

Chapter 7

Prenatal β_2 -Adrenergic Receptor Signaling and Autism: Dysmaturation and Retained Fetal Function

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Abstract The origins of idiopathic autism are prenatal. During fetal life, the β_2 -adrenergic receptor (B2AR) is important for growth as well as terminal differentiation of cells. Signaling from this receptor serves different purposes at different times in virtually all tissues during prenatal development, and provides modulation for most organ functions in postnatal life. Because the B2AR is one of the earliest appearing receptors in brain development, interference with it over time during gestation can theoretically affect the development of other neurotransmitter systems, as well as later functioning of the CNS and peripheral organs. Prenatal overstimulation of the B2AR has been linked to autism in dizygotic twins, and a higher prevalence of more active B2AR polymorphisms has been found in autism families.

Animal studies in developmental neurotoxicology show abnormal outcomes for brain and tissue function after prenatal administration of B2AR agonists. These studies have also shown that the fetal B2AR normally does not desensitize, and that several tissues can retain a fetal pattern of signaling after prenatal B2AR overstimulation. This type of dysregulated signaling may also be responsible for the differences in function noted in brain and other tissues of autistic children compared to controls. Results from published studies in many areas of autism research can be related to B2AR second messengers such as cAMP levels or to physiological patterns that are present during fetal life.

Prenatal interference with signaling from the B2AR is not likely to act alone in the development of autism. Downstream pathways stimulated by the B2AR can share components from signaling through other receptors, including those for stress hormones and cytokines. Effects on these shared pathways during gestation may lead to final common mechanisms for the development of autism, and may be a reason that single genes and individual environmental factors have not been identified to explain its causation.

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Introduction

Autism is included in a spectrum of neurodevelopmental syndromes with heterogeneous presentation that are currently defined in behavioral terms. The origins of autism are prenatal. Neuroanatomical studies show abnormalities in structures that are well established before birth [1, 2, 3, 4]. Increased levels of neuropeptides have been found at birth in archived blood of children who later developed autism [5]. Both these findings suggest that the underlying alterations in brain development and potential peripheral markers occur long before symptoms become obvious in postnatal life. In fact, maternal risk factors for autism during pregnancy have been published [6, 7]. It is highly likely that the developmental cell programs leading to these disorders are established during gestation.

Nearly every neurotransmitter system, as well as the immune system, has been investigated in autism postmortem brain [8, 9, 10, 11, 12, 13], and data from many studies of peripheral tissues such as the immune, gastrointestinal (GI), and neuroendocrine systems have been published as well [5, 14, 15, 16]. A wide range of results that overlap with normal controls, may change with time, and may or may not correlate with patients' functioning levels has been documented in these studies. Although children with this disorder present with a specific set of core characteristics (Diagnostic and Statistical Manual of Mental Disorders-IV), each individual patient is different, one from another. As well, neuroanatomical studies of the brain show a wide range within each area of abnormality such as the brainstem, cerebral cortex, amygdala, hippocampus, and cerebellum. There have been few consistent or predictive results among these investigations that could apply to all patients with autism, and taken together, the data suggest widespread physiological dysregulation. In addition, the genes involved in autism have been difficult to isolate, though considerable scientific research has been devoted to doing so [17, 18, 19, 20, 21]. When considering the heterogeneity of behavior and genetic findings, as well as dysregulation shown in research data, it is reasonable to conclude that etiologic mechanisms involved in the pathogenesis of autism must occur during gestation and must have the potential to affect and interact with many downstream developmental pathways.

All neurotransmitter systems are important for fetal brain development and interact with each other synchronously to result in normal maturation. A significant abnormality in any of the earliest appearing neurotransmitter systems would impact the development of other systems, could dysregulate development, and lead to a cascade of abnormalities that evolve over time. This chapter will present evidence in support of the theory that during gestation, abnormal signaling in an early appearing transmitter system, that of the

catecholamines and especially including the β_2 -adrenergic receptor (B2AR), contributes to the etiology of autism, and will relate published data to a model of dysregulated B2AR downstream signaling factors and delayed development of cellular physiology.

The β_2 -Adrenergic Receptor

Functions

The catecholamine system is one of the earliest appearing neurotransmitter systems in the human fetal brain [22, 23]. The B2AR is part of this system and is the most studied of the catecholamine cell surface receptors. Cell signaling associated with B2AR stimulation results from the binding of norepinephrine and epinephrine in peripheral tissues and norepinephrine in the CNS as ligands. Although for the great majority of its functions the B2AR couples with the stimulatory G protein, Gs, to activate adenylyl cyclase (AC) and generate cyclic adenosine monophosphate (cAMP), protein kinase A (PKA), and an increase in intracellular calcium levels as second messengers, its activation also stimulates or inhibits MAP kinases to regulate fundamental cell processes such as differentiation, growth, migration, and apoptosis [24, 25]. The cAMP generated influences gene transcription through the cyclic AMP response element (CRE) DNA sequence and its transcription factor or binding protein, CREB (Fig. 7.1). The B2AR is transcribed from its gene on chromosome 5q31–32 as one peptide of 413 amino acid residues [26]. Beta adrenergic receptors (BARs) are widely expressed throughout mammalian fetal tissues including the brain, even in cell types where meager numbers of the receptor will be found in adult life [27]. The B2AR is expressed on mammalian oocytes and preimplantation embryos [28], but whether or not the fetal receptor differs structurally (such as in posttranslational modification) from the mature form is unknown.

Stimulation of the B2AR and the resulting signaling cascades serves different purposes in various tissues and at different times during prenatal development and postnatal life. For example, early in fetal life, B2AR stimulation is coupled to cAMP generation through AC as shown in rat studies [27], and provides signals for growth, but later it promotes differentiation in many tissues and axonal outgrowth in neural cells; in certain tissues, activation can also result in apoptosis or, depending on the degree and duration of stimulation, salvage from apoptosis [27, 29, 30, 31, 32, 33, 34, 35]. In several tissues, stimulation of the B2AR during development causes cells to exit from the cell cycle [36, 37, 38], which is the initial step in the transition from growth by means of cell replication to differentiation and growth as a result of cell enlargement. For that reason, the appearance of the B2AR in various brain regions at different times during development is thought to signal terminal differentiation [39]. Both excesses and decrements in downstream signaling molecules (cAMP)

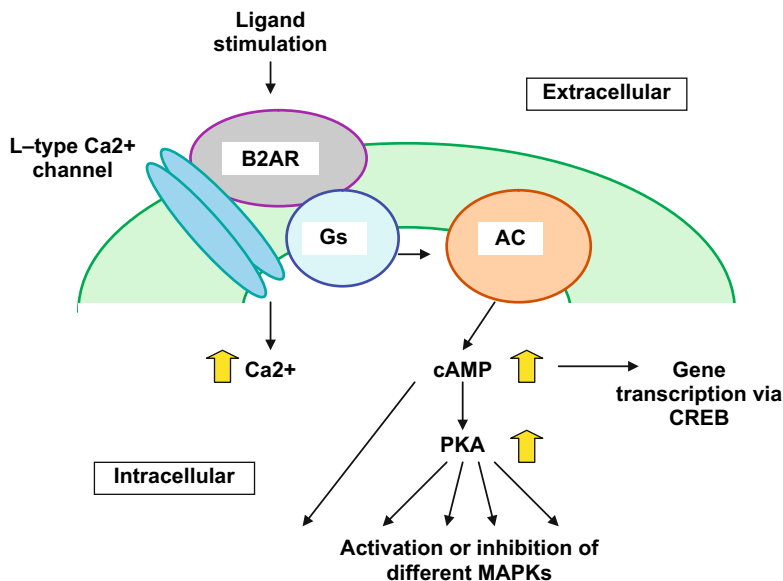


Fig. 7.1 Signaling pathways activated by stimulation of the β_2 -adrenergic receptor (B2AR). The B2AR couples with the stimulatory G protein (Gs), which stimulates adenylyl cyclase (AC) to produce cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA) as second messengers. The cAMP and PKA generated activate or inhibit several mitogen-activated protein kinase (MAPK) pathways involved in cell growth, differentiation, and apoptosis. Cyclic AMP influences gene transcription through cAMP response element-binding protein (CREB). An L-type calcium (Ca^{2+}) channel is also part of this complex, and on B2AR stimulation, it is activated so that intracellular calcium levels rise

cause abnormalities in growth cone formation and function in the developing neurons of lower species such as *Drosophila* [40].

The B2AR system acts as a modulator for cellular signaling in postnatal life. Stimulation of the B2AR facilitates long-term GABAergic transmission to Purkinje cells in the cerebellum [41, 42] and modulates L-type calcium channels in dendritic spines of pyramidal hippocampal neurons in the rat [43]. Presynaptic B2AR stimulation results in long lasting increases in synaptic transmission in rodent amygdala [44], and beta adrenergic activity is essential for enabling glucocorticoid modulation of memory consolidation in the human amygdala [45]. β_2 -Adrenergic receptor signaling activates rodent astrocytes, provides neuroprotection [46], participates in the regulation of a number of cytokines produced by microglia in different situations [47, 48], and increases HLA-DR expression in glioblastoma cells [49]. Signaling through the receptor has many effects on the circulating immune system, such as regulating the amount of IgG1 antibody produced by B lymphocytes in the peripheral blood [50, 51] and regulating immune responses [52, 53]. B2AR second messengers and signaling pathways are also involved in numerous

functions and responses in the GI tract and its immune system, an organ system that, in addition to the brain, has been the subject of many investigations in autism research [15, 54, 55].

Regulation of β_2 -Adrenergic Receptor Signaling

Postnatal signaling by the B2AR is regulated by desensitization (decreased signaling), which can be homologous (involving just the B2AR) or heterologous (involving other receptors that share the same signaling pathway). Homologous desensitization involves two distinct processes: uncoupling of the B2AR from its ability to activate the Gs and, with prolonged receptor stimulation, downregulation (decreased numbers of receptors on the cell surface). The primary mechanism for homologous desensitization involves phosphorylation of the receptor, followed by binding of arrestins to the phosphorylated receptor, which uncouples it from its Gs, ending signal generation. Downregulation is accomplished through events shared with desensitization (phosphorylation and association with arrestins), followed by endocytosis and internalization of receptors, and finally their degradation in lysosomes [56]. Decreasing receptor synthesis and increasing the rate of degradation can also contribute to downregulation. Heterologous desensitization, which also occurs with prolonged B2AR stimulation, involves phosphorylation and uncoupling of other receptors that act through Gs, or loss of function of Gs and AC itself. Together, both desensitization and downregulation terminate cell signaling in the face of excessive input, an essential homeostatic mechanism designed to protect the cell.

Polymorphisms of the β_2 -Adrenergic Receptor

Polymorphisms (single nucleotide substitutions) of the B2AR gene exist in human populations, and three of these code for changes in the amino acid sequence of the receptor that have physiological significance for receptor signaling: glycine at codon 16 (Gly16), glutamic acid at codon 27 (Glu27), and isoleucine at codon 164 (Ile164). Although the Gly16 and Glu27 polymorphisms are associated with enhanced signaling through the receptor, the Ile164 polymorphism results in reduced affinity for ligand binding and lower levels of second messenger formation [57]. Ligand stimulation of Gly16 and Glu27 receptors *in vivo* results in decreased desensitization and downregulation compared to the wild-type variants Arg16 and Gln27 [58, 59].

Polymorphisms of the B2AR gene have been associated with susceptibility to and prognosis in several disease states, including outcome in congestive heart failure [60, 61], medication response in asthma [62], obesity [63], type 2 diabetes [64], Graves' disease [65], myasthenia gravis [66], rheumatoid arthritis [67],

and psychological coping [68]. Because specific combinations of genetic polymorphisms can change the physiology of receptor function and contribute to predispositions for diverse disease states, and because the B2AR is important for normal brain and organ development, it is probable that certain polymorphisms that increase or decrease signaling could become genetic risk factors during gestation for neurodevelopmental disorders, in a similar way as those linked to disease in peripheral organs.

Animal Studies

The functional characteristics of the B2AR have been extensively investigated in the developing rat by Slotkin's group at Duke University. Studies have shown that protective regulatory mechanisms for B2AR signaling are not intrinsic properties of cells, but are acquired during ontogenesis. In fact, the arrival of innervation in target tissues provides a timing signal for the development of receptor desensitization [69]. Fetal and newborn tissues not only are resistant to B2AR desensitization but actually show the opposite: agonist stimulation of the fetal receptor enhances net physiological responses instead of producing desensitization, as in adult tissues [70].

Work in rodents has clarified the mechanisms that underlie fetal sensitization of continued B2AR signaling, and although the earliest studies utilized neonatal cardiac tissue, further research resulted in similar findings in the central nervous system in several mammalian species [71, 72, 73, 74]. Changes in signal transduction after overstimulation of the fetal B2AR depend primarily on changes in receptor coupling and response elements downstream from the receptor, rather than on receptor numbers [75]. Enhanced fetal responses involve increased expression of membrane-associated Gs (which is stimulatory for AC), decreased expression of Gi (which is inhibitory for AC), increased concentration of a more active splice variant of the alpha subunit of Gs, and elevated expression of AC molecules [76, 77, 78]. In addition, the expression of muscarinic type 2 cholinergic receptors (m2AChR) that couple with Gi, as well as their ability to inhibit AC, is decreased, at least in the heart [79]. These differences in fetal tissue promote signaling through AC and decrease its inhibition, resulting in increased production of second messengers such as cAMP and PKA. It is also important to note that sensitization in the fetus is "heterologous," meaning that downstream signaling generated from activation of other receptors that, like the B2AR, utilize Gs and AC (such as the glucagon and β_1 -adrenergic receptors) is enhanced as well [70] (Fig. 7.2).

The signaling changes described above in rodent studies, resulting from overstimulation of the B2AR, do not occur uniformly throughout all brain regions at all ages. As in many other studies involving manipulation of gestational cell signaling [80, 81, 82], these responses depend upon the region investigated, gender, and maturational stage at exposure, and they change with age.

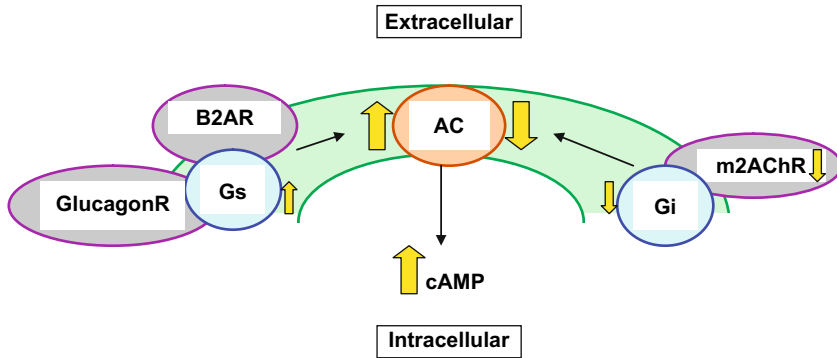


Fig. 7.2 Cellular mechanisms for enhanced adenylyl cyclase (AC) signaling in the fetus. Stimulatory G protein (Gs) function/expression is enhanced, as is the expression of AC molecules, both of which increase AC function. Sensitization is heterologous: signaling from stimulation of other receptors that couple with Gs (such as glucagon) is enhanced as well. In addition, the expression/function of the inhibitory G protein (Gi) is reduced, as is its ability to inhibit AC. The expression of at least one receptor that couples with Gi, the muscarinic cholinergic receptor (m2AChR), is reduced (in cardiac tissue). Block arrows indicate the direction of expression or function

Maturation stage is the predominant factor determining the net signaling response to B2AR agonist exposure during brain development. For example, fetal exposure to terbutaline, a selective B2AR agonist, during the developmental period equivalent to the early-to-mid second trimester of human pregnancy (GD17–20) [83], results in nongender-dependent, enhanced AC responses in whole brain during the immediate period after treatment, compared to controls [71]. Later administration of terbutaline to neonatal rats (PN 2–5), equivalent to the late second and early third trimester in human pregnancy, produces similar changes, but only in specific regions that follow a maturational timetable of susceptibility [71]. After this neonatal exposure schedule, by PN 45 (the end of adolescence in the rat), significant increases in AC responses in males and reductions in females are found in the cerebellum, the last brain structure to develop [71]. Other areas such as the brainstem and striatum show decreases in both genders. Thus, in adolescence, components of the pattern seen with fetal (GD17–20) administration of terbutaline, specifically enhanced Gs and AC signaling, persist into the postnatal period in the developing rat according to a regional pattern that reflects the timetable for maturation of each brain area. Other regions at different times show no effect or decreased AC signaling. By adulthood (PN 60), decrements in AC signaling were found in the cerebral cortex, an area that had shown no net changes in adolescence [75]. To date, AC responses after prenatal or neonatal exposure to B2AR overstimulation have not been measured in the rat brain at a time equivalent in human development to young childhood, when behavioral symptoms of neurodevelopmental disorders often emerge.

Excessive B2AR signaling during PN 2–5 in the rat results in abnormalities in AC function that continue in adolescence and adulthood but with regional and quantitative (over- or undersignaling) differences from those found at the outset. Thus, it is probable that overstimulation of the B2AR or factors activating similar mechanisms alters the “program” for development of cell signaling through G proteins and AC. Because AC and cAMP are involved in countless cell processes that include gene expression through cAMP response element-binding (CREB) protein and neuronal function, abnormalities in AC signaling, such as those described, likely lead to alterations in neuronal cell differentiation, cytoarchitecture, and synaptic signaling.

The fetus has little or no protective mechanism to decrease effects from prolonged B2AR signaling, and exposures during pregnancy that increase B2AR signaling, or overstimulate the receptor, could have widespread effects, the severity of which may depend upon the dose, timing, and duration of interference in the specific brain regions and organs affected. Although decreases in receptor binding can occur in some regions of the CNS and in peripheral tissues of the fetus with excessive B2AR stimulation, it is downstream signaling pathways that are upregulated and provide increased responses [70, 71]. These functional changes occur without differences in form; terbutaline treatment in rats does not affect brain or body weight or rate of growth, characteristics that may be analogous to the situation in autism.

What is the Relationship to Autism?

Prenatal overstimulation of the B2AR, in combination with its more active polymorphisms, likely contributes to the etiology and pathogenesis of autism. Indeed, these two factors have been linked in human studies to this disorder. Exposure for 2 weeks or longer to terbutaline, a selective B2AR agonist that was originally developed for use in asthma and that has been used extensively to arrest or prevent preterm labor [84], has been linked to concordance for autism spectrum disorders (ASDs) in dizygotic twins (relative risk 4.4 in male twins with no family history of ASDs) [85]. This study supports earlier work showing poor cognitive and abnormal psychiatric outcomes in children exposed to B2AR agonists for preterm labor [86, 87]. Terbutaline crosses the placenta and blood–brain barrier and stimulates B2ARs in all tissues of the fetus [27, 88, 89]. In addition, an increased prevalence of the B2AR polymorphisms Glu27 and Gly16 has been found in dizygotic twin sets compared to the general population [85], and the Glu27 homozygous variant has been linked to an increased risk for autism in parent–child trios from the Autism Genetic Resource Exchange (AGRE) population [90, 91].

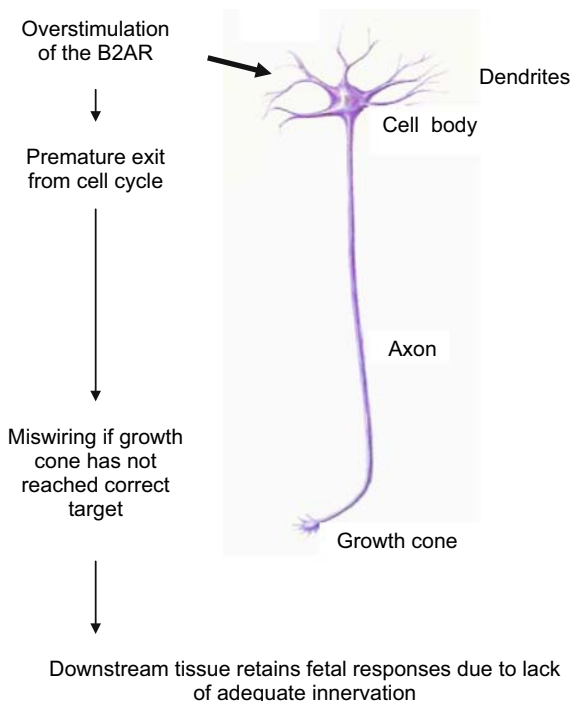
Because signaling through the B2AR contributes to the shift from neural cell proliferation to differentiation, and because the B2AR is part of one of the earliest appearing chemical transmitter systems in brain and tissue development

[23, 92], interference with its function over time during gestation can affect developmental programming and can influence the maturation of other neurotransmitter systems, as well as the later functioning of the CNS and peripheral organs. Rodent studies, in addition to the work cited above, have shown that prenatal overstimulation of the B2AR by administration of terbutaline, on PN 2–5 and 11–14, results in several neuroanatomical abnormalities in the CNS that are analogous to those noted in postmortem autism brain, such as loss of cerebellar Purkinje cells, smaller cells in the sensory cortex, and neuroimmune activation [2, 14, 93, 94]. In addition, prenatal administration of terbutaline has not resulted in abnormalities in form (birth weight and rate of growth were unaffected), but in abnormal function (measured by receptor signaling in membrane preparations) of lungs, liver, heart, and kidneys in the developing rat [72, 79, 95]. Administration of this drug to neonatal rats has also resulted in microglial activation in brain areas that correlate with neuroinflammation in postmortem autism brain, and in juvenile rats at PN 30, it has led to later emerging hyperactivity and auditory sensitivity in preliminary behavioral studies [14, 96].

These results all point to a likely scenario for neurodevelopmental changes, resulting in the pattern seen in autism. Stimulation of the B2AR can cause a premature exit from the cell cycle, a mechanism by which the receptor's signaling decreases cellular proliferation in favor of differentiation [36, 37, 38]. In humans, if excessive BAR stimulation is inappropriately timed and occurs in neural pathways that have not yet completed full innervation of their target tissues, abnormal connections would be formed, and, just as important, the tissues awaiting final innervation and synapse formation might remain in a response state similar to that seen in fetal life, with enhanced AC signaling and decreased inhibition of that enzyme (Fig. 7.3). The net effect of cellular responses at first would be excitatory in pathways that depend upon AC signaling. Later, areas of increased or decreased AC signaling would become region-, gender- and age-dependent, similar to those differences found in the terbutaline animal model. More importantly, patterns of signaling abnormalities would differ among individual patients, since they would depend upon the maturational stage of the CNS at the time of exposure to factors that could overstimulate the B2AR. Later responses and adaptations to the environment that could affect programming of other neurotransmitter systems would be influenced by these early signaling abnormalities, further adding to heterogeneity and disordered maturation.

With this model in mind, the behavioral disorder called autism can be viewed as a biological one marked by dysregulation involving abnormalities in AC function and cAMP formation. Signaling through AC leads to transduction signals shared by numerous neuronal and hormonal pathways. Cyclic AMP is a ubiquitous molecule that not only provides direct signaling within a cell, but also effects gene transcription through the generation of CREB protein. Abnormalities in the AC system, then, could be considered an epigenetic factor that would influence gene expression and developmental trajectory. Autism

Fig. 7.3 Theoretical mechanisms by which overstimulation of the β_2 -adrenergic receptor (B2AR) may cause miswiring, with premature “maturation” in upstream tissues and persistent fetal modes of functioning in downstream tissues. B2AR overstimulation can cause cells to prematurely exit from the cell cycle, decreasing proliferation in favor of differentiation and causing synapse formation at inappropriate targets. The downstream tissues that are targets for innervation under normal conditions continue to show enhanced fetal response patterns (failure to desensitize) since innervation provides a cellular signal for the development of desensitization



may also be a disorder reflecting fetal physiology of the catecholamine system, due to the possibility that the B2AR and its signaling molecules can effect an exit from the cell cycle during the process of innervation. Upstream tissues would then be inappropriately “mature,” while downstream elements might retain fetal responses, and inappropriate, misplaced synapses would create abnormal “wiring” of the CNS. Overall development and functioning would certainly be disordered, as they are in autism.

Relating the Model to Autism Research

Many findings from autism research reflect increased or decreased AC signaling, when parameters being studied are influenced by AC. Alternatively, the findings may reflect a fetal pattern of functioning due to dysregulated AC signaling over time, with some brain regions and other tissues exhibiting sensitized, enhanced responses. Results from these investigations then take on new significance. This section will correlate some of the more recent findings (or those with the greatest impact) in autism literature with these possibilities.

Neuroglial Activation

It is unknown whether the neuroglial activation noted in postmortem autism brain [13] is detrimental, reparative, developmental, or a mixture of all three. Many of the cytokines reported as elevated in cerebrospinal fluid (CSF) and brain tissue by Vargas et al. [13], such as MCP-1 and IL-6, act as growth and differentiation factors during gestation [97, 98], and expression of these two molecules is influenced by cAMP, the former being inhibited by it and the latter being upregulated [99, 100]. When measured by HLA-DR staining, activated and more numerous microglia (the immune cells of the brain) were a major finding in the work by Vargas and colleagues. Microglial HLA-DR expression is present in the human fetal brain from the second trimester and is involved in normal development [101]. Transient overexpression of activated microglia occurs normally in the cerebral white matter of the human fetus [102]; thus the finding of increased expression of HLA-DR in autism brain may reflect dysmaturation. HLA-DR expression *in vitro* is increased in glioblastoma cells in response to cAMP [103], even though B2AR stimulation (and thus increased cAMP) inhibits proliferation of microglia from adult rat brain *in vitro* [104]. Increased numbers of activated microglia may also be a reflection of low AC responses in autism, part of dysregulated AC; norepinephrine (the ligand for the B2AR) depletion in newborn rats results in activated microglia in the cerebellum [105]. Activated microglia may reflect, in part, a developmentally delayed process as well, since in the rodent cerebellum, microglia promote the death of developing Purkinje cells [106], lower numbers of which have been repeatedly noted in cerebellar tissue from autism brain [1, 107, 108].

Increased Cerebral White Matter

Increased white matter on magnetic resonance imaging (MRI) has been documented in children with autism [109]. Some of this increase has been attributed to activated microglia noted in postmortem autism brain [13], since microglia promote myelin formation in cocultures with oligodendrocytes from developing rats [110]. This finding of increased white matter can also be related to enhanced B2AR stimulation and increased cAMP levels, since both processes induce expression of myelin basic protein in immature rodent oligodendrocytes [111, 112]. At a developmental stage immediately preceding the beginning of the active period of myelin synthesis in the rat, the cAMP-dependent pathway that leads to myelin production is stimulated only by B2AR agonists. Thus, delayed or disordered oligodendrocyte maturation may be responsible for the findings by Herbert et al. [109].

Insulin-like Growth Factor-1

Insulin-like growth factor-1 (IGF-1) is a neurotrophic factor that is important in early brain development and axonal assembly at the growth cone [113, 114].

Low levels of IGF-1 in CSF of autistic children [115] may be related to enhanced cAMP signaling, since this molecule inhibits expression of IGF-1 in cultured cells [116] (see Chapter 20 by Riikonen).

Glial Fibrillary Acidic Protein, Bcl-2, and GAD67

Glial fibrillary acidic protein (GFAP) is an astrocytic marker. Astrocytes play important roles in neuronal function, synaptic plasticity, and detoxification [117, 118]. Elevated levels of GFAP in postmortem autism brain [119] correlate with astrogliosis noted by Vargas et al. [13]. They also may reflect increased B2AR signaling, since B2AR stimulation increases the expression of GFAP in astrocytes [120]. In autism brain tissue, decreased expression of Bcl-2, a marker for apoptosis, and GAD67, which catalyzes the conversion of glutamate to GABA [121, 122], can also be related to cAMP signaling. Increased levels of cAMP cause reductions in the expression of GAD67 in C6 glioma cells [123], and levels of Bcl-2 are directly correlated with those of cAMP [124, 125].

Epilepsy

Up to 40% of children with autism develop epileptic seizures, the majority of which have their onset by adolescence [126]. The catecholamines, specifically norepinephrine, have long been known to have anticonvulsant effects in the CNS in animal studies [127, 128]. Work done with the rodent model of prenatal overstimulation of the B2AR described previously resulted in diverse areas of over- and undersignaling through AC that change with age. By adulthood, decrements in cortical AC signaling are apparent in rats after B2AR overstimulation by terbutaline during early development [75]. It is possible that decreased AC signaling may contribute to a propensity to develop seizures in patients with autism as they grow older.

Sulfation, Methylation, and Oxidative Stress

Abnormal sulfation, as it relates to glutathione synthesis and methylation, has been investigated in autism. Lower plasma levels of methionine, *S*-adenosylmethionine (SAM), homocysteine, cystathionine, cysteine, and thus, total glutathione have been found in children with autism compared to controls [129] (see Chapter 10) (Fig. 7.4). Glutathione is a three amino acid molecule that provides the major defense against reactive oxygen species. These values, along with higher levels of *S*-adenosylhomocysteine (SAH), adenosine, and oxidized glutathione, may certainly reflect increased oxidative stress in the peripheral circulation and could reflect impaired methylation capacity. However, these results may also be consistent with additional abnormalities and

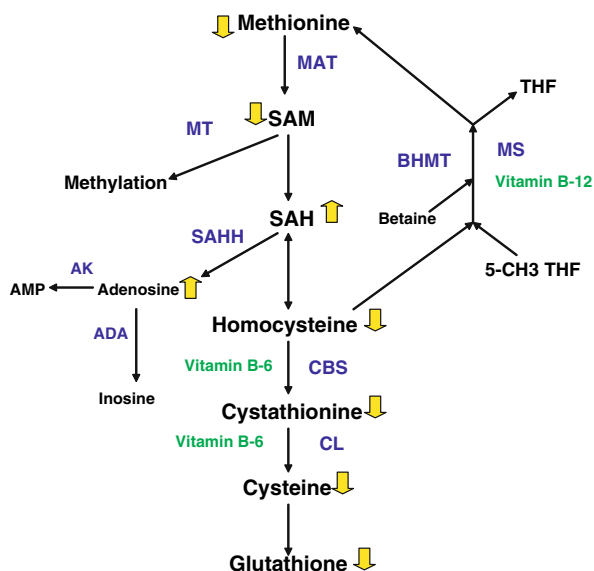


Fig. 7.4 The methionine cycle and generation of glutathione. Homocysteine is remethylated by either of two enzymatic reactions that are folate-vitamin B12-dependent (via methionine synthase, MS) or -independent (via betaine homocysteine methyltransferase, BHMT). After a series of steps involving methyl group transfer and donation for methyltransferase (MT) reactions, methionine metabolites are converted back to homocysteine, which may be metabolized to cysteine through two enzymatic steps involving cystathionine β-synthase (CBS) and cystathionine lyase (CL). Cysteine is incorporated into the tripeptide glutathione, a major defense against reactive oxygen species. Block arrows indicate the direction of plasma levels reported in autistic children by James et al. [129] Other abbreviations: MAT, methionine adenosyltransferase; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; SAHH, SAH hydrolase; AK, adenosine kinase; ADA, adenosine deaminase; THF, tetrahydrofolate; 5CH3THF, 5-methyl THF

fluctuations in cAMP levels as well as dysmaturation. The expression of the enzymes cystathionine β-synthase (CBS) and cystathionine lyase (CL), which catalyze the conversion of homocysteine to cystathionine and cystathionine to cysteine, respectively, are lower in fetal than in postnatal rodent brain and liver, and their expression is cAMP-dependent in human and rat fetal liver [130, 131]. High levels of SAH and low levels of homocysteine would, at first glance, appear to be consistent with decreased S-adenosylhomocysteine hydrolase (SAHH) activity due to high adenosine levels, since increases in adenosine can reduce this enzyme's activity. However, cAMP competes with adenosine to inhibit this enzyme as well, and fluctuations in cAMP (higher levels) could result in similar findings [132, 133]. Finally, increased plasma levels of adenosine found in autistic children [129], and hypothesized to be due to inhibition of adenosine kinase (AK) by oxidative stress, may also reflect low levels of expression of the enzyme, as is found in the fetus and newborn compared to older infants and children [134].

Although oxidative stress is most likely involved in the metabolic functioning of individuals with autism [129, 135, 136, 137], the origin of these findings may not be obvious at first glance. Low activities of antioxidant enzymes such as superoxide dismutase, glutathione peroxidase, and catalase have also been documented in red blood cells from autistic patients [138, 139, 140], as have high levels of nitric oxide [139]. These data make sense when related to fetal functioning since there is a developmental lag in the appearance of superoxide dismutase compared to other antioxidant enzymes in the brain [141], and both glutathione peroxidase and catalase are lower in fetal than in postnatal fibroblasts [142]. In addition, nitric oxide is formed in response to B2AR stimulation in some tissues, such as endothelial cells and platelets [143, 144].

Overstimulation of the B2AR by terbutaline in the fetal rat brain and during the first and second postnatal weeks (equivalent to the second and third trimesters of human gestation) results in an increase in markers of lipid peroxidation compared to nonexposed rats [145], the hallmark of oxidative stress. Similar findings (though using different markers of lipid peroxidation) have been documented in postmortem autism brain [146].

Porphyryns

Increased urinary coproporphyrins have been proposed as a sign of heavy metal toxicity, specifically due to mercury, in children with autism. Geier and Geier [147] and Nataf et al. [148] suggest that high levels of coproporphyrin in both studies, and precoproporphyrin in the latter, are due to heavy metal inhibition of two downstream enzymes in the heme pathway, uroporphyrinogen decarboxylase and coproporphyrinogen oxidase. The inhibitory actions of heavy metals on the functions of these enzymes have been demonstrated in previous literature [149, 150]. An alternate explanation, however, could include a contribution from dysregulated levels of cAMP, since raising cellular levels of this molecule in rodent hepatocyte cultures causes accumulation of coproporphyrins [151].

It is important to note that data from many studies in autism, including those of urinary porphyrins, sulfation, and methylation, are reflections of peripheral organ function, not that of the brain. Although Nataf et al. [148] found that heavy metal chelation tended to normalize the urinary porphyrin levels on paper, it is unknown whether chelation could change the CNS level of mercury. Interestingly, though heavy metal sequestration is a function of the metal carrier metallothionein (MT), and if future research documents heavy metal sequestration in the autistic brain, liver, and bone marrow, this could be a sign of increased levels of MT. Cyclic AMP increases MT levels [152] and induces tissue-specific redistribution of heavy metals for sequestration [153]. The expression of MT in human liver is higher in the fetus than in children over 6 months of age [154, 155]. Thus, increased levels of MT could be

responsible for sequestration of heavy metals such as cadmium and mercury, as a result of higher levels of cAMP or a fetal pattern of physiological functioning.

Plasma and Whole Blood Serotonin

Although whole blood serotonin (5HT) levels are increased in some patients with autism [156, 157], low levels of plasma 5HT that are available for receptor binding have been documented in autistic adult patients compared to normal controls [158], in a study using the platelet and its 5HT transporter (5HTT) as a reflection of serotonin's actions at the synapse. Elevated whole blood serotonin and low-plasma 5HT could be the result of (1) increased 5HT production, or (2) increased expression of the transporter on the platelet surface, or both, among other reasons. Circulating 5HT is produced in, and released from, enterochromaffin cells in the bowel, in a process regulated by neurotransmitter systems that include BARs [159], and is then incorporated into the platelet by the 5HTT. If signaling elements downstream from BARs, such as AC and cAMP, are dysregulated or enhanced, then release of 5HT from the bowel may be disordered or increased. Notably, Vered et al. [160] found a dysregulated response of 5HT levels in platelet-poor plasma from autistic adults after a carbohydrate-rich meal: an initial increase followed by a deficit compared to age-matched normal controls.

Ninety-nine percent of circulating serotonin is contained in platelets as a result of the function of the 5HT transporter [161]. The promoter region of the human serotonin transporter gene contains a CRE motif, and the promoter is inducible by cAMP signal transduction pathways [162, 163]. In addition, an increased density of platelet 5HTT expression has been documented in autism, using paroxetine binding as a specific label [164]. This could be the result of dysregulated AC functioning (leading to enhanced cAMP production) that may have been part of cell programming in bone marrow megakaryocytes before platelet fragmentation. This scenario would be expected after prenatal overstimulation of the B2AR. Because of this, increased transport of 5HT into the platelet could occur, partly due to increased production of 5HT in the bowel as above, and partly because of the increased expression of the 5HTT, both resulting from increased BAR activation. This may therefore contribute to increased platelet levels of serotonin and decreased levels of 5HT in the plasma of autistic patients.

Oxytocin

Oxytocin (OT) is a neuropeptide that mediates complex social and emotional behaviors [165]. Several studies have proposed that excess OT or OT abnormalities play a role in the etiology of autism [166, 167, 168]. Higher levels of

the unprocessed, C-peptide-extended prohormone, OT-X, compared to the completely processed OT have been found in plasma from autistic children compared to age-matched normal controls [169]. A higher ratio of unprocessed: processed OT is similar to that seen in studies of fetal animals [170, 171]. Increased levels of OT-X in autism may therefore reflect the persistence of fetal physiology associated with B2AR dysregulation.

The Gastrointestinal Tract

The GI tract has been a subject of great research interest in autism. Findings of reflux esophagitis and disaccharidase deficiency [55] can be correlated with fluctuations or dysregulation in cAMP and fetal/neonatal functioning. Gastroesophageal reflux is common in premature human infants and neonates, and experimentally increased cAMP levels relax the lower esophageal sphincter in animal models [172, 173]. Disaccharidase expression in the human fetus is relatively low during midgestation and increases toward term [174]. Lactase levels in preterm infants are very low, especially in those younger than 37-weeks gestation [175], making it understandable that these enzymes might be deficient in children with autism, if their intestinal physiology is developmentally delayed and similar to that found in the fetus.

Abnormal motility contributes to diarrhea and constipation, symptoms often reported by caregivers of young children with autism [176, 177]. Peristaltic contractions of the GI tract begin in the stomach and are regulated by “pace-maker” cells, the interstitial cells of Cajal (ICC) [178, 179]. The ICC produce “slow waves,” or the basic electrical rhythm of the smooth muscle layers, upon which electrical action potentials must be superimposed in order to generate true contractions. Human ICC are present from the second trimester of gestation, though they are not well-networked, even at term [180]. If ICC were delayed in their development in autism, peristalsis may be abnormal as a result.

Increased intestinal permeability to lactulose has been documented in 43% of a small group ($N = 21$) of children with high-functioning autism compared to controls [54]. D’Eufemia and colleagues point out that because lactulose is absorbed through the paracellular pathway, its increased absorption may be evidence of damage to intercellular tight junctions, as may be seen with inflammation. However, none of the patients in this study had GI symptoms, such as one might expect from the inflammation documented in later investigations [55, 181], suggesting an alternative explanation for increased permeability. In relating this research to delayed physiology or dysregulated AC signaling, it is known that intestinal permeability to sugars is high in neonates [182], and that intracellular cAMP levels are related to the integrity of intracellular tight junctions [183, 184].

Inflammation in the GI tract has been well-documented in autism, though its impact on the course of the disorder is unknown. The findings in the bowel [185] and stomach [186] in autistic children can be explained only by the interaction

of many factors since the inflammatory process involves the immune and GI systems, both of which are constantly in a state of stimulus–response reactivity. Explanations for this process in relationship to the B2AR theory are beyond the scope of this chapter. A predisposition to inflammation, however, may be inherent in bowel functions of autistic children, as it is in the premature intestine; stimulated fetal enterocytes produce more interleukin-8 (IL-8), a proinflammatory cytokine, than those of older children [187], and transcription of IL-8 is increased by stimulation of B2ARs [188]. DeFelice et al. [189] found no increase in the basal production of IL-8 from endoscopic intestinal biopsies in patients with pervasive developmental disorders, but the measurements were performed with unstimulated enterocytes.

The Immune System

Dysregulated AC cell programming and signaling, along with delayed physiology in the bowel and its immune system, would have far-reaching consequences, because these two organ systems interact continuously to respond to exposures from the external “environment” of the gut lumen. In this situation, an immature, disordered immune system would attempt to respond to stimuli along with an equally dysregulated system of intestinal responses, the results of which could become virtually impossible to predict as static measures, and would change over time. It has become apparent that dysregulated immune responses characterize the inflammation documented in the bowel in autism, as well as the circulating immune system [15], at times reflecting tendencies to T helper type 2 (TH2) responses [181, 190], both TH1 and TH2 activation [191], innate-type immune responses [192], and, less often, normal results [193]. Although predicting one consistent “snapshot” of immune functioning is unattainable given this theory, and indeed, seems impossible in general in autism research, many parameters in the circulating immune system relate to fetal/neonatal functioning or fluctuations in B2AR or AC signaling, and several will be explained here.

Low levels of expression of the TH2 cytokine IL-2 on T lymphocytes [194], “incomplete activation” of T cells, or increased DR+ T cells without a corresponding increase in IL-2 [195], and low intracellular levels of IL-2 and IFN- γ [196] may be explained by enhanced AC signaling, since cAMP inhibits IL-2 production and expression on T helper cells [197], and B2AR stimulation inhibits the production of IFN- γ by T lymphocytes [198]. In addition, cord blood lymphocytes express lower levels of IL-2 receptors [199] and produce lower amounts of IL-2 [200] and IFN- γ than lymphocytes from older children [201], supporting a relationship between these parameters in autism and delayed physiological functioning.

Investigations of serum antibodies in children with autism have revealed many targets for binding in rodent and human CNS tissue [202, 203, 204, 205, 206, 207, 208, 209], suggesting that this disorder may be an autoimmune disease.

However, none of these sera was tested against autism brain. In the more recent work mentioned above, serum antibodies of autistic patients were produced at higher levels than in controls. Interestingly, stimulation of the B2AR regulates the production of immunoglobulin G1 (IgG1) and IgE from B lymphocytes, as well as the number of B cells secreting IgM [210, 211, 212], so that an alternate explanation for these findings might relate them to fluctuations in signaling downstream from the B2AR. In addition, immature CD5+ (or B1) B cells may contribute to increased antibody production in children with autism. These lymphocytes are the predominant B cell during fetal and early neonatal life, and are reactive against autoantigens [213]. They generally produce low-affinity auto- and polyreactive antibodies (usually IgM), including those directed against single-stranded DNA [214, 215], as has been found in children with ASDs and Landau-Kleffner syndrome [204]. Higher levels of antibody binding to proteins of specific molecular weight may be the result of these B-cell effects, which in turn may have been “programmed” by prenatal B2AR overstimulation.

If the immune system in young children with autism is physiologically delayed, the T-cell receptor (TCR) may be immature as well. The CDR3 region of the TCR provides fine antigen recognition and is shorter in length during fetal life compared to that in neonates [216]. The enzyme necessary for lengthening the CDR3 region (terminal deoxynucleotidyl transferase) is ontogenically expressed [217] and is cAMP-dependent [218]. In turn, deficient fine antigen recognition may contribute to the formation of polyreactive autoantibodies in autism.

The Autonomic Nervous System

Both published research [219, 220, 221, 222] and anecdotal reports (urinary retention, sluggish pupillary response to light, and clammy extremities) have noted differences in autonomic nervous system function in patients with autism compared to controls. Ming et al. [223] measured baseline cardiovascular autonomic function in children with autism (ages 4–14) and age-matched healthy controls, using a device that can simultaneously measure heart rate and blood pressure, derive a continuous index of cardiac vagal tone (CVT), and monitor cardiac sensitivity to baroreflex (CSB), in real-time. Both CVT and CSB parameters reflect parasympathetic activity. The CVT and CSB were significantly lower in association with a significant elevation in heart rate, mean arterial blood pressure, and diastolic blood pressure in children with autism compared to controls. Levels of CVT and CSB were lower in autistic children with symptoms of autonomic dysfunction compared to those without, and were not related to age. These low levels suggest impaired cardiac parasympathetic activity, with unrestrained and perhaps hyperactivity of the sympathetic nervous system.

The findings of Ming et al. [223] can be correlated with results from overstimulation of BARs in fetal (GD17–20) or newborn rats (PN 2–5; equivalent to the second trimester of human gestation). Garofolo et al. [79] administered isoproterenol, a combined β_1 and β_2 agonist, and terbutaline, a selective B2AR agonist, to rats during these two time periods. Both treatments caused downregulation of m2 type acetylcholine receptors (m2AChRs) as well as a loss of the ability of m2AChR stimulation to inhibit AC in the heart, which could reduce effects from parasympathetic input. Premature exposure of the developing heart to BAR agonists also promotes the function and sensitization of signaling through AC [70], enhancing sympathetic input. These effects may parallel changes found in sympathetic and parasympathetic tone in autistic children [223].

Differences in Signal Transduction

If autism is a biological disorder characterized, in part, by dysregulated AC functioning that leads to disordered development, then markers of increased AC signaling should be expressed at higher levels in some tissues in patients with autism compared to those in controls. Examples are the enzymes involved in the cAMP-dependent second messenger pathways such as PKA and cAMP. Chauhan et al. [224] have studied signal transduction in children with autism from the AGRE database and found increased expression of PKA in membrane preparations of lymphoblasts from the autistic children compared to normal sibling controls. This finding lends support to the B2AR/AC theory. Further studies investigating levels of cAMP and AC in lymphoblasts are underway by this group. It will be interesting and important to study the expression of other molecules that could be predicted from Theodore Slotkin's previously described rat model, such as a decrement in Gi proteins and deficits in their ability to inhibit AC [77, 78]. This would provide support for a hypothetical physiological signaling imbalance due to prenatal overstimulation of the B2AR.

Different Mechanisms, Similar Result

No single prenatal insult, such as overstimulation of the B2AR during critical periods of brain development, can be responsible in all cases for a complex neurodevelopmental disorder with heterogeneous presentations such as autism. Prenatal B2AR overstimulation in the previously described rat model leads to enhanced signaling through AC early in development, and to gender-dependent dysregulation (enhancement and deficit) of AC signaling as development progresses, in various regions of the brain. It is possible that this sort of downstream signaling abnormality that has the potential to affect numerous processes and cell programming through CREB protein and other components

is a “final common pathway” in the development of autism. Similar findings have been documented in animal models that have been used to investigate mechanisms of insults different from β_2 receptor overstimulation during development.

Several of these mechanisms have been linked to autism in human studies, such as maternal stress [225], or have been theoretically associated in animal models such as the “stress” of infections during pregnancy [226, 227] (see Patterson chapter). Stress itself elevates levels of epinephrine and norepinephrine (B2AR ligands) in the pregnant mother, but these do not cross the placenta effectively; Moreover, stress also causes increases in endogenous levels of glucocorticoids, which do cross to the fetus. Glucocorticoid excess in maternal stress can result from psychological, infectious, or physical causes. Prenatal administration of the glucocorticoid, dexamethasone, to rats results in early, enhanced AC activity in the developing brain and liver [228] that persists, at least in the forebrain, as increased activity of the AC catalytic subunit in postnatal life [229]. After pregnant guinea pigs are exposed to synthetic glucocorticoids, the offspring exhibits alterations in pituitary–adrenal function and responses to endogenous glucocorticoids throughout life, as well as differences in activity levels compared to controls [230, 231]. Effects of maternal adversity (as opposed to exposure to synthetic glucocorticoids) have been studied in the guinea pig as well. Male offspring of stressed dams develops altered basal plasma cortisol concentrations and exhibits increased anxiety compared to controls [232]. Interestingly, the male offspring of glucocorticoid-exposed dams developed elevated plasma testosterone levels [230], which could be analogous to the elevated plasma testosterone levels reported in autistic children [233].

Subtoxic maternal exposure to organophosphate pesticides, which represent half of all the insecticide use in the world, may be another factor in the development of autism. The most used organophosphate, chlorpyrifos, though prohibited now from use in the home in the United States, is still widely applied in the chemical preparation of homesites and throughout agriculture, and thus nearly the entire population is exposed to this agent. In a rodent model, prenatal exposure to this pesticide results in enhanced AC signaling in the brain that is region- and sex-dependent and persists into adulthood [82, 234], results that converge with the outcomes after terbutaline treatment. It is possible that the study of such prenatal disturbances that result in dysregulated AC function will lead further to defining some of the mechanisms involved in the etiology of autism.

Prenatal β_2 -Adrenergic Receptor Overstimulation and Sensitization to Other Factors

It may not be that one process alone, namely B2AR overstimulation during critical periods for prenatal brain development, consistently in all cases leads to a complex neurodevelopmental disorder with heterogeneous presentation

such as autism. The presence of polymorphisms of the B2AR that amplify this tendency could further increase the chances that the disorder might develop in an individual child. It is also possible that prenatal B2AR overstimulation sensitizes the CNS and peripheral tissues to other, later factors that could affect development. For example, in rats, B2AR overstimulation by terbutaline during the neurodevelopmental equivalent of the second trimester in humans results in dysregulated AC signaling as described previously. However, when these same neonatal rats are later exposed at PN 11–14 to the organophosphate insecticide, chlorpyrifos, larger alterations in more widespread areas of the developing brain are found than are seen with either agent alone [75]. A good deal of attention is understandably paid to genetic etiologies of differential susceptibility to environmental toxicants, but it is equally plausible that nongenetic factors such as prior chemical or drug exposure in mothers, children, and fetuses may also define subpopulations that are especially vulnerable to alterations in developmental trajectories.

Effects on Other Neurotransmitter Systems

During postnatal life, B2AR signaling interacts with and affects all other neurotransmitter systems in various tissues. The same may be true for brain development during fetal life. Prenatal B2AR overstimulation not only leads to dysregulation of development in the catecholamine/AC signaling system, but also influences the development of other neurotransmitter systems, such as serotonin (5HT). Overstimulation of the B2AR during PN 2–5 in the rat elicits global increases in 5HT receptors and the 5HT transporter in brain regions possessing 5HT cell bodies (midbrain and brainstem) as well as in the hippocampus [235, 236]. Slotkin and Seidler [236] found that the changes in the 5HT system were demonstrable after this exposure, as late as adolescence. Interestingly, lower total serotonin content was noted in the brains of treated rats compared to controls [236]. This may be analogous to lower levels of brain serotonin synthesis over time in autistic patients compared to controls [237] (see Blue chapter 5).

The Role of Maternal Factors

In considering prenatal factors that may result in the dysregulation of AC functioning and disordered development, one must take into account that effects on the fetus are the result of, and influenced by, the physiology of the pregnant mother and her adaptive responses to the environment. It may be that downstream cell signaling that results from factors such as inflammatory (cytokine) responses in the mother [227], maternal antibodies [238, 239], and stress [225] shares components that are also elicited by overstimulation

of the B2AR. Additional factors may worsen the tendency for abnormal developmental trajectories, such as low maternal plasma serotonin [240] and genetic polymorphisms that could increase or decrease cell responses. Because abnormalities in development induced by the pregnancy environment can become transgenerational [241], studies of mothers and grandmothers of children with autism may be necessary in the future.

Prenatal β_2 -Adrenergic Receptor Understimulation

The opposite case, i.e., blocking BAR function or adrenergic denervation during early development, also produces unique patterns of receptor responses in animal studies. In comparison with prenatal B2AR overstimulation, which would lead to dysregulation of AC signaling, after adrenergic denervation with 6-hydroxydopamine, developing rats exhibit a delay in the onset of desensitization in the heart (PN 25 instead of PN 15) and lack of any desensitization in the liver, even at PN 25 [242]. The resulting signal enhancement continues as an immature pattern of sensitization for a prolonged period compared to controls [242]. Effects can be permanent: receptors in some tissues never acquire some of their essential responses [243]. Denervation in this context, then, shifts a developmental change (one from sensitization to desensitization) toward an older age and creates a physiological developmental delay. This scenario of understimulation may relate to maternal antibodies that can potentially block receptors [238, 239] and viral infections such as influenza in the pregnant mother [227] or postnatal herpes [244], both of which decrease AC signaling and lower cAMP production [245, 246].

Speiser et al. [247] treated pregnant rats during GD8–22 with the BAR antagonists propranolol (a β_1 and β_2 receptor blocker) and atenolol (a specific β_1 receptor blocker). The offspring of rats treated with propranolol demonstrated increased motor activity and poor performance in the active avoidance test, whereas the offspring exposed to atenolol during gestation exhibited no behavioral changes, showing that blocking B2ARs during brain development can be linked to behavioral abnormalities in animals. Blocking BARs in rats during an age equivalent to the second trimester has not been studied as extensively as has B2AR overstimulation in the same period, but there are two studies pointing toward alterations in neural cell development that may be relevant to neurodevelopmental disorders such as autism. In newborn rats, destruction of presynaptic noradrenergic terminals with the neurotoxin 6-hydroxydopamine results in blunting of the development of the ability of B2ARs to elicit cellular responses [248], again showing the importance of a critical period in which the appropriate exposure of receptors to the natural neurotransmitter ligand “programs” the development of cell responses. Similarly, chronic administration of propranolol to pregnant rats throughout gestation delays the development of B2AR coupling to cellular responses [248]. In the

long run, interference with B2AR transmission during pregnancy produces a permanent change in the responsiveness to noradrenergic input in the brain [249]. Since these mechanisms result in abnormalities in AC signaling, they all are potentially important in autism pathogenesis.

Conclusion

Historically, there seem to be several stages in unraveling the etiology of a neurodevelopmental disorder. After recognition that a specific disorder exists (for example, Rett syndrome was first described in 1966), a prolonged period ensues, marked by a plethora of research studies that describe the physiology of the disorder, and proposes mechanisms of pathogenesis as well as candidate genes involved in it. Finally, the cause is discovered and research into treatment begins (the abnormality in the MECP2 gene was discovered in 1999). At this time in history, autism research is still in the descriptive phase of this process, but with cohesive theories on which to base research efforts, it will move from description to discover causes. Autism is clearly heterogeneous in its clinical presentation, with biological research results that show widespread dysregulation. The theory of prenatal B2AR signaling outlined in this chapter, relating environmental influences with genetic predispositions to dysregulation in an enzyme system that is ubiquitous and influences a multitude of developmental pathways, accounts for multiple disparate findings and disordered development. This type of approach will help to unravel the etiologies of autism.

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