NEUROANATOMICAL AND BEHAVIORAL CHARACTERIZATION OF MICE DEFICIENT IN HEPARIN-BINDING GROWTH-ASSOCIATED MOLECULE (HB-GAM)

by

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Abstract

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Heparin-binding growth-associated molecule (HB-GAM) is an extra-cellular matrix-associated protein involved in a variety of neurodevelopmental processes that has neurotrophic and neuroprotective effects. Previous studies suggest that HB-GAM knockout mice exhibit cognitive inflexibility, anxiety, and motor impairment and that the brains of these animals possess increases in cortical neuronal density. Collectively, these features are most similar to the pervasive developmental disorders (PDDs). Therefore, the current studies sought to further characterize the neuroanatomical and behavioral phenotype of HB-GAM knockouts within the context of the hypothesis that these animals might serve as an animal model of the PDDs. Consistent with this hypothesis, HB-GAM knockouts demonstrated cognitive inflexibility, heightened anxiety, and both a contextual and social neophobia. In addition, the knockouts' brains were shown to possess cortical neuronal area decreases and cortical neuronal packing density increases. These data suggest that multiple abnormalities similar to those observed in individuals with PDDs characterize the phenotype of HB-GAM knockouts. The validity and limitations of HB-GAM knockouts as an animal model of the PDDs are discussed, as are suggestions for future studies of these animals.

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CHAPTER 1. GENERAL INTRODUCTION

Heparin-binding growth-associated molecule (HB-GAM). Heparin-binding growth-associated molecule (HB-GAM), also known as pleiotrophin, is an extracellular matrix-associated protein implicated in a variety of processes integral to mammalian nervous system development (Rauvala & Peng, 1997). HB-GAM was first recognized through investigations attempting to identify factors that enhance neurite outgrowth in the rat brain (Rauvala, 1989). Indeed, HB-GAM has been implicated in mitogenesis and neurite outgrowth (Hampton, Marshak & Burgess, 1992; Li, 1990; Milner et al., 1989; Raulo, Julkunen, Merenmies, Pihlaskari & Rauvala, 1992), which are vital to the early development of neural tissue. HB-GAM contributes to these processes by binding at the neuronal surface with N-syndecan (Raulo, 1994), which further employs the cortactin/src-kinase pathway to enhance neurite extension (Kinnunen, 1998). Cell motility appears to be mediated via activity of HB-GAM through the same pathway (Rauvala et al., 2000).

Further study has indicated that HB-GAM is involved in several other key processes of nervous system development. HB-GAM reportedly arrests cellular proliferation (Szabat & Rauvala, 1996) via inhibition of fibroblast growth factor (FGF) (Hienola, Pekkanen, Raulo, Vanttola & Rauvala, 2004). Interestingly, other studies have suggested that HB-GAM promotes cellular differentiation (Szabat & Rauvala, 1996). HB-GAM has also been implicated in processes related to early presynaptic (Dai & Peng, 1996) and postsynaptic specialization (Peng et al., 1995), as well as development of neural vasculature (Christman et al., 2005; Yeh, He, Xu, Hsu & Deuel, 1998).

Possible post-developmental roles for HB-GAM have been suggested by studies that indicate the protein has neurotrophic and neuroprotective effects. HB-GAM is up-regulated in the degenerating substantia nigra of individuals with Parkinson's disease and augments dopaminergic neuron survival and outgrowth in ventral mesencephalic cultures (Marchionini et al., 2007). Similarly, HB-GAM is up-regulated in acutely denervated distal sciatic nerves, protects spinal and facial motor neurons against chronic excitotoxic injury and cell death, and enhances regeneration of myelinated axons in experimentally transected rat sciatic nerves (Mi, Chen & Hoke, 2007).

HB-GAM expression in the non-pathological nervous system is known to decline as constitutive developmental processes approach completion (Rauvala, 1989). However, HB-GAM expression persists beyond development in specific neuronal populations (Takeda et al., 1995; Wanaka, Carroll & Milbrandt, 1993). HB-GAM is highly expressed in pyramidal neurons of the CA1 field of the hippocampus, and evidence suggests that HB-GAM might repair neuronal connections after injury (Takeda et al., 1995). In addition, HB-GAM has been implicated as a modulator of synaptic plasticity in the adult rodent brain. High frequency stimulation sufficient to induce long-term potentiation (LTP) has been shown to result in concomitant increases in HB-GAM expression in the CA1 region (Lauri, Taira, Kaila & Rauvala, 1998). Further, application of exogenous HB-GAM to the CA1 field inhibits the early stages of LTP induction in glutamatergic synapses of the CA1 field (Lauri et al., 1998; Pavlov, Rauvala & Tiara, 2006).

HB-GAM Knockout Mice. Given sufficient evidence that HB-GAM somehow aids in the modulation of hippocampal synaptic function, additional studies have sought to determine the structural and functional effects of an absence of HB-GAM. To that end,

mice null for the HB-GAM gene *Ptn* were created (Amet et al., 2001). Study of these animals' brains revealed increased neuronal density in frontal and parietal cortices (Hienola, Kinnunen & Rauvala, 2002; Hienola et al., 2004). In addition, though gross hippocampal structure and basal excitatory synaptic transmission in the CA1 region are normal in HB-GAM knockouts (Amet et al., 2001; Pavlov et al., 2002), hippocampal slices harvested from these animals display a lowered threshold for LTP induction. This irregular threshold is normalized by application of exogenous HB-GAM, suggesting a functional and not a developmental abnormality (Amet et al., 2001). Additional research indicates that LTP is significantly attenuated in animals over-expressing HB-GAM (Pavlov et al., 2002). These data suggest that HB-GAM acts as an inhibitory modulator of hippocampal LTP.

Hippocampal LTP is considered a putative neurobiological correlate of learning and memory (Cooke & Bliss, 2006). Therefore, a few studies have sought to assess the *in vivo* effects of HB-GAM on learning and memory capacity. Pavlov et al. (2002) reported a subtle impairment in the acquisition of spatial information in HB-GAM knockout mice, and similar deficits have been identified in animals null for syndecan-3, a receptor for HB-GAM (Kaksonen et al., 2002).

Interestingly, additional study (Croll, unpublished data) of HB-GAM knockouts' learning and memory suggested that these animals perform as well as wild-type animals in versions of the Morris water maze dependent on spatial and associative learning. However, when exposed to these two distinct versions of the maze consecutively, HB-GAM knockouts' performance was comparable to that of wild types' in the first but not the second version of the maze in which they were tested, whether spatial or associative.

That is, HB-GAM knockouts demonstrated reduced acquisition relative to wild type mice only in the second version of the maze in which they were tested. This pattern of performance is not suggestive of a primary learning deficit but might reflect difficulty adapting to changing contingencies, or, cognitive rigidity.

HB-GAM knockout mice perform within normal limits on measures of home cage behavior, ease of handling, gross reflexes, body weight, climbing behavior, balance, and grip strength (Croll, unpublished data). However, these animals have reportedly demonstrated increased anxiety upon formal testing in appropriate paradigms (Pavlov et al., 2002). In addition, general behavioral assessment of HB-GAM knockouts revealed deficits in balance beam performance (Croll, unpublished data), on which these animals adopted an atypical stance perpendicular to the length of the beam and appeared unwilling to traverse the beam. HB-GAM knockouts typically fell off the beam when attempting to traverse its length, suggesting that a motor coordination and/or balance impairment, and not increased anxiety, was responsible for their performance.

Cognitive rigidity is associated with a number of pathologies, including schizophrenia and major depressive disorder, which are also often accompanied by pathological anxiety (American Psychiatric Association, 2000). However, a syndrome including but not limited to cognitive rigidity, anxiety, and motor impairment in the presence of increased cortical neuronal density is most consistent with the pervasive developmental disorders (PDDs).

The Pervasive Developmental Disorders (PDDs). The PDDs comprise a group of conditions generally characterized by a delay in or deviation from normal development in

two or more of three principal domains: social skills, language and communication, and behavioral repertoire (American Psychiatric Association, 2000).

Deficits in social functioning, the most common observed in the PDDs, often manifest as the inability to infer or make attributions about the mental states of others and thus to form meaningful interpersonal relationships (Volkmar, Chawarska & Klin, 2005). Language impairments in affected individuals usually take the form of minimal, abnormal or repetitive babbling of non-speech sounds or nonsense syllables in young children (Rice, Warren & Betz, 2005) and absent, delayed or inappropriate use of language, as well as peculiarities of tone and prosody and an overall difficulty with the social pragmatics of language, in older affected children (Sadock & Sadock, 2003).

The behavioral aberrations most associated with the PDDs include frequent performance of ritualistic, repetitive actions, heightened anxiety in the face of novelty necessitating adherence to a strict routine, and an abnormally circumscribed repertoire of interests (Bolivar, Walters & Phoenix, 2007). Additional PDD-associated behavioral symptoms include heightened aggression, self-injurious behavior, and hyperactivity (Lecavalier, 2006). Some PDDs also involve physical abnormalities, such as accelerated or arrested head growth (Dissanayake, Bui, Huggins & Loesch, 2006), and motoric anomalies, such as disturbances in gait, coordination, and fine motor skills (Dewey, Cantell & Crawford, 2007).

Five PDDs are included in the most recent edition of the American Psychiatric Association's *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision* (DSM-IV-TR; 2000), and each is characterized by specific variations in the general PDD phenotype. First described by Kanner (1943), *autistic disorder* is associated

with aberrations in all three domains affected in the PDDs that usually begin before the age of three. The intellectual functioning of affected individuals varies from mentally retarded to above average levels. *Rett's disorder* is observed only in females with an onset typically between six months to two years of age. This disorder is characterized by only rudimentary intellectual, language and social functioning, and is frequently accompanied by seizure disorder as well as poor muscle coordination, ataxia, apraxic gait, and a loss of purposeful hand movements in favor of stereotypical motions such as hand wringing. *Childhood disintegrative disorder* is characterized by normal development within the first two years of life, after which affected children can suffer a loss of previously acquired skills in any two or more of the domains of language, social functioning, motor functioning, play behavior, or bladder and bowel control. *Asperger's disorder*, first described by Asperger (1944) as an "autistic psychopathy," is typified by significant impairments in social functioning coupled with repetitive and stereotyped patterns of behavior in the absence of impairment in language or intellectual functioning. A diagnosis of *pervasive developmental disorder – not otherwise specified* (PDD-NOS) is reserved for those children who demonstrate pervasive impairment in all three principle domains affected in the PDDs but who do not meet formal diagnostic criteria for a specific PDD or for other psychiatric disorders, such as schizophrenia or avoidant personality disorder, which are typified by PDD-like features.

Given the significant overlap of symptoms among autistic disorder, Asperger's disorder, and PDD-NOS, many have questioned the validity of existing diagnostic classifications and postulated that individuals with these pathologies are merely experiencing varying forms of a single disorder that could be placed along a continuum

of *autism spectrum disorders* (ASDs) (Eisenmajer et al., 1996). For example, multiple studies (cf. Mayes, Calhoun & Crites, 2001) have questioned the legitimacy of Asperger's disorder as a discrete PDD given both the potential for normal intellectual functioning and the presence of at least mild language deficits in an overwhelming number of individuals demonstrating other PDD-associated abnormalities. This has led many to consider Asperger's disorder as a form of "high functioning autism" (Eisenmajer et al., 1996).

Epidemiology of the PDDs. Data regarding the prevalence of PDDs has suggested a tremendous increase in the incidence of these disorders in recent years. In the early to mid-twentieth century, PDDs were thought to occur in approximately 3 - 4/10,000 individuals, but current estimates range from 1/500 to 1/166 individuals (Herbert et al., 2006). Indeed, between 1991 and 1997 alone, a near five-fold increase in the prevalence of these disorders was observed (Stokstad, 2001). However, estimates of occurrence as a function of gender have stayed relatively stable, consistently favoring males with a ratio of 4:1 (Frombonne, 2005).

Despite that prevalence estimates vary based on the assessment method, sample size, publication year, and geographic location of studies, an unequivocal increase in the occurrence of PDDs is evident (Frombonne, 2005). This increase has been attributed to a number of possible factors, including better-defined diagnostic criteria (Fombonne, 2003), increases in pathogenic environmental factors that interact with genetic precipitants (Herbert et al., 2006; Persico & Bourgeron, 2006), and increases in assortative mating among individuals with PDD endophenotypes, such as ritualized behavior and systematized cognition (Baron-Cohen, 2006).

Etiology of the PDDs. From the time of their first clinical characterization through the middle of the twentieth century, PDDs were attributed to emotional disturbance resulting from disordered early attachment to psychopathological parents (cf. Bettelheim, 1967). In the absence of substantial empirical evidence for such an origin, however, biological etiologies have been investigated in recent years. A number of environmental factors have been implicated in the pathogenesis of the PDDs, including pre- and post-natal viral infections, prenatal exposure to teratogenic drugs, obstetric complications, and side effects from traditional childhood vaccines (Ardnt, Stodgell & Rodier, 2004; Libbey, Sweeten, McMahon & Fujinami, 2005). However, such influences have proven to be of minimal and inconsistent influence on close examination (Newschaffer et al., 2007). PDD-like symptoms have also been associated with a number of other pathologies, from seizure disorder to Duchenne muscular dystrophy, but affected individuals presenting with symptoms of conditions like these represent only a small portion of diagnosed cases (Muhle, Trentacoste & Rapin, 2004).

Alternatively, numerous studies have identified the pathogenesis of the PDDs as strongly genetic. Concordance rates of autism among siblings are markedly greater than prevalence rates in the general population (Chakrabarti & Fombonne, 2001). Further, concordance rates in monozygotic twins are consistently and strikingly greater than concordance rates in dizygotic twins, which approach minimal and sometimes non-existent levels, even under broad phenotypic definition (cf. Bailey et al., 1995). Despite the unequivocal conclusion drawn from these data that PDDs have a genetic basis, the means by which these disorders are inherited is still largely unknown, likely owing to a

polygenic mechanism of inheritance as indicated by a low sibling recurrence risk (cf. Jorde et al., 1990).

Further, attempts to characterize the genetic architecture of the PDDs have been stymied by the etiologic heterogeneity suggested by volumes of discordant research. The complexity of the genetic basis of PDDs is not surprising given the multiplicity of symptom constellations observed in affected individuals, which suggests locus heterogeneity (Veenstra-VanderWeele, Christian & Cook, 2004). Some have postulated that variants in individual genes might underlie discrete features of a PDD, such as social deficits or language impairment, while an aggregation of variants in multiple genes might result in the full phenotypic presentation labeled as a specific disorder (Piven & Palmer, 1999).

Indeed, no single investigation attempting to determine the locus of genetic abnormalities underlying the PDDs has yielded definitive results. Recently, a large genetic linkage analysis yielded suggestive evidence for near universal linkage in the vicinity of chromosomal region 11p12-p13 (The Autism Genome Project Consortium, 2007). However, individual linkage studies have suggested a role for several other regions in both risk for and development of PDDs, including 2q, 7q and 17q (Grice & Buxbaum, 2006). Of note is that the human *Ptn* gene is located on chromosome 7 (Li et al., 1992).

Collectively, other genetic studies have identified no fewer than fifteen genes associated with development of PDDs (Risch et al., 1999), including those that code for serotonin transport mechanisms (McCauley et al., 2004), reelin protein expression (Fatemi, Stary, Halt & Realmuto, 2001), neuroligins (Jamain et al., 2003), and gamma-

amino butyric acid (GABA) receptor A (Cook et al., 1998). Interestingly, HB-GAM's modulation of hippocampal LTP has been owed to $GABA_A$ receptor-mediated inhibition of glutamatergic synapses (Pavlov, Rauvala & Tiara, 2006), suggesting that decreased LTP induction thresholds in HB-GAM knockout hippocampi might be due in part to abnormalities in GABA receptor mechanisms.

Findings linking the PDDs to abnormalities in the aforementioned genes have proven replicable but not consistently so. Further, defects in specific genes have frequently been associated with specific variants in symptom presentation. For example, abnormalities in the serotonin transporter gene have been linked to particularly prominent rigid and compulsive behavioral symptoms (McCauley et al., 2004). Taken together, these facts further argue for the heterogeneity of loci of genetic defects predisposing individuals to manifest either endophenotypes of the PDDs or the full disorders. Further, allelic heterogeneity is thought to underlie the tremendous variability in symptom severity common in PDDs. For example, different mutations in the methyl-CpG binding protein 2 (MECP2) gene have been associated with different symptom presentations in individuals with Rett's disorder (Zappella et al., 2003). The inconsistent findings of investigations into the genetic basis of the PDDs speak to the polygenetic etiology of these disorders and might also explain the tremendous variability in symptoms observed among individuals presenting with features characterizing a single disorder.

Neurobiology of the PDDs. The search for candidate genes underlying PDD development has been hampered primarily by the incomplete understanding of the neurobiology of these disorders (International Molecular Genetic Study of Autism Consortium, 1998). Studies associating PDD-like symptoms with other pathologies such

as seizure disorder have failed to establish a strong causal link between those pathologies and features of the PDDs (Muhle, Trentacoste & Rapin, 2004). However, such studies remain illuminating in that they strongly suggest a neurological basis for PDDs.

Indeed, the PDD phenotype is now widely recognized as one typified by neurobiological as well as behavioral irregularities. Macroscopically, an early increase in overall brain size is widely reported in the PDDs, apparently the result of pathological brain growth in the first year of life followed by an equally abnormal arrest in growth (Redcay & Courchesne, 2005). Consistent with this brain growth phenotype, the percentage of excess brain volume in PDD-affected individuals compared to normal controls, assessed by either head circumference or volumetric analyses conducted via magnetic resonance imaging (MRI), lessens with increasing age from approximately 10% in three to four year olds (Redcay & Courchesne, 2005) to only very small volume excesses in six to seven year olds (Sparks et al., 2002). Brain enlargement is reflective of increases in both cerebral gray and white matter (Hazlett et al., 2005). Such increases in gray and white matter volume are also observed in the cerebellums of individuals with PDDs (Courchesne et al., 2001), but the gray matter of the cerebellar vermis is generally reduced in size (Kaufmann et al., 2003). Interestingly, cortical gray matter hyperplasia has also been observed in HB-GAM knockout mice (Hienola et al., 2002).

The core features of PDDs, such as cognitive and social function deficits, are useful indicators as to the neural regions and systems that might be affected in these disorders, provided an understanding of which structures underlie normal function (Baron-Cohen, 2004). Numerous functional imaging studies (cf. Reiss, Abrams, Singer, Ross & Denckla, 1996) have established strong correlations between neural structure

volume and functional capability. Such correlations have also been demonstrated in individuals with PDDs. Reduced density of the corpus callosum (Manes et al., 1999) and thalamic gray matter (Spencer et al., 2006) have, for example, been correlated with intellectual impairments in autism. In addition, exploratory behavior and rate of stereotyped movement have both been negatively correlated with volume of the cerebellar vermis (Pierce & Courchesne, 2001).

Deficits in discrete components of social cognition have also been associated with structural and volumetric abnormalities in specific neural regions thought to subserve those components. For example, reduced volume of the amygdala has been associated with deficits in the recognition of facial expressions of emotion (Nacewicz et al., 2006). Further, reduced gray matter density in the superior temporal sulcus has been correlated with poor performance on tasks requiring the analysis of socially and communicatively important stimuli produced by others and on other tasks necessitating the incorporation of visual and auditory information vital to the processing of human speech (Pelphrey, Adolphs & Morris, 2004).

A litany of studies utilizing small samples but producing convergent findings have also demonstrated microscopic cellular abnormalities in PDD-affected individuals (for reviews, see DiCicco-Bloom et al., 2006; Polleux & Lauder, 2004), including decreased number of cerebellar Purkinje cells, dysplasia of the brainstem and olives, increased neuronal packing density and smaller neuronal area in limbic structures, hippocampal deformation, cerebral cortical dysgenesis, and neocortical aberrations such as misorientation of pyramidal neurons.

These microscopic abnormalities do not fully explain the gross cerebral enlargement consistently observed in the brains of those affected by PDDs (Courchesne & Pierce, 2005a). Macrocephaly might be better accounted for by an increase in the density and number of cortical neurons, which are also abnormally small (Courchesne et al., 2001). Macrocephaly might also be due to excessively numerous, tightly packed minicolumns of reduced width in areas such as frontal and temporal association cortices, which have been observed in those with PDDs (cf. Casanova et al., 2006).

Increases in projection neuron numbers presuppose a proportional increase in axons, which could underlie the observation of increased white matter in brains of those with PDDs. Such increases could also have adverse implications for the proper organization of neural circuitry (Eigsti & Shapiro, 2003). Some have characterized the PDDs as disorders of cortical inhibitory control (cf. Kana, Keller, Minshew & Just, 2006), which would likely be negatively affected by the imbalance of excitatory and inhibitory inputs engendered by an abnormal increase in cortical neurons. In addition, smaller neuronal area, as has been frequently observed in brains of those with PDDs, implies that assemblages of such cells would experience a greater challenge in relaying signals across relatively expansive neural distances (Kana, Keller, Minshew & Just, 2006; Laughlin & Sejnowski, 2003).

A likely consequence of disordered long distance signal transmission is reduced synchronic activation among structures comprising cortical networks, which has indeed been observed in PDD-affected brains and linked via functional imaging to cognitive functions known to be impaired in these disorders. For example, affected individuals demonstrate reduced activation of a cortical network comprised of the medial prefrontal

cortex, adjacent rostral anterior cingulate cortex, posterior cingulate cortex, and precuneus (Kennedy, Redcay & Courchesne, 2006), which is thought to underlie a variety of higher order socio-emotional processes, such as self and other person judgments and perspective-taking (Cavanna & Trimble, 2006; Kennedy et al., 2006). Similarly, PDD-affected individuals fail to exhibit normal synchronic activation in inferior frontal and superior temporal language areas during sentence comprehension (Just, Cherkassky, Keller & Minshew, 2004), in the putamen and thalamus during semantic categorization (Haznedar et al., 2006), and in frontal and parietal systems during performance of executive function tasks (Just et al., 2007; Koshino et al. 2005).

Interestingly, it has been hypothesized (cf. Baron-Cohen, 2004) that reduced functional connectivity among brain regions comprising neural networks might underlie some affected individuals' fixation with and heightened ability to process discrete components of stimuli at the expense of the ability to integrate and wholly process those stimuli (Frith, 1989). Integrated neural processing is almost indisputably reliant on the concerted activity of interconnected brain regions, and therefore, reduced functional connectivity could form the basis of integrated processing impairments in the PDDs.

Reduced activity in discrete neural regions is also repeatedly observed in those with PDDs (cf. Baron-Cohen, 2004). Such reductions have been associated with disordered structural and functional neural connectivity in associated neural systems. For example, disruption in frontal-parietal connectivity has been linked to hypoplasia of associated regions of the corpus callosum (Just et al., 2007). A variety of other discrete structural activity reductions have been noted in PDD-affected brains and correlated with disruptions in associated cognitive functions. For example, activity of the medial

prefrontal cortex has been negatively correlated with scores on clinical measures of social impairment (Kennedy et al., 2006). Similarly, reduced activity of the posterior superior temporal sulcus, which is involved in perception and attribution of others' gaze, is correlated with deficits in joint attention (Pelphrey, Morris & McCarthy, 2005). These findings have not yet been explicitly linked to abnormalities in neural network connectivity. However, it remains possible that such under-connectivity could lay the foundation for hypoplasia and hypoactivity of specific neural regions (cf. Baron-Cohen, 2004).

Functional imaging further indicates that individuals with PDDs demonstrate unusual patterns of neural activation when performing tasks associated with cognitive processes that are impaired in these disorders. Such data suggest that affected individuals recruit different neural regions than do normal controls when performing certain tasks, which might represent an additional, adaptive consequence of disordered neural connectivity (Courchesne & Pierce, 2005b). For example, autistic individuals show right but not left auditory cortex activation during presentation of both verbal and non-verbal sounds (Muller et al., 1999), as well as abnormal activation of the left auditory cortex during performance of language-related tasks. These irregularities might underlie the language impairments and unusual responses to auditory stimulation observed in those with PDDs (Boddaert & Zilbovicius, 2002). In addition, those with PDDs tend to recruit regions immediately adjacent to the left medial prefrontal cortex (PFC) rather than the left medial PFC itself when performing tasks requiring higher-order cognitive functions, such as the attribution of mental states to others (cf. Happe et al., 1996), and many (cf. Baron-Cohen et al., 1999) have reported that those with PDDs recruit frontal regions less

extensively and fail to show normal activation of the amygdala when performing similar "theory of mind" tasks, such as identifying others' emotional expressions.

Indeed, emotional and cognitive processing of others' facial expressions is commonly impaired in those with PDDs and has therefore been the subject of many studies in affected persons. These studies reveal activation of temporal, mesolimbic and cerebellar regions, but not activation of the fusiform face area (FFA), during facial processing (Critchley et al., 2000). Some have hypothesized that disruption of amygdala development might lead to similar development disruptions in other temporal cortical structures that mediate components of social perception, such as the FFA, and others have suggested these ventral temporal structures also subserve elements of social knowledge such as facts about others (Schultz, 2005). Interestingly, hypoactivity of the temporal lobe (cf. Zilbovicius et al. 2000) and reduced or absent activity in the FFA (cf. Baron-Cohen et al., 1999) have been demonstrated repeatedly in those with PDDs.

Further, data from recent studies attempting to link functional anatomical and cognitive behavioral abnormalities in the PDDs have suggested that aberrations in "mirror neurons," frontal, posterior temporal and parietal neurons that fire while participants view others' behavior or infer affective states, might underlie the deficits in empathic understanding demonstrated by those with PDDs (Iacobani, & Dapretto, 2006). Some have proposed that the neural activation shared by actor and observer, which is putatively made possible by these mirror neurons, might form the biological basis of awareness of others' phenomenological experience of self as well as the capacity for empathic experience (Gallese, Eagle & Migone, 2007), both of which are frequently affected in the PDDs.

Cortical minicolumn abnormalities associated with the PDDs might also adversely affect neural microcircuitry, leading to excessive local connectivity and resultant underdevelopment of long distance cerebral connections (Courchesne & Pierce, 2005a). Indeed, a leading theory of the neurobiological basis of the higher-order cognitive aberrations inherent to the PDDs is that frontal lobe function is hampered by excessive and disorganized local circuit connectivity accompanied by weak and poorly synchronized connectivity with other cortical and subcortical regions, the latter resulting in scant integration of information from other neural systems as well as an inability to adequately process and respond to such information (Courchesne & Pierce, 2005b).

Normal minicolumn morphology has been demonstrated in the primary sensory cortices of PDD-affected brains (Casanova et al. 2006), a finding that complements the clinical observation of intact sensory processing in those with PDDs (de Jonge et al. 2007) relative to gross impairments in the complex integration of sensory stimuli thought to be subserved by those neural regions in which minicolumn pathology is consistently observed (Casanova et al. 2006). Such a correlation between neural structural and functional aberrations associated with the PDDs might begin to explain the initially baffling clinical presentation of those with PDDs as individuals who experience extreme difficulty in vital but ultimately well-constrained functional domains (Baron-Cohen, 2004). In addition, the presence of excessively restricted interests and, in relatively rare cases, specialized savant-like abilities in PDD-affected individuals might reflect the increased information processing power that abnormally numerous, heavily overlapping or highly intra-connected minicolumns would likely provide (Courchesne, Redcay, Morgan & Kennedy, 2005; Casanova et al., 2006). Ultimately, heterogeneity of

minicolumn pathology as a function of brain region (e.g. frontal association versus primary visual cortex) is the result of inconsistent disruptions of neural developmental processes and might account for the tapestry of intact and impaired functions that characterizes the behavioral phenotype of the PDDs.

Developmental Neurobiology of the PDDs. The appearance of most features of the PDDs early in development, and the characteristic developmental delays or regressions observed in some PDDs, strongly suggests that these disorders result from abnormal prenatal and postnatal neural development (DiCicco-Bloom et al., 2006). Neural circuitry underlying those functions impaired in the PDDs requires an extensive period of development, which likely explains the fact that PDD-associated impairments do not manifest until such time as this development would approach completion and the related functions emerge, that is, during the second and third years of life (Courchesne et al., 2005).

In addition, given that indications of neural enlargement do not appear in those with PDDs until well after birth, it is plausible that cellular developmental deviations occur disproportionately or exclusively in those regions and systems that develop relatively late in postnatal life, such as prefrontal and sensory association cortices (Courchesne et al., 2005). Further, brain structures subserving functions that are highly related or interdependent and develop at roughly similar periods do not seem to develop around the same time in those with PDDs. This abnormal developmental dissociation might contribute to findings such as the abnormal correlation between frontal and temporal-parietal gray matter in affected individuals (McAlonan et al., 2005). Such developmental temporal and volumetric correlations are putative indicators of

connectivity among involved structures. Therefore, a lack of these normal correlations could reflect a fundamental disruption of synchronic brain development that might precede the aberrant structural connectivity believed to exist in the PDDs (Lainhart, 2006).

The precise nature of the neural maldevelopment that forms the basis of PDDs has yet to be completely characterized. However, several likely developmental scenarios have been posited to explain those neurobiological features most consistently observed in the brains of individuals affected by PDDs.

Increased cortical neuronal density, which is thought to contribute to observed volume increases in affected individuals, has been attributed to a variety of deviations from normal neural development, including enhanced neuron growth due to elevations in prenatal and early postnatal serotonin (Casanova, Buxhoeveden, Switala & Roy, 2002; Scott & Deneris, 2005), compensatory neurogenesis in response to an as yet unknown early developmental challenge, and a deficit or delay in apoptosis (Polleux & Lauder, 2004). The possibility of disordered apoptosis is further supported by reports of increased levels of molecular promoters of apoptosis in the frontal cortex and cerebellum, two regions that demonstrate neuronal density abnormalities in postmortem autistic brains (Argahi-Niknam & Fatemi, 2003). Further, because apoptosis occurs later in postnatal neural development in an attempt to refine synaptic connections, a failure of normal apoptosis might explain reports of decelerated head growth following earlier periods of normal or accelerated growth in some of the PDDs, such as Rett syndrome (Johnston, Mullaney & Blue, 2003).

In addition, vasculogenesis is developmentally coordinated with neurogenesis, such that developmental vascularization is thought to be regulated by the metabolic requirements of emerging neuronal populations (Louissaint, Rao, Leventhal & Goldman, 2002; Palmer, Willhoite & Gage, 2000). Therefore, neural cellular abnormalities in the PDDs might be associated with or preceded by disordered vascular development. This concept is supported by studies indicating brain hypoperfusion in affected individuals (e.g. Boddaert & Zilbovicius, 2002; Wilcox et al., 2002), an association between prenatal thalidomide exposure (a proposed pathogenic factor in the PDDs) to inhibition of developmental angiogenesis (Hallene et al., 2006), and a link between vascular malformations and mutations of the *HOXA1* gene, which are known to be associated with PDD development (Tischfield et al., 2005). Similarly, intracranial developmental venous anomalies have been identified in those with conditional phosphatase and tensin homologue on chromosome ten (*Pten*) gene mutations, many of whom also possess a PDD behavioral phenotype (Tan et al., 2007). In addition, the PDDs have been associated with enhanced oxidative stress, abnormal platelet activation, and vasoconstriction, which would result in blood flow abnormalities (Yao, Walsh, McGinnis & Pratico, 2006). Despite suggestive evidence that vascular developmental anomalies are associated with the PDDs, a complete understanding of the vascular phenotype of the PDDs has yet to be reached. Of note, however, is that HB-GAM is a mediator of developmental angiogenesis (Christman et al., 2005; Yeh et al., 1998).

Regardless of the etiological mechanism, early vascular deficits would likely result in, among other consequences, disrupted neural cell migration. Such disruptions might also explain the presence of unusually narrow and overly numerous minicolumns

in the brains of those with PDDs. Indeed, postmortem examination of autistic brains has indicated the presence of neuronal migration deficits (Bailey et al., 1998), which have also been observed in mice null for N-syndecan, the receptor for HB-GAM. These animals have a disordered laminar structure of the cerebral cortex secondary to impaired radial migration as well as an impairment of the rostral migratory stream (Hienola, Tumova, Kulesskiy & Rauvala, 2006). In humans with PDDs, neural migration deficits could conceivably alter the vertical integrative features that define cortical minicolumns, resulting in disproportionate neuron number and density as well as a disordered ratio of excitatory pyramidal neurons to inhibitory glia among minicolumns (Courchesne et al., 2005).

Immunocytochemistry performed on PDD-affected brains has revealed marked microglial and astroglial activation with expected increases in neuroglial-derived cytokines. These observations were made in frontal cortex and subcortical white matter but were most striking in the cerebellum, where pronounced glial activation was associated with both neuronal and axonal degeneration (Vargas, Nascimbeme, Krishnan, Zimmerman & Pardo, 2005). Complementary studies have also revealed a pro-inflammatory profile of cytokines in cerebrospinal fluid obtained from PDD-affected individuals without the presence of reactive immunological factors, suggesting that neuroinflammation might be a primary, etiological process in the PDDs (Pardo, Vargas & Zimmerman, 2005).

The role of neuroinflammatory processes such as enhanced glial activation in the development of PDD-associated developmental abnormalities has not yet been fully elucidated. Abnormally increased glial activation could contribute to the white matter

volume increases observed in the brains of those affected by PDDs (Pardo et al., 2005). Further, the pinnacle of glial activation in later postnatal development could promote the period of decelerated or arrested brain growth seen following early postnatal overgrowth in those with PDDs (Courchesne et al., 2005).

The relation between heightened neuroinflammation and increases in cortical neuron number is even less clear. Compensatory processes associated with neuroinflammation might interrupt normal apoptosis of cortical neurons later in development and thus lead to abnormal neuronal increases or, alternatively, neuroinflammation itself might drive increases in both cortical neurons and glia (Vargas et al., 2005).

Primary abnormalities in glia might also precipitate the developmental deviations and resultant neurobiological abnormalities observed in the PDDs. Disruptions in glial processes would likely result in abnormalities in apoptosis, synaptogenesis, and modulation of synaptic connectivity (Ullian, Christopherson & Barres, 2004). Further, irregularities in radial glia would adversely affect neural migration, which is vital to the structural and functional development of cortical minicolumns (Colombo & Reisin, 2004). Though the pathological developmental processes that lead to PDD development are as yet unknown, there exist several plausible candidates that await further examination.

Neurochemistry of the PDDs. Data regarding the neurochemical aspects of PDDs are scant and often inconsistent. Consonant with the established link between abnormalities in the serotonin transporter gene and development of PDDs, several studies have indicated serotonergic abnormalities in those with PDDs, including elevations in

whole blood serotonin (WBS) that decline with increasing age (McDougle, Erickson, Stigler & Posey, 2005) and increased 5-HT production and asymmetrical 5-HT synthesis in affected children (Chugani et al., 1999). Interestingly, autistic children exhibiting decreased 5-HT synthesis in the left hemisphere reportedly have greater language impairments than do those exhibiting either symmetrical or right-biased, asymmetrical 5-HT synthesis (Chandana et al., 2005).

No significant evidence exists for monoaminergic dysfunction in those with PDDs, though some have reported increased metabolites of dopamine in the cerebrospinal fluid, and increased dopamine accumulation in the PFC, of affected individuals. Similarly, most studies have failed to uncover significant noradrenergic abnormalities associated with these disorders (for review, see McDougal et al., 2005).

Elevated peripheral levels of glutamate in those with PDDs have been frequently but not consistently reported (e.g. Aldred, Moore, Fitzgerald & Waring, 2003), and the glutamate receptor 6 (GluR6) gene has been identified as a candidate susceptibility gene for these disorders (Jamain et al., 2002). $GABA_A$ receptor subunit genes (Cook et al., 1998) have been similarly cast, yet there remain no consistent data on levels of GABA in affected persons (Lam, Aman & Arnold, 2006; McDougal et al., 2005).

The neuropeptides oxytocin and vasopressin play major roles in the affiliative and social behavior commonly affected in the PDDs (Domes, Heinrichs, Michel, Berger & Herpertz, 2007; Insel, 1997; Insel, O'Brien & Leckman, 1999; Young, Lim, Gingrich & Insel, 2001) and have therefore garnered attention as candidate transmitters involved in these disorders. Indeed, autism has been associated with oxytocin receptor gene polymorphisms (Jacob, Brune, Carter, Leventhal, Lord & Cook, 2007), and several

studies have revealed abnormally low plasma levels of oxytocin in those with PDDs (e.g. Green et al., 2001; Modahl et al., 1998). Further, oxytocin activity in rodents is inhibited by the opioid beta-endorphin (Ortiz-Miranda, Dayanithi, Custer, Treistman & Lemos, 2005), which is reportedly elevated in those with PDDs (for review, see Tordjman et al., 1997). No evidence of abnormalities in vasopressin has been identified in affected individuals (McDougal, Erickson & Posey, 2005).

Among neurotrophic factors, brain-derived neurotrophic factor (BDNF) has been a focus of study in the PDDs because of its importance for normal neuronal development (Polleux & Lauder, 2004). Elevations of BDNF have been found in both serum and brain tissue of PDD-affected individuals (Tsai, 2005). Such elevation might result in early acceleration of brain development, cortical neuronal hyperplasia and seizure disorder, all of which have been observed in those with PDDs. In addition, BDNF is a potent up-regulator of serotonin, elevated levels of which have been shown in PDD affected individuals (Sodhi & Sanders-Bush, 2004).

Treatment of PDDs. Given the as yet incomplete understanding of the pathogenesis of PDDs, interventions for these disorders have been targeted not at addressing underlying causative factors but at reducing symptoms and managing behavioral abnormalities.

A wide variety of pharmacological interventions have been tried in individuals with PDDs, only a few of which have proven beneficial in reducing a subset of associated behavioral disturbances. Psycho-stimulants and opioid antagonists have yielded little benefit in the treatment of the PDDs (Findling, 2005). Adrenergic agonists provide some improvement in hyperactivity, inattention and agitation in affected persons but often

produce marked sedative effects (Fankhauser, Karumanchi, German, Yates & Karumanchi, 1992). Tricyclic anti-depressants are efficacious in treating compulsive and ritualistic behavior but have had mixed results in treating individuals' aggressive and hyperactive symptoms (Findling, 2005). Selective serotonin reuptake inhibitors (SSRIs) yield some reduction in individuals' compulsive, repetitive, and ritualistic behavior but have less reliable effects on aggression and hyperactivity in those with PDDs (Kolevzon, Mathewson & Hollander, 2006). Mood stabilizers and typical anti-psychotics have been used to address symptoms of emotional instability, aggression, impulsivity and hyperactivity in the PDDs (Findling, 2005), but these often produce undesirable side effects (Gillberg, 2000). Atypical anti-psychotics, the most efficacious of which is risperidone, a dopamine D_2 receptor antagonist, has proven particularly useful as a pharmacological treatment for the PDDs given its effectiveness in treating aggression, self-injury, repetitive motor behavior, and hyperactivity as well as depressive and anxious symptoms (West & Waldrop, 2006).

Many have proposed alternative treatments for PDDs, such as vitamin supplements and special diets. For example, some have claimed improvement in behavioral symptoms of PDD-affected children treated with vitamin B_6 in combination with magnesium (Aman, 2005) and similar improvement in those treated with gluten- and casein-free diets (for review, see Garvey, 2002; White, 2003). However, empirical research into these approaches presents methodological issues and conflicting results that cast doubt on their efficacy in managing PDD symptoms (Aman, 2005).

Non-pharmacological approaches to managing PDD-associated behavioral abnormalities have also been employed, such as applied behavior analysis (ABA), which

seeks to manipulate environmental contingencies to affect the occurrence of specific behaviors (Ogletree & Oren, 2001) and generalize desirable behavior to a variety of settings (Hillman, 2006). ABA has been shown to yield significant, objectively measured gains in intellectual and adaptive functioning, expressive language ability and social skills (e.g. Lovaas, 1987; Sallows & Graupner, 2005). However, many studies (e.g. Sherer & Schreibman, 2005) indicate that affected individuals with low levels of intellectual and language functioning do not respond as well to ABA interventions.

Current treatments for PDDs aim to reduce associated symptoms but cannot address underlying causal factors. These factors appear to be largely genetic, suggesting that treatments aimed at allaying causative factors and not merely managing symptoms should be targeted at addressing genetic abnormalities. However, the manipulation of suspected susceptibility genes and experimentation with potential genetic treatments for these disorders in human participants using true experimental methodology would be practically and ethically prohibitive. Therefore, experimental research using animal models of PDDs provides a feasible means by which to investigate precipitating factors in the development of these disorders as well as to formulate more effective treatments.

Animal Models of the PDDs. Animals with experimentally induced lesions of neural areas known to be abnormal in the PDDs constituted the earliest potential animal models of these disorders (Andres, 2002). Not surprisingly, these animals often manifested some of the aberrant behaviors observed in affected individuals. For example, bilateral temporal lobe ablation in newborn rhesus monkeys resulted in abnormal social interaction, changes in facial and body expression, and the emergence of repetitive behavior (Bachevalier, 1996), effects that have also been induced by lesioning the lateral

and central amygdala in early postnatal rats (Wolterink et al., 2001). Although lesion studies can reproduce some behavioral abnormalities associated with the PDDs, these investigations cannot experimentally induce the microscopic neuroanatomical abnormalities observed in affected individuals, nor can they address the role of possible causal factors in the development of PDDs.

Therefore, other attempts to model these disorders in animals have involved exposing prenatal subjects to the same immunological or teratological challenges as those hypothesized to be pathogenic in affected individuals (Murcia, Gulden & Herrup, 2005). For example, infection of neonatal rats with the Borna disease virus (BDV), a neurotrophic virus thought to play a role in some cases of autism (Libbey, Sweeten, McMahon & Fujinami, 2005), results in cerebellar hypoplasia as well as reduced and abnormal social interaction in a number of independent studies (for review, see Lancaster, Dietz, Moran & Pletnikov, 2007). Similarly, pre- or post-natal exposure to thalidomide and valproic acid (VPA) in rodents has been used in attempts to replicate phenotypic features of PDDs, as exposure to these teratogens *in utero* at the time of neural tube closure has been linked to development of these disorders (Ardnt, Stodgell, & Rodier, 2004). Prenatal rodents treated with thalidomide have demonstrated hyperserotonemia (Narita et al., 2002) and altered distribution of serotonergic neurons, suggesting an abnormality in early serotonergic neuron differentiation and migration that some have proposed to be pathogenic in the PDDs (Miyazaki, Narita & Narita, 2005). Prenatal exposure to VPA in rodents has also been associated with disrupted serotonin synthesis (Ardnt et al., 2004) and elevated levels of serotonin in frontal cortex as well as with cerebellar hypoplasia, developmental delays, disturbances of circadian rythym,

aberrant sensitivity to sensory stimuli, ritualistic behavior and hyperactivity in novel environments, all of which resemble symptoms of the PDDs (e.g. Tsujino et al., 2007; Wagner, Reuhl, Cheh, McRae & Halladay, 2006).

Many others have reported PDD-like features in animals following experimental perturbations of a variety of endogenous biochemicals. Induced neonatal hypothyroidism in rats has been associated with motor hyperactivity, attenuated habitation to novel environments, and an inability to adapt to environmental changes as evidenced by inferior performance in a maze task following modification of the maze's configuration (Sadamatsu, Kanai, Xu, Liu & Kato, 2006). Neonatal blockade of gastrin-releasing peptide, which has been implicated in the formation and extinction of emotional memory as well as in the pathogenesis of neurodevelopmental disorders, reportedly induces reduced social interaction and impaired inhibitory avoidance and novel object recognition in rats (Presti-Torres et al., 2007). Further, newborn rats treated with terbutaline, a beta2-adrenoreceptor agonist associated with increased concordance for autism in dizygotic twins, demonstrate marked microglial activation and hyper-reactivity to novel environments and aversive stimuli (Zerrate et al., 2007). Similarly, reactive gliosis was observed in rodents treated with intracerebroventricular infusions of propionic acid, a short chain fatty acid known to regulate cellular metabolism, along with hyperactivity and repetitive, abnormal motor movements (MacFabe et al., 2007). Despite achieving the development of PDD-like features in rodents, studies of this nature utilize infectious and chemical agents that typically affect the entire central nervous system, making the role of such agents in the development of aberrant behavior difficult to ascertain.

Other studies using chemical perturbations to induce PDD-like characteristics in animals have done so in specific brain regions and, therefore, better elucidate the anatomical and chemical interactions that might lay the foundation for specific symptoms of the PDDs. For example, experimental depletion of forebrain serotonin similar to that observed in autistic individuals has been linked to impulsive, aggressive, repetitive, and socially inappropriate behavior, thus yielding another possible mouse model of autism (Boylan, Blue & Hohmann, 2007). In addition, application of a GABA agonist to the substantia nigra pars reticulata in rats has been shown to induce marked contraversive circling, a putative correlate of the repetitive and stereotyped behavior observed in humans with PDDs, via disinhibition of midbrain afferents to the striatum (Velisek, Veliskova, Ravizza, Giogi & Moshe, 2005).

Yet, perhaps the most compelling possible animal models of the PDDs are genetic knockouts, which allow the association of genotype and phenotype and, therefore, provide a viable means of modeling these largely genetically determined disorders. Such models have arisen both from studies that delete candidate genes for PDD susceptibility in order to examine associated PDD-like features and those that have inadvertently discovered PDD-like phenotypes in genetically altered animals.

Some such animals display neurobiological abnormalities similar to those observed in humans with PDDs. *Hoxa1* knockout mice have significant hypoplasia or complete absence of brainstem structures such as the superior olive, and similar aberrations have been identified in those with PDDs (Rodier, Bryson & Welch, 1997). *Hoxa1* has been implicated as a regulator of early human head growth (Muscarella et al., 2007) and is up regulated by the action of valproic acid (Stodgell et al., 2006), but genetic

linkage studies have yielded conflicting results regarding the association between *Hoxa1* and development of PDDs.

Conversely, Engrailed-2 (*En2*), a gene responsible for pattern formation in the midbrain and hindbrain, has been consistently linked with PDD development (Murcia et al., 2005). *En2* knockout mice exhibit a hypoplasia of the cerebellar vermis similar to those with PDDs that might be attributable to a failure of normal structural and organizational patterning (for review, see Kuemerle, Gulden, Cherosky, Williams & Herrup, 2007). Further, reduction in specific populations of cerebellar Purkinje cells in these animals might result in reduced inhibition of deep cerebellar nuclei and resultant over-excitation of cerebellar efferents, including the thalamus and neocortex, abnormalities of which have been frequently implicated in the PDDs. Ultimately, these animals possess neurobiological abnormalities similar to those observed in the PDDs but have either failed to demonstrate similarly abnormal behavior or are still awaiting detailed behavioral assessment. Therefore, though such animals might prove enlightening regarding the etiology and treatment of PDD-associated neurological aberrations, they cannot be considered complete models of these disorders.

A preponderance of proposed animal models of the PDDs demonstrate behavioral peculiarities reminiscent of PDD symptoms. However, many are limited to emulating social impairments with conceptual analogy to those observed in these disorders. For example, mice deficient for *Dvl1*, one of three mouse homologues of the Drosophila segment polarity gene *dishevelled*, demonstrate deficits in nest building, reduced cuddling with cage mates, and subordinate responses upon social dominance testing. Deficits in prepulse inhibition of acoustic and tactile startle were also evidenced in these

animals (Lijam et al., 1997). Abnormal social interaction was also reported in mice null for the vasopressin V1a receptor gene, which has been associated with development of autism in humans (Egashira et al., 2007). Similarly, Brattleboro rats, which possess vasopressin deficiencies secondary to mutations in other vasopressin-regulating genes, demonstrate reduced social memory (Engelmann & Landgraf, 1994). Oxytocin knockout mice also demonstrate social memory impairments independent of general cognitive or olfactory compromise (Ferguson et al., 2000) in addition to reduced vocalization during periods of separation from their mothers and peers (Winslow et al., 2000). Finally, *Pten* knockouts demonstrate reductions in social interaction, social preference, social learning, sexual behavior and maternal care (Greer & Wynshaw-Boris, 2006). However, data obtained from *Pten* knockouts should be interpreted cautiously given that conditional knockouts involve deactivation of target genes postnatally to avoid lethality that would result from gene deactivation earlier in development. Further, *Pten* deletion in these animals was restricted to post-mitotic neurons, likely yielding a condition more analogous to a neurodegenerative rather than a neurodevelopmental disorder.

Interestingly, though, the *Pten* knockout studies are distinct from their aforementioned counterparts in that the proposed animal model of the PDDs demonstrates multiple behavioral and neurobiological characteristics of affected individuals. Indeed, *Pten* mutant mice also show exaggerated reactions to sensory stimuli, heightened anxiety in most relevant paradigms, decreased learning, seizures, and frontal macrocephaly (Kwon et al., 2006). Interestingly, macrocephaly is a key feature of the PDDs that has been linked to *Pten* mutations in a subset of autistic individuals (Herman et al., 2007).

A similarly broad, abnormal phenotype has been ascribed to mice null for other genes and their products. Though the behavioral phenotype of *Fmr1* knockout mice is somewhat inconsistent across studies, the majority of data indicate that these animals show decreased preference for social novelty, inappropriate social interaction, deficits in reversal learning perhaps secondary to cognitive rigidity, deficits in fear conditioning possibly due to amgydala abnormalities, and seizures (for review, see Bernardet & Crusio, 2006). Mutation of the *Fmr1* gene in humans underlies the development of Fragile X syndrome, which is the most common cause of inherited cognitive impairment and frequently associated with mental retardation and PDD-like features (Hagerman, Ono & Hagerman, 2005).

Animals deficient in calcium-dependent activator protein for secretin 2 (CADPS2), a protein that regulates exocytosis of dense-core vesicles at the ATP-dependent binding step, also demonstrate multiple PDD-like behaviors, including abnormal sleep-wake cycles indicative of circadian rhythm disruption, reduced social interaction, and poor maternal care of offspring. These animals also display home cage hyperactivity but decreased locomotor activity in paradigms such as the open field and radial arm maze, which suggests increased anxiety secondary to the novel nature of these behavioral paradigms. Indeed, CADPS2 knockouts also demonstrate reduced exploration of a novel object in the open field and possess a decreased number of cerebellar Purkinje cells, which might be due to reduced BDNF release secondary to impaired translocation of CADPS2 to the axon terminal. Interestingly, *CADPS2* mRNA is abnormally spliced, resulting in a loss of exon 3, in some autistic individuals (Sadakata et al., 2007).

Mice with mutations in the methyl-CpG binding protein 2 (MECP2) gene, abnormalities of which are responsible for the majority of cases of Rett's disorder, exhibit repetitive forelimb movements reminiscent of the stereotyped hand movements that help to characterize the disorder (Moretti, Bouwknecht, Teague, Paylor & Zoghbi, 2005). These animals also show home cage hyperactivity, reduced social interaction, and impaired social memory. In addition, MECP2 knockouts also possess deficits in spatial learning and fear conditioning, suggesting disruption in the function of both the hippocampus and amygdala perhaps secondary to abnormal long-term potentiation. Interestingly, these learning deficits have been likened to the cognitive impairments observed in Rett-affected individuals, who are frequently mentally retarded (Moretti et al., 2006).

Ultimately, the study of potential animal models of PDDs is directed toward an understanding of the etiology of these disorders and, consequently, the development of effective treatments. Interestingly, a few studies have demonstrated reduction or elimination of PDD-like abnormalities in proposed animal models. For example, induced postnatal ectopic expression of BDNF in animals deficient in MECP2 has been shown to delay onset and slow progression of phenotypic behavioral abnormalities as well as improve motoric function and increase spontaneous firing of cortical layer V pyramidal neurons. These data suggest a value for manipulation of BDNF signaling in the treatment of Rett's disorder (Chang, Khare, Dani, Nelson & Jaenisch, 2006). Further, gradual activation of the *MECP2* gene in knockout mice has been consistently associated with increased lifespan and negation of their phenotypic abnormalities, such as gait

disturbance, stereotyped limb clasping, and reduced LTP, suggesting that not all PDD-like symptoms are irreversible (cf. Guy, Gan, Selfridge, Cobb & Bird, 2007).

Non-pharmacological interventions have also demonstrated efficacy in reducing phenotypic abnormalities in knockout animals. For example, exposure to enriched environments has been associated with reversal of the abnormal social interaction and heightened anxiety observed in rats treated prenatally with valproic acid (Schneider, Turczak & Przewlocki, 2006). The proposed mechanism for this reversal is the cognitive engagement provided by enriched environments. Such environments promote enhanced neuro- and synapto-genesis and provide increased opportunities for animals to learn associations between responses and environmental consequences. Indeed, exposure to cognitive enrichment and secondary learning of response-consequence contingencies are cardinal features of behavioral therapeutic approaches designed to address PDD symptoms (Lovaas, 1987).

Studies demonstrating amelioration of neurobiological and behavioral abnormalities in proposed animal models of the PDDs suggest a potentially beneficial effect of similar treatments in PDD-affected individuals. Perhaps as importantly, such findings bolster the predictive validity of these animal models by mirroring not only PDD symptom presentation but also treatment response.

Despite the *prima facie* evidence that animal models can provide insight into the etiology and treatment of PDDs, the validity of these models has been a matter of protracted debate for a number of reasons. These include the difficulty in identifying direct relationships between genotype and phenotype (cf. Routtenberg, 1995), skepticism about non-human species' ability to model complex cognitive and behavioral processes

or disruptions of these (cf. Hau & Van Hoosier, 2004), the challenges inherent to identifying animal cognitive processes and behavior with conceptual analogy to humans', and the questionable efficacy of some existing paradigms designed to assess cognitive and behavioral abnormalities in rodents (cf. Crawley, 2000, 2004). Still others assert that the PDDs' apparently polygenetic and multi-factorial etiology will continue to elude characterization because of its complexity, which cannot be reliably replicated or manipulated in animals (cf. Watase & Zoghbi, 2003).

Indeed, the genetic complexity thought to underlie the PDDs likely precludes the prospect of complete phenotypic recapitulation in any single genetically altered animal (Moy et al., 2007). Therefore, many (e.g. Moy et al., 2007; Tordjman et al., 2007) have suggested that existing potential animal models of PDDs be re-conceptualized as models of endophenotypes of these disorders just as those attempting to model other neurobehavioral disorders, such as schizophrenia, have acknowledged that no single mutant animal can be expected to fully model a complex human neurobehavioral disorder (Boksa, 2006). That is, there is no such thing as an "autistic mouse," but merely animals with similar albeit incomplete PDD-like biological and behavioral profiles, animals that can, however, prove invaluable to the study of the etiology, nature and treatment of specific features of these disorders.

Ultimately, a review of the literature reveals a plethora of potential animal models of the PDDs. Some of these models demonstrate a limited number of PDD-like behavioral features and fewer possess multiple neurobiological and behavioral traits similar to those exhibited by affected individuals, but an animal has yet to be generated or discovered that manifests the full range of abnormalities that define the PDDs. However,

perhaps examination of each proposed animal model of PDDs followed by comprehensive synthesis of findings across studies would allow researchers to put together the proverbial puzzle of PDDs, as Arguello and Gogos (2006) have written, “one piece at a time.”

The Current Studies. HB-GAM knockouts have previously demonstrated cognitive rigidity, increased anxiety, and motor impairment (Croll, unpublished data; Pavlov et al., 2002), all of which are included in the constellation of PDD symptoms. Further, these animals exhibit cortical neuronal hyperplasia (Hienola et al., 2002, 2004), which is a commonly observed neurobiological feature of these disorders. Therefore, HB-GAM knockouts might manifest a PDD-like phenotype and could, therefore, represent such a piece of the puzzle.

The current studies sought to further delineate the phenotypic characteristics of HB-GAM knockout mice. This was achieved via comprehensive behavioral assessment, with an emphasis on those features known to be abnormal in the PDDs but capable of quantification in mice, specifically, capacity for perseveration, anxiety and behavioral reticence, and social behavior. Most of the behavioral paradigms used have been established as valid for the assessment of PDD-like behavior in animals (for review, see Crawley, 2000, 2004, 2008). Further, because the PDD phenotype appears neurobiological as well as behavioral, examination of these animals’ cerebral cortical microstructure was also conducted. Finally, assessment of HB-GAM knockouts’ cerebral cortical vasculature was performed to determine whether vascular abnormalities exist in these animals given that vasculogenesis is developmentally coordinated with

neurogenesis (cf. Louissaint et al., 2002; Palmer et al., 2000), a process shown to be abnormal in the PDDs.

CHAPTER 2. GENERAL METHODS

Animals. Mice (a 50/50 mix of the 129 and C57B1/6 inbred strains) null for HB-GAM were used from a line created as previously described (Amet et al., 2001). Animals were housed either singly or in small groups, never exceeding five animals per cage, and provided food and water *ad libitum*. The colony room was maintained at 23 degrees Celsius on a 12 hour light-dark cycle (lights off at 19:00). All procedures were conducted with approval from and in strict compliance with the animal welfare policies of the Institutional Animal Care and Use Committee of Queens College of the City University of New York.

The current studies used a total of 24 HB-GAM knockouts and 21 wild type mice balanced for gender and tested in three cohorts. Animals were approximately four to five months of age during behavioral testing and approximately six to seven months of age at the time of sacrifice for neuroanatomical analyses. On occasion, individual animals were excluded from study in the event of either apparent illness or injury due to recent skirmishes with cage mates. Animals were allowed 60 minutes to acclimate to the testing room in their home cage prior to all behavioral testing. All behavior was observed and recorded live by at least two experimenters. Behavioral testing and neuroanatomical analyses were conducted by at least one experimenter blind to animal genotype. The behavioral testing paradigms used have been validated as measures of PDD-like behavior in rodents (for review, see Crawley, 2000, 2004, 2008 unless otherwise referenced).

Data Analysis. Statistical analyses were conducted using the Statistical Package for the Social Sciences (SPSS) software version 11.5 (SPSS Inc., Chicago, Illinois). Most behavioral tests were conducted in at least two cohorts; in these instances, the data

reported are for combined cohorts. Analyses involving two levels of a single factor (e.g. genotype) and one dependent measure were analyzed using t-tests. Analyses involving more than two levels of a factor were conducted using analysis of variance (ANOVA), one-way or factorial where appropriate, followed by *post hoc* Tukey tests to probe significant effects. Multivariate analysis of variance (MANOVA) was conducted when analyzing multiple dependent variables across paradigms that assess a common, conceptually related variable. In addition, because of the gender imbalance in PDD prevalence, factorial ANOVAs assessing the effect of gender were conducted across paradigms, however, when gender did not interact with genotype, results reflect data collapsed across the levels of gender. Chi square analysis was used to determine the independence, or lack thereof, of categorical variables. Pearson's correlation coefficients were calculated to assess the association between variables, and multiple regression was used to determine variables' strength as predictors of a criterion variable, where appropriate. A $p < .05$ was considered statistically significant for all analyses. Statistical outliers, defined as animals whose measures fell two or more standard deviations above or below the mean, were removed from all analyses.

Figures. Data in figures are presented as means and standard error of the means. Asterisks (*****) represent results that are statistically significant at $p < .05$.

CHAPTER 3. SPECIFIC AIM 1

Specific Aim 1 **was to evaluate the presence of perseverative tendencies in HB-GAM knockouts.**

The chief criterion for a valid animal model of the PDDs is expression of a behavioral and cognitive phenotype paralleling that observed in affected individuals. A common component of the PDDs is repetitive behavior (Bolivar et al., 2007), which has been conceptualized and statistically validated as sorting into two clusters: "lower-order" repetitive behavior, such as stereotyped movements, and "higher-order" repetitive behavior, such as rituals, circumscribed interests, and a strict adherence to routine (Cuccaro et al., 2003).

Studies attempting to characterize PDD-like behavioral phenotypes in knockout mice have analyzed animal behavior that is arguably conceptually analogous to repetitive human behavior. For example, mice null for the $GABA_A$ receptor gene *gabrb3* undergo protracted periods of intense circling, and *Hoxb8* gene knockout mice demonstrate excessive grooming to the point of self-injury (Lewis et al., 2007). Such rodent behaviors indeed mirror some of the repetitive motor behaviors observed in those with PDDs.

The presence of PDD-like "higher-order" repetitive behavior, such as adherence to strict routines resulting from an "anxiously obsessive desire for sameness" (Kanner, 1943), has been investigated in potential animal models of the PDDs via assessment of animals' cognitive flexibility. For example, in addition to motor hyperactivity in novel behavioral paradigms, thyroid hormone-deficient mice demonstrate an inability to adapt to environmental changes as evidenced by inferior performance in a maze task following modification of the maze's configuration (Sadamatsu et al., 2006). Similarly, mice null

for *Fmr1*, a gene linked to the development of the PDD-like Fragile X syndrome in humans (Hagerman et al., 2005), demonstrate deficits in water maze reversal learning perhaps secondary to cognitive inflexibility (for review, see Bernardet & Crusio, 2006).

Preliminary data (Croll, unpublished data) suggested that HB-GAM knockout mice demonstrate a similar inability to adapt to changing contingencies. This was evidenced by inferior performance relative to wild type mice in the second but not the first version of the Morris water maze in which they were tested. Further, the knockouts' inferior performance in the second version of the maze was independent of the type of water maze, i.e. spatial or cued, in which they were tested first.

Given the precedents for assessing repetitive behavior, both motoric and cognitive, in rodents, another cohort of HB-GAM knockouts was tested in two versions of the Morris water maze (spatial and cued) in two separate sequences (spatial then cued and cued then spatial) to further investigate the presence of cognitive inflexibility in these animals. In addition, HB-GAM knockouts' capacity for flexible behavior was assessed via use of the Y-maze and hole board paradigms, both of which confer the potential for flexibility and diversity (e.g. alternation of movement and exploration, respectively) in animals' behavior. In addition, to assess the presence of repetitive or hyperactive motor behavior in HB-GAM knockouts, animals' motor behavior, specifically, frequency of grooming behavior and locomotor activity was examined in some behavioral paradigms.

Specific Methods

Morris Water Maze. In a room measuring 1.38 x 2.13 m with various spatial cues kept constant throughout testing, a pool of water measuring 105 cm in diameter and 35 cm in depth was made opaque with non-toxic paint and divided conceptually into four

quadrants, one of which contained an escape platform hidden 2.5 cm beneath the water's surface. Animals were placed in a different, pseudo-randomly selected quadrant at the start of each trial. Animals' latency to escape the maze via discovery of the hidden platform was measured in three trials of one minute per day (a "trial block") over four, five or eight days. Any animal not locating the platform within one minute was assigned a latency to escape of 60 seconds. Normal animals are expected to demonstrate a linear decrease in latency to escape across trials in the water maze, indicating acquisition of the location of the platform over time.

Twenty-four hours after the final day of water maze acquisition testing, animals were returned to the maze for 30 seconds with the platform removed for a test of their retention of the platform's location. In the spatial version of the maze, retention was assessed via a measure of the amount of time animals spent in the quadrant that formerly housed the platform, termed the "goal quadrant." In the cued version of the maze, retention was assessed via a measure of the amount of time animals spent in the quadrant that housed the platform on the last day of acquisition testing, again termed the "goal quadrant." Normal animals are expected to spend more time in the "goal quadrant" than in other quadrants, thus demonstrating retention of the location of the escape platform.

Animals' median latency to escape the maze per trial was averaged to produce a mean latency to escape for each trial block. Animals' swim speed was estimated by dividing latency to escape the maze by the number of maze quadrants crossed per trial. Animals' median swim speed per trial was averaged to produce a mean estimated swim speed for each trial block.

In the *spatial version* of the maze, the location of the hidden escape platform remained constant across trials, forcing animals to rely on extramaze spatial cues to locate the platform. In the *cued version*, the location of the hidden escape platform was varied between days. However, a striped but otherwise clear acetate measuring 30 x 10 cm was affixed to the inside rim of the maze parallel to the escape platform to serve as a distinctive visual cue, compelling animals to learn the location of the hidden platform via association with this cue.

In the first sequence of water maze testing, animals were tested first in the spatial version of the maze and then in the cued version two weeks later. In the second sequence of water maze testing, which occurred 8 weeks after the first sequence, animals were first tested in the cued version of the maze and then in the spatial version two weeks later. Variation in the number of trial blocks both within and between sequences is owed to the fact that animals traditionally learn the cued version of the maze, as well as the first version of the maze to which they are exposed, better than they do the spatial version or the second version to which they are exposed.

Y-maze. This apparatus was composed of an enclosed, black runway arm measuring 30 x 9 x 10 cm, at the end of which were two identical arms, also painted black and measuring 30 x 9 x 10 cm. Animals' home cages were placed beyond the maze to serve as a motivator for animals to traverse the maze. Animals were placed in the runway and given the opportunity to navigate the maze until they traversed at least half the length of one of the maze's other two arms. Once an animal reached such a point, it was removed from the apparatus and then returned to the runway arm after a three second rest. The procedure was repeated over 11 trials for each animal. For each trial, the

animal's choice of arm (left or right, as defined by the animal traversing at least half the length of the arm) was recorded. Normal animals are expected to spontaneously alternate their choice of arm in an attempt to fully explore the apparatus. Animals' time to make an arm choice was also recorded. The proportion of identical, consecutive arm choices (perseverative arm choices) relative to total number of arm choices made was also recorded. No time limit was imposed, but an animal's testing was discontinued if it required more than five minutes to make a choice in two consecutive trials. Animals yielding data for fewer than four consecutive trials were excluded from data analysis.

Hole Board. Animals were placed in a white, box-like apparatus measuring 43.2 cm x 43.2 cm enclosed by walls 25.4 cm high. Each quadrant of the floor of this apparatus contained a circular hole 2.5 cm in diameter, for a total of four holes. Animals were allowed to freely explore the apparatus for five minutes, during which behavioral measures were taken. The number of head dips into any of the four holes made by each animal, and the proportion of consecutive head dips into the same hole (perseverative head dips) relative to total number of head dips, were recorded. A head dip was defined as an animal's head surpassing the depth of the rim of a hole, and is traditionally considered an exploratory act. In an attempt to further quantify perseverative head dipping behavior, animals' "favored hole," that hole into which each animal dipped its head most frequently, was identified and the proportion of each animal's number of head dips into that favored hole relative to its total number of dips was recorded.

Motor Behavior. Animals' capacity for repetitive motor behavior, operationally defined as frequency of face and/or body grooming, was measured, as was animals'

capacity for hyperactive motor behavior, as measured by estimated swim speed in the Morris water maze and the number of grids crossed in the open field.

Results

Morris Water Maze: First Sequence. In the first sequence of Morris water maze testing, animals were tested in the spatial version of the maze over 8 days and then, 2 weeks later, tested in the cued version of the maze over 4 days.

Spatial Version. HB-GAM knockouts and wild type mice demonstrated a significant decrease in latency to escape across trials (F (25, 175) = 17.51, p = .000), reflecting that animals of both genotypes successfully learned the location of the escape platform. The genotypes did not differ significantly in mean latency to escape across trials (F (1, 18) = 2.97, p = .998), and no significant genotype by trial interaction was found (F (25, 175) = .670, p = .697, Figure 1A), suggesting that animals of both genotypes learned the location of the escape platform at a similar rate. In the retention trial, HB-GAM knockouts and wild type animals did not differ significantly in time spent in the maze quadrant that previously housed the escape platform (t (24) = .464, p = .647, Figure 2A), suggesting similar delayed memory for the location of the escape platform between the genotypes.

Cued Version. HB-GAM knockouts and wild type mice demonstrated a significant decrease in latency to escape across trials (F (25, 175) = 8.45, p = .000), indicating that animals of both genotypes gradually learned the association between the visual cue and the location of the escape platform. However, HB-GAM knockouts demonstrated a significantly greater mean latency to escape across trials as compared to wild type mice (F (1, 18) = 4.81, p = .041), suggesting that the knockouts demonstrated

delayed acquisition of the association between the cue and the platform location. No significant genotype by trial interaction was found (F (25, 175) = 1.30, p = .254, Figure 1B), indicating that HB-GAM knockouts demonstrated overall reduced acquisition of the association between the cue and platform location relative to wild type mice, but rates of learning were similar between the genotypes. In the retention trial, HB-GAM knockouts and wild type mice did not differ significantly in time spent in the maze quadrant that previously housed the escape platform (t (24) = .608, p = .549, Figure 2B), suggesting that HB-GAM knockouts eventually learned the association between the cue and platform location as well as wild type mice.

Morris Water Maze: Second Sequence. The second sequence of water maze testing occurred 8 weeks after the first sequence. In this sequence, animals were tested in the cued version of the maze over 5 days and then, 2 weeks later, tested in the spatial version of the maze over 4 days.

Cued Version. HB-GAM knockouts and wild type mice demonstrated a significant decrease in latency to escape across trials (F (14, 140) = 3.58, p = .000), reflecting that animals of both genotypes successfully learned the location of the escape platform. The genotypes did not differ significantly in mean latency to escape across trials (F (1, 10) = .290, p = .602), and no significant genotype by trial interaction was found (F (14, 140) = 1.45, p = .138, Figure 1C), suggesting that animals of both genotypes learned the location of the escape platform at a similar rate. In the retention trial, HB-GAM knockouts and wild type mice did not differ significantly in time spent in the maze quadrant that previously housed the escape platform (t (13) = .410, p = .691,

Figure 2C), suggesting similar delayed memory for the location of the escape platform between the genotypes.

Spatial Version. HB-GAM knockouts and wild type mice demonstrated a significant decrease in latency to escape across trials (F (3, 45) = 13.04, p = .000), indicating that animals of both genotypes gradually learned the location of the escape platform. However, HB-GAM knockouts demonstrated a significantly greater mean latency to escape across trials as compared to wild types (F (1, 15) = 3.96, p = .012), suggesting that the knockouts demonstrated delayed acquisition of the platform location. A significant genotype by trial interaction was found (F (3, 45) = 3.775, p = .017, Figure 1D), likely due to similar performance between the genotypes during some but not all trial blocks. In the retention trial, HB-GAM knockouts and wild type mice did not differ significantly in time spent in the maze quadrant that previously housed the escape platform (t (14) = .1.38, p = .187, Figure 2D), suggesting similar delayed memory for the location of the escape platform between the genotypes.

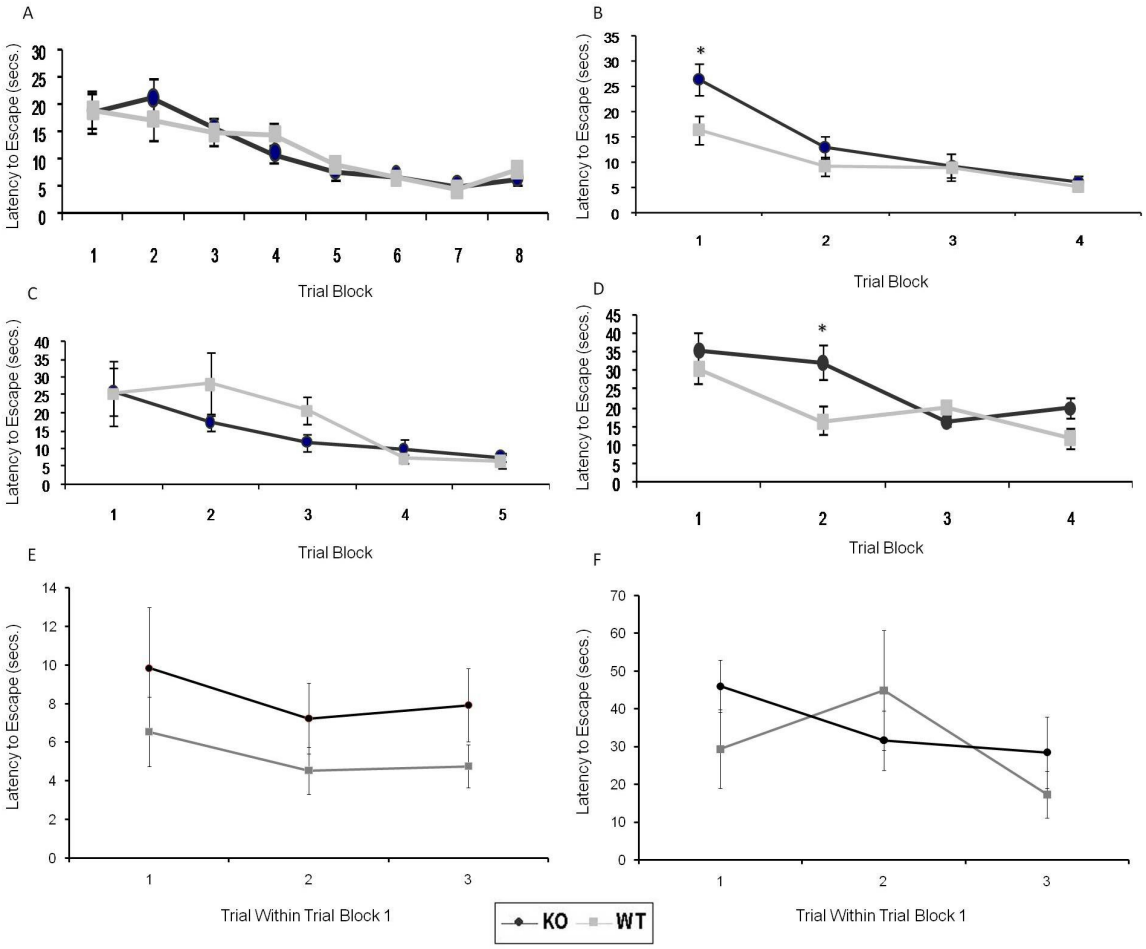

Figure 1. Latencies to escape the Morris water maze for HB-GAM knockouts and wild type mice during the first sequence of testing (KO *n* = 13, WT *n* = 13): spatial (A) then cued (B), during the second sequence of testing (KO *n* = 7, WT *n* = 5): cued (C) then spatial (D), and during the first three trials (comprising trial block 1) for the second version of the maze in which animals were tested for both the first (E) and second (F) sequence of testing.

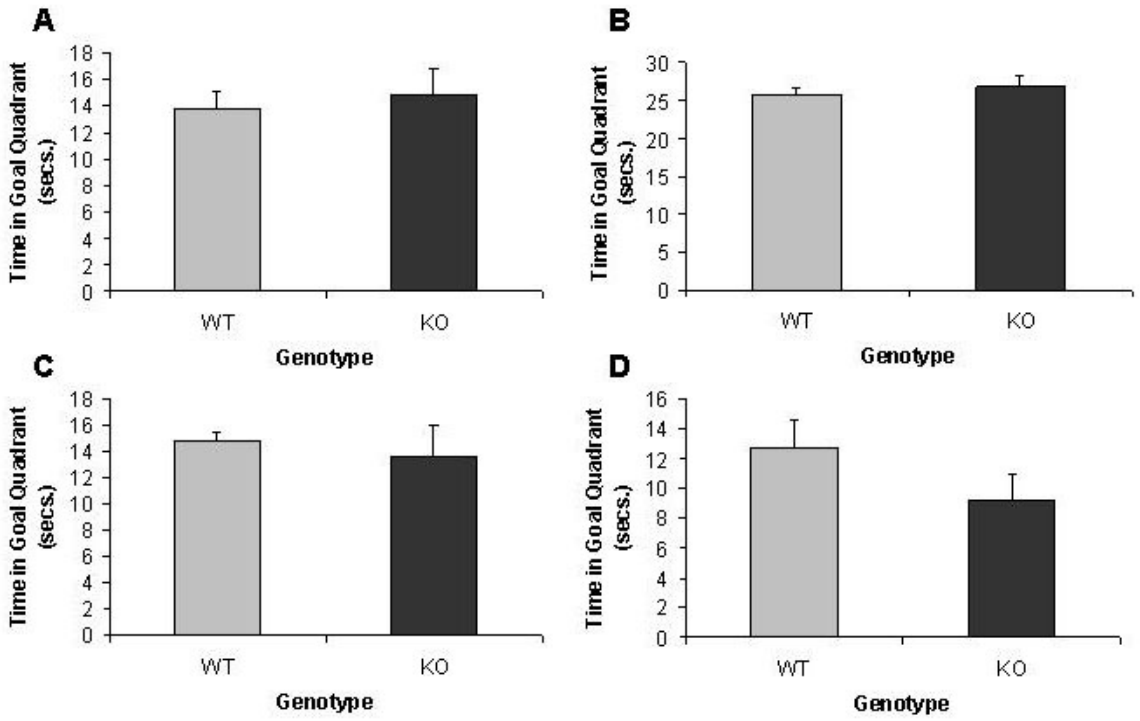

Figure 2. Time spent in the goal quadrant of the Morris water maze for HB-GAM knockouts and wild type mice during the first sequence of testing (KO n = 13, WT n = 13): spatial (A) then cued (B), and during the second sequence of testing (KO n = 7, WT n = 5): cued (C) then spatial (D).

Y-Maze. HB-GAM knockout mice made a significantly greater percentage of perseverative arm choices than did wild type mice (t (31) = 2.81, p = .008, Figure 3), suggesting a reduction in spontaneous alternation in the knockouts.

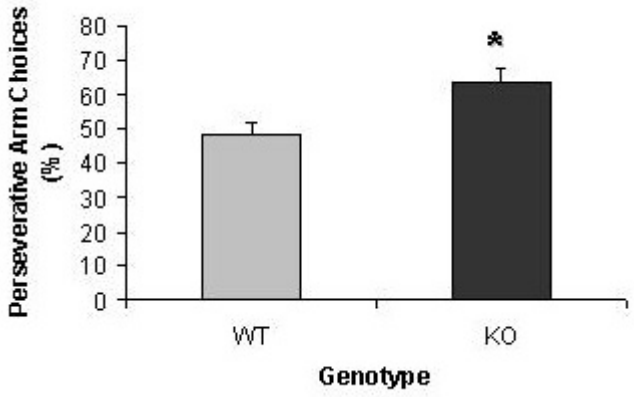

Figure 3. Percentage of perseverative arm choices made by HB-GAM knockouts (*n* = 15) and wild type mice (*n* = 18) in the Y-maze.

Hole Board. HB-GAM knockouts made significantly more head dips in the hole board than did wild type mice (t (14) = 2.91, p = .015). A significant genotype by gender interaction was found (F (1, 12) = 5.28, p = .040, Figure 4A), which indicated the extent to which the knockouts made more head dips than did wild type mice was significantly greater in males. However, HB-GAM knockouts and wild type mice did not differ significantly in the percentage of perseverative head dips made (t (14) = .342, p = .737, Figure 4B). Similarly, the genotypes did not differ significantly in the proportion of head dips into animals' favored hole to total head dips (t (14) = .957, p = .355, Figure 4C).

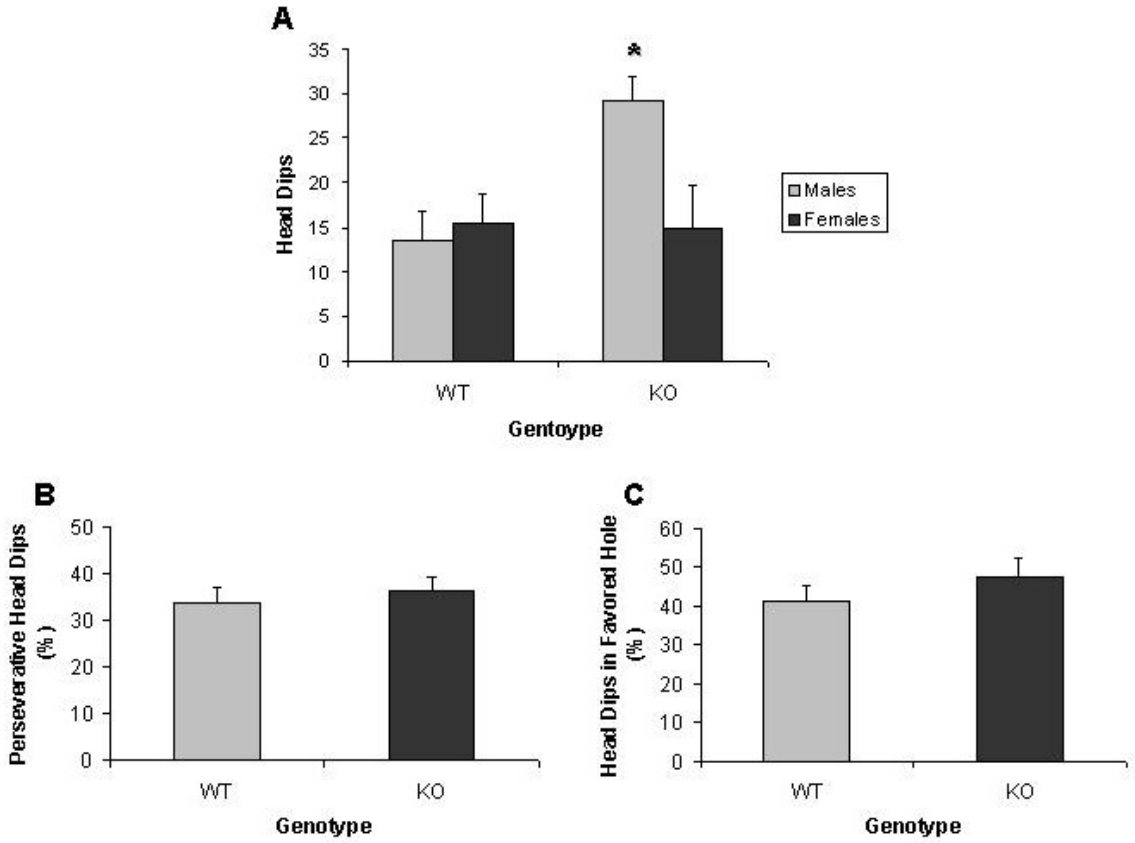

Figure 4. Number of head dips (A), percentage of perseverative head dips (B), and percentage of head dips into animals' favored hole (C) made by HB-GAM knockouts (n = 8) and wild type mice (n = 8).

Motor Behavior. HB-GAM knockouts and wild type mice did not differ significantly in the number of grooms demonstrated in the open field (F (1, 14) = .099, p = .758, Figure 5A), suggesting no tendency toward compulsive motor behavior or repetitive motor stereotypy in the knockouts. Similarly, HB-GAM knockouts and wild type mice did not differ significantly in overall number of grid crossings made in the open field (F (1, 14) = 1.35, p = .265, Figure 5B), again suggesting no motor hyperactivity in the knockouts. Further, HB-GAM knockouts and wild type mice did not differ significantly in estimated swim speed in either the cued (F (1, 10) = .002, p = .963,

Figure 5C) or spatial (F (1, 15) = 2.466, p = .137, Figure 5D) version of the Morris water maze during the second sequence of water maze testing.

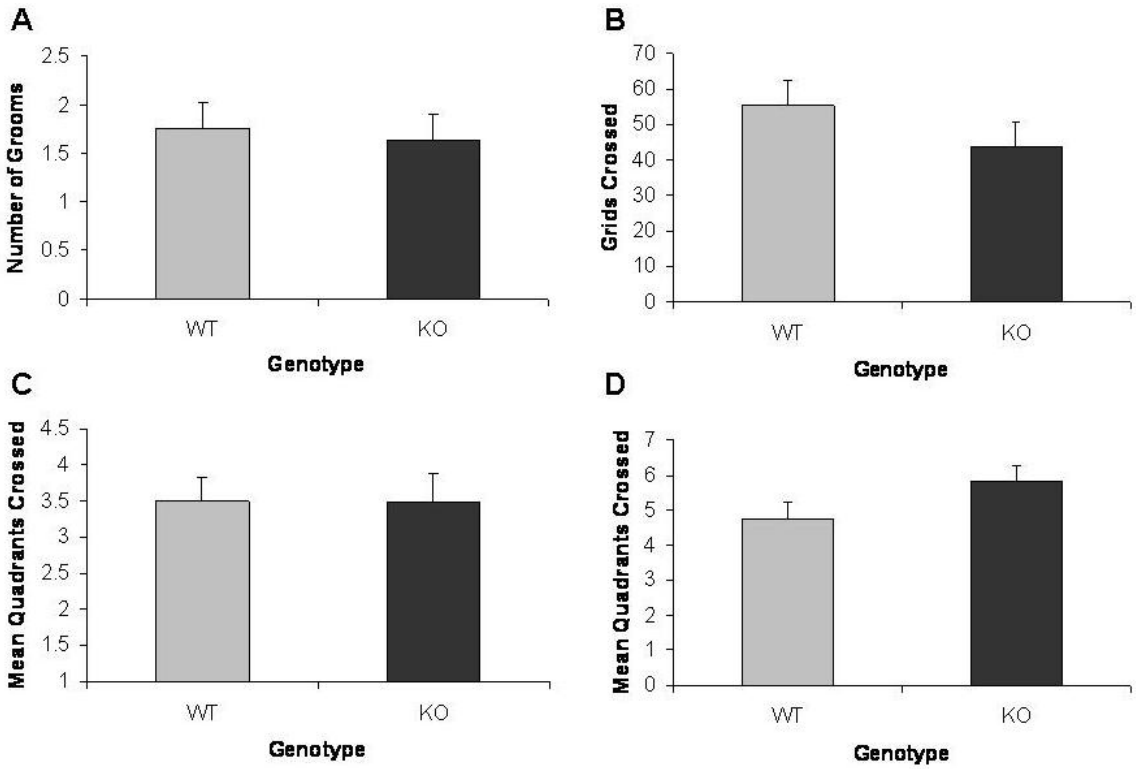

Figure 5. Number of grooms (A) and number of total grid crossings (B) made by HB-GAM knockouts (n = 9) and wild type mice (n = 5) in the open field, and estimated swim speed (latency to escape/quadrants crossed) in the cued (C) and spatial (D) versions of the Morris water maze for HB-GAM knockouts (n = 7) and wild type mice (n = 5) during the second sequence of water maze testing.

Discussion

In an attempt to assess HB-GAM knockouts' validity as a potential animal model of the PDDs, these animals' capacity for perseverative behavior was assessed because such behavior is commonly observed in these disorders. This assessment consisted of

examination of the knockouts' capacity for both motor stereotypy and hyperactivity, or "lower-order" perseverative behavior, and cognitive inflexibility, resulting in "higher-order" perseverative behavior.

Assessment of animals' capacity for "lower-order" repetitive behavior revealed no appreciable difference between HB-GAM knockouts and their wild type counterparts. HB-GAM knockouts did not differ significantly from wild type mice in frequency of grooming in the open field, and no qualitative observations of increased grooming behavior were made when animals were tested in behavioral paradigms in which grooming behavior is not traditionally quantified.

Increased grooming behavior is traditionally considered an indicator of motor stereotypy in rodents and analogous to repetitive, compulsive motor behavior, e.g. rocking or hand flapping, in humans with PDDs (Crawley, 2000, 2004). Indeed, several potential models of the PDDs have been identified based on the presence of repetitive motor behavior, such as rodents prenatally exposed to VPA (Wagner et al., 2006) and those treated with experimental depletion of forebrain serotonin (Boylan et al., 2007), in addition to mice with mutations of the *MECP2* gene (Moretti et al., 2005) and those null for the *Hoxb8* gene (Lewis et al., 2007). Contraversive circling, another putative rodent correlate of repetitive motor behavior in humans observed in potential animal models of the PDDs (e.g. Lewis et al., 2007; Velisek et al., 2005), is also absent in HB-GAM knockouts.

Similarly, HB-GAM knockouts did not demonstrate an increased capacity for motor hyperactivity relative to wild type mice. The knockouts and wild type mice did not

differ significantly on measures of motor speed, specifically, the overall number of grid crossings in the open field and estimated swim speed in the Morris water maze.

Hyperactive motor behavior in animals has been deemed analogous to hyperactive motor behavior associated with the PDDs (Crawley, 2000, 2004) and has been observed in some other potential animal models of the disorders, such as rodents prenatally treated with VPA (Wagner et al., 2006) and those with induced neonatal hypothyroidism (Sadamatsu et al., 2006). The absence of stereotyped and hyperactive motor behavior in HB-GAM knockouts suggests that these animals do not possess an increased capacity for motor stereotypy or hyperactivity and, consequently, do not model these features of the PDDs.

Interestingly, however, examination of HB-GAM knockouts' capacity for "higher-order" repetitive behavior indicative of cognitive inflexibility (Crawley, 2000, 2004, 2008) revealed notable abnormalities in these animals. HB-GAM knockouts displayed no significant learning impairment in either the spatial or cued versions of the Morris water maze relative to wild type mice. However, the knockouts' performed comparably to wild type mice only in the first version of the water maze in which they were tested across two sequences of testing. That is, the knockouts demonstrated significantly reduced overall learning in the second version of the maze in which they were tested, regardless of whether that second version was spatial or cued. These data are consistent with those obtained previously (Croll, unpublished data).

HB-GAM knockouts' visual acuity is reportedly comparable to that of wild type mice (Croll, unpublished data; Pavlov et al., 2002), and both previous (e.g. Pavlov et al., 2002) and current measures of motor speed in these animals do not suggest the presence

of motoric deficits. Further, HB-GAM knockouts demonstrated water maze learning similar to their wild type counterparts in both the spatial and cued versions when tested in these respective versions first, a finding that does not indicate discrete deficits in either spatial or associative learning in HB-GAM knockouts. Instead, because the knockouts performed significantly worse than wild type mice only in the second version of the water maze in which they tested, regardless of the version across two testing sequences, it is most plausible that these animals experience difficulty adapting to changing contingencies in the form of tasks with similar but not identical demands. Interestingly, however, HB-GAM knockouts and wild type mice did not differ significantly in time spent in the maze quadrant that previously housed the escape platform upon testing in the retention phase of the paradigm. These data indicate that, though the knockouts demonstrated consistently reduced overall learning in the second version of the maze in which they tested, they eventually learned the location of the escape platform with efficacy comparable to that of normal animals.

Decreased or delayed learning but relatively intact retention of information is commonly observed in psychopathologies associated with anxiety (DSM IV-TR; 2000), and similar findings have been made in animals with anxious phenotypes (cf. File, 2001). Because HB-GAM knockouts' overall learning was reduced compared to that of wild type mice in only the second version of the maze in which they were tested, it is possible that their inferior performance – and the cognitively inflexibility this suggests – might reflect an underlying or associated novelty-induced anxiety.

Such cognitive inflexibility has also been ascribed to mice deficient in thyroid hormone (Sadamatsu et al., 2006) and those null for the *Fmr1* gene (for review, see

Bernardet & Crusio, 2006). These animals demonstrate an inability to adapt to changes in environmental contingencies in maze tasks, as evidenced by inferior performance relative to controls only after initiation of such changes. The cognitive inflexibility suggested by these data has been used to qualify these animals as potential models of the restricted repertoire of behavior commonly associated with the PDDs.

Unlike thyroid hormone-deficient mice and *Fmr1* knockouts, however, HB-GAM knockouts apparently demonstrate additional perseverative tendencies in the cognitive domain. Specifically, the knockouts made a significantly greater percentage of identical, consecutive arm choices in the Y-Maze than did wild type mice. Because HB-GAM knockouts did not demonstrate qualitative abnormalities in movement patterns across behavioral paradigms, their lack of normal spontaneous alternation in the Y-maze further suggests "higher order" perseverative behavior and not exclusively "lower-order," or repetitive motor, perseverative behavior *per se*.

Contrary to their performance in the Y-maze, HB-GAM knockouts did not demonstrate perseverative tendencies in the hole board paradigm, as assessed by percentage of identical, consecutive head dips and percentage of head dips into animals' favored hole. However, HB-GAM knockouts made significantly more head dips overall than did their wild type counterparts, a difference that was itself significantly greater in male mice. Mice possess a strong tendency toward head dipping or nose poking into holes as a means of exploration in an attempt to locate appetitive stimuli, such as food or mates (Crawley, 2000). However, given the perseverative tendencies demonstrated by the knockouts in both the water maze and Y-maze, and these animals' normal exploratory behavior in the open field and other behavioral paradigms, their significantly increased

head dipping behavior might represent a perseverative behavior and not increased exploratory drive. If so, the possibility of receiving appetitive rewards as a result of exploration via head dipping might have been sufficient to negate a perseverative quality to their head dipping but not sufficient to counteract overall more frequent, repetitive head dipping.

Alternatively, increased head-dipping behavior in the hole board has been attributed to animals' attempts to escape the apparatus because of heightened anxiety, a claim supported by decreases in head-dipping behavior following anxiolytic treatment in anxiety-prone animals (cf. Saitoh et al., 2006). The possibility that HB-GAM knockouts' increased head dipping behavior is secondary to heightened anxiety is plausible given that these animals have previously demonstrated increased anxiety (Pavlov et al., 2002). In addition, cognitive rigidity, as demonstrated by the knockouts in the water maze and Y-maze, is often associated with heightened anxiety in both human psychopathologies (DSM-IV-TR; 2000) and in putative animal models of those pathologies (cf. Sadamatsu et al., 2006).

Though the implications of increased head dipping behavior for HB-GAM knockouts' behavioral phenotype are uncertain, these animals' behavior in the water maze and Y-maze paradigms clearly suggest a phenotype that includes cognitive rigidity. A frequent neurological explanation for cognitive rigidity in rodents is disequilibrium between direct and indirect cortical-basal ganglia pathway activity, though such an abnormality has also been frequently associated with motor stereotypy (Lewis et al., 2007). Therefore, the presence of cognitive but not motoric aspects of perseverative

behavior in HB-GAM knockouts argues against such an etiology for these animals' cognitive rigidity.

Alternatively, an *in vivo* absence of HB-GAM might best explain the finding of cognitive inflexibility in the knockouts. HB-GAM is believed to act as a suppressor of synaptic plasticity in the hippocampus. Consequently, HB-GAM's absence *in vivo* has been associated with a reduced threshold for hippocampal LTP induction (Lauri et al., 1998; Amet et al., 2001). The resulting enhancement in synaptic strength in HB-GAM knockouts might, therefore, account for the cognitive and behavioral rigidity that would likely underlie an inability to adapt behavior, either in response to shifting contingencies in the water maze or to an innate tendency toward flexible behavior in the Y-maze. That is, HB-GAM knockouts' capacity for premature induction of LTP could lend the knockouts' first behavioral response in a given paradigm a permanence of sufficient strength to preclude adaptation or amendment of that response. Therefore, the first contingency learned by HB-GAM knockouts in the water maze, as well as specific arm choices in the Y-maze, might become synaptically "overlearned."

An alternative explanation for the knockouts' water maze performance is that the loss of HB-GAM's inhibitory effect on LTP resulted in increased capacity for LTP saturation in these animals. Indeed, induced saturation of hippocampal LTP has been shown to impair spatial learning in rodents (e.g. Moser, Krobert, Moser & Morris, 1998). The synaptic networks required to adequately meet the demands of the spatial and cued versions of the water maze are likely not entirely identical. However, the possibility that the knockouts' difficulty adapting to changing contingencies is due to LTP saturation is still valid given evidence that other mechanisms by which LTP is attenuated, such as

long-term depression (LTD), can act heterosynaptically (e.g. Huang & Hsu, 2001; Muller, Hefft & Figurov, 1995).

The possibility that alterations in capacity for synaptic depotentiation or LTP saturation underlie the knockouts' cognitive inflexibility suggests that these animals might possess a reduced threshold for, or be predisposed to a potentiation of, the synaptic changes underlying the development of stimulus-reward associations. If so, the knockouts' strong tendency to make perseverative arm choices in the Y-maze might reflect a greater capacity for re-enforcement of even a seemingly neutral action, such as a random choice of maze arm, thereby increasing the likelihood that the behavior would be repeated. To investigate this possibility, future studies might examine these animals' capacity for developing stimulus-reward associations using appropriate paradigms, such as the conditioned place preference test.

Regardless of the mechanism underlying HB-GAM knockouts' apparently perseverative behavior, further testing is advisable to more fully delineate the extent of these animals' capacity for cognitive inflexibility. Though the Morris water maze is commonly used to assess animals' capacity to adapt to changing contingencies and learn reversals, the task is known to be especially anxiety provoking (cf. Crawley, 2000). Therefore, further assessment of the knockouts' ability to demonstrate cognitive and behavioral flexibility could be undertaken using less anxiogenic paradigms amenable to alteration or those traditionally used to test reversal learning, such as the T-maze (e.g. Sadamatsu et al., 2006), attention set-shifting tasks (e.g. Birrell & Brown, 2000), and associative learning tasks that can include measures of cognitive adaptability, such as the

interactive touchscreen system that allows animals to learn flexible associations between pictorial icons and the receipt of food (Morton, Skillings, Bussey & Saksida, 2006).

The current data indicate that HB-GAM knockouts demonstrate a behavioral phenotype characterized by cognitive rigidity as manifested by an inability to adapt to shifting contingencies in the water maze and a failure to make variable, flexible choices in favor of perseverative responses in the Y-maze. In addition, increased head dipping in the hole board might provide further evidence of either perseverative behavior or increased anxiety in HB-GAM knockouts. However, the knockouts did not exhibit a tendency toward motor stereotypy or hyperactivity. These data suggest that HB-GAM knockouts possess a behavioral phenotype that mirrors the cognitive but not the motoric manifestations of perseveration associated with the PDDs.

Given the suggestion of increased anxiety in HB-GAM knockouts by both previous and current data, further behavioral testing was conducted to assess capacity for anxiety in HB-GAM knockouts. In addition, because cognitive rigidity is associated with and perhaps predicated on novelty-induced anxiety, these animals' responses to novel environments and novel stimuli were also assessed.

CHAPTER 4. SPECIFIC AIM 2

***Specific Aim 2* was to evaluate HB-GAM knockouts' behavior in response to novel environments and novel stimuli.**

The perseverative tendencies and restricted routines and interests observed in PDD-affected individuals might be the result of heightened novelty-induced anxiety, which is frequently seen in these persons (DSM-IV-TR; 2000). Not surprisingly, attempts have been made to identify anxious or aberrant behavior in the face of novel stimuli in potential animal models of the PDDs. Prenatal VPA exposure, which has been implicated in the pathogenesis of the PDDs, has been associated with hyperactivity in novel environments in rodents as well as with other symptoms resembling those of the PDDs, including elevated levels of serotonin in frontal cortex, disturbances of circadian rhythm, aberrant sensitivity to sensory stimuli, and ritualistic behavior (Tsujino et al., 2007). Similarly, induced neonatal hypothyroidism in rats has been associated with increased time to habituate to novel environments in addition to motor hyperactivity and, perhaps more vital to the argument that these animals represent a model for PDD-like features, an inability to adapt to changing environmental contingencies (Sadamatsu et al., 2006).

In addition, animals null for CADPS2, the mRNA of which is abnormally spliced in some autistic individuals, demonstrate home cage hyperactivity along with locomotor hypoactivity in behavioral paradigms, suggesting anxiety elicited by novel environments. Consistent with a neophobic phenotype, these animals also display reduced exploration of novel objects in the open field. Interestingly, CADPS2 knockout mice display multiple other PDD-like features, including but not limited to reduced social interaction and poor maternal care of offspring (Sadakata et al., 2007). Similarly, animals null for *Pten*,

mutations of which have been linked to macrocephaly in humans with PDDs (Herman et al., 2007), also exhibit heightened anxiety upon behavioral testing (Kwon et al., 2006).

Novelty-induced anxiety is a major component of the PDD behavioral phenotype, and potential animal models of these disorders that demonstrate multiple PDD-like features also exhibit apparent novelty-induced anxiety. Taken together, these facts suggest that assessment of the behavior exhibited in novel environments by any potential animal model of the PDDs is advisable. Therefore, HB-GAM knockouts' capacity for anxiety was assessed in the light-dark paradigm and elevated plus maze. In addition, because rodent anxiety can manifest in behavioral hypoactivity (e.g. Kalueff & Tuohimaa, 2005) as well as in freezing, hiding, and excessive grooming (Dunn, Guild, Kramarcy & Ware, 1981), animals' behavioral initiation in the visual cliff, open field, Y-maze and novelty-suppressed feeding paradigms was investigated, as was these animals' readiness to accept to a novel food flavor in the gustatory neophobia paradigm.

Specific Methods

Elevated Plus Maze. This apparatus was composed of a platform measuring 15 x 15 cm elevated 114 cm on a central pole. Joined to this platform were four arms, each measuring 61 x 15 cm, arranged in a cross-like configuration and equal in height relative to the platform. In direct opposition were two white, open arms and two black arms enclosed by walls measuring 30 cm high. Animals were placed on the platform and allowed to freely explore the maze for five minutes. The number of seconds animals spent on the platform and in the open and closed arms was recorded. Increased time spent in the closed arms is traditionally considered indicative of heightened anxiety, as rodents tend to avoid open, brightly lit areas that are unfamiliar or might expose them to danger,

such as airborne predators. The number of open and closed arm entries made by animals was recorded as a measure of motor activity and exploratory behavior. Further, animals' latency to first arm entry was recorded as a measure of behavioral initiation.

Light-Dark Exploration. Animals were placed in a rectangular apparatus measuring 45 x 20 x 20 cm, half of which was white and open at the top (the "light" side), and half of which was black and covered (the "dark" side). Animals were placed in the center of the light side and allowed to freely explore the apparatus for five minutes. The amount of time animals spent in the light and dark sides, respectively, was recorded. As in the elevated plus maze, increased time spent in the dark, enclosed portion of the apparatus is considered indicative of increased anxiety. Animals' latency to enter the dark side of the apparatus was recorded as a measure of behavioral initiation.

Visual Cliff. A clear, Plexiglas sheet measuring 45 x 20 cm, the exact length and width dimensions of the light-dark apparatus, was placed atop that apparatus, creating the appearance of a 22.5 x 20 cm black platform atop the dark side of the apparatus and a 22.5 x 20 cm chasm from the open, light side of the apparatus. Animals were placed on the platform and allowed to freely explore both the platform and transparent Plexiglas sides for three minutes. The amount of time each animal spent on the platform and transparent sides, respectively, was recorded, as was animals' latency to approach the cliff as a measure of behavioral initiation. Normal animals are expected to approach the cliff side cautiously, as uninterrupted movement across the cliff would indicate a lack of perception of the apparent cliff and, therefore, visual impairment.

Open Field. Animals were placed into a white, box-like apparatus measuring 48 x 48 x 24 cm. The inside floor was divided into nine grids, each measuring 16 x 16 cm.

Animals were placed in the center grid and allowed to freely explore the maze for two minutes. This procedure was repeated five times in succession, for a cumulative 10-minute period termed a "trial." Animals were tested in the open field in two trials separated by 24 hours. Animals' latencies to groom and to exit the center grid were recorded. These measures are thought to assess anxiety-like behavior because grooming latency can reflect animals' sense of safety in an environment, and rodents' natural inclination is to hug the walls of an apparatus rather than stand exposed at its center as a protective mechanism against predators. In addition, frequency of grooming was recorded as a measure of animals' capacity for repetitive motor behavior. Further, the number of total grid crossings made by animals was recorded as a measure of general locomotor and exploratory behavior.

Novelty-Suppressed Feeding. Following a 24-hour period of food deprivation, animals were placed in a black, open field measuring 40 cm x 40 cm. After a five-minute habituation period, a pellet of the animals' standard food (PicoLab Mouse Diet 20, LabDiet, St. Louis, MO) was introduced, and animals' latency to feed on the food pellet was recorded. Increased latency to feed on the food pellet is considered indicative of increased anxiety secondary to the novel nature of the environment.

Gustatory Neophobia. Because accidental water deprivation during these studies once proved especially fatal to HB-GAM knockouts, animals were not water-deprived prior to testing in this paradigm. In their home cages (individually, if group-housed), animals were presented with 100 ml of water sweetened with 1% sucrose (a novel but particularly appetitive stimulus) and 100 ml of normal tap water (a familiar stimulus) in separate, graded bottles. The amount of each kind of water drunk by animals, measured

in ml, was recorded one, two, three, and four hours after initial presentation. Avoidance of drinking the sweet water is considered indicative of a neophobic reaction sufficient to negate the appetitive value of the sweet water.

Latency to Behave Across Paradigms. A MANOVA was conducted to analyze animals' latency to behave across behavioral paradigms because latency measures taken across paradigms were assumed to assess a single, conceptually related variable. In the rare instance of unequal *n*s among behavioral paradigms, missing values were interpolated using the mean for a missing animal's genotype on the variable being interpolated.

Motor Activity. In some paradigms, animals' motor activity (e.g. operationally defined as number of arm entries in the elevated plus maze, number of grids crossings on the open field) was measured to assess the contribution of motor activity to animals' performance in these paradigms.

Results

Elevated Plus Maze. HB-GAM knockouts spent a significantly greater percentage of time in the closed arms of the elevated plus maze than did wild type mice (t (12) = 2.38, p = .035, Figure 6A) and made a significantly smaller percentage of open arm entries than did wild type mice (t (12) = 2.44, p = .031, Figure 6B), both of which are suggestive of increased anxiety in the knockouts. However, HB-GAM knockouts did not differ significantly from wild type mice in percentage of closed arm entries (t (12) = .908, p = .382, Figure 6B), percentage of platform entries (t (12) = .126, p = .902, Figure 6B), or total number of arm entries (t (12) = 1.31, p = .213, Figure 6C), indicating similar locomotor activity between the genotypes. HB-GAM knockouts and wild type mice did

not differ significantly in latency to first arm entry (t (12) = 1.148, p = .273, Figure 6D), suggesting similar behavioral initiation between the genotypes in this paradigm.

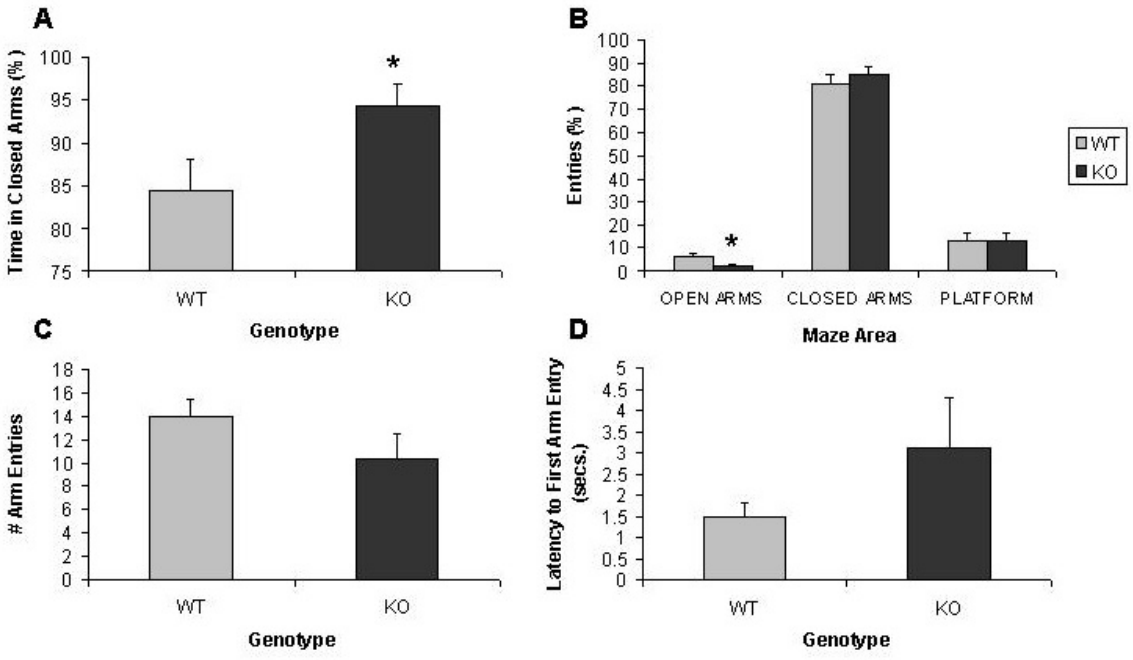

Figure 6. Percentage of time spent in the closed arms (A), percentage of open arm, closed arm, and platform entries (B), number of arm entries (C), and latency to first arm entry (D) for HB-GAM knockouts (n = 8) and wild type mice (n = 6) in the elevated plus maze.

Light-Dark Exploration. HB-GAM knockouts and wild type mice did not differ significantly in percentage of time spent in the dark side of the light-dark apparatus (t (35) = .079 p = .938, Figure 7A), suggesting no appreciably greater anxiety in HB-GAM knockouts in this paradigm. Similarly, HB-GAM knockouts and wild type mice did not differ significantly in latency to approach the dark side of the apparatus (t (34) = .717, p = .479, Figure 7B), suggesting similar behavioral initiation between the genotypes in this paradigm.

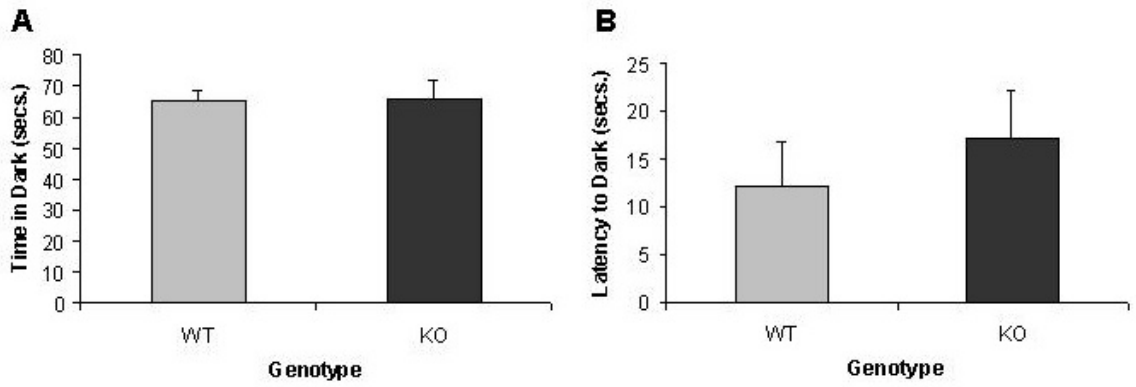

Figure 7. Percentage of time spent in the dark side (A) and latency to enter the dark side (B) of the light-dark apparatus for HB-GAM knockouts and wild type mice. Note that outliers were removed: time in dark (KO n = 19, WT n = 18) and latency to dark (KO n = 21, WT n = 15).

Visual Cliff. HB-GAM knockouts spent a significantly greater percentage of time on the platform side of the visual cliff apparatus than did wild type mice (t (31) = 2.26, p = .037, Figure 8A). Given that HB-GAM knockouts' visual acuity appears to be comparable to that of wild type mice, this result might suggest increased reticence to approach the cliff because of its novel nature. Indeed, HB-GAM knockouts demonstrated a significantly increased latency to approach the cliff of the apparatus as compared to wild-type mice (t (32) = 2.43, p = .014, Figure 8B), suggestive of reduced behavioral initiation in the knockouts in this paradigm.

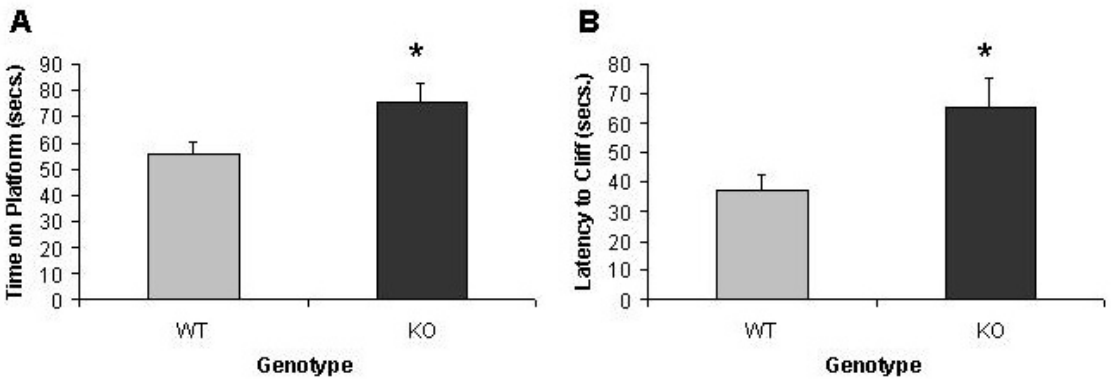

Figure 8. Percentage of time spent on the platform side (A) and latency to approach the cliff (B) of the visual cliff apparatus for HB-GAM knockouts and wild type mice. Note that outliers were removed: time on platform (KO n = 19, WT n = 18) and latency to cliff (KO n = 16, WT n = 17).

Open Field. HB-GAM knockouts demonstrated a significantly greater latency to groom than did wild type mice across both trials in the open field (F (1, 12) = 12.12, p = .005, Figure 9A), which might suggest anxiety–induced hypoactivity in the knockouts. In addition, HB-GAM knockouts demonstrated a significantly increased latency to exit the center of the open field than did wild type mice in the first but not the second trial in the open field, as evidenced by a significant genotype by trial interaction (F (1, 13) = 4.86, p = .046, Figure 9B), which might suggest hypoactivity secondary to neophobia in the knockouts. HB-GAM knockouts and wild type mice did not differ significantly in the total number of crossings made in the open field across trials, however, HB-GAM knockouts made significantly fewer total grid crossings in the first but not the second trial in the open field, as evidenced by a significant genotype by trial interaction (F (1, 14) = 11.41, p = .005, Figure 9C), another result suggestive of hypoactivity secondary to neophobia in the knockouts. HB-GAM knockouts and wild type mice did not differ

significantly in the percentage of outer grid crossings made in either trial in the open field (F (1, 13) = .402, p = .537, Figure 9D), suggestive of overall comparable motoric activity and exploratory drive between the genotypes.

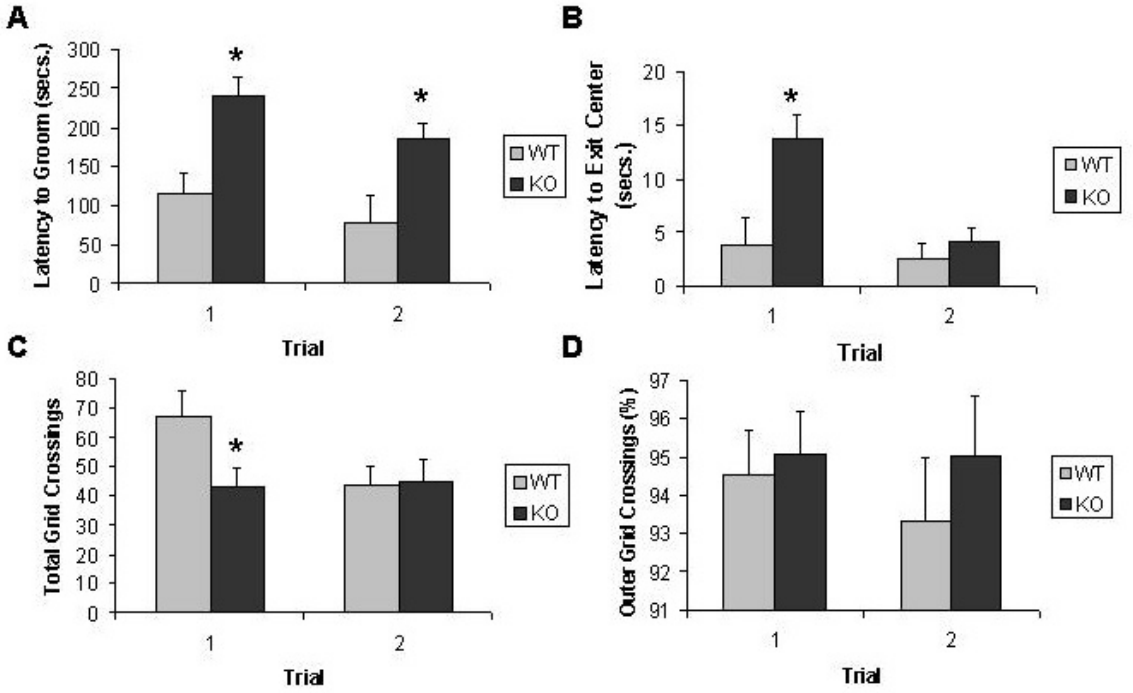

Figure 9. Latency to groom (A), latency to exit the center grid (B), total grid crossings (C), and percentage of outer grid crossings (D) for HB-GAM knockouts and wild type mice in two trials in the open field. Note that outliers were removed: latency to groom (KO n = 9, WT n = 5), latency to exit the center grid (KO n = 8, WT n = 7), total grid crossings (KO n = 8, WT n = 8), and percentage of outer grid crossings (KO n = 8, WT n = 7).

Y-Maze. HB-GAM knockouts and wild type mice did not differ significantly in latency to first arm choice (t (31) = .493, p = .626, Figure 10A) or in mean latency to arm choice across trials (t (31) = .425, p = .674, Figure 10B). By itself, this result is

suggestive of similar behavioral initiation between the genotypes in this paradigm. However, animals demonstrating extreme behavioral hypoactivity in this paradigm (as defined by making fewer than four consecutive arm choices) were excluded from analysis. Ultimately, 25% of HB-GAM knockouts and 5.5% of wild type mice failed to make this requisite number of consecutive arm choices. A 2 x 2 Chi Square analysis revealed that this was a significant difference (x^2 (4, N = 39) = 44.06, p = .000, Table 1), suggesting an overall greater frequency of behavioral hypoactivity in the knockouts in this paradigm.

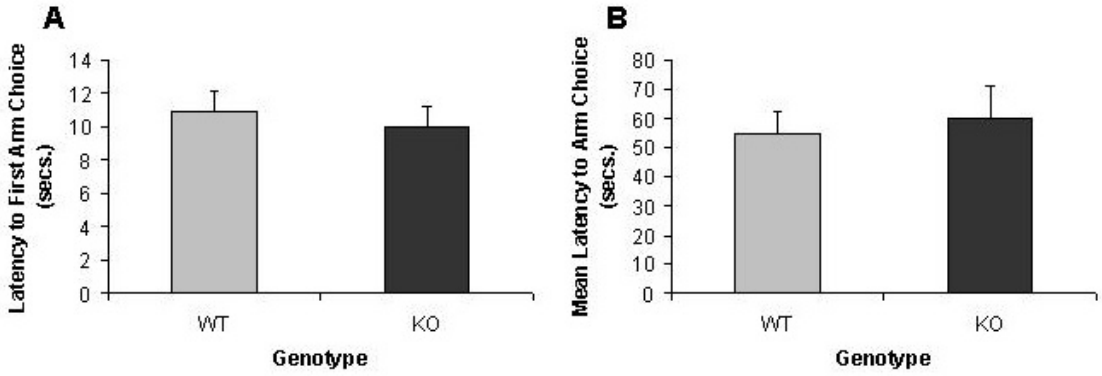

Figure 10. Latency to first arm choice (A) and mean latency to arm choice (B) for HB-GAM knockouts (n = 15) and wild type mice (n = 18) in the Y-maze.

Table 1

Frequency of Number of Consecutive Arm Choices Made by HB-GAM Knockouts and Wild Type Mice in the Y-maze

Number of Consecutive Y-maze Arm Choices	Genotype	
	Wild Type Mice	HB-GAM Knockouts
Fewer than four	1	5
Equal to or greater than four	18	15

Novelty-Suppressed Feeding. HB-GAM knockouts and wild type mice did not differ significantly in latency to feed in the novelty-suppressed feeding paradigm (t (17) = 1.14, p = .270, Figure 11), suggesting similar behavioral initiation between the genotypes in this paradigm.

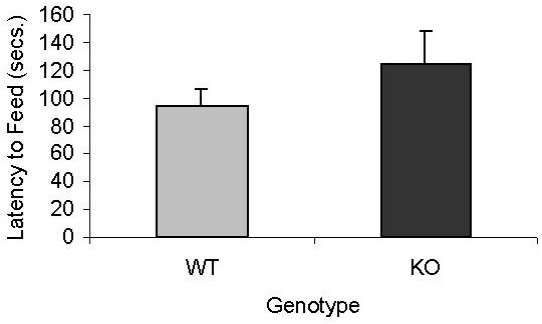

Figure 11. Latency to feed in the novelty-suppressed feeding paradigm for HB-GAM knockouts (n = 9) and wild type mice (n = 10).

Gustatory Neophobia. HB-GAM knockouts and wild type mice did not differ significantly in the total amount of either sweet water (t (10) = .279, p = .736) or regular water drunk (t (10) = .750, p = .470). As expected, a significant linear trend in the mean amount of water drunk across trials was evidenced for both sweet water (F (3, 30) = 60.66, $p < .001$) and regular water (F (3, 30) = 40.92, $p < .001$). However, no genotype by time interaction was found for either the amount of sweet water (F (3, 30) = .129, p = .942, Figure 12A) or regular water (F (3, 30) = .845, p = .480, Figure 12B) drunk. These data suggest similar adaptability to a novel flavor between the genotypes.

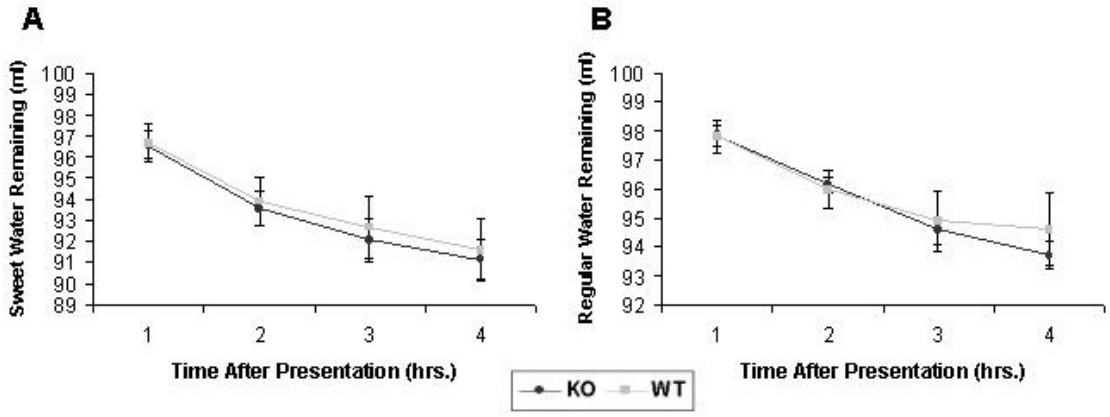

Figure 12. Amount of sweet water (A) and regular water (B) drunk by HB-GAM knockouts (n = 7) and wild type mice (n = 5) across a four hour period in the gustatory neophobia paradigm.

Latency to Behave Across Paradigms. MANOVAs were performed on latency to behave measures across relevant paradigms in two cohorts. These analyses indicated an effect of genotype in both cohorts, such that HB-GAM knockouts' overall latency to behave was significantly greater than that of wild type mice. In the first cohort (KO n = 11, WT n = 9), the overall effect of genotype on latency to behave was statistically

significant (p = .004). The effect of genotype was significant for animals' latency to the cliff in the visual cliff paradigm (p = .014), latency to exit the platform of the elevated plus maze (p = .011), and latency to first groom in the open field (p = .002), however, no significant effect for genotype was found on animals' latency to enter the dark side of the light-dark apparatus (p = .961), latency to first arm choice in the Y-maze (p = .368), or latency to exit the center grid of the open field in the first trial (p = .591).

In the second cohort (KO n = 11, WT n = 9), the overall effect of genotype on latency to behave was also statistically significant (p = .001). The effect of genotype was marginally significant for latency to approach the cliff in the visual cliff paradigm (p = .091) and for latency to first arm choice in the Y-maze (p = .090). However, no significant effect of genotype was found on animals' latency to enter the dark side of the light-dark apparatus (p = .533) or on animals' latency to feed in the novelty-suppressed feeding paradigm (p = .744).

Discussion

Anxiety is commonly observed in the PDDs, and the possibility of heightened anxiety in HB-GAM knockouts has been reported previously (Pavlov et al., 2002). Therefore, these animals' behavior was assessed in the elevated plus maze and the light-dark apparatus, paradigms that are traditionally used to assess anxious behavior in mice (Crawley, 2000, 2008). In addition, because PDD-associated anxiety is often accompanied by, and perhaps founded on, neophobia (DSM-IV-TR; 2000), HB-GAM knockouts' behavior in response to novel environments and novel stimuli was also assessed.

HB-GAM knockouts' behavior in the elevated plus maze suggests increased anxiety in these animals. Specifically, the knockouts spent significantly more time in the closed arms of the maze and made significantly fewer entries into its open arms, findings consistent with those of Pavlov et al. (2002). Interestingly, however, HB-GAM knockouts did not differ from their wild type counterparts in time spent in the dark side of the light-dark apparatus, a finding inconsistent with increased anxiety and the animals' performance in the elevated plus maze.

These paradigms have been validated as measures of rodent anxiety because they both present animals with a prototypical, naturalistic conflict between the desire to explore a novel environment and the aversion associated with brightly lit, open areas that might lead to exposure to predators (cf. Lister, 1987). However, the elevated plus maze could be considered the more anxiety-provoking of the two paradigms because of additional features that are anxiogenic for mice but lacking in the light-dark paradigm: open arms that are also elevated and narrow (Crawley, 2000). Therefore, HB-GAM knockouts' increased anxiety in the elevated plus maze but not the light dark paradigm might indicate that these animals indeed possess a capacity for heightened anxiety, but only in more intensely anxiety-provoking situations in which their innate desire to explore does not outweigh the aversive nature of the maze's configuration.

This supposition is further supported by HB-GAM knockouts' behavior on the visual cliff, where these animals spent significantly more time on the platform of the apparatus. The visual cliff paradigm is traditionally used to grossly assess animals' visual capability and depth perception, and failure to hesitate approaching the cliff and/or pause before attempting to cross the cliff is considered reflective of decreased visual perceptual

ability (Fox, 1965). Therefore, HB-GAM knockouts' increased time spent on the platform of the apparatus suggests that these animals possess visual perceptual ability at least comparable to that of wild type mice, a conclusion supported by their similar performance relative to wild type mice in the visually-cued version of the Morris water maze when they were tested in that version first.

Indeed, the knockouts' preference for the platform of the visual cliff apparatus is more likely attributable to anxiety elicited by the elevated and open nature of the apparatus, which is similar to that of the open arms of the elevated plus maze that the knockouts also demonstrated a strong tendency to avoid. Consistent with this inference, HB-GAM knockouts demonstrated a significantly increased latency to approach the cliff of the apparatus. The appearance of a cliff, as created by a clear Plexiglas covering over a deep chasm, likely represents a novel stimulus for mice. Given the apparent absence of abnormal visual perception and the presence of anxious tendencies in HB-GAM knockouts, it is plausible that these animals' anxiety is elicited not only by particularly anxiogenic circumstances, e.g. elevated, open areas, but also by novel stimuli, such as the illusion of a cliff.

Interestingly, the knockouts' behavior in the open field is consistent with hypoactive anxiety (e.g. as indicated by freezing) stimulated by novelty. These animals demonstrated a significantly increased latency to exit the center square of the open field in their first but not second exposure to the paradigm. Consistent with this finding, the knockouts made significantly fewer grid crossings than did wild type mice in their first but not second open field exposure. Normal mice prefer to hug the walls enclosing an apparatus rather than remain in its open center in an attempt to avoid perceived dangers

associated with brightly lit, open areas, such as predators. Therefore, increased latency to exit the center of the open field could be considered an indicator of reduced anxiety (Crawley, 2004, 2008). However, it is also possible that behavioral reticence secondary to anxiety could be responsible for the knockouts' increased latency to exit the center of the open field. Further, because the knockouts' latency to exit the center of the open field, and total number of grid crossings, were comparable to that of wild type mice during their second exposure to the paradigm, it is possible that this behavioral reticence occurred in response to the novelty of the open field at the time of their first exposure when the apparatus was unfamiliar. These data suggest that HB-GAM knockouts might possess a contextual neophobia.

Further, HB-GAM knockouts demonstrated a significantly increased latency to groom in both trials in the open field. Grooming is traditionally considered an anxiety-associated behavior when exhibited early during exposure to a behavioral paradigm (Dunn et al., 1981). However, a delay in onset of grooming following exposure to a novel environment has also been characterized as hypoactivity secondary to anxiety (Kalueff & Tuohimaa, 2005). Given the anxiety-associated behavior exhibited by the knockouts in the open field and other behavioral paradigms, the latter attribution seems more supportable. Indeed, hypoactive anxiety might also explain HB-GAM knockouts' increased latency to behave appropriately in the visual cliff and open field paradigms.

HB-GAM knockouts' comparable latency to make their first arm choice and mean time to choice of arm in the Y-maze as compared to wild type mice is seemingly inconsistent with these findings. However, those data were collected in animals that made the number of consecutive arm choices considered sufficient for inclusion in data

analysis. Excluding animals that made fewer consecutive arm choices might have increased the validity of analysis of animals' capacity for perseveration and behavioral reticence. However, this method removed from analysis those animals demonstrating the most behavioral hypoactivity. Interestingly, a significantly greater number of knockouts were excluded because of failure to make the requisite number of consecutive arm choices, suggesting an overall greater frequency of behavioral hypoactivity, perhaps secondary to anxiety, in these animals.

Nevertheless, HB-GAM knockouts exhibited comparable latency to first arm entry in the elevated plus maze and latency to the dark side of the light-dark apparatus, findings not indicative of behavioral hypoactivity. The elevated plus maze and light-dark apparatus provide animals with the opportunity to enter a dark, enclosed space, an attractive choice for rodents (cf. Lister, 1987), and one consistent with the knockouts' demonstrated anxiety. Therefore, HB-GAM knockouts' behavioral reticence in these paradigms might have been negated by the opportunity to enter an environment they perceived as safe. Indeed, a significantly increased latency to behave appropriately in the knockouts was consistently observed in those paradigms offering no such safe environment – the visual cliff and open field – and, more generally, the Y-maze.

HB-GAM knockouts did not demonstrate significantly increased latency to feed in the novelty-suppressed feeding paradigm, nor did they differ significantly from their wild type counterparts in the amount of novel, sweet water drunk in the gustatory neophobia paradigm. The appetitive nature of these stimuli might have ameliorated the knockouts' behavioral reticence despite their inherent novelty. Such a conclusion is consistent with the aforementioned notion that HB-GAM knockouts' capacity for

anxiety, despite being heightened relative to wild type mice in some paradigms, is capable of negation in moderately but not severely anxiogenic situations.

Overall, a phenotype including behavioral reticence in some novel circumstances can be ascribed to HB-GAM knockouts given the significant effect of genotype revealed by multivariate analyses across those two cohorts in which measures of latency to behave were taken. Indeed, most of the significant between-subjects effects in individual paradigms were preserved in these multivariate analyses.

HB-GAM knockouts did not differ significantly from their wild type counterparts in the number of elevated plus maze arm entries or number of outer grid crossings in the open field. These findings provide further evidence that HB-GAM knockouts do not possess impairments in locomotion or exploratory drive. Instead, these data support the notion that HB-GAM knockouts' increased latency to behave appropriately in some paradigms is due to anxiety induced by either extremely anxiogenic features or the novelty inherent to those paradigms.

Anxiety and abnormal behavior in the face of novelty has been identified in other potential animal models of the PDDs, such as rats exposed prenatally to VPA (Tsujino et al., 2007), and both *Pten* (Kwon et al., 2006) and CADPS2 knockouts (Sadakata et al., 2007), in addition to neonatal rats with induced hypothyroidism, which interestingly also demonstrate an inability to adapt to changing environmental contingencies (Sadamatsu et al., 2006) as do HB-GAM knockouts. Though an unequivocal understanding of the physiological mechanisms underlying these phenotypic abnormalities has yet to be reached, the presence of these features have been used to qualify these animals as potential models of the PDDs.

Physiological explanations for anxious and reticent behavior in HB-GAM knockouts, whether or not in response to novelty, are limited. Previous research suggests that disruption of midkine, a protein homologous to HB-GAM, is associated with heightened anxiety in mice (Nakamura et al. 1998). Additional research corroborates the suggestion that molecules such as HB-GAM are responsible for early developmental processes that modulate the activity of neurobiological systems underlying fear and anxiety in addition to those considered responsible for learning and memory (Manabe et al., 1993; Stork et al., 1999).

Regardless of the underlying mechanism, the possibility of novelty-induced anxiety in HB-GAM knockouts is further supported by the presence of cognitive rigidity and behavioral inflexibility in these animals, as perseveration and neophobic anxiety are co-morbid in both human psychopathologies (DSM-IV-TR; 2000) and in putative animal models of those pathologies (cf. Sadamatsu et al., 2006), facts that strongly suggest phenotypic association between these features. Indeed, neophobia in the knockouts complements the cognitive inflexibility these animals demonstrated in the second version of the Morris water maze in which they were tested and in the Y-maze because normal performance in these paradigms requires the use of new behavioral and exploratory strategies.

Further assessment of anxiety-related behavior in HB-GAM knockouts using specific paradigms sensitive to novelty-induced anxiety might more fully determine whether these animals' heightened anxiety is based on, associated with, or exacerbated by novel contexts and stimuli. Such paradigms include the novel object test and conditioned fear paradigms that might demonstrate enhanced contextual fear in the knockouts (e.g.

van Gaalen & Steckler, 2000). Given their demonstrated cognitive rigidity, HB-GAM knockouts' ability to learn and re-learn context-contingency pairings is also worthy of future investigation.

The current assessment of HB-GAM knockouts' capacity for anxiety and behavior in the face of novelty indicates that these animals possess anxiety and behavioral reticence in the presence of anxiogenic circumstances but not in situations where a ready means of escape to a perceived safe environment or an appetitive stimulus is present. Further, because HB-GAM knockouts' apparent anxiety seems to vanish upon extended exposure to some paradigms such as the open field suggests that their behavioral reticence might be due to neophobia. These data, therefore, support additional phenotypic behaviors associated with the PDDs in HB-GAM knockouts. Given this further indication of a similarity in behavior between HB-GAM knockouts and humans with PDDs, assessment of the social behavior of these animals was conducted because impairments in social functioning are among the most prominent in the PDDs.

CHAPTER 5. SPECIFIC AIM 3

***Specific Aim 3* was to investigate the social behavior of HB-GAM knockouts.**

Deficits in social functioning are among the most commonly observed in the PDDs. These include the inability to infer or make attributions about the mental states of others and deficits in the ability to form meaningful interpersonal relationships (Volkmar et al., 2005).

Social behavior has, therefore, been intensively studied in virtually all potential animal models of the PDDs, and many such models have indeed been so classified on the basis of the presence of social deficits. Many early primate studies (e.g. Bachevalier, 1996) reported social impairments following lesioning of the amygdala, but these studies failed to replicate other PDD-like features in these animals.

Later studies attempting to experimentally manipulate endogenous biochemicals in animals have reported social deficits as well as other PDD-like characteristics in such animals. For example, BDV infection, a putative immunological cause of PDDs, reportedly results in abnormal, reduced social interaction in addition to cerebellar hypoplasia in neonatal rats (for review, see Lancaster et al., 2007). Similarly, inappropriate or reduced social behavior has been reported in conjunction with an additional PDD-like feature in experimental animals, such as those treated with a neonatal block of gastrin-releasing peptide (with reduced novel object recognition; Presti-Torres et al., 2007) and those with experimentally depleted forebrain serotonin (with impulsive, aggressive, and repetitive behavior; Boylan et al., 2007).

A multitude of potential knockout animal models of the PDDs demonstrate social impairment but no other PDD-like features, including mice deficient for *Dvl1* (abnormal

home cage social behavior, reduced cuddling and nest building; Lijam et al., 1997), the vasopressin V1a receptor gene (abnormal social interaction; Egashira et al., 2007), and oxytocin-promoting genes (social memory impairment; Ferguson et al., 2000; reduced vocalization during period of maternal and peer separation; Winslow et al., 2000).

Interestingly, a number of other potential knockout animal models of the PDDs demonstrate social deficits in addition to other anomalies reminiscent of those observed in the PDDs. Among these are *Pten* knockouts, who demonstrate reductions in social interaction, social preference, social learning, sexual behavior and maternal care (Greer & Wynshaw-Boris, 2006) in addition to exaggerated reactions to sensory stimuli, heightened anxiety, decreased learning, seizures, and frontal macrocephaly (Kwon et al., 2006). In some PDD-affected persons, *Pten* mutations have been linked to macrocephaly (Herman et al., 2007). Similarly, animals null for *Fmr1*, another gene shown to be abnormal in some individuals with PDDs, show decreased preference for social novelty and inappropriate social interaction along with cognitive rigidity, deficits in fear conditioning, and seizures (for review, see Bernardet & Crusio, 2006). Similar social deficits in conjunction with other PDD-like traits have been observed in CADPS2 knockout mice (with neophobia and cerebellar hypoplasia; Sadakata et al., 2007) as well as in animals null for the *MECP2* gene, mutations of which are the putative cause of most cases of Rett's disorder (with repetitive movements and learning impairments; Moretti et al., 2005).

In order to further establish the validity of HB-GAM knockouts as a potential model of the PDDs, these animals were exposed to paradigms assessing social behaviors shown to be abnormal in other potential models of these disorders. Specifically, animals

were tested in the social interaction paradigm, which assesses the quality, quantity, and appropriateness of social behavior, and in the social approach paradigm, which assesses animals' sociability, social memory, and preference for social novelty.

Specific Methods

Social Interaction. In one of many variations on this basic paradigm (for review, see File & Seth, 2003), animals were placed on an apparatus, colored black and without delimiting walls, measuring 48 x 48 cm. The surface was divided into 25 grids, each measuring 9.6 x 9.6 cm. Fifteen seconds prior to animals' introduction to the apparatus, a novel, wild type mouse was placed in the center of the apparatus. The test and novel animals were allowed to freely explore the apparatus for five minutes. The number of face to face and face to body contacts initiated by animals was recorded. The number of aggressive acts initiated and instances of cuddling (animals lying together closely and non-aggressively) were also recorded, as was following behavior (considered inappropriate behavior, and quantified by the number of grids across which one animal followed the other). Test animals' latency in seconds to initiate first contact with the novel animal was recorded in a subset of animals as a pilot measure. Because of occasional difficulty in determining which of the two animals initiated an action leading to social contact or an aggressive exchange, only those observations that were agreed on by more than one experimenter were included in statistical analyses.

Social Approach. As described by Moy et al. (2004), this apparatus consisted of three chambers, each measuring 20 x 40.5 x 22 cm and separated by clear Plexiglas walls with small, closeable portals measuring 3.5 cm in diameter. In the *sociability test*, animals were placed in the center chamber and allowed to freely explore this section of

the apparatus for five minutes, during which access to the adjoining chambers was not permitted. After this acclimation period, a novel wild type mouse (stranger 1) was placed in one of the two adjoining chambers. This placement was systematically alternated between trials. The stranger was enclosed in a cylinder of wire mesh measuring 11 cm high and 10.5 cm in diameter with bars spaced 1 cm apart to permit nose contact between the test and novel animals. A small weight (10 g) was placed atop the mesh cylinder to provide stability. The stranger had been habituated to the cylinder for five minutes prior to its introduction to the apparatus. An identical but empty wire mesh cylinder and stabilizing weight were placed in the adjoining, empty chamber to balance the novelty of the two chambers. The portals to the adjoining chambers were then opened and test animals were allowed to freely explore the entire apparatus for 10 minutes. The amount of time animals spent in, and the number of entries made into, the center, empty, and stranger 1 chambers, respectively, was recorded. Normal animals are expected to demonstrate a preference for social interaction as evidenced by increased time spent in the stranger 1 chamber relative to the center and empty chambers.

Following this test of sociability, animals were briefly returned to the center of the apparatus and the portals to the adjoining chambers were closed for the *social novelty test*. At this time, a second novel wild type mouse (stranger 2), also enclosed in and adequately acclimated to a wire mesh cylinder as previously described, was placed in the previously empty chamber. Again, the portals to the adjoining chambers were opened and animals were given 10 minutes to freely explore the apparatus. In order to assess animals' preference for interaction with a new stranger over the stranger previously presented in the sociability test housed in the opposite chamber, the amount of time animals spent in,

and the number of entries made into, each chamber (that housing either stranger 1 or stranger 2) was recorded, as was the amount of time animals spent in the center chamber. Consistent with rodents' demonstrated preference for social novelty, normal animals are expected to spend more time in the chamber housing the more novel second stranger than in the center chamber or the chamber housing the less novel first stranger. In addition, the number of entries made into the empty and novel animal chambers was recorded during both phases of testing in the social approach paradigm as a measure of locomotor and exploratory behavior.

Results

Social Interaction. HB-GAM knockouts and wild type mice did not differ significantly in the number of face-to-face contacts (t (35) = .084, p = .933, Figure 13A) or face-to-body contacts (t (35) = .393, p = .697, Figure 13B) initiated with the novel animal. No cuddling, aggressive behavior, or inappropriate behavior (e.g. the test animal climbing over the novel animal) was observed. Following behavior was observed in only a very small number of animals (~ 20%) and, therefore, was not analyzed. Overall, these data suggest similar quality and quantity of social interaction among HB-GAM knockouts and wild type mice. Among the subset of animals (n = 7) for which latency to first contact with the novel animal was recorded, HB-GAM knockouts did not require significantly more time to initiate their first contact with the novel animal than did wild-type mice, though a statistical trend in the expected direction was found (t (5) = 1.95, p = .108, Figure 13C).

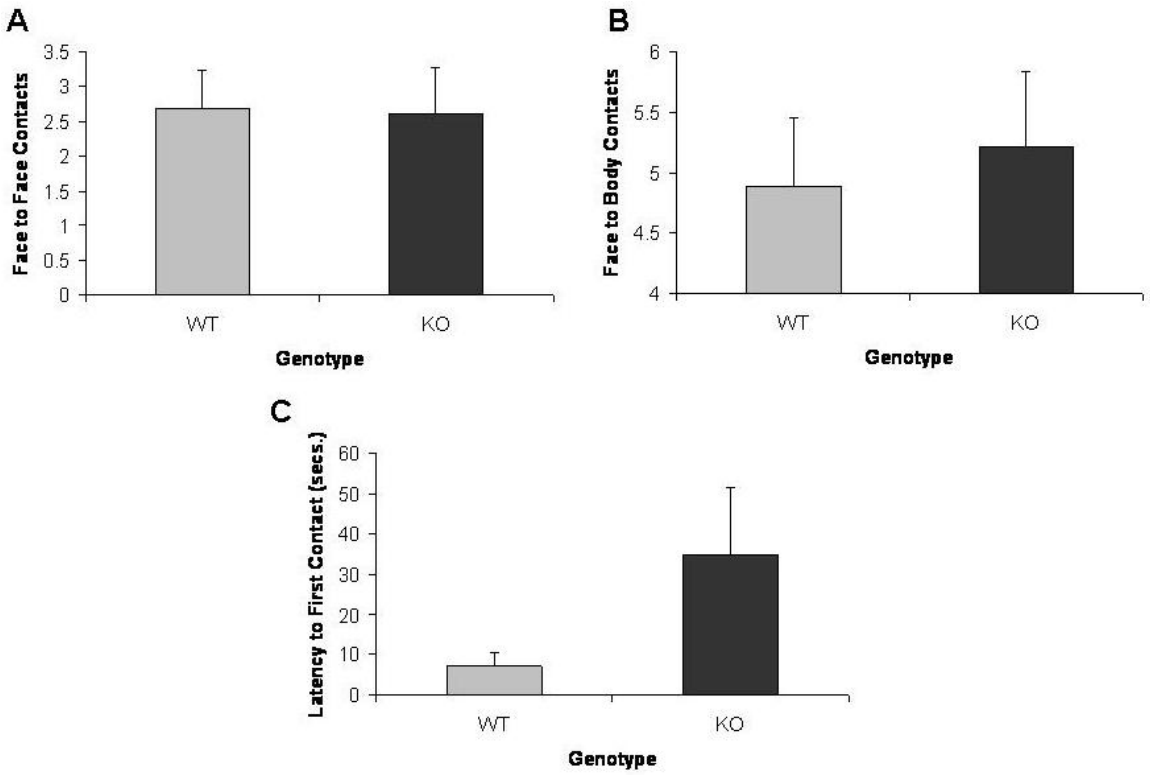

Figure 13. Number of face-to-face contacts (A) and face-to-body contacts (B) initiated by HB-GAM knockouts (n = 18) and wild type mice (n = 19), and the mean latency to initiate first contact (C) for a subset of HB-GAM knockouts (n = 3) and wild type mice (n = 4), in the social interaction paradigm.

Social Approach: Sociability Test. HB-GAM knockouts spent significantly less time in the stranger chamber (t (18) = 2.73, p = .011, Figure 14A) than did wild type mice, however, HB-GAM knockouts and wild type mice did not differ significantly in time spent in the empty chamber (t (21) = .233, p = .819, Figure 14B). HB-GAM knockouts spent significantly more time in the center chamber (t (18) = 2.45, p = .021, Figure 14C). These data suggest that HB-GAM knockouts demonstrated the greatest preference for the center chamber, to which they were acclimated prior to testing, at the expense of time exploring the adjoining, novel chambers. HB-GAM knockouts and wild

type mice did not differ significantly in total number of chamber entries (t (18) = .707, p = .488, Figure 14D), suggesting similar locomotor and exploratory behavior between the genotypes.

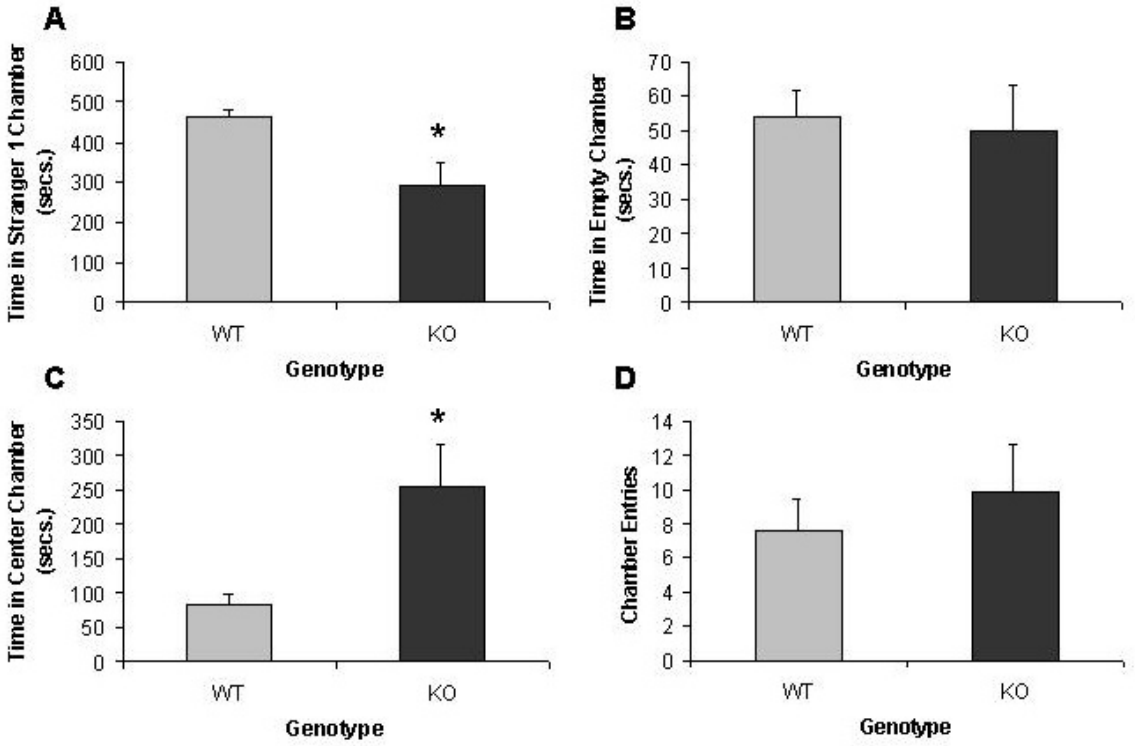

Figure 14. Time spent in the stranger chamber (A), empty chamber (B), and center chamber (C), and the total number of chamber entries made (D), during the sociability test in the social approach paradigm by HB-GAM knockouts (n = 11) and wild type mice (n = 9). Note that outliers were removed: total chamber entries (KO n = 10, WT n = 9).

Social Approach: Social Novelty Test. HB-GAM knockouts and wild type mice did not differ significantly in time spent in the stranger 1 chamber (t (18) = 1.37, p = .188, Figure 15A) or in time spent in the center chamber (t (18) = 1.40, p = .183, Figure 15C). These data suggest increased exploration of the previously novel first stranger, and

the apparatus in general, by HB-GAM knockouts upon second exposure to the social approach apparatus relative to first exposure. HB-GAM knockouts and wild type mice did not differ significantly in amount of time spent in the stranger 2 chamber (t (18) = 1.88, p = .079, Figure 15B), though a statistical trend in the expected direction was found, suggesting the possibility of reduced preference for social novelty in the knockouts. HB-GAM knockouts and wild type mice did not differ significantly in total number of chamber entries (t (18) = .035, p = .973, Figure 15D), suggesting similar locomotor and exploratory behavior between the genotypes.

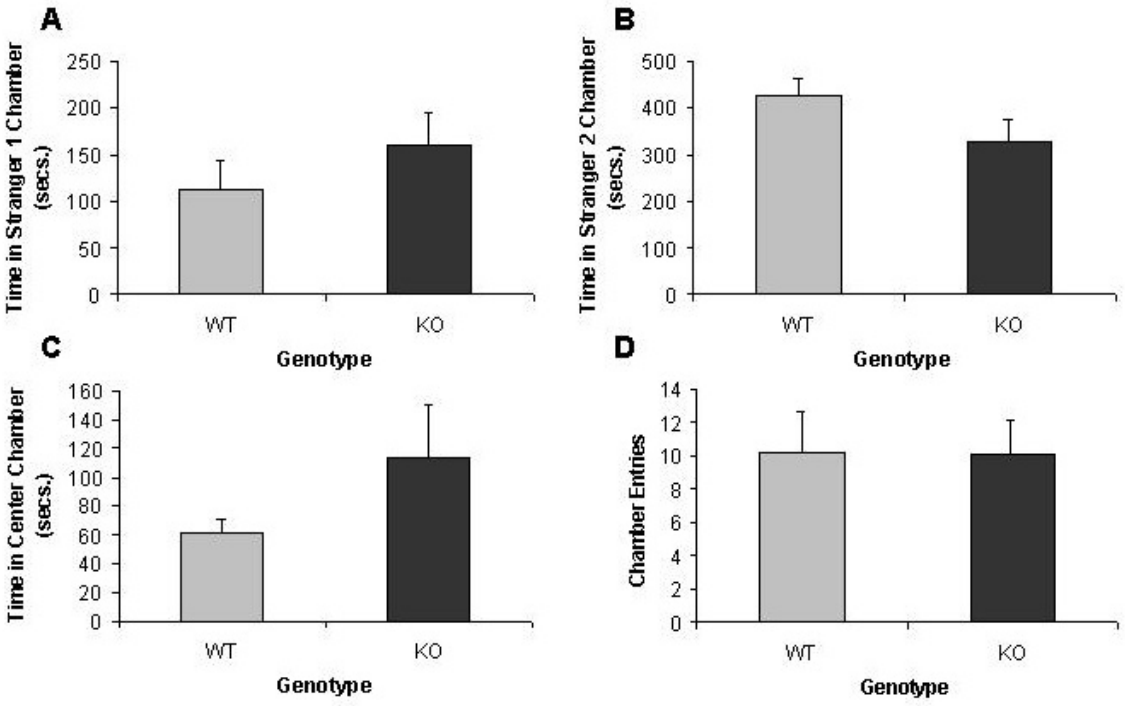

Figure 15. Time spent in the stranger 1 chamber (A), stranger 2 chamber (B), and center chamber (C), and the total number of chamber entries made (D), during the social novelty test in the social approach paradigm by HB-GAM knockouts (n = 11) and wild type mice (n = 9).

Discussion

Because abnormalities in social behavior, such as decreased social memory and decreased preference for social novelty, are commonly associated with the PDDs (DSM-IV-TR; 2000), HB-GAM knockouts' social behavior was assessed. Specifically, animals' quality, quantity, and appropriateness of social behavior were assessed in the social interaction paradigm, and animals' sociability, social memory, and preference for social novelty were assessed in the social approach paradigm.

HB-GAM knockouts and wild type mice did not differ significantly in those behaviors assessed in the social interaction paradigm. The comparable number of face to face and face to body contacts initiated by animals of both genotypes suggests that the knockouts do not possess appreciable abnormalities in the frequency of behaviors traditionally associated with social interaction and exploration of a novel conspecific. Similarly, the comparable absence of following and other inappropriate social behavior in both the knockouts and wild type mice indicate that HB-GAM knockouts' behavior in this paradigm is consistent with the conventions of rodent social behavior.

Interestingly, however, the knockouts' latency to initiate their first contact with a novel conspecific in the social interaction paradigm was greater than that of their wild type counterparts, though this difference was only marginally significant. However, animals' latency to first social contact is not traditionally recorded in this paradigm, and so these data were recorded in only 16 percent of animals as a pilot measure because of HB-GAM knockouts' increased latency to behave appropriately in some other behavioral paradigms. Given this small sample size, even the knockouts' marginally significant increase in latency to first social contact is notable and suggests that further testing with a

larger sample might yield significant findings. Indeed, these data are consistent with HB-GAM knockouts' increased latency to act appropriately in those other novel paradigms, such as the visual cliff, involving an open environment and/or novel stimuli.

Therefore, the knockouts' increased latency to initiate social contact in the social interaction paradigm suggests that a novel conspecific was a stimulus sufficiently novel to induce behavioral reticence in these animals. Further, the knockouts' social behavior in this paradigm was otherwise comparable to that of wild type mice, suggesting that the knockouts' behavior might normalize upon extended exposure to novel environments and novel conspecifics, findings also consistent with these animals' behavior in other paradigms like the open field.

Upon sociability testing in the social approach paradigm, which provides animals a choice as to whether to engage in social interaction, HB-GAM knockouts spent significantly less time in a chamber containing a novel conspecific than did wild type mice. These findings seem to imply a decreased preference for social interaction in the knockouts. However, HB-GAM knockouts spent significantly more time in the center chamber, to which they had been acclimated prior to testing, but they spent a statistically comparable amount of time in the adjoining, empty chamber.

These data indicate that HB-GAM knockouts had the greatest preference for the center chamber, a moderate preference comparable to that of wild type mice for the empty chamber, and the least preference for the chamber housing a novel conspecific. Perhaps not coincidentally, the knockouts' chamber preferences were directly proportional to the degree of novelty of each chamber. That is, acclimation to the center chamber prior to testing made this chamber most familiar to the animals. Further, the

empty chamber, though previously unexplored by the animals, more closely resembled the familiar center chamber than the chamber containing a novel conspecific, which possessed the greatest novelty because of the presence of another, strange animal. Therefore, HB-GAM knockouts' activity during sociability testing in the social approach paradigm might be more attributable to reticence in the presence of novelty than to a decreased preference for social interaction.

Such a conclusion is supported by HB-GAM knockouts' behavior during social novelty testing in the social approach paradigm, which occurred immediately following sociability testing, a total of 15 minutes after test animals' introduction to the apparatus. During this test, HB-GAM knockouts and wild type mice did not differ significantly in time spent in the center chamber or in time spent in the chamber housing the first novel conspecific placed in the apparatus during the sociability test.

These findings support the notion that HB-GAM knockouts exhibited neophobic tendencies in the social approach paradigm, as the first novel conspecific in the adjoining chamber was no longer as novel during this second phase of testing in the paradigm. Interestingly, HB-GAM knockouts spent less time in the chamber housing the second, more novel conspecific than did their wild type counterparts, a difference that was marginally significant. This finding is also consistent with the hypothesis that HB-GAM knockouts' behavior in the social approach paradigm was affected primarily by behavioral reticence in the presence of novelty and not by reduced preference for social interaction *per se* or a social deficit such as reduced social memory.

Further, HB-GAM knockouts did not demonstrate impairments in locomotion or general exploration in either portion of testing in the social approach paradigm, as

evidenced by a statistically comparable number of chamber entries made by the knockouts and their wild type counterparts. These data are consistent with the knockouts' performance in other behavioral paradigms and contraindicate the attribution of differences between the genotypes on social approach measures to motor or exploration deficits.

Assessment of social behavior in HB-GAM knockouts indicated that these animals do not demonstrate reduced frequency of normal social behavior, inappropriate social behavior, or lack of preference for social interaction *per se*. A plethora of other possible animal models of the PDDs indeed exhibit these abnormalities, such as rats infected with BDV (Lancaster et al., 2007), those treated with a neonatal block of gastrin-releasing peptide (Presti-Torres et al., 2007), and those with experimentally depleted forebrain serotonin (Boylan et al., 2007) as well as animals null for *Pten* (Greer & Wynshaw-Boris, 2006), the *Fmr1* gene (Bernardet & Crusio, 2006), CADPS2 (Sadakata et al., 2007), and the *MECP2* gene (Moretti et al., 2005). However, the knockouts' increased latency to first social contact in the social interaction paradigm, and their reduced time spent in those chambers housing novel conspecifics in the social approach paradigm, suggest behavioral reticence to engage in social contact and social exploration when a conspecific is novel. Therefore, these data are suggestive of neophobia manifested in the social domain. Again, neophobia has also been observed in a number of potential animal models of the PDDs (e.g. Sadakata et al., 2007; Sadamatsu et al., 2006; Tsujino et al., 2007).

The social approach paradigm (as created by Moy et al., 2004) is ideally suited to assess in animals some of the social abnormalities associated with the PDDs, such as

reduced preference for social interaction and social novelty. Indeed, the social approach paradigm was developed based on clinical observation of autistic children's social behavior (Dennis, 2005), which includes reticence to engage in social contact and anxiety when presented with strangers (DSM-IV-TR; 2000). Therefore, the behavior of HB-GAM knockouts in this paradigm might have some implications for the etiology of social function abnormalities in the PDDs. That is, these data support the notion that some of the social impairments observed in PDD-affected individuals might be secondary to neophobia.

Nevertheless, social behavior in HB-GAM knockouts requires further examination. HB-GAM is strongly expressed in both limbic cortex and olfactory bulb (e.g. Amet et al., 2001), regions that play vital roles in neural circuits underlying rodent social behavior (e.g. Ricceri, Moles & Crawley, 2007). Therefore, the hypothesis that an absence of HB-GAM throughout development and adulthood could lead to disruption of normal social or affiliative behavior is worthy of further testing.

Such a hypothesis cannot be fully addressed with the data obtained in the social interaction and social approach paradigms, despite their standard use as part of test batteries assessing social behavior in rodents (Crawley, 2000, 2004, 2008). For example, naturalistic social and affiliative behavior in rodents can be abnormal in the presence of normal social behavior in standard social testing paradigms (Crawley, 2000, 2004; Ricceri et al., 2007). Therefore, observation of abnormal social behavior in rodents is arguably more feasible via assessment of naturalistic behavior rather than animals' responses to presented stimuli in experimental paradigms. For example, assessment of the knockouts' home cage behavior at various points during their development and adulthood

might reveal abnormalities in social behavior not readily evidenced in less ecologically valid paradigms. In addition, testing in social behavioral paradigms that are both unobtrusive and ecologically valid, such as the visible burrow system (Arakawa, Arakawa & Blanchard, 2007) could characterize the knockouts' behavior in a naturalistic social environment as well as their role in the social hierarchy of that environment. Similarly, because rodents make extensive use of ultrasonic vocalizations (UVs; for brief review, see Ricceri et al., 2007) that might modulate social behavior across a variety of relationships, examination of UVs in HB-GAM knockouts might reveal abnormalities that could be associated with specific social impairments.

Indeed, qualitative data collected during these studies indicated that pups born to HB-GAM knockout dams survived less frequently than did those born to wild type dams. This finding is not attributable to pups' failure to thrive because HB-GAM knockout newborns survived in numbers comparable to that of their wild type counterparts when cared for by their heterozygous mothers or cross-fostered to wild type dams. Further, observations of defensive burying and avoidance of pups by knockout dams were made repeatedly during the current studies. Therefore, quantitative examination of HB-GAM knockouts' maternal care behavior, as well as their courtship and mating habits, might also yield illuminating data. Indeed, impaired sexual behavior and reduced maternal care have been identified in some possible animal models of the PDDs (e.g. Greer & Wynshaw-Boris, 2006).

Existing data regarding the social behavior of HB-GAM knockouts indicate that these animals do not differ from their wild type counterparts in the quality or quantity of social interaction, nor do these animals exhibit a decreased preference for social

interaction *per se*. However, an increased latency and decreased desire to explore novel conspecifics was evidenced in the knockouts, data that are further suggestive of neophobia.

Though HB-GAM knockouts did not demonstrate some of the social deficits commonly associated with the PDDs, e.g. reduced frequency of social interaction or inappropriate social behavior, the neophobia these animals demonstrated when presented with novel conspecifics is consistent with a behavioral phenotype similar to that observed in humans with PDDs.

Because the PDD phenotype is characterized by neurobiological as well as behavioral aberrations (for reviews, see DiCicco-Bloom et al., 2006; Polleux & Lauder, 2004), and a valid animal model of a human disorder should manifest multiple phenotypic features of the modeled disorder (Fisch, 2007), assessment of HB-GAM knockouts' cerebral cortical microstructure was conducted to more fully assess the phenotypic similarity between these animals and humans with PDDs.

CHAPTER 6. SPECIFIC AIM 4

Specific Aim 4 **was to examine the entorhinal cortical microstructure of HB-GAM knockouts.**

The general PDD phenotype is typified by neurobiological as well as behavioral irregularities. Larger overall brain size is widely observed in those with PDDs during the first four to five years of life (Redcay & Courchesne, 2005), but these increases lessen as affected children mature (Sparks et al., 2002). These data suggest that the PDDs are associated with early pathological brain growth followed by an equally abnormal arrest in development (Redcay & Courchesne, 2005).

Early brain enlargement in the PDDs reflects increases in both the gray and white matter of the cerebral cortex (Hazlett et al., 2005) and cerebellum (Courchesne et al., 2001). PDD-associated macrocephaly might also be due to the excessively numerous, tightly packed cortical minicolumns of reduced width that have been observed in those with PDDs (cf. Casanova et al., 2006).

Microscopic neural cellular abnormalities have also been identified in PDD-affected individuals, including decreased cerebellar Purkinje cells, dysplasia of the brainstem and olives, hippocampal deformation, cerebral cortical dysgenesis, and misorientation of neocortical pyramidal neurons (for reviews, see DiCicco-Bloom et al., 2006; Polleux & Lauder, 2004). These microstructural aberrations cannot fully account for PDD-associated macrocephaly. Instead, macrocephaly in the PDDs is more likely due to an increase in cortical neuron density and number, which has been observed in affected individuals (Polleux & Lauder, 2004). Interestingly, HB-GAM knockouts possess a similar neuronal hyperplasia in frontal and parietal cortices (Hienola et al., 2002, 2004).

The PDDs have also been associated with abnormally small neurons in the cerebral cortex (Courchesne et al., 2001) and limbic structures (DiCicco-Bloom et al., 2006), findings consistent with the presence of cortical hyperplasia but largely normal overall brain volume in affected individuals. The brains of adult HB-GAM knockout mice are grossly normal in size (Amet et al., 2001), suggesting that the cortical neuronal hyperplasia they possess would be accompanied by abnormal reduction in neuron size, as in humans with PDDs.

Neuroanatomical assessment of proposed animal models of the PDDs is indeed sensible given the large body of research associating these disorders with neurological abnormalities. Some potential animal models of the PDDs display cerebellar hypoplasia similar to that observed in affected individuals. Specifically, rodents infected with BDV postnatally (Lancaster et al., 2007), those treated with VPA either pre- or post-natally (cf. Wagner et al., 2006), and *En2* knockout mice (Kuemerle et al., 2007) have been identified as potential PDD animal models because of these cerebellar anomalies.

However, potential animal models of PDDs that demonstrate both neurobiological and behavioral phenotypic characteristics of these disorders are scarce. However, mice null for *Pten*, mutations of which have been linked to PDD-associated macrocephaly in a subset of affected individuals (Herman et al., 2007), demonstrate learning impairments and heightened anxiety as well as frontal macrocephaly (Kwon et al., 2006). *Pten* knockouts are unique among animal models of the PDDs because they display both neurobiological and behavioral features of these disorders. However, an animal should ideally demonstrate multiple phenotypic features of a condition in order to have full construct validity as a model of that condition (Hau & Van Hoosier, 2004).

HB-GAM knockouts indeed demonstrate both behavioral and cortical neuronal abnormalities reminiscent of those observed in the PDDs. HB-GAM knockouts exhibit grossly normal brain size (Amet et al., 2001) but increased neuronal density in frontal and parietal cortices (Hienola et al., 2002, 2004), findings similar to those in adults with PDDs (e.g. Courchesne et al., 2001; Courchesne & Pierce, 2005a). In an attempt to further characterize the neural cellular phenotype of HB-GAM knockouts, assessment of these animals' cerebral cortical microstructure, specifically, neuronal area, inter-neuronal distance, and cortical thickness, was conducted. These assessments were conducted in the deep layers of the lateral entorhinal cortex because structural anomalies in this area that correlate with behavioral impairments have been identified in those with PDDs (for review, see Salmond et al., 2005). In addition, this region maintains robust connectivity with the amygdala (for brief review, see Hoistad & Barbas, 2008) and has been implicated conceptually (e.g. Gray & McNaughton, 2000) and empirically (Otto, Cousens & Herzog, 2000) as a contributor to rodent affective responses. Therefore, a structural analysis of the deep layers of the lateral entorhinal cortex was indicated by the increased anxiety and neophobia demonstrated by HB-GAM knockouts.

Specific Methods

Animal Sacrifices. Animals were deeply anesthetized and overdosed with chloral hydrate-pentobarbital, transcardially exsanguinated with heparinized isotonic (0.9%) saline, and perfusion fixed with 4% paraformaldehyde, first in low pH buffer (acetate, 100 ml) and then in high pH buffer (borate, 100 ml). After fixation, brains were removed and placed in 30% sucrose in borate buffer for three to seven days at 4 °C.

Tissue Processing. Following fixation and immersion in borate buffer, mouse brains were frozen and sectioned at 30 μm on the coronal plane using a sliding microtome. Frozen sections were stored at –20 °C in cryoprotectant solution as in Watson, Wiegand, Clogh & Hoffman (1986) until mounted on gelatin-coated slides and stained with cresyl violet for evaluation of entorhinal cortical microstructure.

Tissue Analysis. Brain sections were placed under an Olympus BX51 microscope, and the Neurolucida image analysis software (version 8.001, MicroBrightfield BioSciences, Williston, VT) was used to obtain neuronal area in squared micrometers in layers IV and V of the lateral entorhinal cortex. Approximately 10 – 20 pseudo-randomly selected cells were measured per frame bilaterally (~ 20 – 40 cells per animal).

Assessment of inter-neuronal distance was made using Image J software (National Institutes of Health, Bethesda, MD), and those images were used for quantification of neuronal area. A standard number of neurons (approximately 20 per animal) met by points on a stereology grid were randomly chosen for measurement. Inter-neuronal distance was defined as the distance in micrometers between the center of a randomly chosen neuron to the center of its closest neighboring neuron in the same plane of focus.

To assess lateral entorhinal cortical thickness, three images per animal were acquired by a Diagnostic Instruments 320 video camera (Diagnostic Instruments, Sterling Heights, MI) attached to a Nikon microscope (Morell Instruments, Melville, NY). Images were transferred to a computer using Spot Digital Imaging software (Diagnostic Instruments, Sterling Heights, MI) and analyzed using Image J software (National Institutes of Health, Bethesda, MD). All measures were taken by experimenters blind to animal genotype.

Predictors of Cortical Thickness. Pearson's correlation coefficients were calculated to assess the association between entorhinal cortical thickness and the following variables: entorhinal cortical neuronal areas in layers IV and V, respectively, and entorhinal cortical inter-neuronal distance in layers IV and V, respectively. In addition, a multiple regression was used to determine these variables' combined strength as predictors of entorhinal cortical thickness.

Results

Neuronal Area. Figure 16 shows representative photomicrographs of cells in layers IV and V of the entorhinal cortex in an HB-GAM knockout and a wild type mouse. Neuronal area in layer IV of the entorhinal cortex was significantly smaller in HB-GAM knockouts than in wild type mice (t (16) = 3.519, p = .003, Figure 17A). However, neuronal area in layer V of the entorhinal cortex did not differ significantly between the genotypes (t (17) = .125, p = .902, Figure 17B).

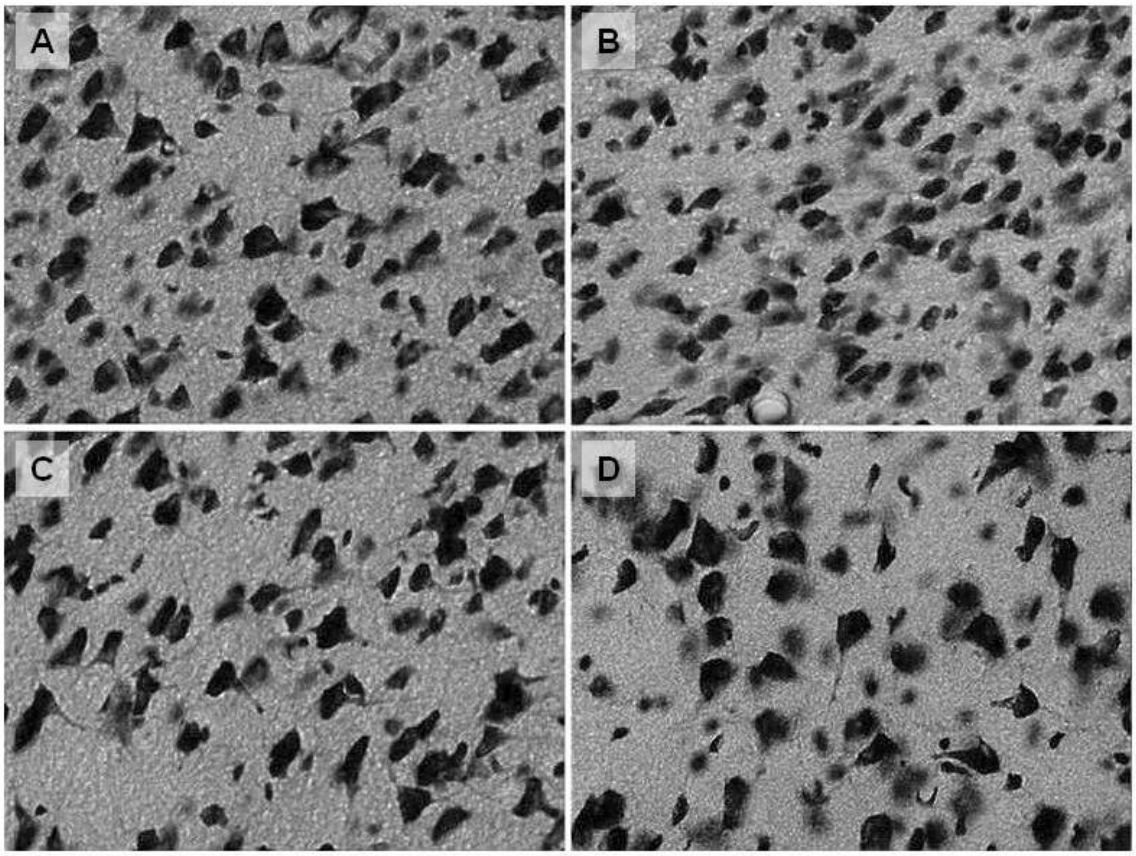

Figure 16. Nissl-stained sections of layer IV (wild type mouse, A; HB-GAM knockout, B) and layer V (wild type mouse, C; HB-GAM knockout, D) of entorhinal cortical tissue.

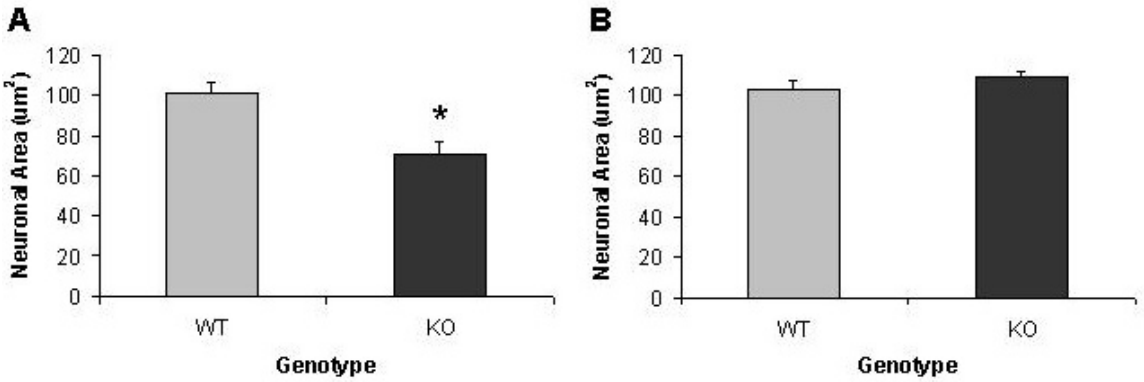

Figure 17. Neuronal area in layer IV (A) and layer V (B) of the entorhinal cortex in HB-GAM knockouts (n = 9) and wild type mice (n = 10).

Inter-neuronal Distance. Inter-neuronal distance in layer IV of the entorhinal cortex was significantly smaller in HB-GAM knockouts than in wild type mice (t (17) = 2.24, p = .038, Figure 18A). Similarly, inter-neuronal distance in layer V of the entorhinal cortex was also significantly smaller in HB-GAM knockouts than in wild type mice (t (17) = 3.221, p = .007, Figure 18B). These data suggest the presence of increased neuronal packing density in layers IV and V of the entorhinal cortex in HB-GAM knockouts.

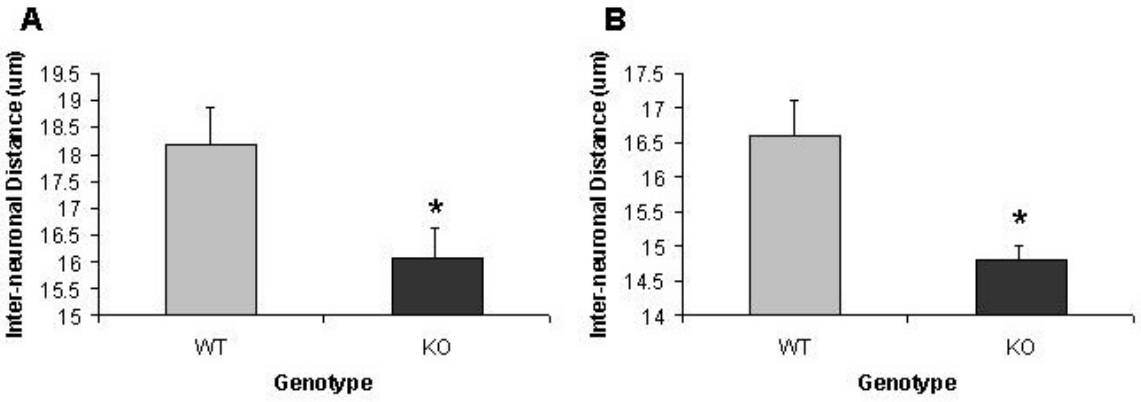

Figure 18. Inter-neuronal distance in layer IV (A) and layer V (B) of the entorhinal cortex in HB-GAM knockouts (n = 9) and wild type mice (n = 10). Y-axis values were chosen based on the range of inter-neuronal distances observed.

Cortical Thickness. Figure 19 shows representative photomicrographs of entorhinal cortex in an HB-GAM knockout and a wild type mouse. Entorhinal cortical thickness did not differ significantly between the genotypes (t (14) = 1.252, p = .232, Figure 19C).

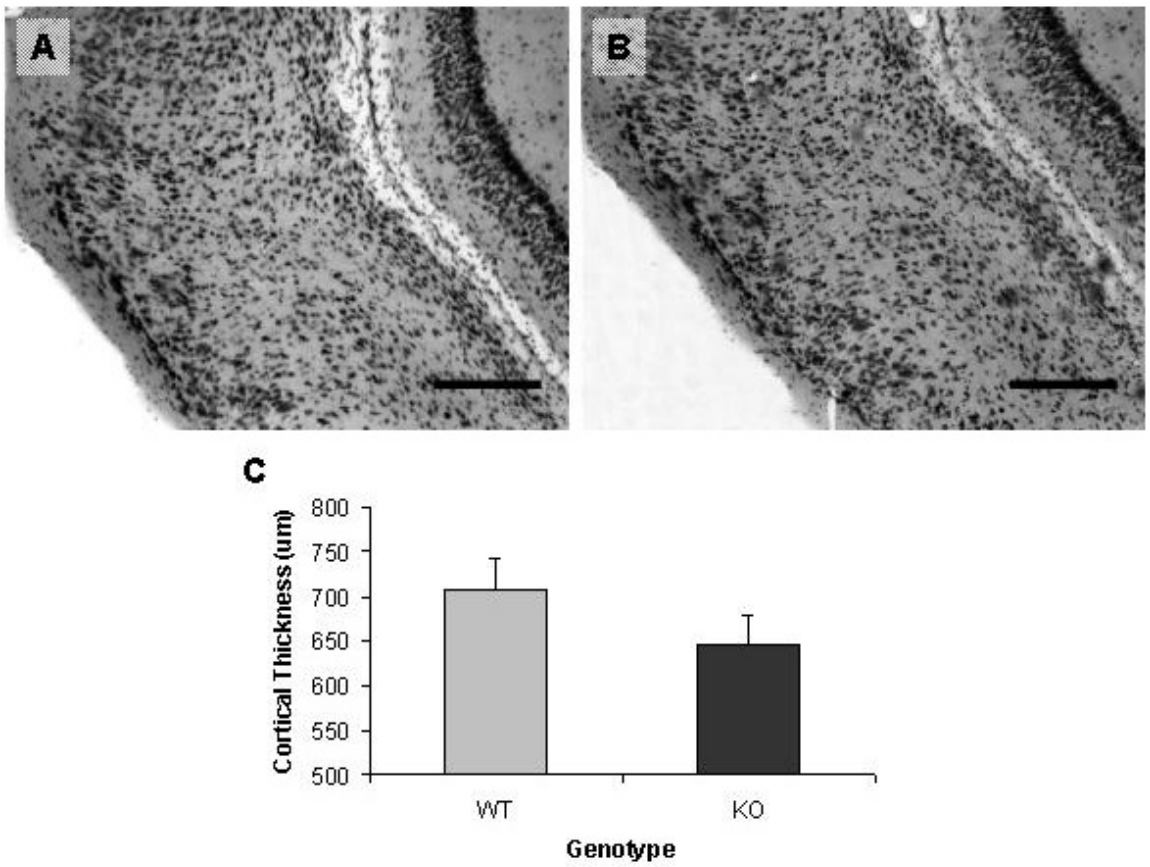

Figure 19. Nissl-stained sections of entorhinal cortical tissue from a wild type mouse (A) and an HB-GAM knockout (B). Scale bar = 250 μm. Entorhinal cortical thickness (C) of HB-GAM knockouts (n = 8) and wild type mice (n = 8).

Predictors of Cortical Thickness. Pearson's correlation coefficients indicated no significant correlations between entorhinal cortical thickness and neuronal area in layer IV (r = .376, p = .151) and layer V (r = .298, p = .263) of the entorhinal cortex. However, significant correlations were revealed between entorhinal cortical thickness and inter-neuronal distance in layer IV (r = .732, p = .001) and layer V (r = .706, p = .002) of the entorhinal cortex. These data suggest that neuronal packing density was a stronger contributor to entorhinal cortical thickness than was neuronal area.

A multiple regression using the combination of neuronal area and inter-neuronal distance in layers IV and V as predictors of entorhinal cortical thickness was significant (r^2 = .823; F (4, 11) = 12.78, p < .000), indicating that 82.3% of the variance in entorhinal cortical thickness was accounted for by the combination of these factors. The strongest predictors of entorhinal cortical thickness were inter-neuronal distance in layer IV (p = .002) and layer V (p = .003), as suggested by bivariate correlations. Neuronal area in layer IV was also a significant predictor (p = .039) of entorhinal cortical thickness, though neuronal area in layer V was not (p = .315).

Discussion

Microscopic neural cellular abnormalities are associated with the PDDs (for reviews, see DiCicco-Bloom et al., 2006; Polleux & Lauder, 2004; Salmond et al., 2005). Further, abnormalities in specific structures, e.g. hypoplasia of the amygdala, have been linked to impairments in functions subserved by those structures, e.g. facial expression recognition (e.g. Nacewicz et al., 2006; Reiss et al., 1996).

Structural anomalies in the entorhinal cortex correlate with affective disturbance and affective processing deficits in PDD-affected individuals (for review, see Salmond et al., 2005). Similarly, this region has been implicated as a contributor to rodent affective responses (e.g. Gray & McNaughton, 2000; Otto, Cousens & Herzog, 2000). Therefore, assessment of entorhinal cortical microstructure in HB-GAM knockouts was conducted because of the anxious and neophobia-related behavior exhibited by these animals.

The current data indicate that HB-GAM knockouts possess a significant, mean 30% decrease in neuronal area and significantly decreased inter-neuronal distance in layer IV of the entorhinal cortex as compared to their wild type counterparts. No

significant difference in layer V neuronal area was found between the genotypes. However, layer V inter-neuronal distance was also significantly decreased in the knockouts.

HB-GAM knockouts did not differ significantly from wild type mice in overall entorhinal cortical thickness. However, a multiple regression indicated that a large (~ 82%) portion of the variance in entorhinal cortical thickness was accounted for by neuronal area and inter-neuronal distance in layers IV and V. This result suggests that there might exist different but proportional neuronal area and inter-neuronal distance irregularities in other layers of the HB-GAM knockouts' entorhinal cortex or that these animals possess abnormalities in these layers exclusively. The finding of comparable overall entorhinal cortical thickness between the knockouts and wild type mice might suggest the latter and indicate the presence of subtle, layer-specific neuronal irregularities that are not detectable via gross measures of overall cortical morphology.

Collectively, these data are consistent with the observation of grossly normal brain size (Amet et al., 2001) but increased neuronal density in the frontal and parietal cortices of HB-GAM knockouts (Hienola et al., 2002, 2004). Perhaps more interestingly, decreased neuronal area and increased neuronal density have been observed in a number of structures, including the frontal and entorhinal cortices, in those with PDDs (e.g. Courchesne et al., 2001; Courchesne & Pierce, 2005a). In addition, the gray matter density of rhinal structures has been positively correlated with ratings of the severity of PDD symptoms, particularly those with an affective component (e.g. Salmond et al., 2005).

Indeed, in addition to playing a strong role in declarative memory (cf. Zola-Morgan, Squire, Amaral & Suzuki, 1989) via its hippocampal connections (e.g. van Groen , Miettinen & Kadish, 2003; Witter & Amaral, 1991), the entorhinal cortex might participate in a number of affective processes. In primates, the entorhinal cortex maintains reciprocal connections with the amygdala, which itself projects to the orbitofrontal cortex (e.g. Hoistad & Barbas, 2008). In addition, the entorhinal cortex receives input from unimodal sensory cortices, polymodal sensory areas, and the orbitofrontal cortex. This anatomical organization suggests multiple roles for the entorhinal cortex in modulation of emotional memory formation via its amygdalar and hippocampal connections, affective labeling of stimuli via projections to and feedback from the amygdala, and modulation of behavioral responses to affective stimuli via projections to the amygdala-orbitofrontal cortical duo (e.g. de Curtis & Pare, 2004; Hoistad & Barbas, 2008; Meunier, Cirilli & Bachevalier, 2006).

Spatial learning impairments have been repeatedly demonstrated following entorhinal cortex lesions in mice (Hardman et al., 1997), though these observations constitute the virtual limit of available empirical data on the cognitive and behavioral effects of such lesions in these animals. Indeed, empirical data on the role of the entorhinal cortex in affective processes in mice is limited to data indicating disrupted recognition of pup UVs in mouse mothers that is suggestive of impaired processing of affectively relevant stimuli (Koch & Ehret, 1991).

Similarly, data obtained in primates suggests that entorhinal lesions result in subtle alterations of behavioral responses to affectively salient stimuli, including decreased approach and increased defensive behaviors in response to affectively positive

and neutral stimuli independent of memory impairment. Consequently, primates with entorhinal lesions apparently tend to make negative evaluations of affective stimuli in general, overestimating the risk inherent to interacting with these stimuli (Meunier et al., 2006). Disruption of rhinal areas' modulation of hippocampus-amygdala interactions was postulated as underlying this aberration based on the notion that the hippocampus inhibits approach to affective stimuli by increasing the weight of affectively negative information during approach-avoidance conflicts (Gray & McNaughton, 2000). This idea is supported by observations that anxiety-induced hyperalgesia is associated with entorhinal-hippocampal circuit activation in humans (Ploghaus et al., 2001).

The entorhinal cortex receives a variety of neural inputs underlying sensory perception, memory, and affective states, and might therefore modulate these inputs to form a cognitive appraisal of the affective meaning of stimuli that, in turn, guides behavioral responses to those stimuli. Therefore, decreased neuronal area and inter-neuronal distance in layers IV and V of the entorhinal cortex in HB-GAM knockouts might contribute to the heightened anxiety and neophobia exhibited by these animals.

Cell assemblages comprised of undersized neurons might be less able to relay signals across neural distances, resulting in disruptions in neural integration that would likely be associated with absent, delayed, and/or inappropriate behavioral responses to stimuli (Kana et al., 2006; Laughlin & Sejnowski, 2003). Similarly, disordered neural cellular organization, which is associated with increased neuronal density, might also lead to excessive local connectivity at the expense of long distance cerebral connectivity (Courchesne & Pierce, 2005b). Given the suggested role of the entorhinal cortex in rodent affective processes, the microscopic entorhinal abnormalities identified in HB-

GAM knockouts might lead to disruption of neural processes that allow for appropriate appraisals of, and behavioral responses to, affectively salient stimuli and situations like those experienced by these animals in novel behavioral paradigms.

Further, because the knockouts' apparently anxiety-driven behavioral reticence appears to vanish upon extended exposure to some paradigms, these animals might need more time to process novel stimuli and contexts, generate a cognitive appraisal of these inputs, and behave accordingly. Such increased processing time would indeed be needed if relevant neural circuits were disrupted, which would be expected if the entorhinal cortex were unable to engage in normal neural signal integration and transmission. Testing this hypothesis would require experimental manipulation of the entorhinal cortex in mice to determine whether disruption of this area results in unusual behavior similar to that observed in HB-GAM knockouts.

Regardless of the functional consequences of the microstructural cortical abnormalities observed in HB-GAM knockouts, investigation into the etiology of these irregularities is advisable. Neurotrophic and neuroprotective roles for HB-GAM have been proposed, both during development (Hienola et al., 2002, 2004, 2006) and in pathological states (Marchionini et al., 2007; Mi et al., 2007). Reduced neuronal area in layer IV of the entorhinal cortex in HB-GAM knockout brains might, therefore, reflect a loss of HB-GAM's trophic and/or protective effects.

In addition, HB-GAM is known to inhibit cellular proliferation, play a role in radial migration of neural cells during development, and induce differentiation of FGF-responsive cells toward a neuronal phenotype (Hienola et al., 2004, 2006). Therefore, neuronal packing density increases in HB-GAM knockout brains could conceivably be

due to an increase in early cellular proliferation but a decrease in differentiation of these cells, resulting in larger numbers of hypotrophic neurons. Interestingly, however, HB-GAM knockouts have not been shown to possess an increased number of neurons in frontal or parietal cortices, where their neuronal density increases have been observed (Hienola et al., 2004, 2006). These data suggest a more likely role for the loss of HB-GAM's pro-migratory effects in the abnormal neuronal organization observed in the knockouts. Alternatively, increased neuronal density could result from a deficit or delay in apoptosis (Polleux & Lauder, 2004), however, HB-GAM has not been shown to affect apoptotic cell death (Hienola et al., 2004).

Compensatory neurogenesis and elevations of serotonin during development have also been implicated as driving cortical hyperplasia in the PDDs (e.g. Scott & Deneris, 2005). Interestingly, evidence indicating disturbance of those neurodevelopmental processes in which HB-GAM plays a role have been reported in the brains of PDD-affected individuals (e.g. Bailey et al., 1998; Courchesne et al., 2005a; Courchesne & Pierce, 2005). Considering the shared neurobiological phenotype of individuals with PDDs and HB-GAM knockouts, these facts suggest future investigation into whether compensatory neurogenic processes or early elevations of serotonin are present in the developing brains of HB-GAM knockouts.

Similarly, the disruptions in minicolumn structure, number, and density in PDD-affected brains (e.g. Casanova et al., 2006) suggests that analysis of HB-GAM knockouts' cortical minicolumn morphology might reveal further evidence indicative of phenotypic similarity between these animals and individuals with PDDs. In addition, though a number of neural cellular abnormalities have been reported in affected brains

(for reviews, see Polleux & Lauder, 2004; DiCicco-Bloom et al., 2006), cerebellar abnormalities are among the most consistently reported (e.g. Courchesne et al., 2001; Kaufmann et al., 2003), suggesting the importance of future examination of the knockouts' cerebellar microstructure. Further, because the PDDs are also commonly associated with accelerated and then decelerated brain growth in early life (e.g. Redcay & Courchesne, 2005; Salmond et al., 2005; Sparks et al., 2002), study of HB-GAM knockout brains at multiple points during pre- and post-natal development might also be at once enlightening and further supportive of these animals' validity as a model of the PDDs.

Another commonly reported neurobiological feature of the PDDs is reduced or abnormal activation of neural regions and lack of synchronic activation among associated structures (e.g. Baron-Cohen et al., 1999; Zilbovicius et al., 2000). Functional neuroimaging is becoming more feasible and more common in studies using small animals (e.g. Obenaus & Jacobs, 2007). Therefore, functional imaging of HB-GAM knockout brains might help to more fully characterize these animals' neurobiological phenotype and further assess their similarity to PDD-affected individuals. Indeed, the entorhinal neuronal abnormalities these animals possess suggest the possibility of disordered intra- and/or inter-connectivity of this structure. Functional imaging could reveal evidence indicative of such abnormalities while illustrating disruptions in neural circuitry that might underlie the behavioral disturbances observed in these animals.

Analysis of HB-GAM knockouts' entorhinal cortical microstructure revealed significantly decreased neuronal area and significantly decreased inter-neuronal distance in entorhinal cortical layer IV, and no significant neuronal area abnormality but

significantly decreased inter-neuronal distance in entorhinal cortical layer V. These data are not surprising given the suggested developmental roles of HB-GAM, however, it is most likely the loss of HB-GAM's role in neural migration early in prenatal neural development to which the knockouts' neuronal abnormalities should be attributed. Nevertheless, these findings are also consistent with the frontal and parietal cortical microstructural abnormalities reported previously in HB-GAM knockouts and might serve to explain these animals' anxious and neophobic behavior.

These data indicate that HB-GAM knockouts exhibit phenotypic traits similar to individuals with PDDs in both the neuroanatomical and behavioral domains. Among the plethora of proposed animal models of the PDDs, only a limited number share this distinction (e.g. *Pten* knockouts, Kwon et al., 2006; CADPS2 knockouts, Sadakata et al., 2007). Further characterization of HB-GAM knockouts' neuroanatomical phenotype was accomplished via assessment of these animals' cerebral vasculature in light of HB-GAM's involvement in developmental angiogenesis (Christman et al., 2005; Yeh et al., 1998) in addition to findings (e.g. Louissaint et al., 2002; Palmer et al., 2000) suggesting that neuronal abnormalities might be preceded by anomalies of vascular development.

CHAPTER 7. SPECIFIC AIM 5

***Specific Aim 5* was to examine the cerebral vasculature of HB-GAM knockouts.**

Recent work (e.g. Louissaint et al., 2002; Palmer et al., 2000) has indicated that vasculogenesis is developmentally coordinated with neurogenesis, a process that is likely disrupted in those with PDDs as evidenced by the microstructural neuroanatomical abnormalities that have been consistently reported in these individuals (for reviews, see DiCicco-Bloom et al., 2006; Polleux & Lauder, 2004). Though little data exists as to the mechanism by which the synchronization of cerebral vascular and cellular development is orchestrated, developmental vascularization is thought to occur until the metabolic requirements of emerging neuronal populations are satisfied (Louissaint et al., 2002; Palmer et al., 2000).

Therefore, it is possible that neurodevelopmental disorders such as the PDDs are manifestations of associated disruptions in vasculogenesis and neurogenesis or, perhaps, abnormalities in vascular development underlie the disordered neurobiological phenotype of the PDDs. However, understanding of the vascular phenotype associated with the PDDs is currently incomplete. Numerous neuroimaging studies (e.g. Boddaert & Zilbovicius, 2002; Wilcox et al., 2002) indicate cerebral hypoperfusion in those with PDDs, though this finding might represent the result of functional connectivity abnormalities and resultant under-utilization of specific neural regions and not a primary vascular abnormality. Several suspected pathogenic factors in the PDDs have been associated with vascular abnormalities, including prenatal thalidomide exposure with inhibition of developmental angiogenesis (Hallene et al., 2006), mutations of the *HOXA1* gene with vascular malformations (Tischfield et al., 2005), and *Pten* gene mutations with

developmental venous anomalies (Tan et al., 2007). In addition, enhanced oxidative stress, which is associated with abnormal blood flow, has been reported in the PDDs (Yao et al., 2006).

Despite the suggestion that aberrations in vascular development are associated with the PDDs, significant vascular abnormalities have either not been investigated or not demonstrated in potential animal models of these disorders (e.g. Tan et al., 2007). However, convincing data exists to substantiate a link between neural structural development and vasculogenesis, thereby suggesting the possibility that vascular deficits are indeed part of the neurobiological phenotype of the PDDs. In addition, HB-GAM's role in developmental angiogenesis (Christman et al., 2005; Yeh et al., 1998), coupled with findings of neuronal abnormalities in HB-GAM knockouts, further suggests examination of these animals' cerebral vasculature. Therefore, animals' cerebral vasculature, specifically, vascular density and diameter, was assessed in frontal and temporal cortices. These areas were chosen for vascular assessment given the previous finding of significant neuronal abnormalities in frontal cortex (Hienola et al., 2004), and the current data suggestive of temporal cortical abnormalities, in HB-GAM knockout brains.

Specific Methods

Animal Sacrifices. Animals were anesthetized and sacrificed, and their brains removed, as described earlier.

Immunocytochemistry. Mouse brains were stained with alpha collagen-IV (primary antibody: rabbit-anti-mouse alpha collagen-IV, 1:500, Chemicon, Temecula, CA; secondary antibody: goat anti-rabbit, 1:500, R & D Systems, Minneapolis, MN) to

visualize cerebral vasculature basement membranes as in Franciosi et al. (2007), using the general immunostaining protocol described in Morse et al. (1993). Stained sections were then mounted on gelatin-coated slides, which were dehydrated via immersion in ethyl alcohol and cover-slipped after subsequent immersion in xylenes.

Tissue Analysis. Stereology to assess vascular perimeters was performed on alpha collagen IV-stained brain sections after acquisition of three images per region per animal by a video camera (Diagnostic Instruments, Sterling Heights, MI) attached to a Nikon microscope (Morell Instruments, Melville, NY). Images were transferred to a computer using Spot Digital Imaging software (Diagnostic Instruments, Sterling Heights, MI) and analyzed using Image J software (National Institutes of Health, Bethesda, MD).

Vascular density was assessed via point-count stereology, that is, an acetate containing points in a grid pattern was superimposed on images captured from layers V – VI of the frontal cortex (primary motor cortex) and layers V – VI of the temporal cortex (lateral entorhinal cortex). All points that fell on a vessel were counted. Vascular density was defined as the proportion of points falling on alpha collagen IV-positive tissue across all three images to the total number of points in the grid multiplied by three.

To assess blood vessel diameter, the same acetate was overlaid on those images utilized for quantification of vascular density. A standard number of vessels (approximately 20 – 30 per animal) met by points on the grid were randomly chosen for measurement, which was performed using Image J software. All measures were taken by experimenters blind to animal genotype.

Results

Vascular Density. Figure 20 shows representative photomicrographs of vasculature in layers V – VI of the entorhinal cortex in an HB-GAM knockout and a wild type mouse. The density of frontal cortical vasculature did not differ significantly between HB-GAM knockouts and wild type mice (t (11) = 1.46, p = .173, Figure 21A). Similarly, the density of temporal cortical vasculature did not differ significantly between the genotypes (t (11) = .386, p = .707, Figure 21B).

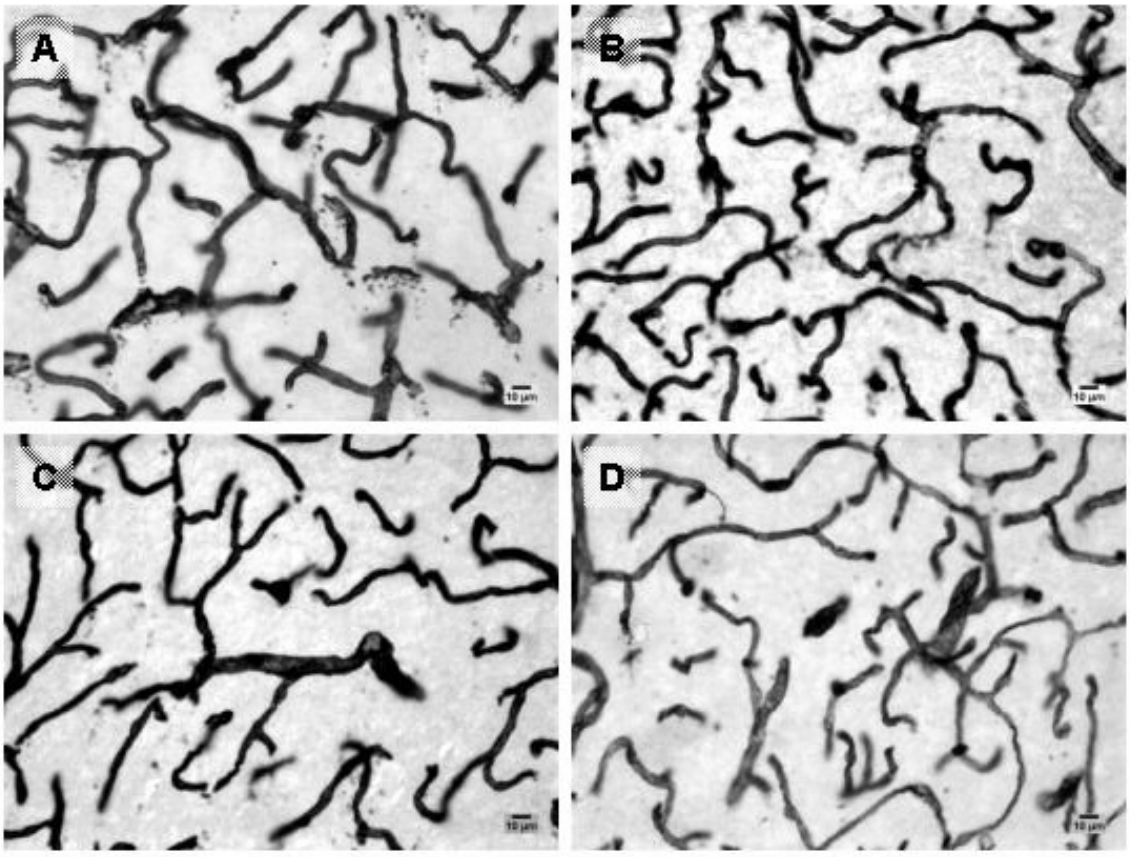

Figure 20. Alpha collagen IV-stained sections of frontal cortical tissue (wild type mouse, A; HB-GAM knockout, B) and temporal cortical tissue (wild type mouse, C; HB-GAM knockout, D). Scale bar = 10 μm.

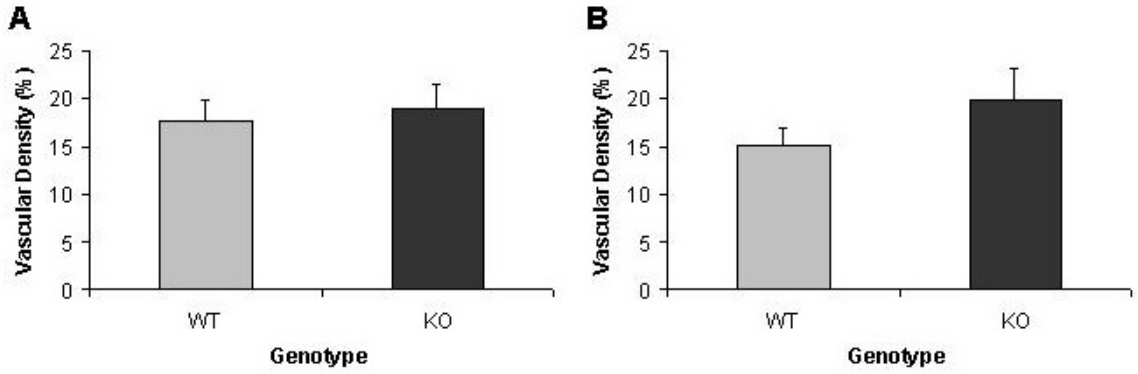

Figure 21. Vascular density in frontal cortex (A) and temporal cortex (B) in HB-GAM knockouts (n = 5) and wild type mice (n = 8).

Vascular Diameter. The diameter of frontal cortical blood vessels did not differ significantly between HB-GAM knockouts and wild type mice (t (11) = .350, p = .733, Figure 22A). Similarly, the diameter of temporal cortical blood vessels did not differ significantly between the genotypes (t (11) = .760, p = .463, Figure 22B).

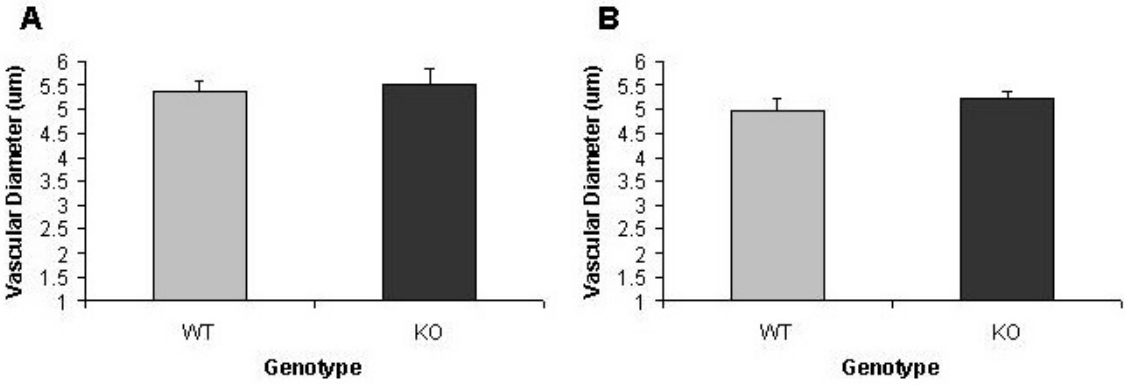

Figure 22. Vascular diameter in frontal cortex (A) and temporal cortex (B) in HB-GAM knockouts (n = 5) and wild type mice (n = 8).

Discussion

The apparent link between neural structural development and vasculogenesis (e.g. Louissaint et al., 2002; Palmer et al., 2000) suggests the possibility that the PDDs could be associated with vascular defects that either underlie or accompany the neural structural abnormalities observed in these disorders. Given the similarity between the neuronal abnormalities observed in the brains of those with PDDs and HB-GAM knockouts, it is possible that such irregularities in these animals are accompanied by vascular anomalies. HB-GAM's known role in developmental angiogenesis (Christman et al., 2005; Yeh et al., 1998) further warrants study of cerebral cortical vasculature in HB-GAM knockouts. Therefore, animals' frontal and temporal cortical vasculature was assessed because of indications of neuronal abnormalities in the frontal (Hienola et al., 2004) and temporal (see Chapter 6) cortices of HB-GAM knockouts.

Measures of vascular density and diameter did not differ significantly between HB-GAM knockouts and their wild type counterparts in either the frontal or temporal cortex. Similarly, no qualitative observations of gross vascular differences, e.g. in blood vessel tortuosity, between the genotypes were apparent. These data are somewhat unexpected given HB-GAM's role in developmental angiogenesis (Christman et al., 2005; Yeh et al., 1998). However, the notion that some as yet unknown compensatory mechanism aids in the preservation of vascular development in the absence of HB-GAM is certainly feasible.

The finding of relatively normal cerebral vasculature in HB-GAM knockouts is also noteworthy in the context of the apparent association between neurogenesis and vasculogenesis (e.g. Louissaint et al., 2002; Palmer et al., 2000). The condition of

vasculature is thought to have a primary impact on progenitor cell proliferation and neuronal differentiation in both developmental and pathological states (e.g. Teng et al., 2008). Therefore, the presence of normal cerebral cortical vasculature but increased neuronal packing density and decreased neuronal area in specific cortical areas of the knockouts' brains indicates that the latter irregularities are driven by subtle and/or transitory vascular defects or are independent of the state of cerebral vasculature. Therefore, these data suggest that the neuronal abnormalities observed in HB-GAM knockouts are more likely the result of the absence of HB-GAM's effect on radial migration and cellular differentiation during development. Finally, further examination of HB-GAM knockouts' cerebral vasculature could be conducted in other neural regions, particularly those in which neural abnormalities have been identified in the PDDs, such as the cerebellum (Courchesne et al., 2001).

The absence of cerebral vasculature irregularities in HB-GAM knockouts does not negate their phenotypic similarity to humans with PDDs. Cerebral hypoperfusion has been commonly reported in the PDDs (e.g. Boddaert & Zilbovicius, 2002; Wilcox et al., 2002), but these findings might reflect under-utilization of, or abnormal functional connectivity among, neural regions rather than a primary vascular abnormality. Consistent with the lack of substantial empirical data demonstrating abnormal vasculature in the PDDs, analysis of cerebral vasculature in potential animal models of these disorders has either not been performed or not revealed vascular defects (e.g. Tan et al., 2007). Analysis of cerebral cortical vasculature in HB-GAM knockouts, though not suggestive of phenotypic vascular irregularities, might serve to limit the number of possible explanations for the neuronal abnormalities seen in these animals and suggests

that these deviations are due to the loss of HB-GAM's role in radial migration and differentiation of nascent neural cells.

CHAPTER 8. GENERAL DISCUSSION

Previous, pilot studies of mice deficient in HB-GAM, a protein involved in a variety of neurodevelopmental processes, suggested the presence of cognitive rigidity, anxiety, and motor impairment as well as cortical neuronal hyperplasia in these animals. The PDDs include these abnormalities and have a strong neurodevelopmental basis. Therefore, further characterization of the behavioral phenotype of HB-GAM knockouts was performed with an emphasis on features known to be abnormal in the PDDs but capable of quantification in mice, that is, capacity for perseveration, anxiety and behavioral reticence, and social behavior. In addition, characterization of knockouts' neuroanatomical phenotype focused on the microstructure of the lateral entorhinal cortex, a region with anomalies that correlate with symptom severity in PDD-affected individuals, and on cerebral cortical vasculature because of the possible link between neuronal development and vascular development.

The HB-GAM Knockout Phenotype. Assessment of the behavior and neuroanatomy of HB-GAM knockouts indicated that these animals possess a phenotype characterized by: (1) perseverative tendencies in the cognitive domain, as manifested by difficulty adapting to shifting contingencies and a failure to make variable, flexible choices in favor of perseverative behavioral responses, but not by perseverative tendencies in the motor domain, as suggested by the absence of motor stereotypy and hyperactivity; (2) heightened anxiety in the presence of anxiogenic environments not offering a ready means of escape; (3) behavioral reticence in novel paradigms without an appetitive component that vanishes upon extended exposure, suggestive of contextual neophobia; (4) social neophobia, as evidenced by reduced preference for interaction with

a novel conspecific, without deficits in frequency or quality of social interaction; (5) decreased neuronal area and increased neuronal packing density in layer IV, and normal neuronal area but increased neuronal packing density in layer V, of the lateral entorhinal cortex; and by (6) frontal and temporal cerebral cortical vasculature comparable to that of wild type mice.

Validity of the HB-GAM Knockout Model. The current studies attempted to characterize the neuroanatomical and behavioral phenotype of HB-GAM knockouts within the context of the hypothesis that these animals might serve as a model for some of the anomalies associated with the PDDs. The relatively recent proliferation of proposed animal models of human psychopathologies has necessitated much discussion regarding the theoretical and practical processes by which these potential models are validated. Validation of animal models of pathologies like the PDDs is most often comprised of assessing three separable features of these potential models, that is, their (1) *face validity*, the extent to which a model is similar to the entity it is proposed to model; (2) *construct validity*, the extent to which features of the model and the modeled entity have the same or similar etiology; and (3) *predictive validity*, the extent to which the same treatment has a similar effect in the model and the modeled entity (for review, see van der Staay & Steckler, 2001).

The face validity of HB-GAM knockouts to serve as an animal model of the behavioral features of the PDDs is bolstered by the cognitive perseveration, anxiety, and neophobia they exhibit upon testing. Further, the increased neuronal density and decreased neuronal area present in some cerebral cortical regions of the knockouts' brain supports their face validity as a model of those neurobiological features of the PDDs.

The apparent absence of motor stereotypy and hyperactivity in these animals is not consistent with a PDD-like phenotype. However, the significant heterogeneity in symptom profile and severity observed among PDD-affected individuals might extend to animals with similar phenotypic traits. Indeed, the absence of repetitive motor behavior does not by itself exclude diagnoses of PDDs in humans (DSM-IV-TR; 2000). Further, though HB-GAM knockouts lack abnormalities in the quality and quantity of social interaction, these animals demonstrate a reduced preference for social novelty perhaps secondary to neophobia, qualities indeed reminiscent of the PDDs.

The construct validity of HB-GAM knockouts as a model of the PDDs is more difficult to assess given the incomplete understanding of the etiology of these disorders. Though the *Ptn* gene is located on chromosome 7q (Li et al., 1992), which has been associated with PDD development in genetic linkage studies (Grice & Buxbaum, 2006), the state of pleiotrophin in humans with PDDs has not been investigated.

Similarly, the lack of reliably effective treatments for PDD symptoms, whether pharmacological or behavioral, makes judgments as to the predictive validity of these animals as a PDD model equally difficult. However, investigation into whether the abnormal phenotypic features demonstrated by HB-GAM knockouts can be reduced or reversed via experimental interventions might suggest potential treatments for PDD-affected individuals. Indeed, gradual activation of the *MECP2* gene in knockout mice reportedly results in reduction of these animals' phenotypic abnormalities (Guy et al., 2007). Therefore, future work might examine the effect of similar activation of *Ptn* in HB-GAM knockouts.

In addition, exposure to enriched environments has been linked to reversal of the abnormal behavioral phenotype of rats treated prenatally with valproic acid, a result attributed to enhanced neuro- and synaptogenesis and opportunities for animals to learn associations between responses and environmental consequences (Schneider et al., 2006). Accordingly, these mechanisms have been implicated in the efficacy of behavioral therapeutic approaches designed to address PDD symptoms, especially anxiety and social deficits, in humans (cf. Lovaas, 1987). Therefore, examining the effect of cognitive enrichment on HB-GAM knockouts' neophobic tendencies might yield data that could bolster their predictive validity as models of the PDDs.

HB-GAM knockouts demonstrate multiple phenotypic features, both neurological and behavioral, associated with the PDDs. Many animal models of these disorders have been proposed, but few (e.g. *Pten* knockouts, Kwon et al., 2006; CADPS2 knockouts, Sadakata et al., 2007) have been empirically characterized as exhibiting multiple PDD-like irregularities of neuroanatomy and behavior. Therefore, the face validity of HB-GAM knockouts as a model of the PDDs demonstrated by the current studies urges further study to elucidate the construct and predictive validities of these animals to serve as such a model.

Limitations and Future Directions. The ambiguity regarding the construct validity of these animals as a PDD model is not limited to HB-GAM knockouts, as a number of cogent criticisms regarding animal models of human neurobehavioral disorders have been raised in response to the recent proliferation of potential animal models of these disorders.

Chief among issues relating to the validity of animal models of PDDs is that the association between genotype and phenotype is often dubious. Counterfactual reasoning dictates that phenotypic abnormalities in genetic knockouts result from a lack of the deleted gene and, consequently, a lack of that gene's products (Hernandez & Blazer, 2006). However, some (cf. Routtenberg, 1995) have argued that knockout animals are essentially "reactionisms," organisms wherein gene deletion and gene product deficiency likely results in altered activation of other genes and their products. Indeed, though HB-GAM is involved in a wide variety of processes vital to development of the nervous system, animals null for HB-GAM, and animals null for its receptor, N-syndecan (e.g. Hienola et al., 2006), survive development into adulthood with grossly normal central nervous system structure. This suggests activation of genetic compensatory mechanisms in HB-GAM knockouts that might have implications for these animals' phenotype.

That is, genetic compensatory mechanisms might contribute to abnormal phenotypic features in knockout animals in general, and HB-GAM knockouts in particular, or prevent expression of phenotypic traits that would otherwise result from a loss of a deleted gene's products. Accordingly, genetic compensatory mechanisms have frequently been implicated in studies that have failed to demonstrate expected phenotypic abnormalities following genetic manipulation in animals. For example, postnatal blockade of gastrin-releasing peptide reportedly induces reduced social interaction, impaired inhibitory avoidance and novel object recognition in rats (Presti-Torres et al., 2007), but others (e.g. Yamada, Wada & Wada, 2000) have reported increased social interaction and intact memory in gastrin-releasing peptide knockout mice.

The possibility of phenotypic effects differing as a function of the method by which an endogenous biochemical, such as HB-GAM, is depleted should therefore be investigated in any proposed genetic knockout model of the PDDs. Indeed, future investigations might ascertain the presence of genetic compensatory mechanisms in HB-GAM knockouts, and more precise developmental roles for HB-GAM as a result, by assessing the phenotypic features of mature HB-GAM "knockdown" animals. Such an approach might be invaluable in ascertaining whether the *in vivo* absence of HB-GAM is directly related to behavioral abnormalities by examining the behavior of these animals both before and after depletion of HB-GAM.

Proposed animal models of the PDDs are in actuality attempting to mirror developmental disorders that are likely polygenetic in origin. For that reason, though complex genetic compensatory mechanisms might yield mediating genetic alterations that are difficult to identify, such mechanisms might also occur in the pathogenesis of the PDDs (Hochgeschwender & Brennan, 1995). Consequently, knockout animals could be considered ideal organisms in which to attempt the modeling of complex developmental disorders. Therefore, study of possible compensatory mechanisms in HB-GAM knockouts and other potential PDD models might reveal valuable information about these mechanisms in humans and also support the construct validity of these proposed animal models.

Indeed, the concept of parallelism between the biology underlying the behavior of humans and other animals is critical to the argument that animals can be used to model human neurodevelopmental disorders. However, the similarity of both biology and behavior between humans and other animals has been questioned since the earliest

recorded use of animals to study human diseases and disorders in the first century A.D. (Hau & Van Hooiser, 2004). The concept of Darwinian evolution dictates that such similarities exist, and the presence of homologous genes in species of varying genetic complexity, from *drosophila* to *homo sapiens*, has been observed repeatedly (Tordjman et al., 2007).

However, the effect of genetic manipulation is not always identical between humans and other animals despite the presence of homologous genes among species. For example, mutation in the hypoxanthine phosphoribosyltransferase (HPRT) gene causes the X-linked recessive disorder Lesch-Nyhan syndrome (LNS) in humans. LNS is characterized by cognitive impairment, self-injurious behavior and critical reductions in striatal dopamine, all of which are considered the result of excessive production and excretion of purines secondary to the loss of HPRT (Sadock & Sadock, 2003).

Knockout animals deficient for HPRT, however, do not demonstrate a biological or behavioral phenotype similar to LNS-affected individuals (Engle et al., 1996). Despite conservation of the *HPRT* gene and its function between humans and rodents, differences exist in each species' metabolic reaction to HPRT loss during development. Indeed, only when treated with an inhibitor of adenine phosphoribosyltransferase (APRT), the second enzyme involved in the murine salvage pathway, did HPRT knockout mice manifest self-injurious behavior (Wu & Melton, 1993). Similarly, though the *Ptn* gene is conserved between mice and humans with an amino acid sequence homology of 99% (Rauvala & Peng, 1997), the consequences of its absence *in vivo* might not be identical between these species. Therefore, the *prima facie* similarity between HB-GAM knockouts' characteristics and some features of the PDDs must be considered cautiously, as should

all comparisons between PDD-affected individuals and animal models that exhibit similar abnormalities.

Indeed, despite the conservation of genes and behavior between *mus musculus* and *homo sapiens*, many question whether these animals can truly serve as a model of human neurobehavioral disorders, which are typified by abnormalities of complex behavior and cognitive processes that are considered uniquely human. This criticism seems validated by the most accepted definition of a valid animal model of a human disorder, that is, an animal that manifests all of the abnormalities associated with the modeled disorder. Therefore, a valid animal model of the PDDs would manifest neurobiological aberrations similar to those with PDDs as well as abnormalities in the three principle domains affected by these disorders: social functioning, language and communication, and behavioral repetroire (Fisch, 2007).

Though some doubt the ability of animals to manifest behavior and cognitive processes that are analogous to humans' even when broadly and liberally defined, others acknowledge that an animal model of most any human disorder is by definition an abstract, approximate, and reductive entity and need not mirror that disorder in complexity but rather only in quality (Tordjman et al., 2007). Animal behavior with conceptual analogy to that of humans can be measured, and interpreted within the context of species-typical differences, in the domains of social behavior (cf. Moy, Nadler, Magnuson & Crawley, 2006) and behavioral repetroire (as measured via cognitive and behavioral flexibility, e.g. Bernardet & Crusio, 2006; Hagerman et al., 2005; Sadamatsu et al., 2006).

Nevertheless, modeling in animals deficits commonly ascribed to PDD-affected individuals, such as those in "theory of mind," is difficult given that such processes seem unique to humans. However, methods have been proposed to examine analogous functions in mice, such as via measurement of an animal's ability to locate buried food by observing the strategy of a successful conspecific, or the social transmission of food preference task, wherein normal animals choose a new flavor of food because of social interaction with a conspecific that has eaten food with that new flavor (Crawley, 2004, 2008).

Similarly, assessing the presence of PDD-like language deficits in animals presents obvious challenges. Ultrasonic vocalizations (UVs) represent a form of a rodent communication across different relationships, including mother-pup dyads, in which dams' UVs accompany attention and care of pups and pups' UVs urge maternal behavior, and mating pairs, in which UVs of varying frequency serve specific purposes, such as mate summoning. However, whether these vocalizations are primarily the result of physiological processes, such as coughs, sneezes, or attempts to maintain homeostasis, or they represent a form of communication with rudimentary linguistic properties (e.g. Holy & Guo, 2005) has been a matter of protracted debate.

Should the latter be possible, a valid rodent model of the PDDs would be expected to demonstrate abnormalities in UV production, content, or timing of expression, given the repetitive nonsense speech observed in some affected children (Saddock & Saddock, 2003) and the contextually inappropriate speech and abnormal tone, rhythm and prosody often seen in affected adolescents and adults (Rice et al., 2005). However, UVs have not been systematically investigated in proposed animal models of

the PDDs (Ricceri et al., 2007), perhaps because the validity of abnormal ultrasonic vocalization in rodents as a recapitulation of language impairments in humans with PDDs remains highly questionable. Nevertheless, investigation of UVs in any potential rodent model of the PDDs, including HB-GAM knockouts, might yield data to explain, or at least complement, observations of abnormal social behavior and maternal care in these animals and others with PDD-like characteristics.

Utility of Animal Models of the PDDs. The use of animals in the attempt to model complex neurodevelopmental disorders characterized by abnormal behavior seems contraindicated by the understandable skepticism and criticism surrounding the practice, as well as by the significant challenges inherent to identifying animal behavior with conceptual analogy to humans', designing paradigms that validly measure such behavior, and accurately interpreting findings in the context of interspecies differences in biology and behavior. However, considering the practical and ethical restrictions on investigating the etiology of the PDDs and possible treatments for these disorders in humans, animal models provide the best available alternative. Indeed, though the practice of using animals to model these disorders is still in its proverbial infancy, some studies using animal models have already yielded data with obvious potential benefit for humans with PDDs. For example, study of MECP2 knockout mice has already demonstrated the benefits of gradual reactivation of the *MECP2* gene (Guy et al., 2007) and conditional transgene-induced BDNF expression (Chang et al., 2006) in the rescue of these animals' abnormal neurobiological and behavioral phenotype, treatments that might eventually be applied to humans.

Conclusion. These studies sought to further characterize the neuroanatomical and behavioral phenotype of mice deficient in HB-GAM within the context of the hypothesis that these animals might serve as an animal model of the PDDs. Behavioral assessment revealed that these animals exhibit cognitive inflexibility, heightened anxiety, and both a contextual and social neophobia. In addition, HB-GAM knockout brains possess cortical neuronal packing density increases and cortical neuronal area decreases. These data suggest that multiple abnormalities similar to those observed in individuals with PDDs characterize the phenotype of HB-GAM knockouts. Therefore, these animals might be a promising organism in which future investigations could examine possible etiologies of and treatments for these disorders. Indeed, with further study, HB-GAM knockouts might add another piece to the proverbial puzzle of the PDDs.

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