et al., 1999; Friocourt et al., 2003). (ii) Physical and chemical guides for neuronal migration (radial glia, Cajal–Retzius cells, and reelin) are affected by ethanol. (iii) It appears that the warts are formed by cells that are nearing the end of their migration. Warts appear within eight h of ethanol treatment. The MZ is ~20 μm wide. In the presence of ethanol, cell migration in ethanol-treated cultures proceeds at a mean rate of 3.5 μm/h (Siegenthaler and Miller, 2004). Hence, it takes ~6 h for a cell to traverse and move beyond the MZ. (iv) Warts do not contain any BrDU-labeled cells. Thus, it is not an ectopic site of cell proliferation and cells forming the wart must have been generated elsewhere and migrated into the wart.

Ethanol affects the active process of neuronal migration. It alters the rate of migration and the expression of cell adhesion proteins (CAPs) in cultured cortical preparations (Hirai et al., 1999; Siegenthaler and Miller, 2004). Ethanol also induces CAP expression by dissociated primary neural cells and neural cancer cells (Miller and Luo, 2002a,b) and alters the actions of CAPs (Charness et al., 1994; Ramanathan et al., 1996; Wilkemeyer and Charness, 1998; Bear et al., 1999). The data from these culture studies concur with findings from in vivo studies. Prenatal exposure to ethanol delays the onset of neuronal migration and retards the rate of migration (Miller, 1993), alters the structure of radial glia (Miller and Robertson, 1993; Miller, 2003) and affects CAP expression (Miñana et al., 2000). The net result is that ethanol causes neurons to end their migration at ectopic sites throughout cortex (e.g. Miller, 1986, 1988c; Miller et al., 1990; Sakata-Haga et al., 2002; Mooney and Miller, 2003). Interestingly, patterns of ectopic neurons are also evident in mice with conditional knockouts of integrin β1 (Graus-Porta et al., 2001). These animals exhibit infiltration of neurons into the MZ in regions where the extracellular matrix is degraded.

Figure 3. Factors influencing the appearance of warts. (A) The effect of the length of exposure to ethanol (400 mg/dl) on the frequency of slices exhibiting a wart is described. Slices (n = 54) taken from a dozen litters were treated with ethanol for 2, 8, 16, 24, or 32 h. (B) The effect of ethanol concentration on the frequency of warts is shown. Bars depict the percentage of slices that have warts caused by treatment with ethanol (0, 200, 400 and 800 mg/dl) for 24 h. The solid and open portions of the bars represent the ratios of peduncular and appositional warts, respectively. n = 31 slices from seven litters.
Figure 4. Doublecortin labeling in a ventricular surface wart. (A) Doublecortin immunolabeling in the cerebral wall is highest in the IZ. Reduced labeling is evident in the SZ, CP and MZ, and none is detected in the VZ. (B) Treatment with a high concentration of ethanol induces a wart at the ventricular surface. Most cells in this wart are doublecortin-positive. Scale bars = 50 µm.

Figure 5. Cells in wart are viable and Neu-N positive. (A) Few cells in the cerebral wall or the wart are TUNEL-positive (solid arrowheads) or have condensed chromatin (open arrowheads). Thus, the majority of cells are viable. The inset is a high magnification image depicting the two types of cells. (B) Most cells in the CP and the wart are Neu-N immunolabeled (solid arrows). Only a few cells in the wart are Neu-N-negative (open arrows). Scale bars = 50 µm for the low magnification images (A, B) and 10 µm for the inset.

Figure 6. MZ is compromised beneath wart region. (A) Calretinin-positive somata (crossed, solid arrows) and processes (solid arrows) are distributed in both the MZ and subplate. The immunolabeling in the MZ is interrupted beneath the wart and calretinin-negative cells (open arrows) are in the breach. (B) Reelin immunolabeling (open arrows) is common in the MZ. Labeled cells tend to be aligned in the MZ. This array is noticeably absent below the wart, however, reelin immunolabeling is evident toward the limit of the wart (solid arrows). (C) Most cells in a suprapial wart express doublecortin. Scale bars = 50 µm.
Suprapial Warts

The migration defects underlying neuronal infiltration of the MZ and wart formation are not artifacts of the preparation. Instead, they must be caused by ethanol. After all, neither structural abnormality occurs in control preparations and both occur in ethanol-treated slices in a concentration-dependent manner. Moreover, warts emanate from the middle of the slice, that is, they are not growing from the cut surfaces of the slice. Why then do warts form? Some insight comes from studies in which the pia is mechanically damaged. Such damage results in ectopic cells in the MZ and in disruption of the radial glial fibers near the wound (Rosen et al., 1992; Marin-Padilla, 1996, 1999). In addition, mechanical insult leads to defects in cortical lamination which are akin to those caused by ethanol (e.g. Clarren et al., 1978; Miller, 1986, 1988c).

Warts in the present study are not caused by mechanical damage to the pia or the MZ. The most compelling reason for this is that warts are not present in control slices. Ethanol-induced warts are solid clusters of cells with definite borders, e.g. as shown in the reelin and nestin immunostaining near the edge of the wart (see Figures 6 and 8).

Figure 7. Cumulative BrdU labeling. BrdU-labeled cells are confined to the proliferative zones of the cerebral wall, the ventricular zone (VZ) and the subventricular zone (SZ)(open arrows). The wart is devoid of BrdU-labeled cells. Scale bar = 50 µm.

Figure 8. Radial glial fibers. (A) Nestin-immunoreactive radial glial processes extend into the superficial cerebral wall. These processes extend into an appositional wart (black arrows). The breach of the MZ is identified by an asterisk. (B) The normal and abnormal termination of radial glia are shown in a whole-mount preparation. End-feet of radial glia (crossed arrows) are commonly located in the MZ. Radial glia enter and ramify in the wart (white arrows). (C) A drawing of this wart shows the passage of nestin-positive fibers from the MZ into the wart (black arrow) and the disorganized array of fibers and glial end-feet in the wart. Scale bars = 50 µm.
**Ventricular Zone Warts**

In rare instances, warts are evident at the ventricular surface. VZ warts, also known as periventricular heterotopias, are like the suprapial warts in that they are bounded nodules of cells. They contain doublecortin- and nestin-positive elements, which implies that the cells in the VZ warts are actively migrating along radial glia. This finding contrasts with the conclusions of others (Santi and Golden, 2001) who state that periventricular heterotopias form from a failure of cells to migrate from the VZ and result from disrupted radial glia.

The VZ is the site of origin for most migrating neurons. VZ cells are dynamic. The nuclei of these cells move pially and ad-pially as the cells pass through the stages of the cell cycle (Sauer, 1935, 1936; Seymour and Berry, 1975; Tramontin et al., 2003). Presumably, VZ warts form after the ventricular surface is abrogated at a particular point possibly by a focal disruption of tight junctions among adjacent VZ cells (Sakata-Haga et al., 2002). Radial migration is bidirectional (e.g. Hatten, 1990, 2002; T.F. Haydar, personal communication). Although the prevailing route is away from the ventricular surface, some cells may begin their migration in an ad-pial direction and pioneer the formation of a VZ wart if they pass through an ethanol-induced breach in the ventricular surface.

**Ethanol-induced Damage to the Pial Surface**

Basement and pial membranes at the ventricular and pial surfaces, respectively, constitute physical barriers between neural tissue and surrounding environments. The PM provides a contact point for radial glial thus forming the glial limitans (Sievers et al., 1994). The basement membrane at the ventricular surface provides an attachment point for proliferating progenitors undergoing interkinetic nuclear migration (Sauer, 1935, 1936; Seymour and Berry, 1975). Components of the membranes – laminin, collagen and nidogen – are synthesized and released by meningeal cells (Sievers et al., 1994).

Ethanol-induced alterations in the distribution of calretinin- and reelin-immunoreactivity and inappropriate placement of radial glia suggest that ethanol alters the PM and glial limitans. This conclusion is supported by studies showing (i) that ethanol-induced warts are linked to defects in the glial limitans (Komatsu et al., 2001) and (ii) that ethanol causes the abnormal degradation of laminin (Liesi, 1997). Moreover, mice expressing a mutant laminin exhibit disruptions in the extracellular matrix of the PM, over-migration of cells into the MZ, and often have warts (Halfter et al., 2002) like those in ethanol-treated tissues. Interestingly, cellular infiltration of MZ occurs without accompanying defects in the integrity of the glial limitans or in laminin immunostaining (Sakata-Haga et al., 2002).

**Role of Growth Factors**

Growth factors regulate many processes in cortical development (Bibel and Barde, 2000; Huang and Reichardt, 2001). Two neurotrophins involved in the migration of cortical neurons are brain-derived neurotrophic factor (BDNF; Knusel et al., 1994; Behar et al., 1997; Ringstedt et al., 1998; Polleux et al., 2002) and neurotrophin-4 (NT-4; Behar et al., 1997; Brunstrom et al., 1997; Polleux et al., 2002). Altered expression of these neurotrophins results in heterotopic MZ cells (Brunstrom et al., 1997; Ringstedt et al., 1998). Apparently, BDNF negatively regulates reelin expression (Ringstedt et al., 1998) and intraventricular injection of NT-4 results in ectopic cells in the suprapial space at the site of injection (Brunstrom et al., 1997). The latter likely results from aberrant migration and physical damage to the pia.

Another key regulator of neuronal migration is transforming growth factor (TGF). Both TGFβ1 and TGFβ2 are expressed by elements of the migratory system (migrating cells and radial glia, respectively) in the developing cortex (Flanders et al., 1991; Pelton et al., 1991; Miller, 2003). Exogenous TGFβ1 fosters cell migration (Siegenthaler and Miller, 2004). It increases the rate of migration by 74% and promotes the expression of CAPs (specifically, neural cell adhesion molecule and isoforms of integrin) in a concentration-dependent manner.

**Wart Formation**

We propose that wart formation is a three-step process. Initially ethanol interferes with growth factor regulation. For example, ethanol induces an increase in cortical BDNF expression (Heaton et al., 2003) and a decrease in cortical TGFβ1 content (Miller, 2003). The second step is a change in the molecular microenvironment in the MZ and superficial CP. Increased BDNF reduces reelin expression (Ringstedt et al., 1998). Hence, the inhibitory effect of reelin is inhibited. This permits the infiltration of the MZ by migrating neurons. Concurrently, ethanol alters TGFβ1-promoted neuronal expression of CAPs (Siegenthaler and Miller, 2004). The result is that the physical neuron–radial glia interaction which normally ends in the superficial CP, is abnormally retained. This movement of neurons into the MZ is a widespread phenomenon.

The final step is wart formation. This results from a compromise of the pial–subpial glial barrier. Focal damage allows ectopic neurons in the MZ to move beyond the pial surface. At first the warts have a peduncular appearance and then, as their size increases, warts become appositional. Thus, the dynamics of wart formation appear to involve a cascade of chemical and then physical changes.

**Notes**

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