

Neural Development, Cell-Cell Signaling, and the “Two-Hit” Hypothesis of Schizophrenia

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Abstract

To account for the complex genetics, the developmental biology, and the late adolescent/early adulthood onset of schizophrenia, the “two-hit” hypothesis has gained increasing attention. In this model, genetic or environmental factors disrupt early central nervous system (CNS) development. These early disruptions produce long-term vulnerability to a “second hit” that then leads to the onset of schizophrenia symptoms. The cell-cell signaling pathways involved in nonaxial induction, morphogenesis, and differentiation in the brain, as well as in the limbs and face, could be targets for a “first hit” during early development. These same pathways, redeployed for neuronal maintenance rather than morphogenesis, may be targets for a “second hit” in the adolescent or adult brain. Furthermore, dysregulation of cell-cell signaling by a “first hit” may prime the CNS for a pathologic response to a “second hit” via the same signaling pathway. Thus, parallel disruption of cell-cell signaling in both the developing and the mature CNS provides a plausible way of integrating genetic, developmental, and environmental factors that contribute to vulnerability and pathogenesis in schizophrenia.

Keywords: Development, signaling, schizophrenia, genetics, teratogenesis, neuronal circuits.

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Schizophrenia is a common but complex mental disorder that causes disruptions in thought processes, perceptions, and emotions. Although the effects of this disorder are profound, there does not appear to be a single neurobiological cause, such as the widespread neural degeneration observed in Alzheimer’s disease, or a specific lesion in a single brain region like the degeneration of the substantia nigra observed in Parkinson’s disease. In the absence of a discrete cellular or anatomical pathology, the attention of schizophrenia research has turned to more subtle anomalies. Histopathological, pharmacological, and molecular

expression studies suggest that the integrity of neuronal circuitry in the brain may be altered in schizophrenia. However, the pathological mechanisms that mediate these changes remain unclear.

Although several hypotheses suggest that aberrant embryonic development can contribute to schizophrenia, the onset of symptoms generally occurs many years after this process is completed. Thus, any disruptions to developmental processes must result in long-term consequences that leave mature neurons or circuits vulnerable to subsequent pathology. In addition, because the illness is often associated with a progressive decline in functioning, it is important to consider what kinds of pathogenic processes might continue to compromise abnormal neural circuits. Accordingly, it may be useful to consider how normal mechanisms of circuit construction and maintenance in the brain might be disrupted, and how this might lead to pathological changes thought to occur in schizophrenia. We will discuss one set of candidate mechanisms, the cell-cell signaling mechanisms that regulate the initial patterning and formation of neuronal circuits as well as the survival, growth, and plasticity of these circuits in the mature brain. We will then consider how these mechanisms might be compromised in ways consistent with their roles in neuronal development and maintenance as well as the natural history and clinical progression of schizophrenia.

The Elusive Pathogenesis of Schizophrenia: Its Causes and Effects

The Causes of Schizophrenia. Decades of research into the pathogenesis of schizophrenia have examined dozens of distinct genetic and environmental risk factors that can be correlated with the natural history of the disorder; however, no single candidate mechanism has emerged.

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Instead, each factor may contribute to an increased vulnerability to schizophrenia. Depending on the combination of these risk factors, a pathologic threshold may be met, thus leading to the emergence of disease symptoms. This conclusion is supported by several observations.

Schizophrenia is common. Schizophrenia is a common psychiatric disorder. Estimates of lifetime prevalence approach 1 in 100. This high frequency suggests either that the cause of the disorder is common or that there are multiple causes that disrupt essential and vulnerable biological functions and lead to symptom onset.

Schizophrenia is linked to many genetic risk factors. Adoption, family, and twin studies provide strong evidence of the heritability of schizophrenia (Gottesman and Wolfgram 1991). In addition, at least 15 genetic loci have been demonstrated to have weak to moderate linkages to schizophrenia (Riley and McGuffin 2000). Any model for the disorder, therefore, must account for linkage to multiple genes.

Schizophrenia is not strictly genetic. Genetics alone does not predict development of the disease. The concordance rate of schizophrenia is only about 50 percent in identical twins (Cardno and Gottesman 2000). Furthermore, these concordance rates may be reduced in monozygotic twins who do not share a common amniotic environment as compared to those who do (Davis et al. 1995). Thus, although genetic factors clearly can increase the risk for schizophrenia, they cannot alone account for the disease.

Schizophrenia is linked to environmental and non-genetic risk factors. Numerous environmental factors have been linked to schizophrenia. Births in the winter-spring months (Torrey et al. 1997) increase schizophrenia risk possibly because of maternal nutritional deficiencies (Hoek et al. 1996) or the prevalence of winter viral infections (Yolken and Torrey 1995). In addition, the risk of developing schizophrenia is correlated with demographic factors, such as family size and locality, and the availability of an adequate maternal diet (Hulshoff Pol et al. 2000). Nevertheless, these factors alone are not sufficient to predict the development of schizophrenia in any particular individual.

The Consequences of Schizophrenia. Although the specific cause of schizophrenia is not yet clear, its consequences are well documented. These consequences do not appear to include widespread neurodegeneration or cell death (Sapolsky 1993; Falkai et al. 1999); rather, a set of subtle changes to the neuronal circuitry of the brain appears to underlie the psychiatric symptoms that are observed during the progression of the disease. This conclusion is supported by the following observations.

Schizophrenia is not accompanied by consistent neuroanatomical anomalies. General markers of brain pathology, such as changes in ventricular size (Jones et al.

1994; Buchsbaum et al. 1997; Wright et al. 2000), have been associated with schizophrenia. Such changes, however, are also associated with a number of neurological and psychiatric diseases and thus are not reliable predictors of schizophrenia. Alterations in specific cortical cell populations have been reported, such as a loss of neuropil or changes to γ -aminobutyric acid-ergic cells (GABA-ergic cells). There is, however, significant variability in each of these findings within the schizophrenia population (Benes et al. 1991; Selemon et al. 1995; Beasley and Reynolds 1997; Selemon et al. 1998). Thus, neuropathological markers associated with the schizophrenia population are not absolute predictors of the disease in any particular individual.

Molecular changes have been observed in the brains of schizophrenia patients. There is an extensive catalog of molecules whose expression levels change in the brains of schizophrenia patients. These include reelin (Impagnatiello et al. 1998), SNAP25 (Thompson et al. 1998), and neurotransmitter receptors (Joyce et al. 1993a; Akbarian et al. 1995a; Eastwood et al. 1995; Goldsmith et al. 1997). In addition, changes in adhesion molecules, cytoskeletal proteins, neurotrophins, and other cell-cell signaling molecules have been correlated with schizophrenia (Arnold et al. 1991; Vawter et al. 1998; Miyaoka et al. 1999; Takahashi et al. 2000). Diversity, rather than obvious functional relationships between specific molecular families, is the primary characteristic of this list. Thus, the molecular changes seen in the brains of schizophrenia patients may represent multiple final targets for pathology instead of a record of a single pathological mechanism.

Schizophrenia affects the function of neuronal circuits. In addition to these neuroanatomical changes, pharmacological manipulations suggest that synaptic transmission in some brain circuits may be compromised, particularly those involving dopaminergic, GABA-ergic, and glutaminergic neurotransmitters. Agents that increase dopaminergic tone (e.g., L-dopa, cocaine, or amphetamines), when chronically administered, can elicit hallucinations and delusions that resemble the positive symptoms of schizophrenia (Bell 1965; Snyder 1972; Post and Kopanda 1976; Lieberman et al. 1987; Brady et al. 1991; Friedman and Sienkiewicz 1991; Young et al. 1997). Similarly, agents that act as partial antagonists at glutamate receptors (e.g., phencyclidine, ketamine) may also evoke psychotic symptoms (Javitt and Zukin 1991; Krystal et al. 1994; Rosse et al. 1994). In contrast, positive symptoms in schizophrenia patients have been treated for many years with pharmacological agents that antagonize dopamine activity in the CNS (Creese et al. 1976; Meltzer and Stahl 1976; Haracz 1982).

Schizophrenia affects synaptic transmission. The effects of schizophrenia on neuronal circuits are further

supported by evidence of changes in the localization and function of neurotransmitters, including reduced expression of dopamine receptor subtypes or dopaminergic synaptic function in brain regions affected by schizophrenia (Goldsmith et al. 1997; Laruelle 1998). In addition, reduced GABA synthesis (Akbarian et al. 1995b) and glutamate receptor hypofunction have been associated with the disease (Olney and Farber 1995; Mohn et al. 1999).

Schizophrenia symptoms generally appear during late adolescence. It is widely accepted that schizophrenia has its origin during the early stages of neurodevelopment (Murray and Lewis 1987; Weinberger 1987; Weinberger 1995). Subtle attentional, motor, and social problems are reported during childhood in people who are later diagnosed with schizophrenia, although these problems are frequent in the general population and do not reliably predict the onset of the disease (Walker et al. 1996; Erlenmeyer-Kimling et al. 2000). Overt symptoms of schizophrenia generally manifest themselves shortly after the onset of puberty. The onset of symptoms generally occurs at later ages for women than for men, suggesting that gonadal steroid hormones may play at least some role in the disease.

Schizophrenia is a progressive disorder. Often, the first overt manifestations of the illness are attenuated hallucinations and delusions that become more severe and persistent over the course of weeks or months (Haas and Sweeney 1992; Beiser et al. 1993). Early treatment with antipsychotic medications is often effective; however, a longer duration of untreated psychotic symptoms is associated with more severe symptoms during psychotic episodes. These subsequent symptoms are resistant to treatment and more likely to recur (Loebel et al. 1992; Lieberman et al. 1996; Sheitman and Lieberman 1998; Robinson et al. 1999). This suggests that psychotic symptoms themselves “sensitize” the brain and increase the pathology associated with the illness.

Built To Fail: The “Two-Hit” Hypothesis of Schizophrenia

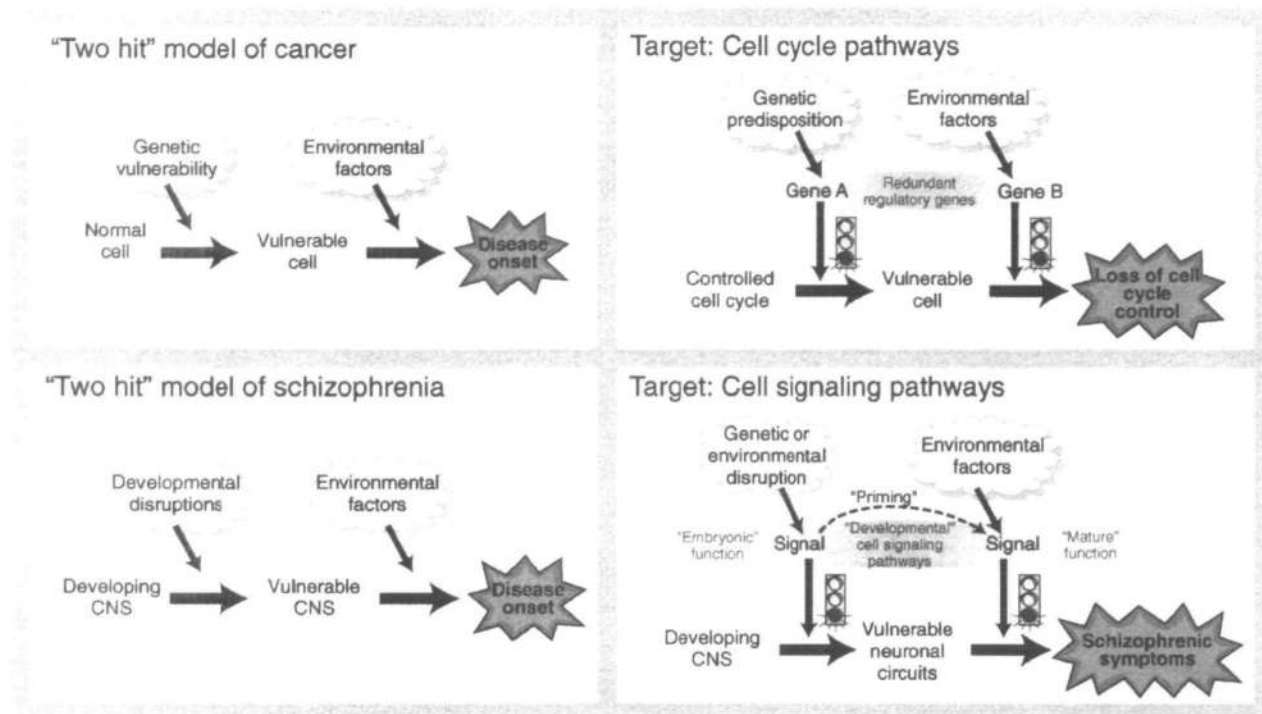
In the absence of a singular genetic or environmental pathogenic agent for schizophrenia, attention has turned to disease models involving multiple factors. One such model is the “two-hit” hypothesis (as described in Bayer et al. 1999). Two-hit models for schizophrenia are similar to those proposed for other complex diseases, such as cancer (figure 1, top). Like schizophrenia, cancer is linked to numerous genetic and environmental factors, although no single mechanism can account for all cases. In addition, although genetic risk factors are present from birth, cancer often does not become apparent for many years. The

two-hit model for cancer provides a valuable framework for assessing relationships between cell division, growth, survival, and death mechanisms that contribute to oncogenesis. Although the loss of a single regulatory mechanism could theoretically lead to a loss of cellular control, there is a relatively high level of redundancy and resiliency in the regulation of cellular functions. Thus, a complete breakdown in cellular control often occurs only after the disruption of multiple regulatory mechanisms. A genetic mutation might make a particular cellular pathway vulnerable for transforming a specific cell type. Subsequently, an environmental carcinogen or a somatic mutation event might disrupt redundant or compensatory mechanisms, resulting in transformation and tumorigenesis.

The two-hit hypothesis for schizophrenia suggests that a prenatal genetic or environmental “first hit” disrupts some aspect of brain development, and establishes increased vulnerability to a second hit that may occur later in life (figure 1, bottom). Neither insult by itself is sufficient to induce schizophrenia. Instead, the first hit “primes” the nervous system for the second, which then precipitates disease symptoms. Because the first hit is thought to occur during embryonic development, it seems candidates for this hit should involve disruption of a mechanism that is (1) susceptible to numerous genetic and environmental perturbations, and (2) capable of producing long-term significant changes. In contrast, a second hit might go unnoticed in a brain that had not been previously “primed” to respond in an aberrant way. One general mechanism stands out as being both susceptible to many perturbations and capable of producing long-term changes: cell-cell signaling pathways initially involved in induction and morphogenesis of the CNS (and other structures), and later in ongoing maintenance of CNS neurons and circuits, are ideal targets for two hits that might result in schizophrenia vulnerability and pathogenesis.

Lessons From the Minor Physical Anomalies: Inductive Signaling and Schizophrenia

Many severe neurological deficits, such as those observed in Down’s syndrome patients, are accompanied by physical malformations. These anomalies include abnormal proportions or defects in the structures of the head and face, limbs and digits, and internal organs. Quantitative assessments of these anomalies (termed the “minor physical anomalies”) (Waldrop et al. 1968; Waldrop et al. 1978) show that they are correlated with psychological and behavioral problems. Minor physical anomalies are observed more often among adult schizophrenia patients than within the general population (Gualtieri et al. 1982; Guy et al. 1983;

Figure 1. Schematic representations of two-hit models for cancer and schizophrenia

Note.—The two-hit model of cancer (top left) suggests that a first hit, such as a genetic predisposition to the disease, produces a vulnerability within specific cell populations, whereas a second hit, probably from environmental factors, induces the onset of the disease. This model is complementary to the growing understanding of the molecular mechanisms that underlie the disorder (top right). At the molecular level, the first hit likely corresponds to the loss of one gene within a set of redundant regulatory genes. The failure of one regulatory gene is likely compensated for by other parallel mechanisms, so a loss of cell cycle control likely occurs only after the subsequent loss of additional mechanisms. In a similar fashion, a two-hit model for schizophrenia (bottom left) would suggest that disruptions in the development of the central nervous system (CNS) produce a vulnerability to the disorder but that the onset of symptoms in a vulnerable brain would be triggered by environmental factors. At the molecular and cellular levels, this model may represent how disruptions in early cellular signaling events may leave some neuronal circuits vulnerable to disruptions in the signaling events that occur in the mature CNS (bottom right).

Ismail et al. 1998). In particular, schizophrenia patients are more likely to have deviations in craniofacial proportions, including distance between the eyes, placement and symmetry of the ear pinnae, and cranial circumference. Similarly, malformations of the digits of the hands and feet (including anomalous lengths or shapes of the digits and partial syndactyly of the toes) are more frequent in schizophrenia patients.

Malformations of limbs or facial structures are presumably not the direct cause of schizophrenia. There may be, however, a similar set of “minor brain anomalies” that develop in parallel to the visible defects in the limbs and face. For instance, the olfactory bulbs appear reduced in size in some schizophrenia patients, and olfactory impairments are often seen (Moberg et al. 1999; Turetsky et al. 2000). It is likely that both the minor physical anomalies and the minor brain anomalies are the result of the same aberrant developmental

processes, which may be involved in the pathogenesis of schizophrenia. Indeed, a single developmental mechanism, nonaxial mesenchymal/epithelial induction (LaMantia 1999; LaMantia et al. 2000), is essential for initial morphogenesis and differentiation at all of these sites.

Mesenchymal/Epithelial Induction, the Minor Physical Anomalies, and the Forebrain

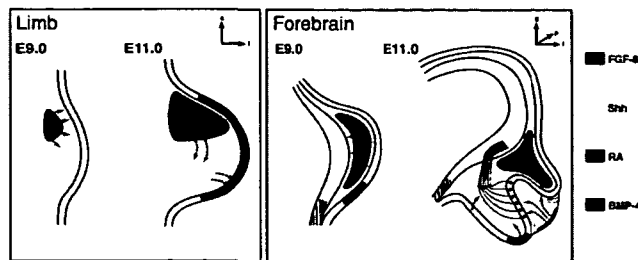
During the first trimester of embryogenesis, morphogenesis of the limbs, heart, face, and forebrain relies on a complex set of cell-cell signaling interactions known as mesenchymal/epithelial induction. These interactions result from the apposition of two cell groups—epithelia, sheets of cells that constitute the inner and outer surfaces of the

embryo, and mesenchyme, the loosely arrayed accumulations of cells in the interstices between epithelial layers. The apposition of these two tissue compartments facilitates the exchange of short- and long-range signals that in turn regulate the differentiation of both epithelial and mesenchymal derivatives. Although the mechanisms involved in mesenchymal/epithelial induction are [quite] complex, experimental studies in the limb and forebrain provide insight into these signaling processes, including the identity of many [of the] important molecular components. Parallels between the limb and the forebrain suggest that mesenchymal/epithelial induction may be a potential target for a first hit in the two-hit model of schizophrenia.

The most complete picture of the cellular and molecular components of mesenchymal/epithelial induction has emerged from studies of the developing limb (reviewed by Rubin and Saunders 1972; Tabin 1995; Johnson and Tabin 1997). As soon as limb buds are recognizable, local axes can be identified within the mesenchyme and epithelium. These axes define the anterior/posterior, dorsal/ventral, and proximal/distal dimensions of the limbs and reflect regional differences in mesenchymal or epithelial tissues. The axial coordinates dictate the direction of limb growth and the position of digits (Tickle et al. 1975; Smith et al. 1978). The embryological definition of these epithelial and mesenchymal interactions facilitated the molecular identification of short-, medium-, and long-range signals that mediate initial patterning, morphogenesis, and differentiation. These molecules include retinoic acid (RA), sonic hedgehog (Shh), and members of the fibroblast growth factor (FGF) and bone morphogenetic protein (BMP) families of signaling molecules (Tabin 1995; Johnson and Tabin 1997), each available from discrete loci in the limb mesenchyme or epithelium. Their expression, activity, and morphogenetic effects depend on a complex network of regulatory interactions facilitated by the apposition and exchange of signals between mesenchymal and epithelial cells (figure 2).

This combination of mesenchymal/epithelial apposition, local axes, and molecular signals operates during the early development of the forebrain and limbs. Thus, the initial formation of the olfactory pathway depends on mesenchymal/epithelial interactions mediated by RA, FGFs, Shh, and BMPs. Each of these signals participates in defining local axes that then constrain the further development of the olfactory epithelium and nerve (LaMantia et al. 2000) (see figure 2, right). Similar signals are available to the developing forebrain neuroepithelium (LaMantia et al. 1993; Shimamura and Rubenstein 1997) and may influence the initial morphogenesis of the olfactory bulb (Belloni et al. 1996; Anchan et al. 1997) and other rudimentary forebrain regions. Accordingly, nonaxial mesenchymal/epithelial induction and the signaling

Figure 2. Comparison of signaling mechanisms involved in the patterning of the limb and forebrain



Note.—In the limb (left), signaling by retinoic acid (RA) in the mesenchyme initially induces the expression of other patterning genes, such as sonic hedgehog (Shh) in the posterior of the limb bud. Subsequently, Shh and RA modulate the expression of other patterning signals, such as fibroblast growth factor (FGF)—8 along the edge of the limb ectoderm, and bone morphogenetic protein (BMP)—4 in the posterior portion of the mesenchyme and in a restricted stripe along the edge of the ectoderm. The boundaries of these expression patterns are regulated through interactions between each of the signals; for example, interactions between Shh and BMP—4 regulate the expression of both, such that their expression domains do not overlap. In the forebrain (right), signaling from RA in the mesenchyme also patterns the expression of other genes, such as Shh and BMP—4, and the interactions between these signals guide the morphogenesis of the frontonasal region, including the olfactory epithelium and nerve. It should be noted that neither of these schematics is completely inclusive because numerous other genes, such as additional members of the FGF and BMP families, are also involved. The arrows in each figure illustrate where a molecular signal is involved in patterning the development of adjacent regions. The information in this figure is derived from numerous sources, including Tabin 1995 and LaMantia et al. 2000.

molecules that mediate this process contribute to morphogenesis and differentiation of forebrain regions and pathways.

Disruptions in mesenchymal/epithelial induction, therefore, could constitute a first hit for schizophrenia vulnerability. The reliance of these inductive processes on signaling between distinct cell types, the need for ligand/receptor interactions that might be compromised by environmental factors (including teratogens or drugs of abuse), and the complex hierarchy of genes necessary to guide induction and morphogenesis suggest several vulnerable targets. The consequences of disrupted induction could include changes in forebrain neurons or circuits accompanied by parallel changes in limb, heart, or facial structures. These changes may predispose the brain to subsequent pathogenic processes that converge on a threshold that, once reached, leads to schizophrenia.

Developing Risk: Potential Molecular Targets for a First Hit in Schizophrenia

Several genetic loci and environmental factors closely linked to schizophrenia can be associated with specific

molecular and cellular mechanisms that contribute to mesenchymal/epithelial induction. Three of these—embryonic disruptions of retinoid signaling, haploinsufficiency of chromosome 22q11, and genetic anomalies of notch signaling pathways—are not only linked to schizophrenia, but also associated with minor physical anomalies (figure 3). Thus, a closer examination of these disruptions in cell-cell signaling related to induction may help to identify specific molecular mechanisms involved in producing a first hit during neural development that establishes long-term vulnerability to schizophrenia.

Retinoid Signaling. RA is an important developmental signaling molecule critically involved in induction of the limbs, heart, branchial arches, and forebrain (Linney and LaMantia 1994; LaMantia 1999). Embryonic RA exposure leads to a constellation of developmental deficits that resemble the minor physical anomalies in schizophrenia patients. Thus, it has been suggested that embryonic signaling through this pathway may also be compromised in schizophrenia patients (Goodman 1998; LaMantia 1999). Moreover, many genetic loci linked to schizophrenia are close to the sites of important genes in the retinoid signaling pathways (Goodman 1995). Apparently, RA signaling is an important factor for neural development, and early dysregulation may contribute to CNS dysfunction.

Retinoid signaling, like signaling of all members of the steroid-thyroid superfamily of hormones and receptors, involves a variety of transduction mechanisms (figure 4, left). In addition to the ligand RA (the acidic form of vitamin A or retinol), there are two multigene families of receptor molecules: the retinoic acid receptors (RARs) and the retinoid-x-receptors (RXRs) (Yang et al. 1991; Mangelsdorf et al. 1992; Allenby et al. 1993). When a cell is exposed to RA (which is highly lipid soluble), the ligand enters the cell and binds to nuclear RARs and RXRs. These receptors routinely heterodimerize upon binding RA (thus, two separate receptor types, such as an RAR and an RXR, bind to one another). Subsequently, in conjunction with a number of corepressors and co-activators (Minucci and Ozato 1996), RA ligand-receptor complexes transcriptionally activate specific genes (figure 4, left). This activation depends on direct binding of the activated receptors to genomic regulatory sites known as RA response elements (RAREs).

The details of RA signaling indicate several steps where this process could be disrupted, leading to developmental anomalies that might confer schizophrenia vulnerability. Teratogenic exposure to RA or RA deprivation resulting from a restricted maternal diet can alter development. For example, RA exposure during early fetal life leads to gross malformations of several structures including the limbs, face, heart, and CNS (Shenefelt 1972;

Lammer et al. 1985; Anchan et al. 1997). More subtle deficits in behavior have been reported after later fetal exposure to RA (reviewed by Adams and Lammer 1993).

Experimentally induced mutations in RA receptors and cofactors have been more difficult to interpret. Single null mutations in several genes that contribute to RA signaling produce few detectable neural phenotypes, presumably because of functional redundancy and overlapping spatial distribution of various RA receptors and binding proteins (Perez-Castro et al. 1989; Gustafson et al. 1993; Sucov and Evans 1995; Krezel et al. 1996). An exception to this trend is seen in the consequences of inactivating a major retinoid synthetic enzyme, retinaldehyde dehydrogenase 2 (RALDH2), which disrupts embryogenesis at a very early stage (Niederreither et al. 1999). In addition, inactivation of the RAR β gene results in subtle but detectable changes in long-term potentiation in the hippocampus (Chiang et al. 1998). Because of the potential for functional redundancy, other studies have examined the result of inactivating two receptors simultaneously. The absence of both RAR β and RXR γ leads to a dramatic behavioral change, where both long-term potentiation and long-term depression are diminished in the hippocampus (Chiang et al. 1998). In addition, the removal of both genes leads to significant deficits in locomotion, perhaps resulting from disrupted expression of dopamine receptors (Krezel et al. 1998). Together, these results demonstrate that retinoid signaling can influence some aspects of higher order cognitive functions. The current data, however, do not address whether RA-sensitive behavioral and cognitive changes reflect altered RA-dependent neural development, ongoing RA signaling required for mature brain function, or both.

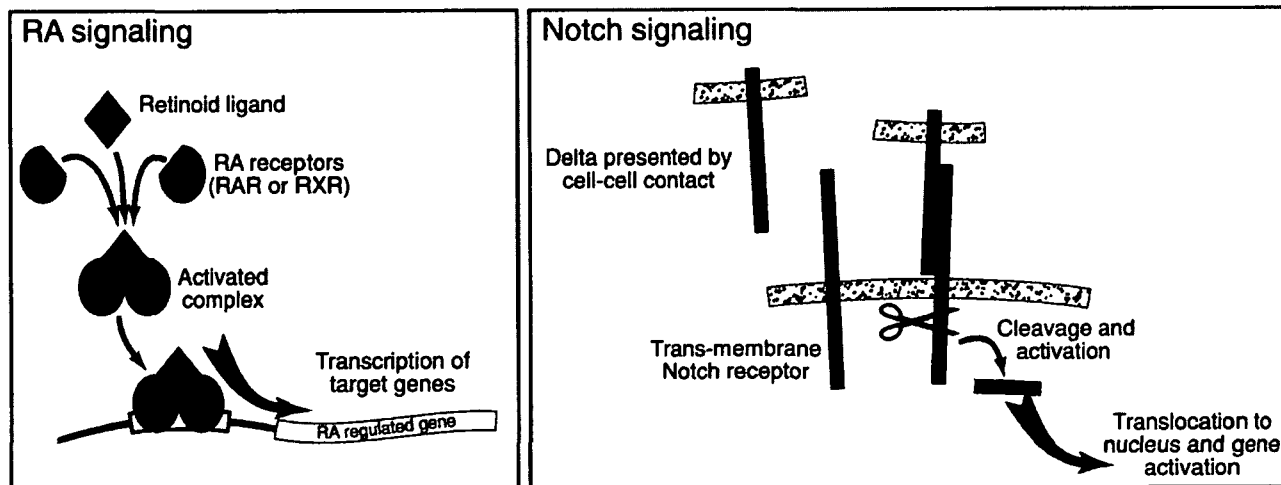
22q11 Deletion Syndrome. Haploinsufficiency of regions of chromosome 22q11 leads to a constellation of developmental and behavioral anomalies (Goldberg et al. 1993; Driscoll 1994; Ryan et al. 1997; Shprintzen 2000), collectively known as the 22q deletion syndrome (22qDS, also known as velocardiofacial syndrome [VCFS] or DiGeorge syndrome). This deletion syndrome is one of the best characterized genetic conditions linked to an increased risk for schizophrenia (Cohen et al. 1999; Murphy et al. 1999). The phenotype of 22qDS is highly variable, even within families (Vincent et al. 1999), but like RA-exposed individuals, patients with 22q11 deletions have a characteristic set of heart, limb, and craniofacial anomalies that mirror the “minor physical anomalies” frequently observed in schizophrenia patients. Many people with 22qDS have cognitive problems (Golding-Kushner et al. 1985; Moss et al. 1999; Swillen et al. 1999); behavioral or psychiatric difficulties are also common (Papolos et al. 1996; Carlson et al. 1997a;

Figure 3. Cellular signaling pathways are linked with the "minor physical anomalies"

Disruption to Developmental Signaling		species	Linked to Schiz?	Craniofacial	Limb and digit	Heart	Thymus	Olfaction/bulb	Other CNS
Retinoid Signaling	RA exposure	hu	√	√	√	√	√	√	√
	Pax6 ^{3ey}	mo	-	√		√		√	√
	Gli3 ⁺	mo	-	√	√			√	√
VCFS	22q11 deletion	hu	√	√	√	√	√		√
Notch Signaling	Notch 4	hu	√						
	Jagged 2	mo	-	√	√		√		
Cell Death	APAF-1	mo	-	√	√				√
	Bcl2/Bcl2L	mo	-		√		√		√
Inductive Signaling	BMP 4	mo		√	√	√		√	√
	Shh	mo		√	√	√		√	√
	FGF 8	mo		√	√	√		√	√

Note.—Previous studies have demonstrated that schizophrenia patients often have a distinctive set of congenital defects, termed the "minor physical anomalies." Studies of two groups of patients at substantially increased risk for developing schizophrenia, those with velocardiofacial syndrome and those who have been prenatally exposed to teratogenic doses of retinoic acid (RA) agonists, demonstrate that these patients display a similar set of congenital anomalies, including defects in limb, heart, craniofacial, and central nervous system (CNS) structures. Studies of mouse models demonstrate that the same signaling mechanisms are responsible for the formation of each of these structures. Thus, the same mechanisms that serve to sculpt the limbs and craniofacial features, including RA signaling, notch signaling, developmentally regulated cell death, and signaling through sonic hedgehog and the bone morphogenetic protein and fibroblast growth factor protein families, are also likely involved in the development of specific CNS structures and circuits.

Figure 4. Schematic of retinoic acid and notch signaling pathways



Note.—Retinoid signaling (left) primarily occurs in the nucleus. Retinoid agonists (such as 13-cis retinoic acid) enter the nucleus, possibly with the assistance of cellular binding proteins, and bind to the retinoid receptor protein. After a receptor has bound to its ligand, it forms a dimer with another retinoid receptor, and this dimerized protein in turn can bind to specific regulatory elements within the genome and regulate the transcription of target genes. Notch signaling (right) begins at the cell surface, where the extracellular portion of the trans-membrane notch receptor can bind to its ligand, a member of the delta family of proteins (which includes the jagged proteins). Normally, the ligand is expressed on the surface of an adjacent cell, and contact between the two cells induces the binding of the receptor to its ligand. Upon binding to its ligand, the notch receptor is activated (possibly by forming a dimer with another activated notch receptor) and the intracellular portion of the receptor is cleaved away. This intracellular fragment is then translocated into the nucleus, where it appears to activate transcription of target genes, probably by binding to other nuclear localized transcription factors.

Shprintzen 2000). Most strikingly, between 10 percent and 40 percent of affected patients develop symptoms of schizophrenia (Shprintzen et al. 1992; Chow et al. 1994; Murphy et al. 1999). These cognitive and behavioral manifestations are likely the result of subtle malformations of the brain. Individuals with 22qDS have significant reductions in brain volume (Eliez et al. 2000), as well as reductions in gray matter when the deletion is present on the maternally derived chromosome. Furthermore, this gray matter reduction may be progressive during adolescence (Eliez et al. 2001).

VCFS generally results from deletions within a "minimal critical region" of 1.5 million to 2 million bases of chromosomal DNA within the 22q11 region. However, it is not yet clear which gene (or genes) within this region are responsible for the characteristic symptoms. Although several interesting candidates are found among the approximately 30 genes contained within this region, genetic mapping of 22qDS patients has not identified a single gene (or set of genes) that is uniquely deleted in all affected patients. Instead, 22qDS patients with similar symptoms have been found to have deletions that do not overlap one another (Amati et al. 1999; McQuade et al. 1999). Additionally, the size and position of the deletions within 22q11 do not appear to correlate well with the severity of the phenotype, suggesting that more severe phenotypes are not simply the result of more genes being deleted (Carlson et al. 1997a; Carlson et al. 1997b; Vincent et al. 1999). Thus it is possible that the complex phenotype of 22qDS results either from the overlapping regulation of several genes within this region or from its concerted participation in a highly regulated process.

The complexity of this deletion syndrome is illustrated by the difficulty of producing a representative animal model. At least 27 of the approximately 30 genes found within the minimal critical deleted regions of human 22q11 have homologues in the mouse. These homologues, however, are not found in a single syntenic region of the mouse genome; thus, a completely parallel deletion cannot be easily obtained. Three separate transgenic strains have been generated that delete syntenic regions of mouse chromosome 16 (Kimber et al. 1999; Lindsay et al. 1999; Puech et al. 2000). Some anomalies in cardiovascular morphology and behavior have been reported in these mice. However, as is observed in humans with 22qDS, there is considerable phenotypic variability observed in these animal models, as each of the phenotypes is only modestly penetrant (Lindsay et al. 1999). Furthermore, none of the deletions generated thus far produces a phenotype that is entirely representative of the spectrum of limb, craniofacial, heart, and neurobiological disruptions observed in humans.

There are several possible reasons why nonoverlapping deletions within the 22q11 locus may lead to appar-

ently similar neurobiological phenotypes. The simplest possibility is that there may be several genes within this region that have similar functions during embryogenesis. There are a variety of inferred functions for genes within the critical region, although it is not immediately clear whether these functions overlap. A more intriguing possibility is that several genes within this region may act in concert. This could be a consequence of one (or more) 22q11 genes regulating other 22q11 genes. For example, one of the genes on the centromeric end of the 22q11 locus encodes a member of the E2F transcription factor family (Gaubatz et al. 1998; Trimarchi et al. 1998). In parallel, two telomeric genes, Htf9c and RanBP1, are cell cycle regulatory genes regulated by E2F transcription factors (Guarguaglini et al. 1997). Thus, centromeric deletions within the locus might dysregulate the expression of telomeric genes.

Alternatively, the 22q11 genes could act in concert because of their regulation by (or participation in) another regulatory pathway. For example, 22q11 genes could be either upstream regulators or downstream targets of mesenchymal/epithelial signaling in the limbs, heart, face, and forebrain. Thus, a set of 22q11 genes might be coordinately regulated by the cell-cell signals that mediate induction, including FGFs, BMPs, Shh, and RA. It is possible, therefore, that at least some of the complexity of the 22qDS phenotype may be due not only to interactions between 22q11 genes, but also to their participation in cell-cell signaling that mediates mesenchymal/epithelial induction.

Notch-Delta Signaling. Polymorphisms in the human Notch4 locus, a gene that encodes a member of the notch family of signaling proteins, have recently been linked to schizophrenia (Wei and Hemmings 2000). The exact function of Notch4 in mammalian development is not yet clear. Nevertheless, Notch4 is a member of a well-established family of receptors (the notches) and their cognate ligands (the deltas and jaggeds). This signaling pathway regulates various aspects of cell fate in the nervous system and throughout the rest of the embryo (see figure 4, right) (Weinmaster 1997; Artavanis-Tsakonas et al. 1999; Miele and Osborne 1999). Thus, notch-delta signaling might be a target for a first hit that contributes to schizophrenia vulnerability by disrupting normal cell fate determination during early neural development.

Notch signaling may be central to the inductive processes that lead to morphogenesis and differentiation at each of the sites of the minor physical anomalies as well as in the forebrain. The targeted deletion of one of the notch ligands, Jagged2, leads to craniofacial and limb anomalies (including syndactyly) that mirror those described in VCFS (Jiang et al. 1998). It is also possible that notch signaling influences morphogenesis in the

limbs, heart, face, and forebrain by regulating the survival of neural crest-associated mesenchymal cells that are essential for normal mesenchymal/epithelial induction. In avian embryos, the early survival, as well as the eventual differentiation, of some neural crest populations appears to be regulated, at least in part, by notch-delta signaling (Maynard et al. 2000). In addition, notch signaling may be directly involved in cell type specification within the developing forebrain. When notch signaling is disrupted in individual cells in the developing mouse or chicken forebrain, these cells adopt different cell fates than they would normally (Wakamatsu et al. 1999; Gaiano et al. 2000). Thus, disruptions of notch signaling can directly or indirectly alter development at several sites associated with schizophrenia vulnerability.

It is currently difficult to assess the role that Notch4 might play in developmental disruptions that contribute to the disease. Notch4 is primarily found in endothelial tissues and is expressed at only low levels in the developing and mature CNS (Uytenndaele et al. 1996). Thus, expression patterns give little insight into Notch4's contribution to schizophrenia pathology. The Notch4 polymorphisms linked to schizophrenia suggest that changes in the availability or localization of the gene, rather than a simple loss of function, may contribute to the increased risk of schizophrenia. The two candidate Notch4 polymorphisms seen in schizophrenia pedigrees are near the start of the gene: one lies within the first exon of the 30-exon gene, whereas the second lies within the upstream regulatory region (Wei and Hemmings 2000). The first exon does not encode a domain involved in the function of the processed protein. Instead, it may act as a “signaling peptide” that biases how the protein is processed and inserted into the cell membrane (Li et al. 1998). Thus, mutations in this region may alter the compartmentalization of the protein within individual cells. Similarly, mutations upstream of the Notch4 gene are unlikely to alter protein function. They may, however, produce significant changes in expression patterns or levels. Accordingly, in affected patients, alterations to the Notch4 gene may not lead to loss of function of the gene product, but to changes in the expression pattern or localization of Notch4.

The Two-Hit Nature of Schizophrenia May Reflect Neurodevelopmental “Priming”

Cell-cell signaling—via retinoid dysregulation, haploinsufficiency of 22q11 genes, or disruptions in the notch-delta pathway, among other mechanisms—provides a plausible target for a first hit for schizophrenia vulnerabil-

ity. Two questions arise from these observations: first, what sort of disruptions might constitute a second hit; and second, is there a significant relationship between first and second hits?

A second hit must compromise the functional integrity of CNS neurons or circuits. Accordingly, much of the apparent synaptic dysfunction that emerges with schizophrenia—for example, altered dopaminergic modulation—may reflect the consequences of a second hit. In addition, some of the neuropathological changes associated with the disease—including ventricular enlargement or reduction in neuropil—may be precipitated or exacerbated by a second hit. There is no clear consensus on what sorts of mechanisms might cause such functional or structural changes immediately before the onset of disease symptoms. Based on the potential consequences of disrupted cell-cell signaling during development, it is plausible that the identities of second hits might be clarified by considering the nature of the hits that they follow.

Developmental cell-cell signaling mechanisms have two fairly consistent features that are potentially relevant to second hits in schizophrenia pathogenesis. First, these signals are used both in the embryo and in the adult to influence cell identity, maintenance, and function. Second, they can regulate their own function. This self-regulation often attenuates the effectiveness of the ligand by downregulating receptors or upregulating catabolic cofactors. For both the RA and the notch signaling pathways, however, ligand-mediated receptor stimulation enhances signaling via upregulation of receptors and related cofactors (Sucov and Evans 1995; Artavanis-Tsakonas et al. 1999). If these responses are sustained, a major consequence of disrupted embryonic cell-cell signaling might be a silent and subtle—but nonetheless significant—change in the responses of mature cells to similar signals. Thus, a first hit may directly prime cell-cell signaling pathways to respond aberrantly to a second hit to the CNS that exceeds a pathological threshold, leading to schizophrenia.

“Developmental” Signaling Continues in the Adult CNS

The signaling mechanisms described previously were initially characterized by their roles in embryogenesis. Thus, they are often considered “developmental” signals. Nevertheless, members of each of these developmental signaling pathways—including retinoids and their receptors, 22q11 genes, and notch receptors and ligands—are expressed in adult tissues, including the mature CNS (Zetterstrom et al. 1994; Higuchi et al. 1995; Berezovska

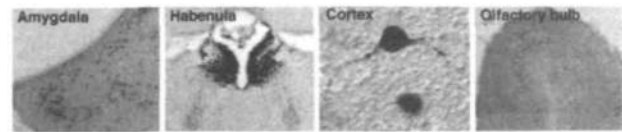
et al. 1998; Krezel et al. 1999). It is still far from clear what roles each of these signals may play in the adult, and specifically in the adult brain. At least two possibilities exist: these pathways may be redeployed for unique purposes that do not reflect their developmental function, or they may serve similar or identical functions to those they serve in the embryo.

Retinoid Signaling. In addition to its roles during development, RA is required for proper functioning of the mature CNS. Retinoid metabolites are necessary to maintain normal vision by their requirement for phototransduction (Dowling and Wald 1960; Wald 1968). In addition, dietary absence or excess of vitamin A, the precursor to retinoic acid, leads to a diverse set of neurological disturbances (Tansley 1933; Sorsby et al. 1966; Di Benedetto 1967; Feldman and Schlezinger 1970; Harris et al. 1998). As an extreme example, rats reared on a diet deficient in vitamin A develop severe neurological deficits, culminating in paralysis and death (Aberle 1933; Zimmerman 1933). These disturbances are accompanied by neuroanatomical disruptions as well, including severe lesions of the spinal cord. In accordance with this mature function, RA receptors and related signaling molecules continue to be expressed in the adult CNS (Dev et al. 1993; Zetterstrom et al. 1994; Krezel et al. 1999), although the mature pattern of receptor expression is significantly different from the patterns observed during embryogenesis.

Some combination of retinoid receptors appears to be present in virtually every region of the adult brain. However, the expression patterns of RA receptors alone do not indicate whether cell-cell signaling via RA actually occurs. Fortunately, the consequences of RA signaling for transcriptional regulation can be assessed with RA-responsive enhancer/promoter transgenes (Balkan et al. 1992; Colbert et al. 1993). Using genomic sequences of RAREs found in regulatory regions of endogenous genes, specific RA-sensitive enhancer/promoter constructs can be made. These regulatory elements can drive reporter genes, such as β -galactosidase, in cells that express appropriate RA receptors and have access to RA ligands. Thus, in mice carrying a RARE transgene, β -galactosidase expression indicates that individual cells respond to endogenous RA. Furthermore, RA is likely to activate endogenous genes in these cells.

Our recent observations of DR5 RARE-dependent, RA-mediated gene expression in adult RA-indicator transgenic mice show that cells within the amygdala, habenula, cerebral cortex, olfactory bulb, and spinal cord are RA responsive in the mature CNS (figure 5). These cells have morphological and biochemical characteristics of neurons. Thus, they have axonal and dendritic

Figure 5. Retinoid activated cells are present within the mature central nervous system (CNS)



Note.—Studies of transgenic indicator mouse strains (Balkan et al. 1992) demonstrate that specific cells within the CNS continue to respond to retinoid signaling into maturity. Cryostat sections from several CNS regions were immunostained to detect cells that express β -galactosidase under the control of the retinoid-sensitive promoter. Sections from the amygdala, habenula, cerebral cortex, and olfactory bulb are shown. Although the transgenically expressed marker protein is primarily found within the cell body, some faint staining can still be observed in the cellular processes. As such, it appears that the activated cells in the habenula are projection neurons because faintly labeled processes can be observed descending from the intensely labeled cell bodies. In contrast, the cells in the other regions appear to be small, locally projecting neurons, as demonstrated by the high magnification view of a labeled cortical cell.

processes; they are labeled with neuron-selective markers and they are not recognized with glial markers.

Although the exact nature of each of these RA-activated cell populations has not yet been established, two are found at sites known for high levels of cellular plasticity in the mature nervous system. In the olfactory pathway as well as in the dorsal horn of the spinal cord, neuronal populations are capable of rapid changes in gene expression, proliferation, and differentiation, and can form new circuits throughout life (Weiss et al. 1996; Bonfanti et al. 1997; Doetsch et al. 1999). In addition, the coincidence of adult RA activation with that in the embryo in both of these locations (Colbert et al. 1993; LaMantia et al. 1993) suggests that there may be shared vulnerability in some CNS neuronal populations to a first and second hit through the same molecular mechanism. Preliminary observations suggest that the RA-activated neurons in the olfactory bulb and spinal cord as well as in the cortex and amygdala are small GABA-ergic interneurons. Anatomical studies of the brains of schizophrenia patients have demonstrated that similar cell populations within the cortex may be altered (Selemon and Goldman-Rakic 1999; Benes 2000). Thus, there may be some overlap between RA-sensitive cells and GABA-ergic neurons believed to be targets of schizophrenia neuropathology.

Furthermore, RA-activated cells within the amygdala and habenula may participate in circuits that mediate altered behavioral responses in schizophrenia patients. The amygdala can modulate a variety of emotional and behavioral responses, such as conditioned fear responses

and autonomic stress responses (Roosendaal et al. 1991; Oakes and Coover 1997). Similarly, the habenula relays information from forebrain regions to limbic structures of the midbrain (Wang and Aghajanian 1977) and may function in a regulatory feedback loop with dopaminergic cells of the substantia nigra (Vincent et al. 1980; Sasaki et al. 1988; Sasaki et al. 1990). In rodents, lesions to the habenula cause deficits in the ability to produce appropriately measured responses to stimuli, especially under stress (Thornton and Bradbury 1989; Thornton et al. 1990; Vale-Martinez et al. 1997). Forebrain circuits that include habenular connections are sensitive to dopamine agonists and antagonists that enhance or inhibit these behaviors associated with schizophrenia (Ellison 1994; Ellison et al. 1996). Apparently, habenular circuits are sensitive to dopaminergic manipulation that modulates behaviors altered in schizophrenia patients. Thus, RA-sensitive neurons in the amygdala and habenula may be targets for an RA-mediated second hit that compromises forebrain circuits implicated in the behavioral pathology of schizophrenia.

These observations raise two final questions: If RA is the agent of a second hit, where is RA normally made and what agents might alter its availability in the mature brain? The choroid plexus, the secretory epithelium associated with the cerebral ventricles, is a likely site of RA synthesis in the adult CNS. At least two RA biosynthetic enzymes—aldehyde oxidase and the retinaldehyde dehydrogenase RALDH-2—are expressed by choroid plexus cells in the adult and developing brain (Huang and Ichikawa 1994; Yamamoto et al. 1996; Bendotti et al. 1997; Yamamoto et al. 1998). It is also possible that subsets of adult neurons produce RA locally, as is the case in the developing spinal cord, where RALDH2 is expressed in subsets of motoneurons (Sockanathan and Jessell 1998). Fluctuations in dietary vitamin A might alter RA production in the adult brain. This could occur either via changes in synthetic enzyme expression in choroid plexus cells or individual neurons, or by changes in RAR/RXR expression in RA-responsive neurons. A more intriguing possibility is raised by the sensitivity of RA synthetic enzymes to gonadal steroids such as testosterone. Aldehyde oxidase can be regulated by testosterone (Kurosaki et al. 1999). Accordingly, retinoid levels in the brain may vary in response to testosterone levels. Conversely, *in vitro* experiments demonstrate that RA can directly modulate the production of testosterone (Chaudhary et al. 1989). Given the association of schizophrenia onset with puberty and late adolescence, there may be a relationship between steroid levels (like that of testosterone) and an RA-mediated second hit that contributes to schizophrenia pathogenesis.

22q11 Deletion Syndrome. The 22qDS patients, like their non-22q11-deleted counterparts, usually develop schizophrenia symptoms in late adolescence or early adulthood. It is possible that this late onset represents a vulnerability established by the genetic deletion during embryogenesis. Alternatively, at least some of the 22q11 genes may have functions in the mature CNS. Our preliminary observations demonstrate that more than half of the mouse homologues of the typically deleted 22q11 genes are expressed in the adult brain, suggesting that they have as-yet-undefined functions. Thus, haploinsufficiency of the 22q11 region may act as a second hit, either by exacerbating deleterious effects of developmental haploinsufficiency or by compromising the function of some mature neuronal populations.

One candidate gene in the 22q11 minimal critical region that could be involved in both a first and second hit is ARVCF (Armadillo Repeats deleted in VCFS), which is likely involved in cell-cell signaling in both the developing and the mature brain. ARVCF is a member of the catenin gene family, a group of related signaling genes homologous to the *Drosophila* Armadillo gene. The catenin genes mediate cell signaling through the Wnt pathway, which is involved in cell fate decisions, cell adhesion, and cell proliferation. Although ARVCF has not yet been studied in the human brain, it is expressed in the mature mouse brain. In addition, at least two other catenin genes are expressed in the adult human brain. Furthermore, these related genes—beta and gamma catenin—are expressed at significantly lower levels in the hippocampus of schizophrenia patient brain specimens than in normal control brains (Cotter et al. 1998). Thus, it is possible that haploinsufficiency for ARVCF could compromise mechanisms that regulate both the differentiation and the maintenance of neurons and circuits in patients at risk for schizophrenia.

Notch-Delta Signaling. Notch signaling appears to play a role in the regulation or maintenance of synaptic connections in mature neurons (Berezovska et al. 1999; Sestan et al. 1999). Activation of the Notch1 and Notch2 signaling pathways in cultured cortical neurons alters the size and extent of neuronal arborizations, suggesting that notch signaling is at least partly responsible for regulating neuronal connectivity. Although the extension and specification of neural processes is most evident during embryogenesis, similar processes likely regulate the synaptic connections of mature neurons as well. At least two of the notch receptors (Notch1 and Notch2) as well as two of their ligands (Delta1 and Jagged2) are expressed throughout the cerebral cortex of the adult brain (Higuchi et al. 1995; Berezovska et al. 1998). Thus, dysregulation of Notch1 and Notch2 signaling in

the mature brain might be a plausible target for a second hit that contributes to schizophrenia pathogenesis by impairing the normal maintenance of normal cortical axonal and dendritic arbors.

It is unclear how Notch4 may be involved either in neuronal development or in the mature functioning of neurons. The targeted deletion of Notch4 in mice does not produce a detectable phenotype (Krebs et al. 2000). In addition, in the mature brain, Notch4 is expressed at high levels in endothelial cells, but it is not present at detectable levels in mature neurons. However, it is possible that Notch4 may interact with the Notch1 and Notch2 signaling pathways because the targeted deletion of Notch4 appears to dramatically enhance the effects of mutations in the Notch1 gene. Thus, it is possible that alterations in Notch4 may act indirectly, through the Notch1 and Notch2 genes, to facilitate signaling in the developing or mature brain.

It is important to note that each of the signaling mechanisms discussed previously likely interacts with each other. Thus, disruption of any one of these mechanisms may in turn disrupt others as well. For instance, RA can modulate the effects of notch signaling in avian neural crest cell populations (Maynard et al. 2000b). In addition, other signaling mechanisms such as those occurring through classical neurotransmitters—including dopamine, serotonin, GABA, and glutamate—that act both in the developing brain and in the adult (Lauder 1993; LaMantia 1995) may modulate pathogenic processes. Although the “mature” roles for many of these signaling mechanisms is not yet clear, they may work together to regulate the integrity of neurons and circuits in the mature brain. Accordingly, disrupted signaling via these pathways could represent a second hit whose impact can be augmented or prefigured by developmental disruptions in the same pathway.

Second Hits and Subsequent Schizophrenia Pathology

Disruption of cell-cell signaling by a second hit might help to explain the progressive course frequently observed in untreated schizophrenia. It is possible that, when untreated, the prolonged dysregulation of neuronal signaling involved in a psychotic episode may injure or stress individual neurons, which may in turn lead to further pathologic changes. Although such cellular stresses can sometimes activate apoptotic pathways, they also can induce the activation of additional signaling pathways, such as the “stress pathways” involving members of the mitogen activated protein kinase (MAPK) family. One potentially interesting set of downstream targets of these stress pathways is the RXR

retinoid receptors (Lee et al. 2000). Thus, prompt and aggressive treatment may not only attenuate immediate schizophrenia symptoms, but spare additional neurons and circuits from further changes in cell signaling that reinforce or accelerate schizophrenia pathology.

Cell-Cell Signaling and Schizophrenia Pathogenesis: A Vulnerable Mechanism for Multiple Hits

Schizophrenia is unlikely to ever be linked to a single genetic or cell biological mechanism. The two-hit model, however, provides a framework for interpreting the significance of many of the genetic and environmental factors correlated with this disorder. The inability to find a single genetic or environmental cause for cancer reflects the complexity and redundancy of regulatory mechanisms involved in controlling cell proliferation. In a similar manner, the multiple causal correlations seen in schizophrenia populations may reflect the complexity of cell-cell signaling processes that underlie the development and later maintenance of neuronal circuits in the forebrain. Because cell-cell signaling is a complex process that involves many genes and can be interrupted by numerous environmental factors, this process seems particularly vulnerable. Accordingly, the two-hit model of schizophrenia, when considered in light of the cellular mechanisms that are the target of each hit, may have the same utility that the related two-hit model of cancer has had for understanding the relationship between various causal agents and oncogenesis. The processes that underlie these signaling mechanisms are complex, and current data do not permit easy conclusions to be drawn about the role of each of these mechanisms in schizophrenia pathogenesis. Nevertheless, further studies of the developmental and mature roles of cell-cell signaling in the CNS may lead to new insights into the causes of several psychiatric disorders, including schizophrenia.

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