NEURONAL PAS DOMAIN 1 PROTEIN

AND ITS HYPOTHETICAL RELATIONSHIP TO AUTISM

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by

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Neuronal PAS domain 1 (NPAS1) was discovered in 1997 as a brain-specific transcription factor first detected at embryonic day 15 of the developing mouse embryo. Its expression was observed to peak in the early post-natal days, and *in situ* hybridization assays in adult mice revealed expression localized to the cortex, hippocampus, thalamus, hypothalamus and superior colliculus. Subsequent immunohistochemical staining assays employing antibodies to NPAS1 revealed an expression pattern largely restricted to inhibitory neurons in the aforementioned brain regions, as well as localized expression in the subgranular region of the dentate gyrus. NPAS1 and NPAS3 transcription factors are paralogues that have evolved from the *Drosophila* gene *Trachealess*. Despite the high homology in their bHLH, PAS-A and PAS-B domains, the NPAS1 and NPAS3 proteins appear to be endowed with diametrically opposing functional properties. Interestingly, a translocation that disrupts the NPAS3 gene has been

reported in a family suffering from schizophrenia. NPAS3-deficient mice have been reported to have behavioral abnormalities reminiscent of schizophrenia, as well as a distinct deficit in hippocampal neurogenesis. In contrast, several variations in NPAS1 have been found in autistic children. It was found that NPAS1-deficient mice are born with larger than normal brains that contain an over-abundance of neurons in the cortex, as well as a significant increase in hippocampal neurogenesis. The NPAS1-deficient mice also exhibited enhanced sensitivity to acoustic and tactile stimuli. The behavioral and neuroanatomical phenotypes observed in the NPAS1-deficient mice appear to be reminiscent of phenotypes seen in autistic children.

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CHAPTER ONE

Autism Introduction

1.1 Clinical Background

The term “autism” was first coined in 1912, by Eugen Bleuler, who also coined the term “schizophrenia”. The term “autism” was assigned to indicate “escape from reality”. Leo Kanner was the first to clinically describe a set of “autistic” patients. He published a paper in 1943, in which he describes these autistic children (Kanner, 1943). Kanner used this term because he believed these children gave the impression that they were trying to escape from reality. Prior to Kanner noticing and recording this pattern of symptoms, the children would have been classified as emotionally disturbed or mentally retarded. However, Kanner noticed that these children often demonstrated capabilities that could prove that they were not merely slow learners, nor did they fit the patterns of an emotionally disturbed child. Thus he invented a new category of mental disorders called Early Infantile Autism. In his first paper, Autistic Disturbances of Affective Contact, the children Kanner describes do not lack intelligence (Kanner, 1943). In fact, they have “excellent rote memory”. They were said to have a phenomenal memory when it comes to placement of objects (such as blocks, beads, or sticks) and could arrange them in the exact pattern they were originally seen, even if it was a completely random, unorganized order. The children tended to get upset if someone had removed a block, or added a new one, thus emphasizing their need for sameness. It seriously disturbed the children if anything was changed, such as a change in routine, of furniture arrangement, or the order of their daily activities. They were also said to be very skillful and precise in their movements (ie.- not uncoordinated). The “pathognomonic” fundamental disorder was described as the children’s *inability to relate themselves* to people or situations around them. Parents mention that the children were “in their own world”, “oblivious to everything around them”, “giving the impression of silent wisdom”, and “happiest when left alone”. Thus, the children were described to be self-absorbed, or self-sufficient and independent.

Autistic children were also observed to display obsessive behavior and interests. They seem to be focused on objects, much more than the people around them. However, they showed unusual imagination and initiative in their play. In their speech, they tended to be quiet, but often repeated certain phrases, and many displayed symptoms of echolalia, which is when someone echos or repeats what others say to them. For example, instead of answering a question, they would simply repeat the question. They often misused pronouns, such as "you" and "I", referring to themselves as "you" or in the third person. Many autistic children also showed a fear of loud or shrill noises; such as from grinders, beaters and vacuum cleaners. Kanner believed this was due to the children's all-powerful need to be left alone and undisturbed. He also mentioned that many of these children (5 of the11 cases he first describes) displayed enlarged head circumferences.

Presently, the clinical description of autism has expanded to include a distribution of symptoms collectively termed Autism Spectrum Disorders (American Psychiatric Association, 2000). This is a broad expansion of, and at times a far stretch from, what Kanner initially described as autism. The Autism Spectrum Disorders range from a mild form of autism, called Asperger's syndrome, to very severe forms, such as Rett Syndrome and Childhood Disintegrative Disorder. Asperger's was named after the Austrian pediatrician, Hans Asperger, who studied and described children in his practice who had difficulties with social interaction, had restricted and repetitive behaviors and interests, demonstrated little empathy for their peers, and although they maintained verbal skills, they did not communicate well with others (McPartland and Klin, 2006). Asperger's children may want to be social and interact with others, but they do not really understand how to go about it. Asperger's differs from autism in that these children preserve linguistic and cognitive development, and often have above average intelligence.

Childhood Disintegrative Disorder, also known as Heller's syndrome, is on the other end of the spectrum. It is a rare condition characterized by late onset (>3 yrs of age) developmental delays in language, social interaction, and motor skills (Hendry, 2000). It is sometimes considered a "low functioning" form of autism. These children show normal development of age-appropriate verbal and

non-verbal communication, social relationships, motor, play, and self-care skills. However, between the ages of 3 and 10, the skills they have acquired are lost almost completely. This syndrome was originally described by the Austrian physician, Theodor Heller, in 1908, 35 years before Kanner and Asperger described autism, but he used the term *dementia infantalis* (Hendry, 2000). It has been associated with other conditions, such as lipid storage disorders, where there may be a toxic build-up of lipids in the brain and central nervous system; subacute sclerosing panencephalitis, a chronic infection of the brain by a form of the measles virus leading to inflammation of the brain and death of neurons; and tuberous sclerosis (Hendry, 2000).

Rett syndrome, which is now included in the broad category of Autism Spectrum Disorders, is an X-linked disorder caused by mutations in, or a loss of, MeCP2 (methyl-CpG binding protein 2). Some argue that this should not be classified as an autism spectrum disorder because it has a specific etiology. However, it is named in the DSM-IV under the broad category of Pervasive Development Disorders. These children typically have no verbal skills, they are not ambulatory, and they suffer from a failure to thrive. They also have stereotyped behaviors such as hand wringing, and many Rett syndrome patients also suffer from seizures (American Psychiatric Association, 2000).

1.2 Genetic Background:

Autism is believed to have a strong genetic basis. Twin studies performed in the late 1980s, which compared the concordance rates in identical and fraternal twins, showed that autism has heritability indices of 0.85-0.92 (Monaco and Bailey, 2001). Smalley et al. reviewed this work and reported an average concordance for identical twins of 64%, and a concordance of only 9% in fraternal twins (Smalley et al., 1988). Despite evidence of high indices of heritability, finding specific genetic associations, and genotype-phenotype correlations has proven difficult. This may be because there is an extreme heterogeneity among the symptoms in autistic children. However, using standard medical genetic evaluation techniques, a genetic cause can be identified in 20-25% of children on the autism spectrum. The identified genetic causes of autism thus far can be classified as chromosomal abnormalities (~5%),

copy number variants (CNVs) (10-20%), and single gene disorders (~5%). The remaining 75-80% of autism cases have an unknown cause (Miles, 2011). Maternally derived 15q duplication of the imprinted Prader Willi / Angelman region are the most commonly observed chromosome abnormalities in autism, detected in 1-3% of cases (Depienne et al., 2009). There have been CNV (copy number variation) studies done, which have also revealed 15q11.2-11.3 duplications as one of the most common autism-related CNVs (Miles, 2011). Shinawi et al. found 16p11.2 microdeletions and duplications to be common as well. Characterization of the samples with 16p11.2 deletions and duplications, revealed that patients carrying the 16p11.2 deletion were more likely to be macrocephalic ($P<0.002$) and autistic, while 60% of the patients with the duplication had microcephaly and attention deficit hyperactivity disorder (ADHD) (Shinawi et al., 2010). For the 15q region, it is fairly well established that UBE3A is the causative gene for Angelman syndrome. The paternal allele is imprinted, so duplication of the paternal allele, deletions of the maternal allele, or mutations in UBE3A can all cause Angelman syndrome (Schanen, 2006). Large genome-wide association studies and investigations of candidate genes have been performed in an effort to find specific genes that can cause, or increase the risk of developing autism. SFARI (Simons Foundation Autism Research Initiative) Gene is a website (genetic database) where all genes connected to autism, which have been published in peer-reviewed scientific literature, are assembled into an easily accessible database. The genes come from different categories, including genetic association studies, rare single gene mutations, and genes linked to syndromic autism. The genes are annotated with their relevance to autism, and with the knowledge of their molecular functions. Table 1 from Miles et al. shows many of the known and putative genes linked to autism (Miles, 2011). The table is organized by pathogenesis (gene function) in order to highlight the progress being made towards elucidating pathways that could lead to autism.

Table 1.

Table 2 Known and putative autism genes (organized by pathogenesis)

Protein name (function)	Gene symbol/locus	Test availability
Neuronal cell adhesion and/or synapse function		
Neuroligin 3 (synapse formation and function)	*NLGN3X* Xq28	Clinical
Neuroligin 4 (synapse formation and function)	*NLGN4X* Xp22.33	Clinical
Neurexin 1 (transsynaptic binding partner for neuroligins)	*NRXN1* 2p16.3	Research
SH3 and multiple ankyrin repeat domains (organizes post synaptic density and binds neuroligins)	*SHANK3* 22q13	Research
Contactin-associated protein-like 2 (synaptic binding partner for contactin molecules involved in neuronal migration)	*CNTNAP2* 7q36	Research
Contactin 4 and Contactin 3 (neuronally expressed adhesion molecules)	*CNTN4* and *CNTN3* 6p26-p25	Research
Protocadherin 10 (a cadherin-related neuronal receptor: may play a role in the establishment and function of specific cell-cell connections; essential for normal forebrain axon outgrowth)	*PCDH10* 4q28	Research
Neuronal cell adhesion molecule	*NRCAM* 7q31	Research
Neuronal activity regulation		
Methyl CpG-binding protein 1 (CAN methylation-dependent transcriptional repressor)	*MECP2* Xq28	Clinical
Ubiquitin protein ligase E3A	*UBE3A* 15q11-q13	Clinical
Deleted in autism	*DIA1* (c3orf58) 3q	Research
Ataxin 2-binding protein 1	*A2BP1* 16p13	Research
Neurodevelopmental genes		
Engrailed 2 (homeobox gene involved in midbrain and cerebellum development)	*EN2* 7q36	Research
Homeobox A1 (involved in hindbrain development)	*HOXA1* 17p15.3	Clinical
Homeobox B1 (involved in hindbrain development)	*HOXB1* 17q21-q22	Research
Reelin (signaling protein involved in neuron migration)	*RELN* 7q22	Research
WENT2 (signaling proteins involved in embryonic patterning, cell proliferation, and cell determination)	*WNT2* 7q31	Research
FOXP2 (transcription factor involved in embryogenesis and neural functioning)	*FOXP2* 7q31	Research
ARX homeobox gene	*ARX* Xp22.13	Clinical
Patched domain containing 1 gene	*PTCHD1* Xp22.11	Research
Sodium channel		
Sodium channel, voltage-gated, type VII	*SCN7A* 2q	Research
Na+/H+ exchanger isoform 9	*SLC9A9* (*NHE9*) 3q24	Research
Calcium channel		
Calcium channel, voltage-dependent, L type, alpha 1C subunit (Timothy syndrome)	*CACNA1C* 12p13.3	Clinical
Calcium channel, voltage-dependent, alpha 1H subunit	*CACNA1H* 16p13.3	Research
Calcium channel, voltage-dependent, L type, alpha 1F subunit	*CACNA1F* Xp11.23	Clinical
Neurotransmitter genes		
GABA receptor subunits (major inhibitory transmitter receptors in the brain)	*GABRB3, GABRA5, GABRG3* 15q11.2-q12	Research
Serotonin transporter	*SLC6A4* 17q11.1-q12	Clinical
Mitochondrial		
Mitochondrial aspartate/glutamate transporter (mitochondrial function and maintaining ATP levels)	*SLC25A12* 2q24	Research
Other genes		
Oxytocin receptor	*OXTR* 3p26.2	Research
Laminin beta 1	*LAMB1* 7q31.1	Research
RING finger protein 8 (ubiquitin ligase and transcriptional coactivator)	*RNF8* 6p21.3	Research

Adapted from GeneReviews, http://www.genetest.org. Copyright, University of Washington, Seattle 1997–2010.

One example of a possible pathway leading to the development of autism is related to abnormalities of synaptic connections. As seen in Table 1, several genes have been identified in this pathway, such as Neuroligin 3 and 4 (needed for synapse formation and function), Neurexin and Shank3 (which bind Neuroligins), Contactin 3 and 4 (neuronally expressed adhesion molecules), Contactin-associated protein-like 2, Protocadherin 10, and Neuronal Cell Adhesion Molecule (NRCAM).

Another pathway that has been implicated in autism is related to neurotransmission. Specifically, it is believed that there could be an alteration of excitatory/inhibitory (E:I) balance. This mechanism not only includes the GABA receptor subunits and serotonin transporter, as listed in the table; but also includes transcription factors that control inhibitory neuron development. For example, mutations have been found in the Dlx homeodomain transcription factors, which control development of forebrain GABAergic neurons, and are expressed in mature cortical interneurons. Mice lacking Dlx1 suffer from degeneration of a subset of cortical interneurons, which results in reduced inhibitory tone and the development of epilepsy (Cobos et al., 2005). Arx is a transcription factor that is downstream of Dlx, and mutations in Arx have also been found in autistic children with epilepsy (Brooks-Kayal, 2010). NPAS1 is also expressed in inhibitory neurons. Its potential role in autism and brain development will be discussed later.

Another autism candidate gene is Engrailed 2 (En2). This homeobox transcription factor lies downstream of FGF-signaling, and mutations in En2 may support the theory that the FGF pathway, leading to growth of the frontal cortex, may be involved in the pathogenesis of autism (Rubenstein, 2010). Also, mutations in genes that suppress growth pathways, such as PTEN and TSC1/TSC2, have been linked to autism (Butler et al., 2005; Goffin et al., 2001; Wiznitzer, 2004; Zori et al., 1998).

One of the most intriguing concepts to explain the complexity and difficulty with finding or understanding the genetic causes of autism, or any complex disorder, is that of epigenetics. Epigenetics can be considered to be any factor which can change the expression of genes without changing the actual DNA sequence (for example – DNA cytosine methylation, RNA editing, and post-translational histone modifications). Since the actual DNA sequence is not changed by these epigenetic mechanisms,

it means that these factors would not be picked up by standard genome-association studies. Thus, there would be an explanation for the lack of specific genetic causes. Also, epigenetics could explain an attenuated genetic concordance between monozygotic twins.

The Autism Spectrum Disorders have many connections with epigenetics, especially imprinting and methylation. For example, the Prader-Willi / Angelman syndrome region is in the imprinted domain on chromosome 15q11-13, which is also the most common recurrent region for cytogenetic abnormailities in ASD. Also, Fragile X syndrome, which includes patients considered to be autistic, is caused by an expansion of a CGG repeat in the promoter region of FMR1 (fragile X mental retardation protein), and this repeat gets methylated, causing the FMR1 gene to be silenced. Fmrp is an RNA binding protein that may be involved in mRNA transportation and translation (O'Donnell and Warren, 2002). In addition, Rett syndrome is caused by mutations or deletions in MeCP2, which is a key regulator involved in the pathway of epigenetic modifications (Amir et al., 1999). MeCP2 binds methylated CpGs and can recruit other proteins to help repress and/or activate gene transcription. Schanen et al. composed a table showing all of the regions linked to autism and their imprint status (Schanen, 2006) (see Table 4). This compilation gives evidence that many of the genetic loci linked to autism are situated within imprinted regions (Morison et al., 2005).

Table 4.

Table 1. Summary of genome-wide linkage screens in ASD and relationship with known imprinted domains (135)

Cytogenetic location	Linked marker(s)	Position (cM)	Imprinted domain (cM)	References
1p13.1–21.1	D1S1631–D1S1675	136.88–149.2	NR	(2,39)
1p12–25	D1S1677	175.62	NR	(36)
1q21–22	D1S1653	149.2–164.09	NR	(39)
1q23.1–23.2	D1S2624–D1S2771	162.57–168.52	NR	(39)
2p12–13.1	D2S1351	103.04	NR	(43)
2q24.3–31.3	D2S2330–D2S364	169.41–186.21	NR	(46)
2q31–31.3	D2S2314–D2S2310	182.24–185.13	NR	(35)
2q33.1	D2S116–D2S309	198.65	NR	(45)
3p25.3	D3S3691	29.19	NR	(47)
3p25.3	D3S3594–D3S3589	32.36	NR	(136)
3p24–26	D3S3691	29.19	NR	(36)
3q13.12–26.1^{word}	D3S3045–D3S1763	124.16–176.54	NR	(114)
3q25–27	D3S3715–D3S3037	178.9–190.43	NR	(39)
4q21-31	D4S1591	106.89	Yes (∼98)	(36,135)
5p13.1	D5S2494	58.91	NR	(37,38)
6q14-21	D6S1021	112.20	NR	(36)
6q16.3	D6S283	109.19	NR	(40)
7q21.3	D7S1813	103.63	Yes (∼104–110)	(135,137)
7q21.2–q31.31	D7S2409–D7S480	110.57–125.95	Yes (∼104–110)	(41,135)
7q22.3–q31.1	D7S496–D7S2418	119.81–122.48	Yes (∼104–110)	(135,138)
7q22.1–22.2 (P > M)	D7S477–D7S2453	111.79–115.96	Yes (∼104–110)	(35,135)
7q32.2	D7S530–D7S684	134.55–147.22	Yes (134.5–138)	(43,135)
7q22-q32	N/A	N/A	Yes (134.5–138)	(135,139)
7q32.2–32.3 (M > P)	D7S530–D7S640	134.55–137.83	Yes (134.5–138)	(35,135)
7q32-qter	N/A	N/A	Yes (134.5–138)	(135,139)
7q32.2–32.3 (P > M)	D7S2527–D7S495	128.99–144.72	Yes (134.5–138)	(115,135)
7q33–36.1	D7S483	165.18	Yes (134.5–138)	(36,37,135)
7q34–36.2	D7S1824–D7S3058	149.9–173.71	Yes (134.5–138)	(114,116,135)
7q36.1–36.3	D7S1805–D7S550	161.21–178.41	Yes (134.5–138)	(39,135)
8q22–24	D8S1832	132.49	Yes[a]	(36,135)
9p21.3–22.2 (M > P)	D9S157–D9S171	32.24–42.73	NR	(35)
9p21.3–21.2 (P > M)	D9S171–D9S161	42.73–51.81	NR	(35)
10p12–q11.1	N/A	N/A	NR	(139)
11p11.2–13	D11S1392–D11S1993	43.16–54.09	Yes (2–5 & ∼38)	(38,135)
13q12.3	D13S217–D13S1229	17.21–21.51	Yes (∼45)	(135,137)
13q21.33	D13S800	55.31	Yes (∼45)	(135,137,140)
14q12	D14S80	26.59	NR	(43)
15q11.2-12	*GABRB3*	9.85	Yes (∼6–18)	(74,135)
17p13.3–q21.1^{phrase}	D17S1298–D17S1299	10.72–62.01	NR	(114)
17p12–q21	D17S1294	50.74	NR	(36)
17p11.2–q11.1	N/A	N/A	NR	(139)
17q11.2	D17S1800	51.63	NR	(38)
17q11.2	D17S1294	50.74	NR	(47)
17q11–21$^{male\ only}$	D17S1880–D17S2180	53.41–66.85	NR	(141)
17q21.2	D17S1299	62.01	NR	(47)
17q23.1–25.2^{word}	D17S1290–D17S1301	82.0–100.02	NR	(114)
19p13.11–q13.1	D19S930–D19S113	44.41–56.69	Yes (∼100)	(47,135)
19q12[b]	D19S433	51.88	Yes (∼100)	(37,135)
Xp11.3–q21.33	DXS6810–DXS6789	42.75–62.52	NR	(39)
Xq25	DXS1047	82.07	NR	(37)

Loci with lod scores over 2.0 are shown and parent of origin effects on sharing are noted as are endophenotypes included in the analyses. These include items from the Autism Diagnostic Interview—Revised: age at first word (word), age at first phrase (phrase) and gender of the affected sib-pair (male only). Markers at or bounding the linkage peak are noted and genetic positions in cM are relative to the Marshfield map (Center for Medical Genetics, Marshfield Medical Research Foundation). NR, none recorded; NA, not applicable. Reference is a meta-analysis of linkage data.
[a]Region of conserved synteny on mouse chromosome 15 contains imprinted *Peg13* gene but no human ortholog is known (142).
[b]Reference notes position at 19p but the marker mapped to 19q on current genome build. Imprinted loci are positioned relative to the nearest Marshfiield markers and noted if they are <50 cM from the area of the linkage peak.

The 15q11-13 imprinted region is especially interesting, not only because of the presence of Ube3a and Pwcr1 which are implicated in Angelman and Prader-Willi syndrome, but because there are several genes in this region that could be considered functional candidates for development of ASD. For example Necdin (NDN), is expressed in post-mitotic neurons and plays a critical role in specification of

inhibitory neurons via interactions with distal-less 5 (DLX5) which has also been implicated in autism (Stuhmer, 2002). Necdin promotes GABAergic neuronal differentiation (Kuwajima, 2006). The other genes related to autism in this cluster are described in the table below (see Table 5) (Schanen, 2006).

Table 5.

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Table 2. Genes in the imprinted cluster on chromosome 15q11–13

Gene	Imprinting status: human/mouse	Expressed allele: human/mouse	Functional relevance to autism or ASD	References
MKRN3	I/I	Pat/pat	Ubiquitously expressed, single exon gene encoding a RING Zinc finger protein. It is contained within the intron–exon of a paternally expressed gene (*ZNF127AS*) that is transcribed from the antisense strand	(143,144)
ZNF127AS	I/I	Pat/pat	Antisense transcript for *MKRN3*	(143,144)
MAGEL2	I/I	Pat/pat	Single exon gene expressed in brain, particularly strong in the hypothalamus. Has homology to necdin but differentially binds necdin interacting proteins at centrosomal regions. Important in PWS	(64,65,145,146)
NDN	I/I	Pat/pat	Encodes a nuclear protein expressed in postmitotic neurons that acts as a growth suppressor and promotes neurite outgrowth. Interacts with other proteins in centrosomal regions. Promotes GABAergic neuronal differentiation. Important in neurological dysfunction in PWS	(61–63,68, 145,146)
SNRPN-SNURF	I/I	Pat/pat	This long, complex transcript encodes the small nucleolar RNA-binding protein N as well as a group of small nucleolar RNAs	(66,70,147)
UBE3A-AS	I/I	Pat/pat	*UBE3A* antisense transcript	(70)
UBE3A	I/I	Mat/mat	Encodes the E6-AP ubiquitin protein ligase. Loss of function leads to AS. Abnormal expression seen in brain in ASD and in lymphoblasts from dup(15) patients. Linkage to this gene has been detected in ASD but no mutations identified in a small group of subjects	(12,13,57, 60,75,76)
ATP10A	I/I	Mat/mat	Encodes an aminophospholipid translocase, which actively transports phosphatidylserine and phosphatidylethanolamine across the cell membrane. Expressed in hippocampus and olfactory bulb. Linkage to this gene has been detected in ASD but no mutations identified in a small cohort of subjects	(59,76,77,148)
GABRA5	CD/NI	Pat/ni	Encodes the alpha 5 subunit of the $GABA_A$ receptor. GABRA5 containing receptors mediate tonic inhibition in hippocampal neurons. Knockout of this subunit in mice leads to enhanced learning and memory	(78,91,149,150)
GABRB3	CD/NI	Pat/ni	Encodes the beta 3 subunit of the $GABA_A$ receptor. Linkage and association studies of this gene in ASD have given mixed results. This gene was misexpressed in brain in ASD and Rett syndrome. Knockout is associated with cleft palate and neurological abnormalities and frequent early lethality	(12,78–81,91, 92,151–153)
GABRG3	CD/NI	Pat/ni	Encodes the gamma 3 subunit of the $GABA_A$ receptor. Linkage and association of this gene in ASD have had mixed results, largely negative. Knockout of this gene does not have an overt phenotype	(78,91,93, 152,154,155)

I, imprinted; NI, not imprinted; CD, conflicting data.

It has been reported that the extent of 5-methyl cytosine methylation can be modified by the maternal diet during gestation (Wolff et al., 1998). One experimental example that strongly supports this concept was a study done on the viable yellow agouti mouse strain (A^{vy}). The coat color ranges from yellow to agouti and depends on methylation of an IAP element that lies in the promoter region of the Agouti locus. The amount of choline and folate in the mother's diet affects the amount of intracellular SAM (S-adenosyl-

methionine), which is the methyl donor used in DNA methylation. Variation in the amount of intracellular SAM has been hypothesized to correlate with the methylation status of gene regulatory elements (Cooney, 1993). The more choline and folate the mother has in her diet, the more methylated the Agouti locus becomes and the gene is silenced. As such, coat color variations in genetically identical mice can vary as a function of changes in the maternal diet (Wolff, 1998). Thus, DNA methylation and imprinting provide a tangible link between genes and environment.

Two related and interesting theories have been proposed regarding the development and etiology of autism. These are called the 'extreme male brain theory' and the 'imprinted brain theory'. The 'extreme male brain theory' presented by Baren-Cohen (Baron-Cohen, 2002; Baron-cohen et al., 2003) is similar to the 'imprinted brain theory' presented by Badcock and Crespi (Badcock and Crespi, 2006). Both of these teams of investigators have argued that autistic personalities are an extreme variant of male behavior. They suggest females tend to be more empathic, and males are more systematic, etc. However, Badcock and Crespi put an emphasis on imprinted genes, and suggest that autism is a disorder of an "extreme imprinted brain". They hypothesize that the "paternal brain" is developed from cells that express genes that are exclusively expressed from the paternal allele, and these are mainly involved in development of regions such as the limbic system (hypothalamus, amygdala, and other regions that mediate basic drives such as hunger, fear, and aggression). The "maternal brain" then comes from cells that express genes exclusively from the maternal allele, and these cells differentially proliferate in the cortex, striatum, and hippocampus (the 'executive brain', involved in language, social reciprocity, planning, and behavioral inhibition). This perspective was supported by the work of Keverne et al. when they made parthenogenetic (duplicated maternal genome) and androgenetic (duplicated paternal genome) mice (Keverne, 1997; Keverne et al., 1996). Keverne and colleagues also showed an evolutionary trend among primates for a relatively larger forebrain relative to limbic system, and suggested that intragenomic conflict, driven by imprinting in the brain, has been a major factor in the tripling of brain size along the human lineage. In support of their "imprinted brain theory" of autism, Badcock and Crespi also brought attention to many of the imprinted genes linked directly to autism, or

possibly involved in the process of development of autism. For example, they implicated UBE3A, Pwcr1 and the 15q11-13 region. Badcock and colleagues also implicated the DLX genes, since DLX5 is imprinted and interacts with DLX1 and 2, which are in an autism linkage region. Also, the DLX genes regulate ARX, which has been found to have mutations that can lead to mental retardation, seizures and autism. GABA receptors, GluR6 and GluR7, and serotinergic receptors are also mentioned to be imprinted and potentially involved in autism. The homeobox genes involved in brain development, HOXA1 and EN2, are hypothesized to be associated with autism, and are imprinted as well (Laroche et al., 2008).

Although it is an interesting theory, it is strange that Badcock and Crespi would think the 'paternal brain' is more autistic when their adrogenetic mice had smaller brains, while the parthogenetic mice had bigger brains (Keverne, 1996). However, the rest of the theory appears to be reasonable. Badcock and colleagues also refer to a valid and noteworthy comment, that the autism spectrum represents human cognitive diversity rather than simply a disorder or disability (Baron-Cohen, 2000). Indeed, they offer the provocative statement that individuals at the highest-functioning end of this spectrum, including Isaac Newton and William D. Hamilton, may have driven the development of science, engineering, and the arts, through mechanistic brilliance coupled with perseverant obsession (Baron-Cohen et al., 2001; Grandin, 1995, 2004).

An intriguing hypothesis offered by Badcock and colleagues is that the pattern of symptoms seen in paranoid schizophrenia are diametrically opposite to those seen in autism, and involve perceptual and cognitive imbalances towards the maternal brain (Crespi and Badcock, 2008). This hypothesis may be important to remember when I discuss the differences between, and opposite roles of NPAS1 and NPAS3, and how they may be related to autism and schizophrenia, respectively. Even though several of the genes associated with autism are also associated to schizophrenia (for example - the GABA receptors, serotinergic receptors), schizophrenia and autism are very different clinically and pathologically. First, the timing of the onset of symptoms is different. Autism symptoms are seen at a

very young age; typically, before the child is 5 years old. Patients with schizophrenia do not usually show signs or symptoms of their disorder until adolescence, or even later, in their twenties. Second, the neuropathologies seen in these patients are very different. The brain pathology for autism suggests increased head circumference, increased neurogenesis and an overabundance of neurons. On the other hand, the neuropathology in schizophrenia suggests impaired neurogenesis and an attenuation of the number of neurons, and enlarged ventricles seen in many post-mortem brains. In either of these disorders, it would be logical to think that genetic components could cause the underlying abnormalities in brain development and neurogenesis. Thereafter, it is possible that environmental factors could influence the timing and onset of symptoms. Many theories have been advanced concerning environmental factors that could be related to the development of neuropsychiatric disorders (Tsuang et al., 2004).

One study performed in order to evaluate possible environmental factors contributing to autism, was called the CHARGE (Childhood Autism Risks from Genetics and Environment) study. Hertz-Picciotto and colleagues addressed a wide spectrum of chemical and biological exposures and susceptibility factors (Hertz-Picciotto, 2006). However, there have not been many follow up papers since this was published describing links to any environmental causes. This makes me believe that the CHARGE study performed by Hertz-Picciotto and colleagues did not find significant causative factors related to autism.

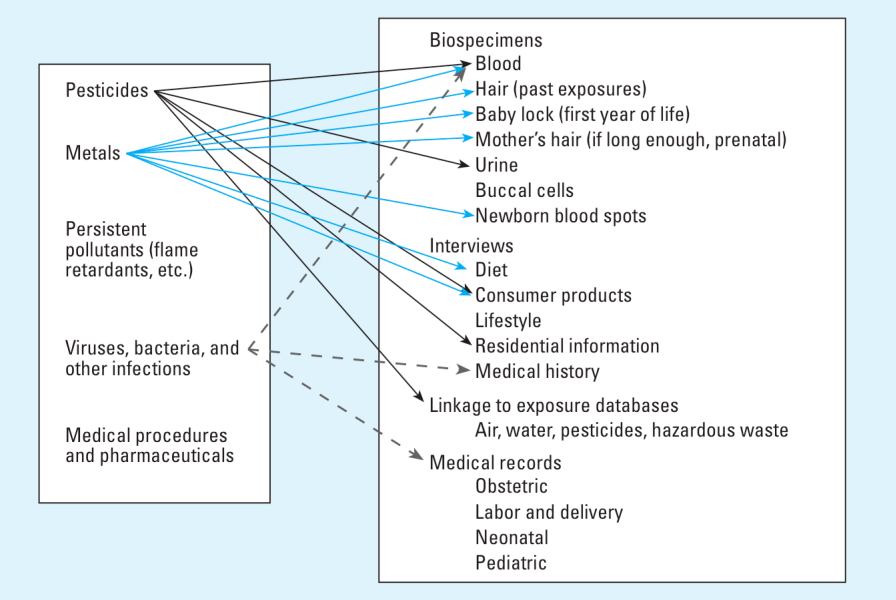

Figure 1. Environmental exposures and sources of information in the CHARGE study. The left-hand box indicates five classes of exposures that are candidates as environmental factors contributing to autism. The right-hand box lists sources of data available on CHARGE study participants. Arrows show a few examples of how specific exposures can be assessed. For example, pesticide exposures and/or their metabolites can be assessed in several ways (black arrows): laboratory assays can be conducted on blood (serum) and urine specimens; the interview collects information on applications in the home and also obtains residential histories that can be linked to exposure databases on commercial pesticide applications in California. Metals (blue arrows) can be measured in hair and in newborn blood spots obtained from the State Genetics Diseases Branch biospecimen bank or assessed by interview questions on fish consumption or use of household products. Exposures to infectious agents (dashed arrows) can be determined from medical records, self-reports, and assays on serum samples to test for seropositivity for antibodies to specific viruses.

Another environmental influence that is a major focus of concern in the media and among autism parent groups, are childhood immunizations. These immunizations are given at around the time of onset of regression in autistic children, which is what has brought on the concern. However, multiple studies and scientific lines of evidence have identified no support for the relationship between immunizations and autism (DeStefano and Thompson, 2004; Taylor, 2006). The original studies by Wakefield and colleagues associating immunizations and autism, have been disproved (Murch, 2004). The work was retracted by *The Lancet*. In fact, in 2010, the British General Medical Council revoked Dr. Wakefield's license to practice medicine (Miles, 2011). One of the tragedies of the fear of an autism epidemic caused by immunizations, was a decreased use of them, leading to an outbreak of measles and childhood

deaths, with no change in the prevalence of autism (Jansen et al., 2003). Hopefully this false belief and fear of immunizations will work its way out of the media soon.

In addition to epigenetic and environmental factors; as mentioned earlier, one of the major impediments to finding genetic causes of autism is that it is a complex, heterogeneous disorder. The disease includes a broad range of behavioral phenotypes, and very few measurable physical phenotypes. It will be important to define and separate the specific endophenotypes of autism, in order to clarify a better understanding of the disorder as a whole. One particular endophenotype, in autistic children which is easily identifiable, is an enlarged head circumference and/or increased brain volume (seen on MRI). It is thought that approximately 35% of children with autism fit into this subgroup. This phenotype is easily measurable and has been the primary focal point of my PhD thesis research. Miles and colleagues (Miles, 2011) offered a table describing various phenotypes that could be used to define specific subgroups of autism. (Table 6)

Table 3 Phenotypic variables that may define discrete autism (ASD) subgroups

Phenotypic variables	Consistently present in ASD populations (% of ASD)
Morphology and growth	
Generalized dysmorphology	Yes (15–20)
Macrocephaly	Yes (~35)
Microcephaly	Yes (~5–15)
Brain malformations	Yes (~20)
Medical/neurologic	
Seizures	Yes (~25)
EEG abn	Yes (~50)
Sleep disorder	Yes (~65)
Savant skills	Yes (~5)
Clinical course	
Age of onset	Yes
Regressive onset	Yes (~30)
Adolescent/adult catatonic regression	Yes (~17)
Significant family history of related disorders	
ASD	Yes (~25)
Alcoholism	Yes (~30)
ADHD	Probably (~70)
Affective disorders	Probably
Bipolar/major affective disorder	Yes (~30)
Functionally defined variables	
IQ	Yes
Adaptive behaviors	Maybe
Outcome measures	Probably (poorly defined)
Response to therapy	Yes
Core autism symptoms	
Social functioning	No (100)
Communication	No (100)
Repetitive, stereotypic behaviors and/or preoccupations, obsessions	Possibly, but must be defined precisely

EEG, electroencephalogram; IQ, intelligence quotient.

Due to our findings in studies of NPAS1-deficient mice, which will be discussed later, we are very interested in the macrocephaly endophenotype. Interestingly, at a Cold Spring Harbor Autism Workshop, Sarah Spence mentioned a genetic association study done on families of autistic children that displayed macrocephaly. Spence and colleagues found a linkage peak at 19q13.2 -3, which is the precise location of the human NPAS1 gene (Figure S3). However, we have not heard anymore from them about this study in the macrocephaly subgroup. It is also known that PTEN mutations can cause macrocephaly and autistic behavior (Butler, 2005; Goffin, 2001; Zori, 1998). TSC1/TSC2 mutations leading to tuberous sclerosis, can also lead to associated macrocephaly and autistic behavior (Fidler et al., 2000). There are other disorders with macrocephaly as a phenotype; such as Sotos syndrome, Weaver syndrome, Cole-Hughes macrocephaly, Neurofibromatosis Type 1 and a subset of Fragile X patients. Courchesne and colleagues (Courchesne and Pierce, 2005) have contributed a table showing syndromes characterized by early brain overgrowth (see Table 7). However, most of these syndromes have known etiology with specific genetic causes, and would not necessarily be considered to be autism spectrum disorders. For example, Sotos syndrome, which is also known as “cerebral gigantism”, is caused by mutations or deletions in NSD1 (nuclear receptor-binding SET domain protein) (Leventopoulos et al., 2009). NSD1 is thought to be involved in histone methylation and transcriptional regulation (Lucio-Eterovic et al., 2010). Also, many of these syndromes are considered “overgrowth syndromes”. Therefore, they often have increased susceptibility and incidence of tumorgenesis and hematopoetic cancers.

Table 7.

Table 1
A selected list of other syndromes characterized by early brain overgrowth

Syndrome	Age of observed macrocephaly	Neurobiological profile	Behavioral and cognitive profile	Physical characteristics	Genetic association	Incidence
Sotos syndrome (''cerebral gigantism'')	Birth (90% of cases (Cohen, 2002); in one case, macrocephaly evident by 31 weeks gestation (Chen et al., 2002)	Enlargement or other abnormality of cerebral ventricles (90%; Cohen, 2003); thinning of the corpus callosum (97%), particularly the posterior region and increased CSF spaces (79%; (Schaefer et al., 1997)	Neonatal hypotonia; early feeding difficulties; clumsiness and poor coordination; mild mental retardation to normal intelligence	Increased birth length and weight; advanced bone age; distinctive facial features such as prominent forehead, receding hairline, pointed jaw and ocular hypertelorism (increased distance between eyes; (Sotos et al., 1964)	Mutations in the NSD1 gene in majority of patients (Douglas et al., 2003; Rio et al., 2003)	Over 300 reported cases (Cohen, 2002). Estimated prevalence 1 in 10,000 to 1 in 50,000 (Sotos, 1997)
Canavan disease	Age of onset inconsistent among patients; macrocephaly occasionally observed at birth, but may not be noted until several months later, sometimes not until 1 year (Traeger and Rapin, 1998)	A degenerative disease causing deterioration of white matter. Death usually occurs within the first few years of life (Gordon, 2001)	Severe psychomotor handicaps present soon after birth; muscular hypotonia and a general failure of cognitive and motor development (Gordon, 2001)	Affected children secrete large amounts of N-acetylaspartic acid in their urine (Matalon et al., 1988)	Mutations in the gene for aspartoacylase 1 ocated on chromosome 17; an autosomal recessive mode of inheritance (Sistermans et al., 2000)	Rare in the general population, but more common in Ashkenazi Jews (carrier rate 1 in 40) (Sistermans et al., 2000)
Simpson-Golabi-Behmel syndrome (SGBS)	Pre and postnatal macrocephaly	Syndrome only seen in males (although females can have partial expression) with a high rate of neonatal death	Hypotonia; typically normal intelligence (Neri et al., 1998)	Increased birth weight and length; congenital heart defects, supernumerary nipples; coarse face (Lin et al., 1999)	X-linked syndrome; Glypican 3 (GPC3) mutations (deletions) in some patients (Li et al., 2001)	
Neurofibromatosis, Type 1	Macrocephaly in majority but not all patients, (Cutting et al., 2000; North, 2000)	Lesions (referred to as regional signal hyperintensities) in majority of patients (Cutting et al., 2000; North, 2000). Hyperintensities are observable on MRI T2-weighted images and may represent foci of neural dysplasia or dysmyelination (DiPaolo et al., 1995). Benign and malignant tumors (Arun and Gutmann, 2004)	Learning disabilities in majority (North et al., 1997) including a high incidence of ADHD (Arun and Gutmann, 2004)	Predominantly pigmentary abnormalities including café-au-lait macules, skinfold freckling and iris hamartomas (Arun and Gutmann, 2004)	Mutation in the NF-1 gene; autosomal dominant transmission; equally prevalent in males and females (North, 2000)	1 in 3000 (Arun and Gutmann, 2004)
Cowden syndrome	Macrocephaly in about 39% (Hanssen and Fryns, 1995) to 85% (Starink et al., 1986).	Seizures	Mild to moderate mental retardation delay of motor development (Hanssen and Fryns, 1995)	Multiple neoplasms (tumors), both benign and malignant, of the skin, breast, thyroid and gastrointestinal tract; lesions of the face and limbs; cobblestone like papules on face and body (Goffin et al., 2001)	Mutations in PTEN gene (a tumor suppressor gene) in q22–q23 region of chromosome 10; genetically transmitted cancer syndrome; inherited in an autosomal dominant pattern (Nelen et al., 1996).	
Weaver syndrome (WS)	Macrocephaly	Wide range of abnormalities noted, although not consistently, including cysts on the septum, dilation of ventricles and hypervascularization (Ardinger et al., 1986; Cohen, 1999)		Macrosomy, advanced skeletal age. Low pitched and horse cry (Cohen, 2003)	Genetic links currently unknown. However, WS found in multiple family members, suggesting an autosomal dominant pattern of inheritance in some cases (Proud et al., 1998). Some evidence of NSDI mutation like Sotos, but only in a small percentage (Douglas et al., 2003)	Rare; approximately 30 cases reported

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1.3 Pathology Background:

The neuropathologic findings in autism include increased brain volume (Courchesne et al., 2011), increased numbers and tighter packing of minicolumns (Casanova et al., 2002; Casanova et al., 2006), increased density of neurons (Courchesne and Pierce, 2005; Schmitz personal communication, 2004), cortical and subependymal dysplasia, and defects in neuronal migration (Wegiel et al., 2010). These data all suggest that autistic children may have abnormalities in brain development and neurogenesis. Minicolumns are thalamocortical projections made up of bundles of neurons. Casanova and colleagues (Casanova et al., 2006) reported minicolumn abnormalities in autistic children, and noted the increased density of neurons. He suggested this could cause a local overconnectivity at the expense of distant connections. Figure S4 is a figure from their paper showing the increased density of neurons in the post-

mortem brain tissue of an autistic patient. Courchesne and Schmitz (Courchesne, 2005; Schmitz personal communication, 2004) have performed stereologic studies on autistic brain samples and have found an increased number of neurons in the autistic subjects. They were only counting neuron number globally in the cortex. It would have been informative for these studies to have been extended to specific regions to see if there are regionalized increases, or if it is just a generalized "whole brain" phenomenon. Courchesne and colleagues suggested three possible mechanisms for the excess neuron number. First, it could be a failure of the regulation of neurogenesis. Second, it could be delays or deficits in normal apoptosis. Third, it could be late "compensatory" genesis of neurons and glial cells in response to some adverse conditions (Courchesne et al., 2005). These investigations also offer the hypothesis that the increased number of neurons could possibly underlie the increased cerebral volume seen in autistic brains; and that the increased number of neurons may disrupt connectivity. Courchesne and colleugues recently performed a review of MRIs and brain volumes from several studies done over the past 18 years, and they found that although brain volume is increased in young autistic children, the enlargement seems to normalize as patients progress to adulthood (Courchesne et al., 2011). This observation has been known for quite sometime, but this more recent paper reviewed and collected data from various studies in order to argue that the observed normalization of brain size is indeed true (Courchesne et al., 2011). Wegiel and colleagues reported on neuropathologic studies performed on 13 autistic post-mortem brain samples (Wegiel et al., 2010). They found several subependymal nodules and cortical dysplasias, suggesting dysregulation of neurogenesis, and disruption of neuronal migration in autistic subjects. Therefore, Wegiel and colleagues hypothesized the existence of multiregional focal developmental alterations that lead to defects in brain microarchitecture in autistic patients, offering the idea that there are multiregional problems can help explain the heterogeneity of symptoms seen in autism. These investigations mentioned that there has been a lack of neuropathologic studies, as compared to clinical studies, mainly because of the scarcity of samples of post-mortem brain tissue (Wegiel et al., 2010).

1.4 Mouse Models:

As with many of the complex human disorders, scientists have begun efforts to develop mouse models of autism. However, mimicking phenotypes of autism in a mouse is an extremely difficult task. Mice with mutations in many of the candidate autism susceptibility genes have been made. Some, such as the Neuroligin mice, have been reported to display autistic phenotypes (Tabuchi et al., 2007). We are very interested in the macrocephaly phenotype, again, because of the findings in our studies of NPAS1-deficient mice. Therefore, we wanted to know if there are any other mice with enlarged brains (not necessarily related to autism). If one disregards the mice that develop hydrocephalus, not many mice have been reported to have enlarged brains; the reported mice include: the PTEN-deficient mice, transgenic mice with a stabilized β-catenin, Caspase 9 mutant mice, and FGFR3 mutant mice (Groszer et al., 2001; Chenn and Walsh, 2002; Kuida et al., 1996; Inglis-Broadgate et al., 2005).

Phosphatase and tensin homolog on chromosome ten (PTEN) is a tumor suppressor gene mutated in many cancers (Ali et al., 1999). Individuals with PTEN mutations are prone to tumors but also display brain disorders, including macrocephaly, seizure, Lehrmitte-Duclos disease, and mental retardation (Waite and Eng, 2002). PTEN mutations have also been reported in autistic individuals with macrocephaly (Butler, 2005; Goffin, 2001; Zori, 1998). PTEN has lipid phosphatase activity against the 3' phosphate of phosphatidylinositol 3,4,5 triphosphate (Maehama and Dixon, 1998). Phosphatidylinositol 3-kinase (PI3K) catalyzes the reverse of this reaction, and leads to AKT activation. AKT phosphorylates several substrates including TSC2, GSK3β, and the proapoptotic protein BAD (Luo et al., 2003). Abnormalities in this pathway have been found in many brain disorders. For example, mutations that inactivate PTEN or activate PI3K are found in malignant brain tumors (Ali et al., 2004; Broderick et al., 2004), low AKT levels are associated with schizophrenia (Emamian et al., 2004), and individuals with TSC mutations have CNS disorders, including autism (Wiznitzer et al., 2004). PTEN null mice are embryonic lethal, and heterozygotes are prone to tumors (Stiles et al., 2004). However, PTEN-deficient mice carrying a conditional loss of PTEN in Nestin+ neural precursor cells, have enlarged brains which are doubled in weight compared to wild type mice (Groszer et al., 2001). Further studies by Groszer and colleagues indicated that the increased brain size was due to increased neuronal precursor cell

proliferation, decreased cell death, and enlarged cell size. From *in vitro* studies of PTEN-deficient neural stem/progenitor cells, Groszer and colleagues found that the loss of PTEN in progenitor cells leads to an increased proliferation capacity and self-renewal, due to a shortened cell cycle time (Groszer et al., 2006). Furthermore, loss of PTEN in a discrete population of mature neurons also leads to macrocephaly (Kwon et al., 2006). The mutant mice that Kwon and colleagues described have a selective deletion of PTEN in neuron-specific enolase (Nse+) neurons. Nse is expressed in certain populations of mature neurons in the cerebral cortex and hippocampus. Kwon and colleagues suggested that the macrocephaly in this set of PTEN-deficient mice is due to neuronal hypertrophy. Abnormal dendritic and axonal growth, and increased synapse number were also identified in these mice, as well as altered social behavior and inappropriate responses to sensory stimuli. The abnormal neuron morphology was associated with activation of the Akt/mTor/S6k pathway and inactivation of GSK3β. Thus, Kwon and colleagues suggested that abnormal activation of the PI3K/AKT pathway can lead to macrocephaly and behavioral abnormalities reminiscent of autism (Kwon et al., 2006). Interestingly, a deficiency of PTEN in progenitor cells leads to neuron proliferation, yet a deficiency in mature post-mitotic neurons only leads to hypertrophy of the neurons. However, both studies implicate the AKT and GSK3β pathway as a possible cause of the observed increase in brain size (Groszer et al., 2001; Kwon et al., 2006). In addition, it is known that GSK3β can phosphorylate β-catenin and target it for destruction (Clevers and van de Wetering, 1997). Therefore, if GSK3β is inactivated by increased Akt activity in PTEN-deficient mice, then perhaps β-catenin is less phosphorylated and more stable, which can lead to cell proliferation, in part via the downstream effects of β-catenin, such as interaction with the lymphoid enhancer factor/T cell factor (LEF/TCF) complex to activate transcription of target genes leading to growth or proliferation (Novak and Dedhar, 1999). Furthermore, a study by Easton and colleagues found that a deficiency of Akt can lead to decreased brain size (Easton et al., 2005).

Chenn and Walsh reported studies on a transgenic β-catenin mouse that expressed a stabilized form of β-catenin in Nestin+ neural precursor cells (Chenn and Walsh, 2002). The stabilized form of β-catenin in

this study had a truncated N-terminus, which prevents it from being phosphorylated by Gsk3β, which would target it for destruction. Chenn and Walsh first overexpressed this stabilized β-catenin in the (nestin+) neural precursor cells using a rat Insulin II intron in the construct to increase expression. These mutant mice had enlarged brains embryonically, and abnormal brain structure that would not be compatible with life (Chenn and Walsh, 2002). However, Chenn and Walsh wanted to understand the effects that stabilization of β-catenin would have post-natally, so they removed the rat Insulin II intron from the transgene construct in order to get lower levels of expression of the stabilized β-catenin in neural precursor cells (Chenn and Walsh, 2003). Some of these mice survived into adulthood. The mutants had enlarged forebrains, increased cortical surface area, expanded subventricular zones with subcortical aggregations of neurons, and enlarged, distorted hippocampi. They also had disorganized layering of the cortex. Chenn and Walsh observed an increased number of proliferative precursor cells, and concluded that this could result from increased mitotic rates, decreased cell death, or changes in cell fate choice (whether to differentiate of proliferate). Chenn and Walsh concluded that the β-catenin stabilization allows the neural precursors to re-enter the cell cycle, rather than differentiate, leading to an increase in the number of neural progenitors. This increase in the progenitor pool leads to an increased total number of neurons, and an enlarged brain with increased surface area. Previous work has proven that an expansion of the neural precursor pool can increase the total number of neurons in the cortex (Caviness et al., 1995; Rakic and Caviness, 1995).

Chenn and Walsh discussed, that in their case, the behavioral consequences of having abnormally large numbers of neurons in distorted forebrains, could only be described anecdotally. Although formal behavioral testing was not performed, Chenn and Walsh do offer that from observation, “the transgenic animals were easily startled”, and “were more aggressive … and would attempt to bite the investigator much more frequently than normal mice” (Chenn and Walsh, 2003). The observation that the mutants are easily startled is an interesting finding considering the similar increased startle response phenotype seen in the NPAS1-deficient mice that will be discussed later.

Caspase 9-deficient mice have a reduction of programmed cell death, which causes brain malformations characterized by cerebral enlargement with increased surface area, ectopic growth, and thickened ventricular zones (Hakem et al., 1998; Kuida et al., 1998). Hakem and colleagues observed decreased TUNEL labeling in the Caspase 9 mutants, along with increased BrdU labeling at E12.5 (Hakem et al, 1998). Caspase 9 is upstream of Caspase 3 in the caspase cascade; thus, in the Caspase 9 null mice there was no activation of Caspase 3 in embryonic brains, and reduced apoptosis (Kuida et al., 1998). Caspase 3-deficient mice also display brain malformations, however, the abnormality was far more severe in the Caspase 9-deficient mice (Kuida et al., 1996). Caspase 9-deficent mice often display protrusions of brain mass, stenosis of ventricles, and heterotopias. Kuida and colleagues suggest that Caspase 9-deficient mice display a more severe malformation than the Caspase 3-deficient mice because Caspase 9 may also activate other caspases in the nervous system, such as Caspase 7 (Kuida et al., 1998). Moreover, Kuida and colleagues suggests that a lack of apoptosis can expand the neural proliferative precursor pool; and the size of the precursor pool determines how many cells will exit the cell cycle and migrate to form distinct structures of the brain (Rakic, 1988). The size of these brain structures are subsequently determined by the number of cells which have asymmetrically divided from the precursor cells, which is determined by cell cycle kinetics, rate of cell cycle exit, and rate of programmed cell death of the progenitor cells (Rakic, 1995).

Mice expressing a constituitively active form of Fgfr3 displayed enlarged brains as well; namely, they had increased cortical thickness due to increased progenitor cell proliferation, and a profound decrease in developmental apoptosis (Inglis-Broadgate et al., 2005). The activating, K650E mutation in FGFR3 (K644E in mice) in this study causes perinatal lethal dwarfism, termed Thanotophoric Dysplasia type II (TDII) (OMIM#187600) in humans. The K650M mutation of FGFR3 can cause a slightly milder disorder, Severe Achondroplasia with Developmental Delay and Acanthosis Nigricans. (SADDAN) (Bellus et al., 1999; Tavormina et al., 1999). Macrocephaly is a prominent feature in both disorders (Inglis-Broadgate, 2005).

In summary, it appears that expansion of the neuronal precursor pool via proliferation at the expense of differentiation, can lead to the generation of excess neurons. Alternatively, the precursor pool could be expanded by a decrease in apoptosis. The excess number of neurons can then lead to enlargement of the brain. The brain can also be enlarged due to neuronal hypertrophy, as in the case of the conditional Nse+ PTEN-deficient mice.

It is interesting to note that the Akt pathway and Gsk3β have been implicated in neuropsychiatric disorders, such as schizophrenia, bipolar disorder, and depression (Emamian et al., 2004; Jope and Roh, 2006), as well as in neurodegenerative disorders, such as Alzheimer's disease (Forde and Dale, 2007). However, in these disorders, Akt levels are low, and Gsk3β is thought to be activated, which is the opposite of what has been discussed regarding mice with macrocephaly and increased numbers of neurons. Gsk3β inhibitors, such as Li^+ and other compounds, have even been used therapeutically (Jope et al., 2006). One might then suggest that Gsk3β activators be used as a treatment for macrocephaly, or possibly even for autism. However, I would have to disagree with that mainly because Gsk3β is expressed in many organs and cell types and important for diverse processes. An activator for Gsk3β would most likely have undesirable side effects, which would outweigh its benefits. Alternatively, if one were to find a more specific target, for a protein involved in this pathway expressed only in the neural progenitor pool, then devising this as a therapeutic strategy may become useful. Yet this would still most likely lead to negative consequences, such as decreased neurogenesis. These complications make the therapeutic treatment of autism an extremely difficult task. Perhaps it is best to first understand the disorder, and learn more about the brain through the ?? of autistic processes. Hopefully this science-based approach will eventually lead to means of prevention of autism, rather than curative treatments.

Figure S3.

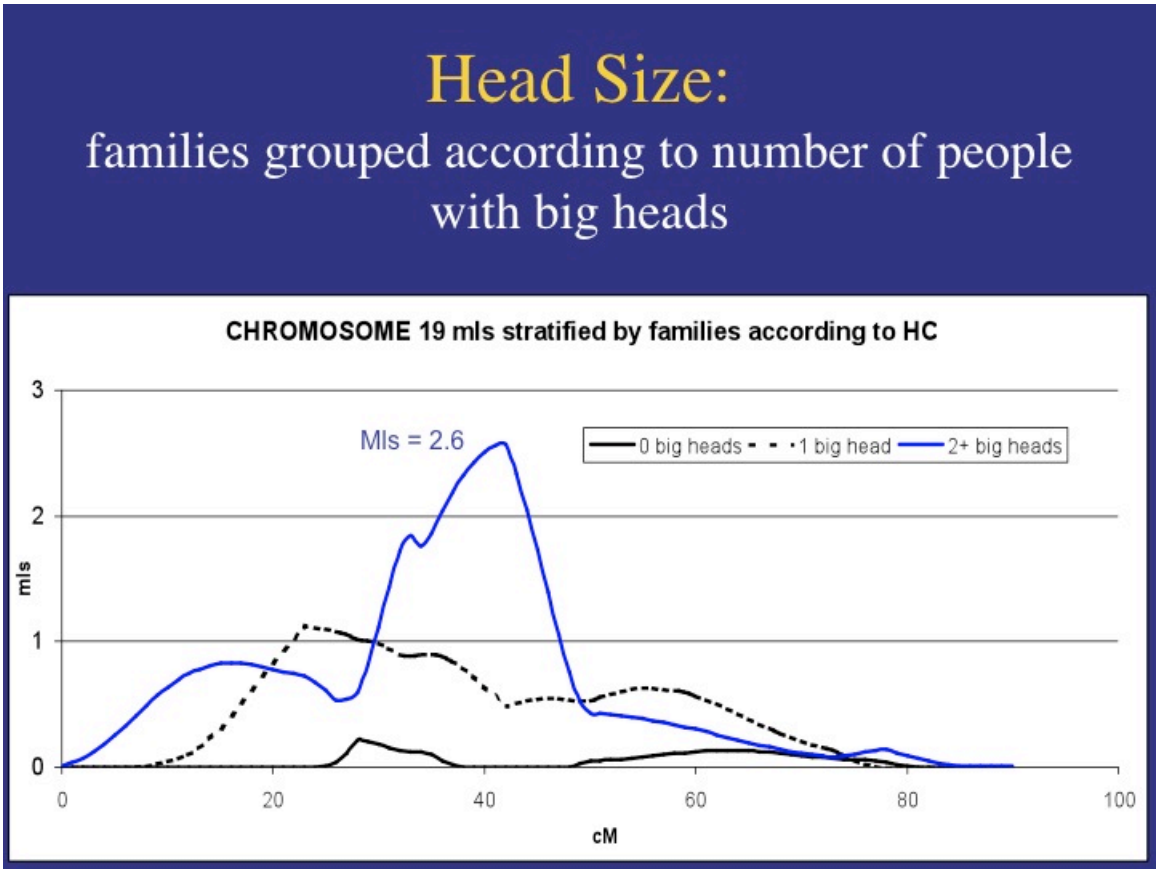
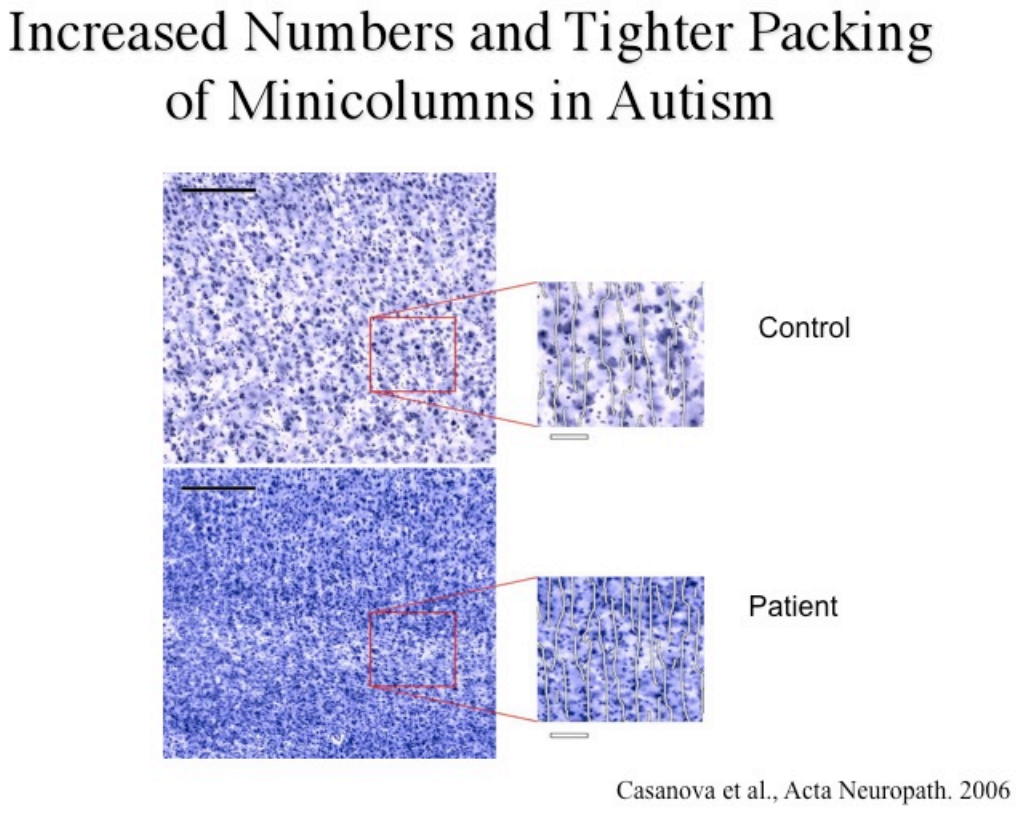

Head Size:
families grouped according to number of people with big heads
CHROMOSOME 19 mls stratified by families according to HC
Mls = 2.6
0 big heads
1 big head
2+ big heads
mls
cM
0
1
2
3
0
20
40
60
80
100

Figure S4.


Increased Numbers and Tighter Packing of Minicolumns in Autism
Control
Patient
Casanova et al., Acta Neuropath. 2006

CHAPTER TWO

Neurogenesis Introduction

2.1 Embryonic Brain Development:

During the third and fourth weeks of development in the human, the ectoderm on the midline of the embryo undergoes neurulation. During this process, the neuroepithelial cells proliferate and the lateral edges of the neuroectoderm rise up. The end result is the production of a fluid-filled neural tube, surrounded by mesenchyme, just underneath the surface ectoderm (see Figure 1) (Blom et al., 2006). At this early stage, the neural tube can be subdivided into four distinct regions. From caudal to cranial, these regions are: the spinal cord, rhombencephalon, mesencephalon, and prosencephalon. These regions are color coded in a longitudinal section in Figure 2. The most anterior part of the tube becomes an enlarged vesicle called the prosencephalon. The wall of this structure is composed of one continuous sheet of neuroepithelial cells that will give rise to all of the structures in the forebrain, including the cerebrum, the hippocampus, and the thalamus. Early growth of the prosencephalon, is highlighted by the expansion of the right and left cerebral hemispheres, as shown in the longitudinal figure (see Figure 2) (http://neurolex.org/wiki/). This expansion is the visible manifestation of a split of the prosencephalon into two regions; the telencephalon, which is the cranial most portion, including the cerebral hemispheres that grow from that region, and the diencephalon, which will give rise to the thalamus, hypothalamus, and epithalamus. Also, the rhombencephalon divides into two regions; the metencephalon, which will form the pons and cerebellum, and the myelencephalon, which will develop into the medulla. Further expansion of the cerebral hemispheres is highlighted by continued expansion anteriorly to form the frontal lobes, and posteriorly to form the parietal and occipital lobes. Yet another outgrowth occurs from a portion of the posterior region of the developing brain; this outgrowth projects first slightly

inferiorly, and then anteriorly, to form the temporal lobe. Also, the cerebellum is formed as an outgrowth of the alar plates of the pons (Hebert and Fishell, 2008). This is a simple overview of the earliest formation of the brain and CNS. However, embryonic neurogenesis and corticogenesis are extremely detailed and spatio-temporally precise processes.

Figure1.

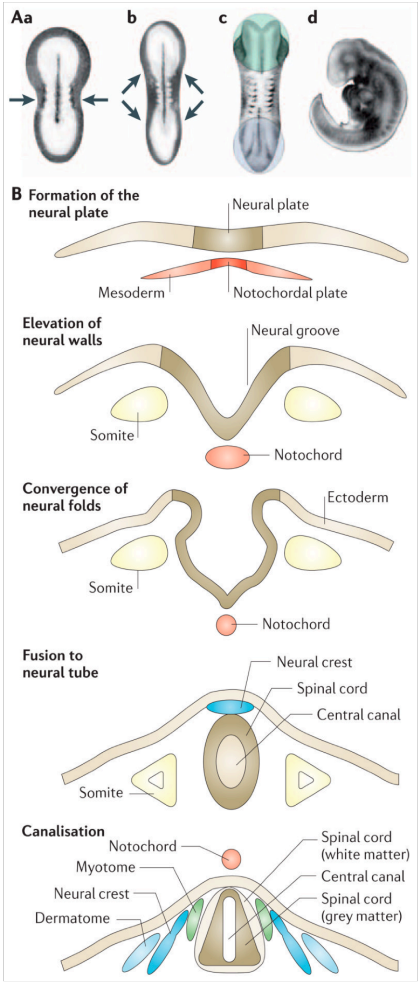
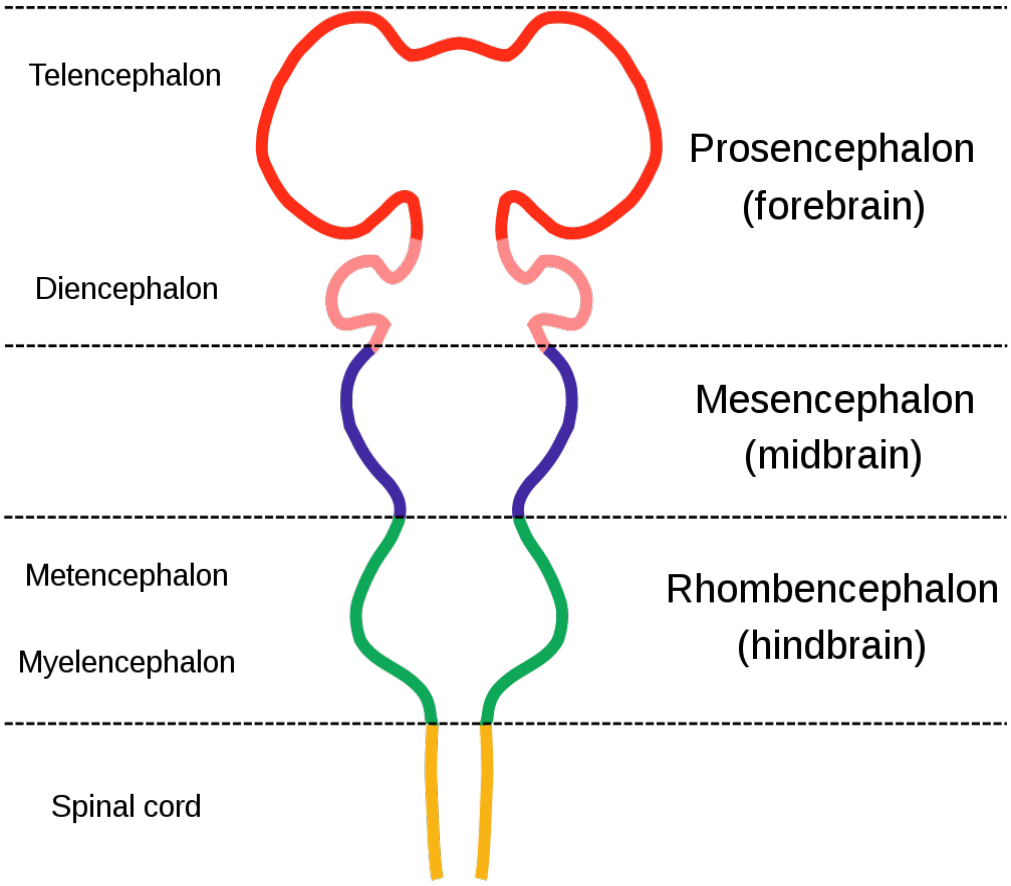


Figure 1. Neural tube closure in the developing mouse

A | Four phases of primary neurulation in neural tube closure. Panels **a**–**c** are dorsal views, whereas panel **d** is a lateral view. **a**–**c** | Bidirectional fusion beginning from the original initiation site in the mid-cervical region (**a**), followed by continued bidirectional fusion (**b** and **c**) rostrally towards the anterior neuropore (blue shading) and caudally towards the posterior neuropore (green shading), which progresses as a result of convergent extension and apical constriction. Panel **d** shows a closed neural tube, with complete fusion over the rhomobencephalon, which has yet to be covered externally. Failure of the posterior neural tube to close can result in spina bifida. Defects in the closure of the anterior neural tube could result in anencephaly. **B** | Stages of neural tube closure in a transverse section. The neural plate elevates to form neural folds, which progressively appose each other, ultimately fusing to create a closed neural tube. This occurs just before the end of the first month of pregnancy. Panel **B** modified, with permission, from REF. [43] © (2001) Society for Experimental Biology and Medicine.

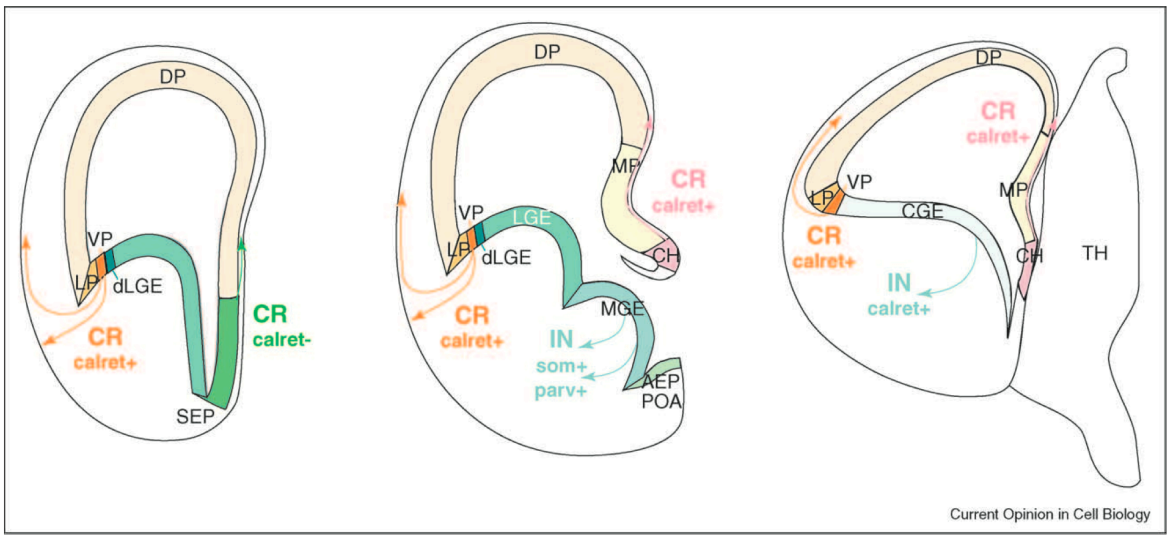
Figure 2.



The mammalian telencephalon, which includes the cerebral cortex and the basal ganglia, is the foundation of motor coordination, emotions, higher cognition, and consciousness in humans (Guillemot, 2005). In the embryo, the telencephalon has two main subdivisions – the dorsal telencephalon (the pallium) and the ventral telencephalon (the subpallium) – which generate very different types of neurons. The germinal zone of the dorsal telencephalon gives rise to the excitatory glutamatergic projection neurons of the cerebral cortex, which undergo radial migration to reach different layers of the cortex. Progenitors of the ventral telencephalon, or subpallium, generate γ-aminobutyric acid-expressing (GABA-ergic) inhibitory neurons. These will develop into basal ganglia neurons, and interneurons that migrate tangentially to contribute to formation of the cortex. The migration of neurons is a complex process, which will be discussed later. Figure 3 shows the subdivisions of the embryonic telencephalon and how the neuronal progenitors are divided into distinct domains that generate specific neuronal subtypes (Guillemot, 2005).

Figure 3.

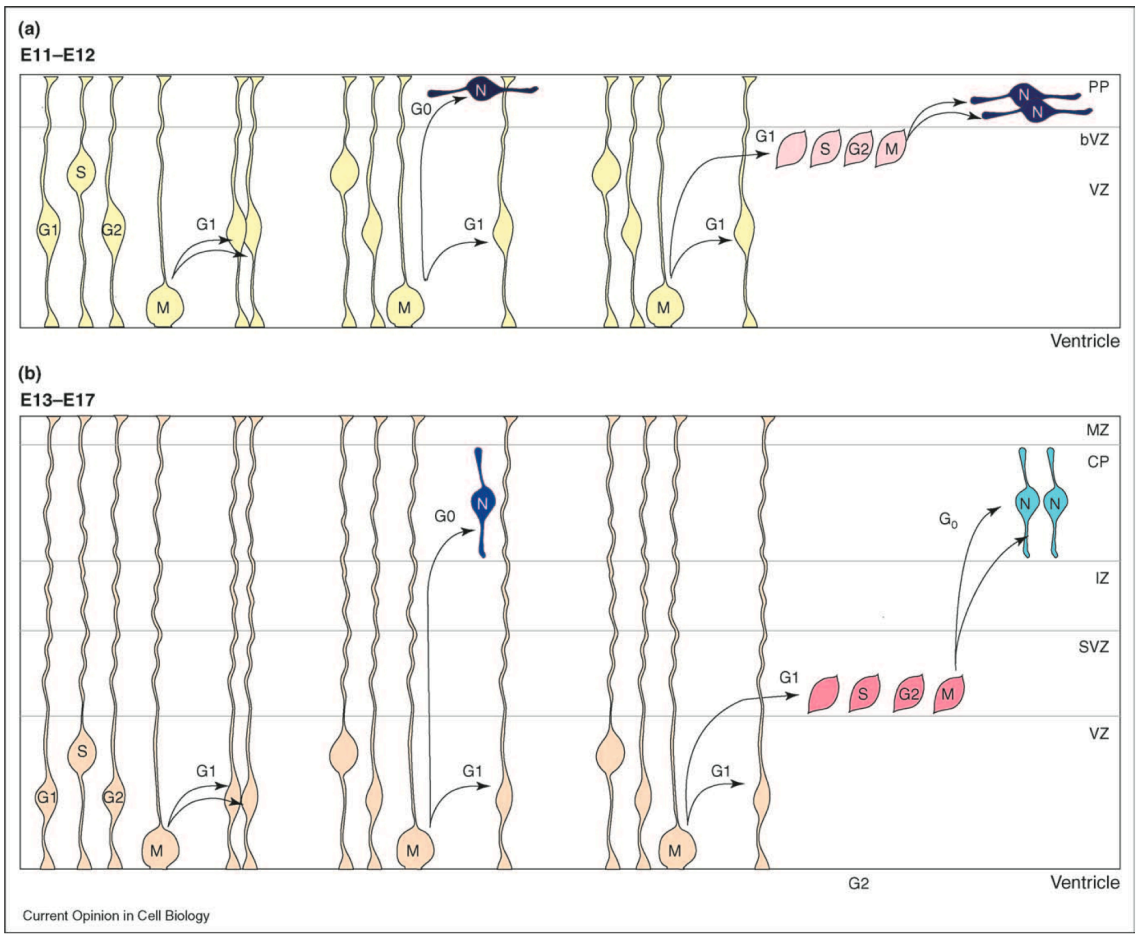
Figure 1



Progenitors in the embryonic telencephalon are organized in distinct domains that generate defined neuronal subtypes. This figure represents frontal sections through the mouse embryonic telencephalon at three rostral–caudal levels (rostral to caudal sections are shown from left to right). The dorsal telencephalon includes the choroids plexus (cpe), the cortical heme (ch), medial pallium (mp), dorsal pallium (dp), lateral pallium (lp) and ventral pallium (vp), while the ventral telencephalon includes the dorsal lateral ganglionic eminence (dlge), ventral lateral ganglionic eminence (vlge), medial ganglionic eminence (mge), septum (sep), and caudally, the caudal ganglionic eminence (cge). Distinct populations of Cajal–Retzius cells (CR; calret+ denotes the presence of caretinin) [20•,21•] and of cortical interneurons (IN; som+, parv+ and calret+ denote the presence of somatostatin, parvalbumin and calretinin, respectively) [18] have been shown to originate from some of these domains. Their migration directions are indicated by arrows.

There are three distinct populations of progenitors in the embryonic telencephalon, which generate neurons and glial cells. The neuroepithelial cells that form the wall of the neural tube at the beginning of neurogenesis give rise to the earliest-born neurons of the cortex, and to two other progenitor populations: radial glial cells and basal progenitors (see Figure 4, Guillemot, 2005).

Figure 4.



Several types of progenitors contribute to neurogenesis in the mammalian cortex [12••–14••]. **(a)** At the beginning of neurogenesis (around E11–E12 in the mouse), neuroepithelial cells located in the ventricular zone (VZ) and undergoing interkinetic nuclear migration either divide symmetrically to generate two new neuroepithelial cells or divide asymmetrically to generate either a neuroepithelial cell and a neuron, which migrates to the preplate (PP), or a neuroepithelial cell and a basal progenitor, which divides symmetrically on the basal side of the VZ (bVZ) to generate two neurons. **(b)** As neurogenesis progresses (around E13–E17 in the mouse), several signalling pathways induce the expression of glial markers by neuroepithelial cells, which thus become radial glial cells. These cells also divide either symmetrically, to generate two radial glial cells, or asymmetrically, to generate a radial glial cell and either a neuron, which migrates through the intermediate zone (IZ) into the cortical plate (CP), or a basal progenitor, which moves to the subventricular zone (SVZ) and divides symmetrically to generate two neurons. MZ, marginal zone.

During each cell cycle, the progenitor cells undergo a distinctive pattern of oscillation in the ventricular zone, called interkinetic nuclear migration. Cells undergo S phase at the basal surface of the ventricular zone, and mitosis (M) at the apical surface. Before the onset of neurogenesis, most progenitor cells undergo symmetric cell division to produce two daughter cells that also adopt the progenitor cell fate. However, as cortical development proceeds, the length of the cell cycle increases, mainly from a longer G1 phase. During this time, the cells begin to undergo asymmetric cell division, and the fraction of cells that begin to differentiate into neurons increases, and the number of cells that remain as progenitors decreases. By the end of cortical development, the majority of neural progenitors give rise to two daughter cells that will

differentiate into neurons, thus leading to the eventual depletion of neural precursors in this region (Caviness and Takahashi, 1995).

Once a progenitor cell has exited the cell cycle, it must migrate out of the ventricular zone, toward its final destination in the developing cortex. At around embryonic day 11 (E11) in the mouse, the first group of neurons that migrates out of the ventricular zone populates the pre-plate. The next wave of neuronal migration (~E13), separates the pre-plate into two layers: the more superficial (or outer) zone, which consists of Cajal-Retzius cells which were born in the first wave, and a deeper subplate, which is made of the rest of the primordial cells (see Figure 5) (Ayala et al., 2007). The development of the cortex continues with successive waves of migration that position neurons in different layers of the cortical plate in an inside-out manner (Hatten, 1999).

Figure 5.

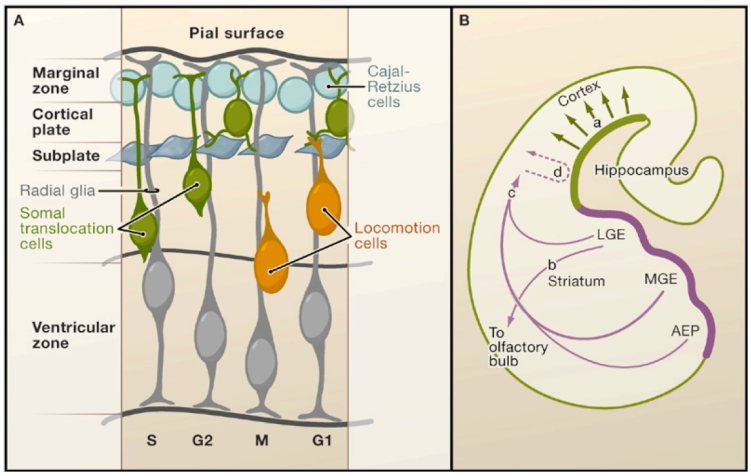


Figure 1. Radial Organization of the Cerebral Cortex
(A) Projection neurons are born from radial glial cells in the ventricular zone and migrate radially along radial glial fibers toward the pial surface. During each cell cycle, the progenitor cells undergo a distinctive pattern of oscillation, termed interkinetic nuclear migration. Cells undergo S phase at the basal surface of the ventricular zone and mitosis (M) at the apical surface. The first cohort of neurons that migrate out of the ventricular zone constitutes the preplate. The subsequent wave of neuronal migration splits the preplate into two layers: the more superficial marginal zone, which consists of the Cajal-Retzius cells; and the deeper subplate. Projection neurons may use any of two distinct modes of radial migration, somal translocation, or locomotion, to arrive at their final position in the cortex.
(B) Primary routes of tangential migration. Projection neurons migrate radially from the dorsal ventricular zone (a). Interneurons expressing GABA originate from the subpallium structures, the LGE, MGE, and AEP, and migrate tangentially into the olfactory bulb (b) or the cortex (c and d). When they arrive at the cortex, some of these neurons are directed toward the ventricular zone before radially migrating into the cortex (d). MGE, medial ganglionic eminence; LGE, lateral ganglionic eminence; AEP, anterior entopeduncular area.

2.2 Neuronal Migration:

Two types of migration have been identified in the development of the forebrain: radial migration, in which cells migrate from the progenitor zone towards the surface of the telencephalon in a radial direction (from the neural tube layout), and tangential migration, in which the cells migrate orthogonally to the direction of the radial migration (Marin and Rubenstein, 2001). Glutamatergic neurons are produced from

the pallium ventricular zone and reach their final destination mainly via radial glia-mediated migration. In contrast, GABA-ergic interneurons and oligodendrocytes are generated from progenitors in the subpallium and reach the cerebral cortex via tangential migration (Marin and Rubenstein, 2001).

Nearly 80-90% of all cortical neurons arise from proliferative zones of the dorsal telencephalon and migrate radially to their final laminar destination. In embryogenesis, as the cortical wall thickens from neuroepithelial cell proliferation, a system of radial glial fibers spans across the radial plane. Each radial glial cell has its soma in the ventricular zone and extends a process across the wall of the neural tube to the pial surface. Recent evidence has shown that radial glial cells are not only providing a pathway for migration, but are also progenitor cells themselves (Kriegstein and Noctor, 2004).

As mentioned earlier, although most cortical neurons reach their final destination via radial migration, the majority of GABA-ergic interneurons and some oligodendrocytes migrate tangentially across the radial glial fibers. The two major tangential routes that have been identified are: (1) from the medial ganglionic eminence (MGE) to the neocortex and hippocampus, and (2) from the lateral ganglionic eminence (LGE) to the neocortex and olifactory bulb (Kriegstein and Noctor, 2004; Marin and Rubenstein, 2001). There are also cholinergic neurons that originate in the anterior entopeduncular area (AEP) and anterior preoptic area (POA) that migrate to the neocortex. Figure 6 displays the origin and migration routes of glutamatergic, GABA-ergic, and cholinergic neurons (see Figure 6) (Marin and Rubenstein, 2001). The route from the LGE to the olfactory bulb persists into adulthood, and that route is often termed the rostral migratory stream originating from the proliferative subventricular zone (SVZ) located in the lateral ventricles (Lois and Alvarez-Buylla, 1994).

Figure 6.

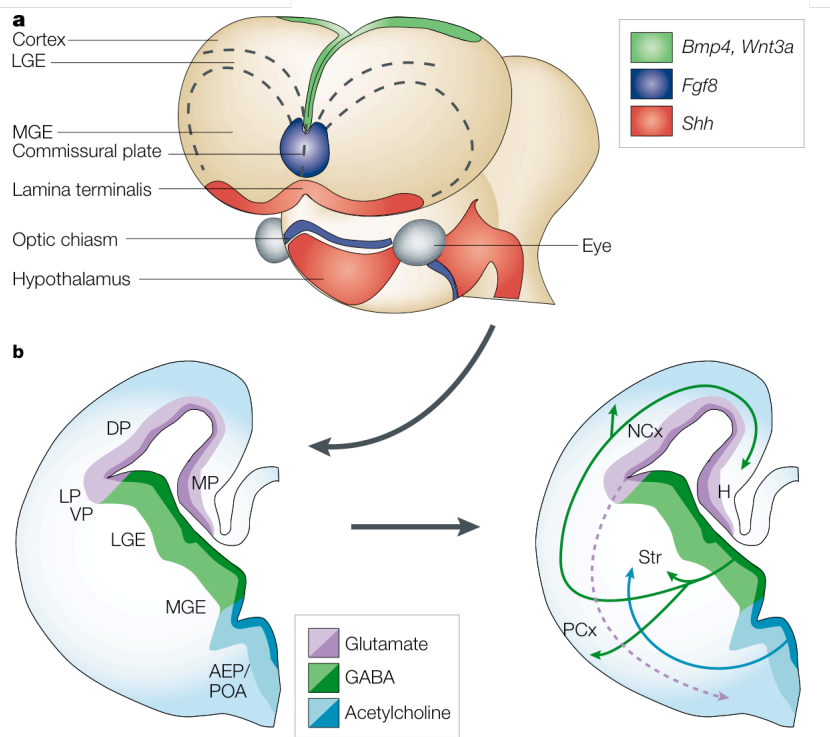


Figure 5 | **Coordination of patterning and migration mechanisms is required to achieve the cellular complexity of the telencephalon. a** | The main known signalling centres of the telencephalon are depicted in this schematic view of the mouse brain at mid-gestation. Patterning molecules include bone morphogenetic proteins (*Bmp4*) and members of the wingless-type MMTV integration site family (*Wnt3a*) (green), fibroblast growth factors (*Fgf8*; blue) and sonic hedgehog (*Shh*; red). **b** | Patterning molecules, alone or in combination, are postulated to regulate the regionalization of the telencephalic progenitor zone through the induction of transcription factors, as depicted in this schema of a coronal hemisection through a telencephalon at embryonic day 14.5. **c** | Distinct neurotransmitter phenotypes seem to be specified in different progenitor populations in the telencephalon. So, it is suggested that glutamatergic neurons are specified in the pallium (VP, LP, DP and MP), whereas GABA and cholinergic neurons are specified in the subpallium (LGE, MGE and AEP/POA). Tangential migration of glutamatergic, GABA (γ-aminobutyric acid) and cholinergic neurons to their final postmitotic destination allows different subdivisions of the telencephalon to increase their cellular complexity (note that radial migrations of projection neurons are omitted for clarity). AEP, anterior entopeduncular area; DP, dorsal pallium; H, hippocampus; LGE, lateral ganglionic eminence; LP, lateral pallium; MGE, medial ganglionic eminence; MP, medial pallium; NCx, neocortex; PCx, piriform cortex; POA, anterior preoptic area; Str, striatum; SVZ, subventricular zone; VP, ventral pallium; VZ, ventricular zone.

There is a tight correlation between the birth-date of a neuron and its laminar fate (layer), even if the neurons originate in different regions. The layers of the cortex are established in an inside-out pattern where the earliest born neurons form the deeper layers of the cortical plate (layers V-VI), while the last wave of neurons born will migrate past the earlier born neurons to form the more peripheral layers II and III (Marin and Rubenstein, 2003). Interestingly, the radially migrating projection neurons and the

tangentially migrating interneurons that are born at the same time, share the same laminar fate. Transplantation experiments suggest that interneurons are capable of sensing layer-fate determinant cues from the cortical microenvironment (Marin and Rubenstein, 2003). Cajal-Retzius (CR) neurons populate the earliest post-mitotic layer or preplate, Cajal-Retzius cells are localized in the most superficial layer of the cortex (marginal zone / layer I), and can play a crucial role in positioning of the younger neurons (in layers II-VI), via expression of the extracellular glycoprotein Reelin (Marin-Padilla, 1998).

The processes involved in the direction of migration of a neuron and its destination are complex because they coordinate the net result of how the neuron behaves in a given microenvironment. Extracellular cues are received through receptors, and signals are transmitted via intracellular signaling pathways, ultimately resulting in changes in the cytoskeleton that can direct the motility of the cell. The complexity of this signaling network can be seen in Figure 7 (Ayala, 2007).

Figure 7.

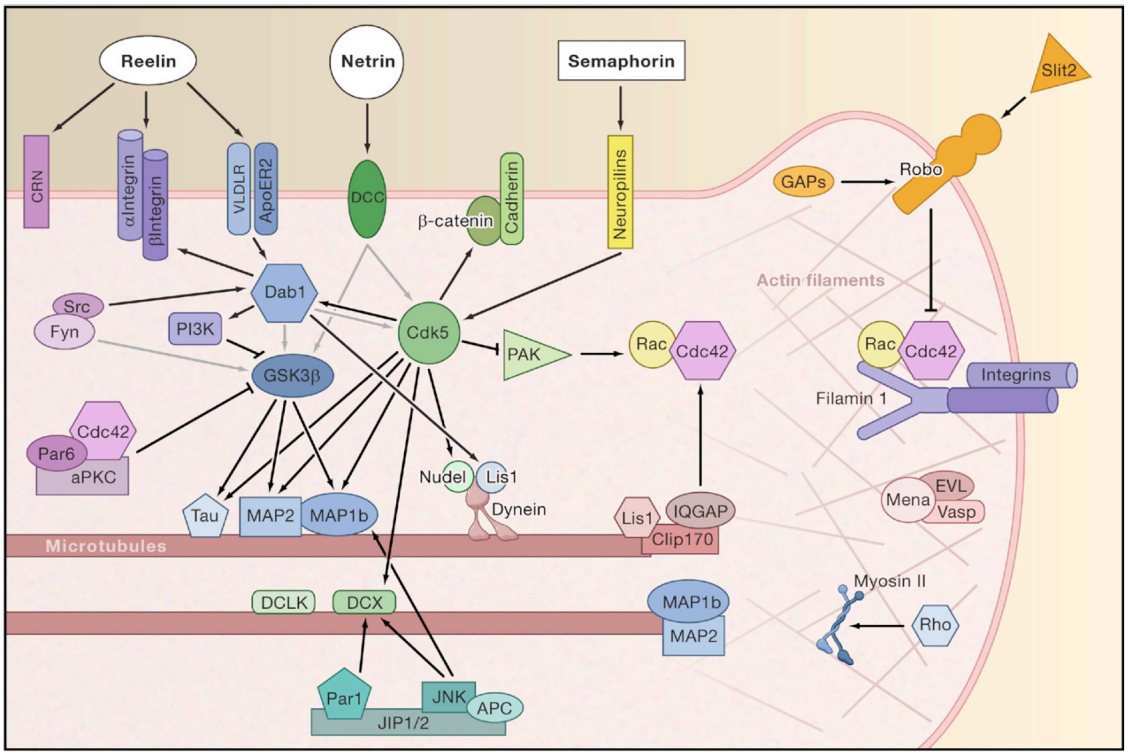


Figure 3. Molecular Network Regulating Neuronal Migration
In order for the new-born neurons to arrive at their correct position, extracellular guidance cues, growth, and neurotrophic factors, and cell adhesion complexes, among others, must trigger a wide range of intracellular signaling cascades and, ultimately, end in the coordinated regulation of cytoskeletal dynamics. Some of these pathways, such as Reelin signaling, are very extensively characterized, where others are just beginning to be elucidated. Black arrows indicate direct interactions between proteins. Gray arrows indicate downstream activation of a pathway without evidence of a direct interaction.

Disruptions in the receipt of guidance cues, in steps of the signaling pathway, or in the cytoskeleton itself, can cause neuronal migration defects, resulting in various disorders, as seen in Table 1 (Ayala, 2007).

Table 1.

Table 1. Genes Responsible for Human Congenital Disorders Featuring Neuronal Migration Defects

Genes	Human Disorders	Gene Loci	Syndromes	Reference
Reelin	Lissencephaly with cerebellar hypoplasia	7q22	Mental retardation, hypotonia, epilepsy, and myopia	Hong et al., 2000
Lis1	Isolated lissecencephaly sequence and its severe form Miller-Dieker Syndrome	17p13.3	Mental retardation, epilepsy, and premature death; Miller-Dieker Syndrome also has craniofacial abnormalities	Ozmen et al., 2000; Reiner et al., 1993
14-3-3 ε	Miller-Dieker Syndrome	17p13.3	Craniofacial abnormalities	Toyo-oka et al., 2003
DCX	Isolated lissecencephaly sequence in males and Subcortical band heterotopia in females	Xq22.3-23	Mental retardation, and epilepsy; less severe in female due to X mosaic inactivation	Lambert de Rouvroit and Goffinet, 2001
Filamin A	Periventricular heterotopia	Xq28	Epilepsy and vascular signs	Fox et al., 1998
Fukutin	Fukuyama-type congenital muscular dystrophy	9q31	Mental retardation, epilepsy and muscular dystrophy	Gressens, 2005
POMGnT1 (protein O-mannose β-1,2-N-acetylglucosaminyltransferase)	Muscle-eye-brain disease	1p32-34	Mental retardation, severe myopia, glaucoma and muscular dystrophy	Yoshida et al., 2001
Disc-1	Schizophrenia	1q42.1	Schizophrenia	Kamiya et al., 2005; Millar et al., 2000

2.3 Radial Unit Hypothesis:

Pasko Rakic noticed that neurons in the embryonic primate cerebrum were organized into geometrically perfect vertical columns. In 1988, Rakic offered the "radial unit hypothesis" to explain the origin of these columns (Rakic, 1988). He hypothesized that each vertical column, or bundle of neurons, originated from a single neural progenitor. Thus, the horizontal coordinates of cortical neurons are determined by the relative positions of their precursor cells in the ventricular zone, while their vertical position (layer) in the cortex is related to their time of origin and arrival in the cortex. Rakic used the term "ontogenetic columns" to describe these radial cortical columns. (Rakic, 1988, and see animated movie at: http://rakiclab.med.yale.edu/RadialMigration.html). Cells within a given radial ontogenetic column

originate from progenitors that share the same birthplace in the mosaic (or protomap) of the proliferative zones (Rakic, 1988). Before the onset of corticogenesis, the founder or neural stem cells divide symmetrically, increasing exponentially the number of potential ontogenetic columns in the cortical plate, indirectly determining the size of the cortical surface in individuals as well as in different species. After their last cell division in the VZ, neurons migrate to the cortical plate along a common radial glial fascicle (Rakic, 1972). Therefore, the neurons in a given "ontogenetic column" are hypothesized to be clonally related, postmitotic neurons.

Many types of cortical columns have been described. For example, Mountcastle and colleagues (Mountcastle et al., 1957), described a "functional column" in the cortex, basing his definition upon the results of single-unit recordings obtained with microelectrodes that entered the cat's somatosensory cortex and went through the layers sequentially from superficial to deep. Mountcastle recorded the responses of the neurons to brief peripheral stimuli, and he wrote as follows: ''… neurons which lie in narrow vertical columns, or cylinders, extending from layer II through layer VI make up an elementary unit of organization, for they are activated by stimulation of the same class of peripheral receptors, from almost identical peripheral receptive fields, at latencies which are not significantly different for the cells of the various layers" (Mountcastle et al., 1957). Thus, the "functional column" described here is defined by the terminations of a group of thalamic fibers that arise from neurons receiving input from the periphery, and the recipient cells in the cortex are vertically arranged so that the first synaptic activity ensuing from their activation spreads almost instantaneously to neurons located in layers above and below them and in a column (Jones and Rakic, 2010). Rakic recently wrote a commentary called "Confusing cortical columns" in PNAS (Rakic, 2008), in which he described the many uses of the term column. Although Rakic agreed that there are "functional columns", he argued that the relationship between ontogenetic columns and the various functional columns has not been adequately investigated. Rakic offered however, that it has been clear from the beginning that any functional column in the adult cerebral cortex must consist of several ontogenetic columns (polyclones), and that neurons from different clones intermix with adjacent columns. Also, the visibility of ontogenetic columns in the developing cortex diminishes

after the arrival of interneurons, glia cells, and afferents; all of which participate in the formation of the neuropile, with various synaptic connections that constitute the diverse types of functional columns (Rakic, 2007). Rakic proposed that the new challenge now is to understand the cellular and synaptic circuits, and devise model cortical columns that are dedicated to each function, in order to determine how deviations from this normal pattern can affect behavior (Rakic, 2008).

2.4 Adult Neurogenesis:

Neurogenesis in the adult mammalian brain was first observed, by Joseph Altman and colleagues in the 1960s, when they reported autoradiographic evidence of new neuron formation in the hippocampal dentate gyrus, olfactory bulb, and cerebral cortex of adult rats (Altman, 1962; Altman and Das, 1965). However, only in the last decade has adult neurogenesis been established and widely accepted to originate from two locations that contain reservoirs of neuronal stem cells: the subventricular zone (SVZ) of the lateral ventricles, and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus (Gross, 2000). Neurons born in the adult SVZ migrate through the rostral migratory stream to become granule neurons and periglomerular neurons in the olfactory bulb (Luskin, 1993). Neurons born in the adult SGZ migrate into the granule cell layer of the dentate gyrus and become dentate granule cells. Furthermore, the surviving neurons mature and participate in hippocampal circuitry, where they are presumed to play a role in learning and memory (Leuner et al., 2006). Whether neurogenesis occurs in areas of the adult mammalian brain other than the SVZ and SGZ remains controversial (Gould, 2007; Rakic, 2002).

Adult neural stem cells (NSCs), or neural progenitors, are cells in the adult nervous system that can self-renew and differentiate into all neural cell types, including neurons, astrocytes, and oligodendrocytes (Gage, 2000). Two types of neural progenitor cells can be identified in the SGZ. Type 1 hippocampal progenitors have a radial process that spans the granule cell layer. These cells express nestin, glial fibrillary acidic protein (GFAP), and the Sry-related HMG box transcription factor, Sox2 (Fukuda et al., 2003; Garcia et al., 2004; Suh et al., 2007). Type 2 hippocampal progenitors have short processes and

do not express GFAP. The transcription factor Sox2 is important for maintaining the "stemness" not only of neural stem cells, but also of embryonic stem (ES) cells. Sox2 can contribute to inducing adult somatic cells into taking a on an ES cell-like fate (Jaenisch and Young, 2008).

Due to the fact that adult neurogenesis has only been consistently found in the SVZ and SGZ, it has been hypothesized that the microenvironments of these two locations may be neurogenic niches, where there are specific factors that are permissive for the differentiation and integration of new neurons (Doetsch, 2003). Classical developmental signals and morphogens involved in embryogenesis like Notch, BMPs, Eph/ephrins, Noggin, and Shh seem to be important for maintaining adult neurogenic niches (Alvarez-Buylla and Lim, 2004). In addition, proliferating cells and putative neural progenitors in both SGZ and SVZ are closely associated with the vasculature, indicating that factors released from blood vessels may have a direct impact on the neural progenitors (Alvarez-Buylla and Lim, 2004; Palmer et al., 2000). Cao and colleagues observed that infusion of vascular endothelial growth factor (VEGF) promoted cell proliferation in the SVZ and SGZ, which can be blocked by a dominant-negative VEGF receptor 2 (Cao et al., 2004). Furthermore, neural progenitors in the SVZ and SGZ can be influenced by neurotransmitters (Jang et al., 2007). The neurotransmitter GABA directly depolarizes type 2 progenitors in the adult hippocampus, which results in calcium ion influx and increased expression of the neuronal differentiation factor NeuroD, suggesting that direct GABAergic input promotes the differentiation of type 2 hippocampal progenitors (Tozuka et al., 2005). Consistent with this observation, calcium ion antagonists and agonists decreased and increased neuronal differentiation in the adult hippocampus, respectively (Deisseroth et al., 2004).

The newborn neurons in the dentate gyrus go through several developmental stages with distinctive physiological and morphological properties (Esposito et al., 2005; Ge et al., 2006; Zhao et al., 2006). Similar to immature neurons in the developing brain, newborn granule cells are initially depolarized in response to GABA because of their high intracellular concentrations of chloride ions. The response to GABA switches from depolarization to hyperpolarization 2-4 weeks after birth of the neuron, which coincides with growth of dendritic spines (Ge et al., 2007). Many newborn neurons die within 4 weeks

after birth, and their survival is influenced by the animals' experience; such as sensory input, learning and exposure to enriched environments (Kee et al., 2007; Tashiro et al., 2007). Therefore, the modulation of neuronal activity in newborn and mature neurons in the circuit plays an important role in the survival of the neurons. In summary, the survival and functional integration of newborn neurons into the circuitry is determined in a critical time period (1-3 weeks after birth) when the neurons are immature and display unique properties. Figure 8 offered by Zhao and colleagues shows the unique properties of newborn neurons, such as their expression of markers, their morphologies, their electrophysiological properties, and their functional integration as they develop during the few weeks after they are born (Figure 8) (Zhao et al., 2008).

Figure 8.

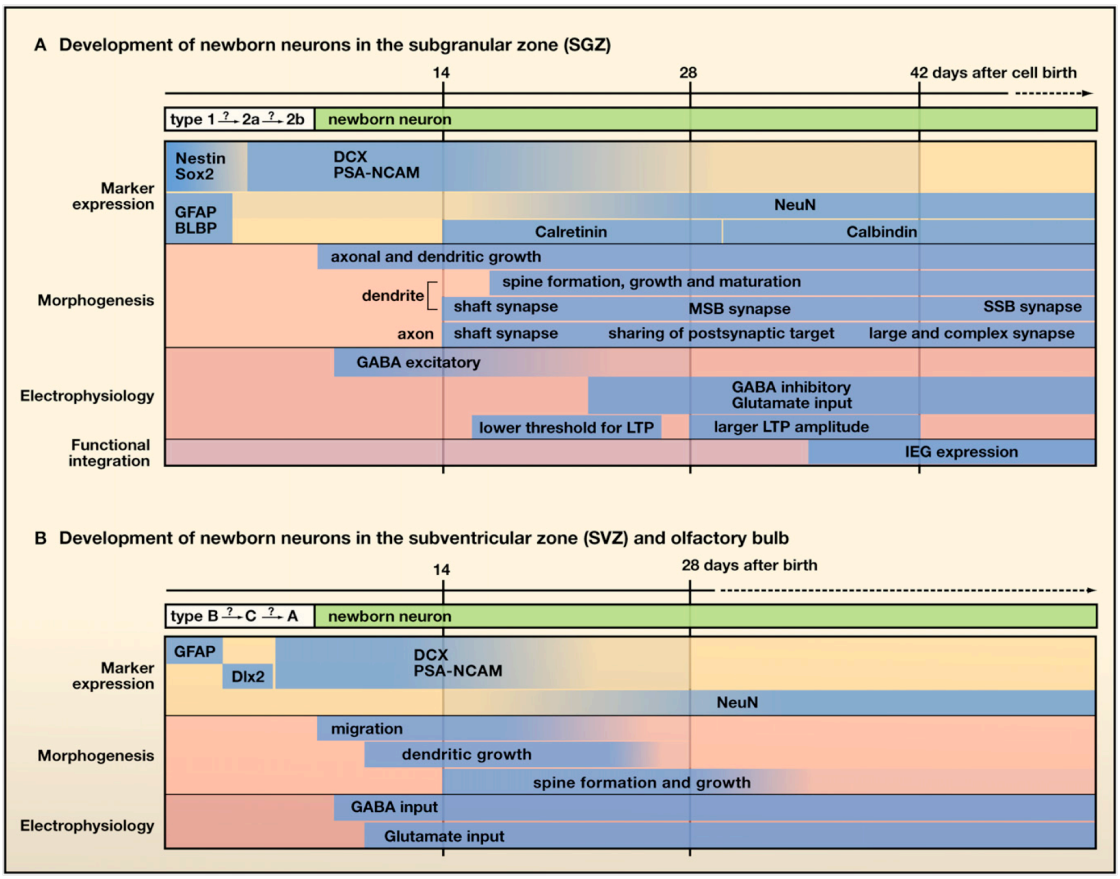


Figure 4. Development of Newborn Neurons in the SGZ and SVZ
Development of newborn neurons in the SGZ (A) and in the SVZ-olfactory bulb (B), as characterized by expression of specific markers, morphogenesis, synapse formation, electrophysiological properties, and functional integration. Abbreviations are as follows: MSB, multiple-synapse boutons; SSB, single-synapse boutons; LTP, long-term potentiation; IEG, immediate early gene.

Many genetic and environmental factors affect adult hippocampal neurogenesis. Mice with various genetic mutations have been shown to have changes in levels of cell proliferation, differentiation, and/or survival that can affect hippocampal neurogenesis (Kempermann and Gage, 2002). In addition, some mutant mice with decreased neurogenesis have impaired performance on hippocampal-dependent

learning tasks, although this is not always the case. Environment has a major impact on SGZ neurogenesis as well (Olson et al., 2006). For example, voluntary running increases SGZ proliferation, and exposure to an enriched environment promotes survival of the 1 to 3-week-old immature neurons as discussed earlier (Kee et al., 2007; Tashiro et al., 2007). In contrast to physical exercise and enriched environment, aging and stress are two negative regulators of adult neurogenesis (Klempin and Kempermann, 2007). Table 2, offered by Zhao and colleagues, reviews many of the genetic and environmental influences that have been observed which can regulate SGZ neurogenesis, and their correlations with cognitive performance (see Table 2) (Zhao et al., 2008).

Table 2.

Table 1. Genetic and Environmental Regulation of Subgranular Zone Neurogenesis and Their Correlation with Cognition

System Regulators	Effects on Neurogenesis	Effects on Cognition	References[a]
Genetic Regulation			
Genetic background	Varied proliferation	Correlated performance with proliferation	Kempermann and Gage, 2002
mbd1$^{-/-}$	Decreased proliferation	Impaired learning and memory	Zhao et al., 2003
fgfr1$^{flox/-}$, *nes-cre*	Decreased proliferation	Defective memory consolidation	Zhao et al., 2007
NT-3$^{flox/-}$; *nes-cre*	Decreased neuron differentiation/survival	Defective learning	Shimazu et al., 2006
neurokinin1$^{-/-}$	Increased proliferation	No improvement in learning and memory	Morcuende et al., 2003
Physical Activity			
Voluntary running	Increased proliferation	Improved learning and memory	van Praag et al., 1999
Hindlimb suspension	Decreased proliferation	Not available	Yasuhara et al., 2007
Enriched Environment (EE)			
Exposure to EE	Increased survival	Improved learning and memory	Kempermann and Gage, 1997
presenilin-1$^{-/-}$	Attenuation of the induction of neurogenesis by EE	Reduced memory clearance induced by postlearning EE	Feng et al., 2001
Stress[b]			
Psychosocial stress	Decreased proliferation	Impaired spatial memory	Ohl et al., 1999
Unexpected chronic mild stress	Decreased neurogenesis	No simple correlation	Minuer et al., 2007
Aging			
Aged rats	Decreased proliferation and neurogenesis	Correlated impairment in learning or memory	Drapeau et al., 2003; Driscoll et al., 2006
Aged rats	Decreased proliferation and neurogenesis	Not correlated to cognition	Merrill et al., 2003; Bizon et al., 2004
Aged animals in EE	Increased neurogenesis[c]	Improved learning and memory	Kempermann et al., 1998
Aged animals with running	Increased neurogenesis[c]	Improved learning and memory	van Praag et al., 2005

[a] See Supplemental References for complete listings.
[b] The relationship between stress and learning is complicated and is subjected to regulations by factors such as gender and task difficulty, etc. (Shors et al., 2004).
[c] Hippocampal neurogenesis in aged rodents that are subjected to environmental enrichment or voluntary running is increased compared to sedentary aged controls.

Interestingly, seizure activity also increases proliferation in both the SVZ and SGZ (Jessberger et al., 2007). Neurogenesis in the SGZ can be elevated for up to 5 weeks after a seizure, but the newborn neurons have abnormal morphologies (Jessberger et al., 2007; Walter et al., 2007). Seizure-induced new neurons integrate into the hippocampal circuitry despite their abnormal connectivity; however, the role of the seizure-associated aberrant neurogenesis in epilepsy is yet to be determined (Zhao et al., 2008).

In conclusion, adult neurogenesis shares many similar mechanisms with embryonic neurogenesis. For example, immature neurons will integrate functionally into the existing circuitry. This integration is especially sensitive to experience (neuronal activity) in the early days and weeks of the neuron. At this critical period, the activities of the newborn, immature neurons are crucial to their survival, maturation,

and integration. Also, in both embryonic and adult neurogenesis, neurons developing from different origins migrate to distinct areas, and acquire unique functions. Furthermore, neurons born at either stage arise from reservoirs of neural progenitor cells that divide asymmetrically. Lastly, abnormalities in any step of the process of either embryonic or adult neurogenesis can lead to neurologic and/or psychiatric disorders, or changes in cognition and behavior.

CHAPTER THREE

Transcription Factors Expressed in the Brain

Many of the transcription factors expressed in the brain during embryonic development are also expressed in the adult hippocampus. In both cases, the various transcription factors appear to be expressed in a series that is designed to allow for properly timed differentiation of neural progenitor cells. This cascade of transcription factor expression leads to gene transcription and protein expression that eventually signal the neural progenitor cell to maintain its "stemness", or to divide, differentiate and mature into a functional neuron, or glial cell. Some transcription factors along the pathway are also meant to determine the fate of the cell (i.e. - whether it will become a neuron or glial cell, what type of neuron or glial cell it will become, and where its final destination will be).

A crtical signaling molecule expressed during development, sonic hedgehog (Shh), plays a key role in dorsal-ventral patterning in both the neural tube, and the telencephalon. An important difference between the role of Shh in the spinal cord and the telencephalon was revealed from *in vitro* experiments where explants were exposed to several concentrations of Shh. These experiments showed that, whereas spinal cord explants respond to Shh in a concentration-dependent manner, the telencephalon responds to Shh signals in a time-dependent manner (Kohtz et al., 1998). This suggested that the dorsal-ventral patterning in the telencephalon requires an additional pathway that acts in parallel with Shh signaling to specify the dorsal-ventral pattern.

To understand the effects of Shh signaling in the telencephalon, it is necessary to consider the three Gli (Glioma-associated oncogene homolog) transcription factors, Gli1, Gli2, and Gli3. The Gli transcription factors all have conserved, tandem C2-H2 zinc finger domains, and they are all mediators of the Shh signaling pathway (Ruppert et al., 1988). Gli1 and Gli2 act principally as activators, whereas Gli3 acts mainly as a repressor (Matise and Joyner, 1999). Interestingly, mutant mice that lack both Gli1 and Gli2 developed relatively normal telencephalons (Park et al., 2000). In contrast, the ventral telencephalon

markers of Gli3 mutant mice expanded dorsally into the cortex (Theil et al., 1999). The fact that opposite telencephalic phenotypes are observed in the Shh and Gli3 mutant mice indicated that the balance between the activities of Shh and Gli3 is crucial in the development of the dorsal-ventral patterning in the telencephalon (Rallu et al., 2002). Rallu and colleagues found that Shh is likely to act through inhibition of Gli3 repressor activity (see Figure 1, Rallu et al., 2002). In addition, Rallu and colleagues observed that in the double Shh/Gli3 mutants, the ventral telncephalic gene expression, that is lost in the Shh mutants, was restored when Gli3 was deficient in combination with Shh. Also, in the absence of both Shh and Gli3 gene function, the level and localization of Nkx2.1 gene expression was specified correctly, which suggested the restoration of the medial ganglionic eminence (MGE), the most ventral part of the telencephalon (Rallu et al., 2002). These results demonstrated that although Shh signaling is important for the dorsal-ventral patterning of the telencephalon, it is dispensable, provided that Gli3 function is also abolished. It also implied that other signaling pathways act in conjunction with the Shh pathway in order to regionalize the telencephalon.

Figure 1.

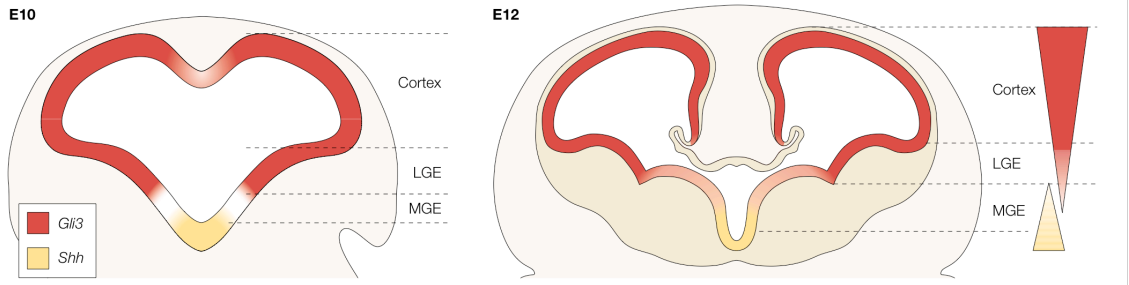


Figure 3 | **Model of genetic interactions between Shh and Gli3 in patterning the mouse telencephalon.** Schematic representation of a coronal section through an embryonic day 10 (E10; on the left) and E12 (on the right) mouse telencephalon, highlighting different domains along the dorsoventral (DV) axis. The expression domains of *Gli3* (in red) and sonic hedgehog (*Shh*; in yellow) are shown at early and late stages of telencephalic development. These genes maintain a complementary pattern of expression throughout development, and genetic analysis shows that their activities strongly antagonize one another. Specifically, in the absence of *Shh* gene function, the telencephalon is strongly ventralized, whereas in the absence of *Gli3* gene function, the telencephalon is strongly dorsalized. In the absence of both *Gli3* and *Shh*, the general aspects of DV patterning are rescued. The notable exceptions are the dorsal midline structures, which are lost in all three mutant genotypes: $Shh^{-/-}$, $Gli3^{-/-}$ and $Shh^{-/-}/Gli3^{-/-}$. These data indicate the existence of an unknown hedgehog-independent pathway that acts in parallel with *Shh* in the establishment of telencephalic DV pattern. MGE, medial ganglionic eminence; LGE, lateral ganglionic eminence.

The other signaling pathways that have been suggested to play a part in patterning of the telencephalon include, BMP, Wnt, and FGF signaling. BMPs are thought to be important for dorsal patterning of the telencephalon. One example of the involvement of BMP signaling, comes from the zebrafish *swirl* mutant, which is a Bmp2b null. The expression of the dorsal telencephalic specific gene, Emx1, is lost in *swirl* mutants (Hebert et al., 2002).

Furthermore, in response to Shh and the other signaling molecules, there are numerous transcription factors that are expressed by progenitor cells, which allow them to adopt specific cell fates. Many of the genes that act in response to these extrinsic cues belong to a set of homeodomain-class transcription factors. Telencephalic subregions can be defined early in development by the expression of a distinct set of gene encoding homeodomain proteins, including Nkx2.1, Gsh1, Gsh2 and Pax6, which are homologues of the *Drosophila* genes *vnd*, *ind*, and eyeless (*ey*), respectively (Marquardt and Gruss, 2002; McDonald et al., 1998; Weiss et al., 1998). These transcription factors provide some of the earliest markers of dorsal (Pax6), intermediate (Gsh2) and ventral (Nkx2.1) regions of the telencephalon (Schuurmans and Guillemot, 2002). In the absence of Nkx2.1, the ventral most aspect of the telencephalon – the MGE – becomes transformed into the more dorsal LGE (Sussel et al., 1999). In *Drosophila*, *vnd* (the Nkx2.1 homologue) and *ind* (the Gsh2 homologue) mutually repress each other (McDonald et al., 1998). However, in vetebrates, Nkx2.1 and Gsh2 do not appear to have any direct effect on each other's expression, despite their complementary pattern of expression. Specifically, the loss of Nkx2.1 does not result in precocious expression of Gsh2 in the ventral region, and loss of Gsh1 and Gsh2 does not result in expansion of Nkx2.1 expression (Corbin and Fishell, unpublished observations). In contrast, Gsh2 and Pax6 have a complementary expression patterns that form a sharp border between the intermediate-dorsal boundary (between the LGE and cortex), and Gsh2 and Pax6 *do* cross-repress each other's expression. Loss of Gsh2 leads to expansion of Pax6 expression (along with other dorsal genes, such as Neurogin2) into the intermediate LGE region, and loss of Pax6 leads to expansion of Gsh2 into the Pax6 region (Yun et al., 2001) (see Figure 2, Rallu et al, 2002).

Figure 2.

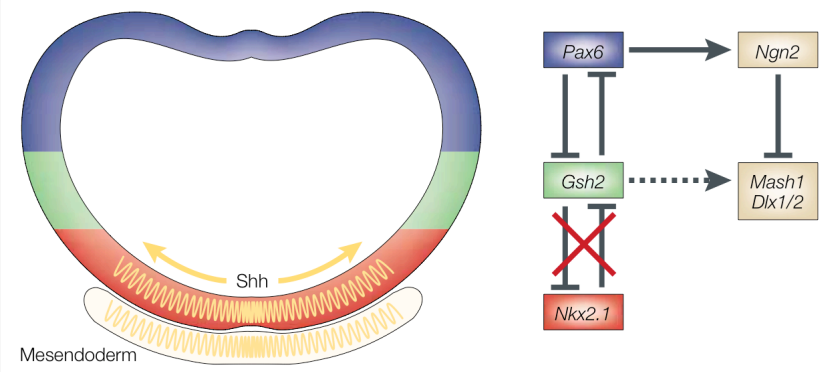


Figure 4 | **Homeodomain and bHLH genetic interactions in telencephalic development.** Schematic of a coronal section of the mouse telencephalon at 26 somites (about embryonic day 9.5), showing the expression pattern of the homeodomain transcription factors Nkx2.1, Gsh2 and Pax6. Sources of both ventral telencephalic and mesendodermal Shh are also shown in this schematic. At this age, shortly after Gsh2 is first detected in the lateral telencephalon, expression of these genes is mostly non-overlapping. Genetic analysis has revealed that while Pax6 and Gsh2 act in a cross-repressive manner, Nkx2.1 and Gsh2 do not. Furthermore, Pax6 may regulate the function of the basic helix–loop–helix (bHLH) transcription factor, Ngn2, which in turn regulates *Mash1* and *Dlx1/Dlx2* gene function.

Pax6 is worthy of further discussion because it is involved in so many aspects of telencephalon development and neurogenesis. It is a highly conserved transcriptional regulator with two DNA-binding motifs, a paired domain and a paired-like homeodomain, which can bind DNA independently or cooperatively (Bopp et al., 1986; Callaerts et al., 1997). The paired box was first identified in the Drosophila gene *paired* and this domain is conserved in other functionally related genes including *gooseberry-proximal, gooseberry-distal*, *Pox meso* and *Pox neuro* (Bopp et al., 1986). Nine Pax members have been identified in both mouse and human, and mutations in these genes are known to cause a number of abnormalities (Chalepakis et al., 1993). Individuals carrying loss-of-function mutations in Pax6 suffer from congenital aniridia (abnormal development of the eyes and brain) (Glaser et al., 1990). *Small eye (Sey)* mice carrying Pax6 loss-of-function mutations have similar phenotypes as seen in the human disorder, with decreased eye size, iris hypoplasia, corneal opacification and cataracts

(Hogan et al., 1988). The brain abnormalities seen in individuals carrying Pax6 mutations include an absent or abnormal pineal gland (Mitchell et al., 2003), an absent or hypoplastic anterior commisure, and the corpus callosum may be reduced in size (Sisodiya et al., 2001). There is also evidence of deficient auditory interhemispheric transfer in anirdia patients (Bamiou et al., 2004). Pax6 has also been implicated in WAGR (Wilm's tumor aniridia genitourinary malformations and mental retardation) syndrome, which is caused by deletion of the 11p12 – p14 region (Hanson and Van Heyningen, 1995; Ton et al., 1991). Moreover, Pax6 has been linked to autism in several studies (Davis et al., 2008; Graziano et al., 2007; Maekawa et al., 2009; Szatmari et al., 2007).

Pax6 is first seen as early as embryonic day 8.5 (E8.5) in the mouse, at the earliest stage of CNS development, when the neural plate consists entirely of proliferating neuroepithelial cells, and the neural tube is in the process of closing. It is widely expressed in the developing CNS, including the forebrain, hindbrain, cerebellum and ventral neural tube (Grindley et al., 1995). It is also expressed in the olfactory system, the lens, cornea and retina of the developing eye. Pax6 is critical for survival since Pax6-null mice die immediately after birth. These mice have an absence of nasal structures and eyes, and the diencephalon fails to innervate the severely malformed cerebral cortex (Pratt et al., 2000; Tyas et al., 2003).

As was discussed earlier, Pax6 is crucial for the regionalization of the embryonic telencephalon into dorsal (pallial) and ventral (subpallial) progenitor domains. The pallial-subpallial boundary is marked by higher expression of transcription factors, such as Pax6, Ngn2, Dbx, Tbr1 and Tbr2 on the pallial side, and Gsh2, Dlx1 and Dlx2, Vax1, Mash1 and Six3 on the subpallial side (Stoykova et al., 2000; Torreson et al., 2000).

In addition, Pax6 plays an important role in neurogenesis. The cortical progenitor cells have two decisions to make: 1) whether to exit or re-enter the cell cycle, and 2) whether to differentiate or proliferate. Evidence has suggested that Pax6 is critical for the regulation of cell cycle exit versus re-entry. Recent analyses have suggested that $Pax6^{-/-}$ mutant cells exit the cell cycle in abnormally large numbers from the onset of corticogenesis and that Pax6 is required to maintain the size of the cortical

progenitor pool (Quinn et al., 2007). The reduced size of the Pax6 mutant progenitor pool provides an explanation for the reduced production of cortical neurons and decrease in the cortical thickness of the Pax6-deficient cortex (Gotz et al., 1998; Quinn et al., 2007).

In the adult SGZ of the hippocampus, Pax6 is expressed in neural stem/progenitor cells. Hevner and colleagues suggested that Pax6 is important for maintaining proliferation of the progenitor pool, and proposed that the transition from Pax6 expression in radial glia-like progenitors to Tbr2 and NeuroD in intermediate stage progenitors, and to NeuroD/Tbr1 in post-mitotic neurons, may represent crucial steps in hippocampal neurogenesis (Hevner, 2006). FABP7, a fatty-acid binding protein, an important downstream molecule of Pax6 (and possibly a direct target), is also expressed in cells with a radial glia-like phenotype in the hippocampus (Feng and Heintz, 1995). In addition, it has been reported that FABP7 functions to keep neural stem/progenitor cells proliferating during hippocampal neurogenesis (Watanabe et al., 2007). Thus, regulation of Fabp7 by Pax6 could be fundamental for both embryonic and adult hippocampal neurogenesis.

As mentioned above, Fabp7 may be a direct target of Pax6. It has also been shown that Neurogenin2 is a direct target of Pax6 (Scardigli et al., 2003). The other possible direct or indirect downstream targets of Pax6 are summarized in the table offered by Osumi and colleagues (see Table 1) (Osumi et al., 2008), along with the probable role of these downstream targets.

Table 1.

Pax6 downstream molecule	Reference	Function			
		Proliferation	Differentiation	Cell adhesion	Patterning
FABP7	[87]	√			
FucT9	[95]	√			
LewisX	[95]	√			
Ngn2	[40]		√		
p27kip1	[54]		√		
L1	[104]			√	
Optimedin	[105]			√	
R-cadherin	[107, 108]			√	
δ-catenin	[110]			√	
Tenascin C	[113]			√	
α5- β1-integrin	[114]			√	
Wnt7b	[141,11]	√	√		√
sFRP2	[115]	√	√		√
Tcf4	[115]	√	√		√
Hoxd4	[117]				√

Figure 2. Multiple functions of Pax6 via different downstream molecules. **(A):** Roles of Pax6 in regulation of proliferation and differentiation of neuroepithelial cells. The cells shown in blue and yellow are a neuroepithelial cell and a neuron, respectively. **(B):** List of Pax6 downstream molecules that control cell proliferation (blue), cell differentiation (yellow), cell adhesion (pink), and patterning (green) and thus govern multiple functions of Pax6.

As discussed earlier, the pallial-subpallial border also displays expression of Dlx1 and Dlx2, and Mash1 on the subpallial side(Stoykova et al., 2000). Mash1 (or Ascl1, a homologue of Drosophila achaete-scute complex) is a basic helix-loop-helix transcription factor. As seen in Figure 2, Pax6 can increase Neurogenin2 (Ngn2, another basic helix-loop-helix transcription factor), and Ngn2 can repress the expression of Mash1, and the Dlx transcription factors (Fode et al., 2000). In the absence of Pax6, Ngn2 expression is lost in the lateral cortex and Mash1 expression expands into this region (Yun et al., 2001). Furthermore, in Ngn1/Ngn2 compound null mice, the normal ventral restriction in the expression of homeodomain genes, such as Dlx2, is disrupted, resulting in their expansion into dorsal regions (Fode et al., 2000). Thus, Mash1, Dlx1 and Dlx2 play important roles in the regionalization of the telencephalon.

In addition to their role in patterning, Mash1, Dlx1 and Dlx2 regulate specific aspects of neurogenesis. Studies pertaining to the distribution of interneurons in mice lacking these transcription factors has revealed their essential role in the timing of interneuron production and differentiation (Marin and Rubenstein, 2001). For example, the loss of Dlx1 and Dlx2 function can inhibit the differentiation and migration of late-born subpallial neurons in the telencephalon. In the Dlx1/Dlx2 double-mutant mice, partially differentiated cells (that can express GABA) fail to migrate, and accumulate as periventricular ectopias (Anderson et al., 1997). However, loss of Dlx1 and Dlx2 does not affect GABA interneurons in

all regions equally. In the Dlx1/Dlx2 double-mutants, the olfactory region maintains a normal number of GABA-expressing interneurons, while the neocortex has a 75% reduction of GABA interneuron number, and the hippocampus has complete loss of GABA interneurons (Anderson et al., 1997). Whereas Dlx gene functions affect late-born subpallial neurons of the telencephalon, Mash1 affects several early-born cell populations. In Mash1 mutant mice, the loss of Mash1 function leads to premature differentiation of the early-born cell populations (Casarosa et al., 1999).

Several other transcription factors have also been proposed to play a role in regulating the differentiation of GABA interneurons. For example, Dlx5 and Dlx6 are expressed in GABA inerneurons after Dlx1 and Dlx2 (Eisenstat et al., 1999; Liu et al., 1997), and may therefore regulate later steps of GABA neuron differentiation and maturation. It has also been observed that the Lim homeodomain proteins, Lhx6 and Lhx7, may participate in the process of interneuron differentiation. Specifically, Lhx6 may contribute to the development of cortical and striatal interneurons (Lavdas et al., 1999). Lhx6 is induced in the medial ganglionic eminence and is maintained in parvalbumin and somatostatin-positive interneurons and is required for the specification of these neurons in the cortex and hippocampus (Liodis et al., 2007). It has also been reported that Lhx6 may be a direct target of the transcription factor Nkx2.1 (Du et al., 2008). On the other hand, Lhx8 has been observed to regulate the development of cholinergic neurons (Zhao et al., 2003).

There are also several basic helix-loop-helix (bHLH) transcription factors expressed during corticogenesis and neurogenesis. Mash1 and Neurogenin1/Neurogenin2 are pro-neural bHLH transcription factors that regulate the differentiation of neurons in the dorsal, and ventral telencephalon, respectively (Schuurmans and Guillemot, 2002). In order to maintain the neural progenitor pool, it is necessary for the progenitor cells to proliferate adequately before they differentiate. Therefore, there are inhibitory mechanisms to block differentiation of the progenitors. Hes and Id are two classes of inhibitory bHLH transcription factors that play a role in antagonizing the pro-neural bHLH transcription factors, in order to maintain the neural progenitor pool.

Hes (hairy and enhancer of split) bHLH transcription factors were first observed to negatively regulate

neurogenesis in Drosophila (Knust, 1997). In mammals, Hes1 and Hes5 have been shown to play key roles in telencephalonic development (Ishibashi et al., 1995). Both Hes1 and Hes5 are expressed in the ventricular zone throughout the telencephalon, where they sustain progenitors in an undifferentiated proliferative state, and inhibit their differentiation into neurons (Ishibashi et al., 1995; Ohtsuka et al., 2001). Loss of Hes1 function in mouse mutants led to premature neuronal differentiation with a 2-fold excess of neurons in the forebrain at E13.5 (Nakamura et al., 2000). Much of our understanding of the functions of Hes1 and Hes5 come from the observation that Hes1 and Hes5 are key target genes that are transactivated by Notch signaling (Iso et al., 2003; Pierfelice et al., 2011) (see Figure 4). Notch, a transmembrane protein, is activated by its ligands such as Delta. Upon activation, the intracellular domain (ICD) of Notch is released from the cell membrane and transferred into the nucleus, where the ICD forms a complex with the transcription factor RBP-J (also known as CBF1). Without ICD, RBP-J represses Hes1 and Hes5 expression by binding to their promoters. However, the complex of RBP-J and the ICD acts as a transcriptional activator. Thus, activation of Notch signaling leads to induction of Hes1 and Hes5 expression (Artavanis-Tsakonas et al., 1999; Honjo, 1996). Disruption of Notch/Hes signaling through the loss of Notch, leads to similar phenotypes as the loss of Hes1 or Hes5, including decreased proliferative ability and premature neuronal differentiation (de la Pompa et al., 1997). Also, in cortical progenitors from mutant mice lacking both Hes1 and Hes5, constituitively active Notch signaling was unable to block neurogenesis (Ohtsuka et al., 1999). Therefore, Hes1 and Hes5 are critical effectors of Notch signaling that act to maintain the proliferative neural progenitor pool.

Id (Inhibitor of DNA binding) bHLH factors have a similar role as Hes1 and Hes5, in that they inhibit precocious differentiation of neural progenitors (Jen et al., 1997). However, these two families have different mechanisms of inhibition. Whereas Hes recruits co-repressors to inhibit transcription of pro-neural genes, Ids inhibit gene transcription by sequestration. Id forms dimers with E proteins, and thus inhibits transcription of genes that require E proteins for activation (which includes Mash, Neurogenin, NeuroD, and Olig family members) (Norton, 2000).

The pro-neural bHLH transcription factors Mash1, Ngn1 and Ngn2 that were discussed earlier have

been suggested to be regulated by glycogen synthase kinase 3 (Gsk3) mediated phosphorylation (Moore et al., 2002). Moore and colleagues found consensus sequences that correspond to Gsk3 phosphorylation sites in each of the pro-neurogenic transcription factors. This would provide a common mechanism for the regulation of the pro-neural genes.

After the first wave of pro-neural bHLH transcription factors induces differentiation, there is a second wave bHLH transcription factors that promote terminal differentiation of the neurons (Kageyama et al., 1997; Lee, 1997). These include NeuroD, NeuroD2 (also called NeuroD related factor, NDRF) and Nex (also called Math2). Similar to the pro-neural bHLH factors, the differentiation bHLH factors bind E-box DNA sequences and when overexpressed, they have been shown to induce cell cycle arrest and differentiation (Farah et al., 2000). One difference however, between the pro-neural bHLH factors and the differentiation bHLH factors is that after pro-neural induction, the cells can become neurons or glia; yet after the differentiation bHLH factors are expressed, the cell is committed to becoming a neuron (Nieto et al., 2001).

Figures 3 and 4, offered by Ross and colleagues, display the pathways that a neuronal progenitor cell can take during differentiation, and emphasizes the bHLH transcription factors involved in these pathways (see Figures 3 and 4) (Ross et al., 2003). Ross and colleagues also offered a table to summarize the bHLH transcription factors involved in corticogenesis (see Table 2, Ross et al., 2003).

Figure 3.

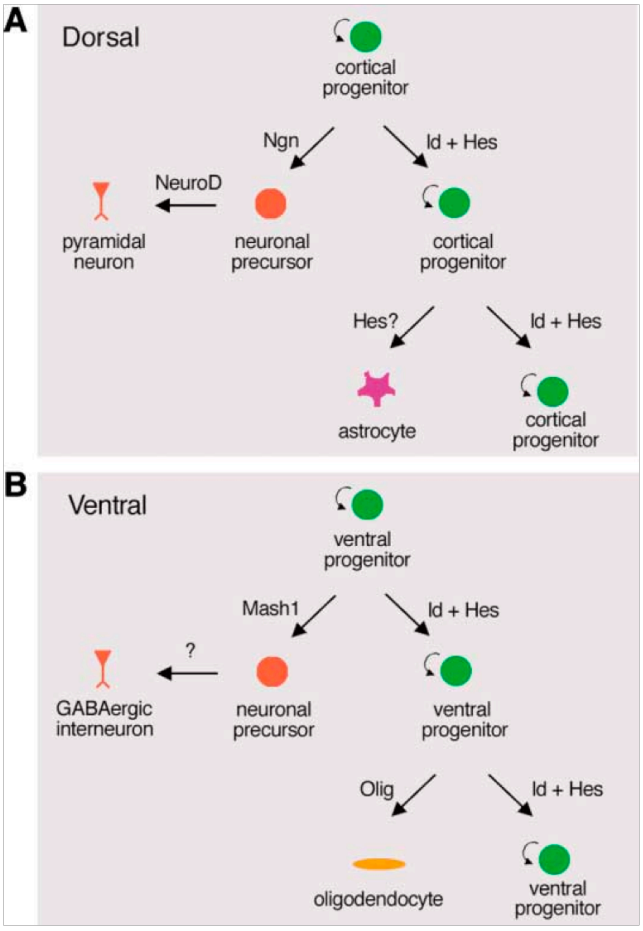


Figure 5. Regulation of Progenitor Specification and Differentiation by bHLH Factors in the Dorsal and Ventral Telencephalon

(A) In the dorsal telencephalon, cortical progenitors are maintained by Id and Hes factors. Members of the Ngn and NeuroD families are involved in early and late phases of neuronal differentiation, respectively, which give rise to pyramidal neurons. Hes factors may also be involved in astrocyte differentiation.

Figure 4.

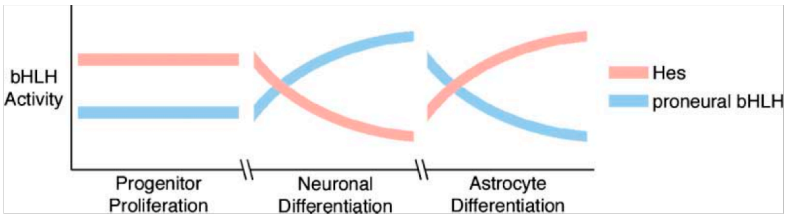


Figure 4. The Timing of Differentiation in the Telencephalon Is Regulated by the Balance of Antagonistic bHLH Factors

Conceptual model based on loss-of-function and gain-of-function studies illustrating the idea that a balance in the activity of proneural bHLH factors and Hes factors is involved in the regulation of progenitor maintenance, neuronal differentiation, and possibly astrocyte differentiation in the neocortex. Oligodendrocyte differentiation may be regulated by an analogous manner by the balance between Ids and Oligs.

Table 2.

bHLH Factor	Related Factor in Drosophila	Function	DNA Element
NeuroD	Atonal*	activate transcription	E box
Ngn	Tap/Biparous		
Olig	Oli	repressor?	
Mash	Achaete-Scute	activate transcription	
E protein	Daughterless		
Id	EMC	sequester E protein	no DNA binding
Hes	Hairy/(E/Spl)	repress transcription	N box

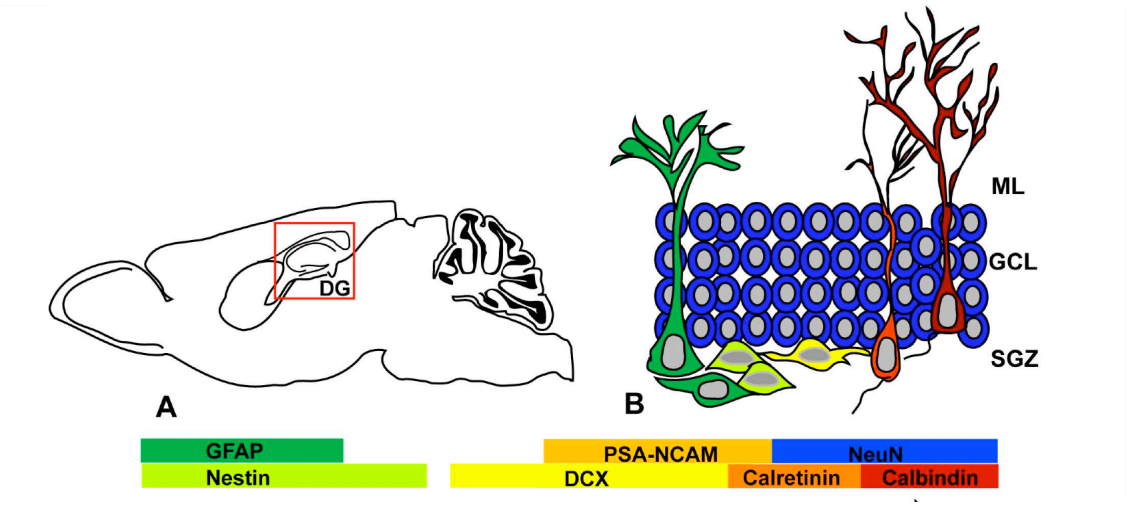
Figure 1. bHLH Factors Involved in Cortical Development

The phylogenetic tree is based on the analysis of Ledent et al. (2002). Note that branch length in the tree is not proportional to distance of relationship between families. Asterisk: strictly speaking, Atonal is the *Drosophila* homolog of Math1, not NeuroD.

Another class of transcription factors expressed during brain development are the POU (Pit-Oct-Unc) domain homeobox proteins. POU factors have been shown to play essential functions in cell-lineage determination (Herr et al., 1988). These transcription factors are sometimes called Oct transcription factors as well because they bind a specific octameric DNA sequence. Class III POU genes are expressed predominantly in the CNS. POU3f3 and POU3f2 are also called Brain1 (Brn1) and Brain2 (Brn2), respectively. POU3f2 first came to our attention when we noticed that its ortholog in Drosophila, *Drifter* (or ventral veinless, vvl), was reported to directly interact with *trachealess* (Zelzer and Shilo, 2000), which is the fly ortholog of NPAS1 and NPAS3. *Trachealess* and *Drifter* were observed to interact through their PAS and POU domains, and they were shown to autoregulate each other, as well as coregulate target genes (Zelzer et al., 2000). Although this study pertained to tracheal development, Johnson and colleagues originally identified Drifter (also called Cf1a) on the basis of its ability to bind a neuron-specific cis-regulatory element, element C of the dopa decarboxylase (Ddc) gene, which is why it was called Cf1a (Johnson et al., 1989). Johnson and colleagues also demonstrated that Drifter was expressed during embryonic development in the midline precursor cells of the developing CNS, in the progeny of other neuroblasts derived from the neurogenic ectoderm, and throughout the tracheal system (Anderson et al., 1995). Also, an analysis of tracheal and CNS development in Drifter mutant embryos suggested a requirement for Drifter in the differentiation and migration of tracheal cells and midline glia in the CNS (Anderson et al., 1995). More recent studies have found that POU3f2 (Brn2) may interact with Mash1, and may promote the differentiation and migration of neurons (Castro et al., 2006; Vierbuchen et al., 2010). POU3f2 was also interesting to us because we later found that its expression was reduced in the NPAS1-deficient mice in our microarray studies. QPCR confirmed this finding often, but not every time. Also, with pull-down experiments, I did not observe any clear interactions between NPAS1 and POU3f2. Nevertheless, POU3f2 (Brn2) seems to be an interesting transcription factor involved in CNS development, and could be looked into further.

Adult neurogenesis in the SGZ of the hippocampus involves several transcription factors that overlap with those expressed during embryonic corticogenesis. During adult neurogenesis, the neural progenitor cells in the SGZ undergo transitions from progenitor (Type 1) cells to intermediate progenitor (IP or Type 2a) cells that express Nestin, to a second subclass of IP (Type 2b) cells, that express Nestin and Doublecortin (Dcx), and then Type 3 cells express further markers of neuronal differentiation, such as PSA-NCAM, and do not express Nestin (Kempermann et al., 2004), and finally post-mitotic and mature neurons that express NeuN (see Figure 5, Kempermann et al., 2004).

Figure 5.



As mentioned earlier, Pax6 is a paired domain and homeodomain-containing trancription factor expressed in the embryonic progenitor cells of the cerebral cortex (Englund et al., 2005). Several studies have shown that Pax6 is expressed in the Type 1 cells of the SGZ, and is not present in post-mitotic neurons (Hodge et al., 2008; Maekawa et al., 2005; Nacher et al., 2005; Roybon et al., 2009).

Tbr1 and Tbr2 are T-box transcription factors that are expressed in both the developing cerebral cortex and the adult hippocampus. Englund and colleagues demonstrated that Tbr2 is expressed in the cortical IP cells during embryonic development (Englund et al., 2005). In the adult SGZ, Tbr2 expression has

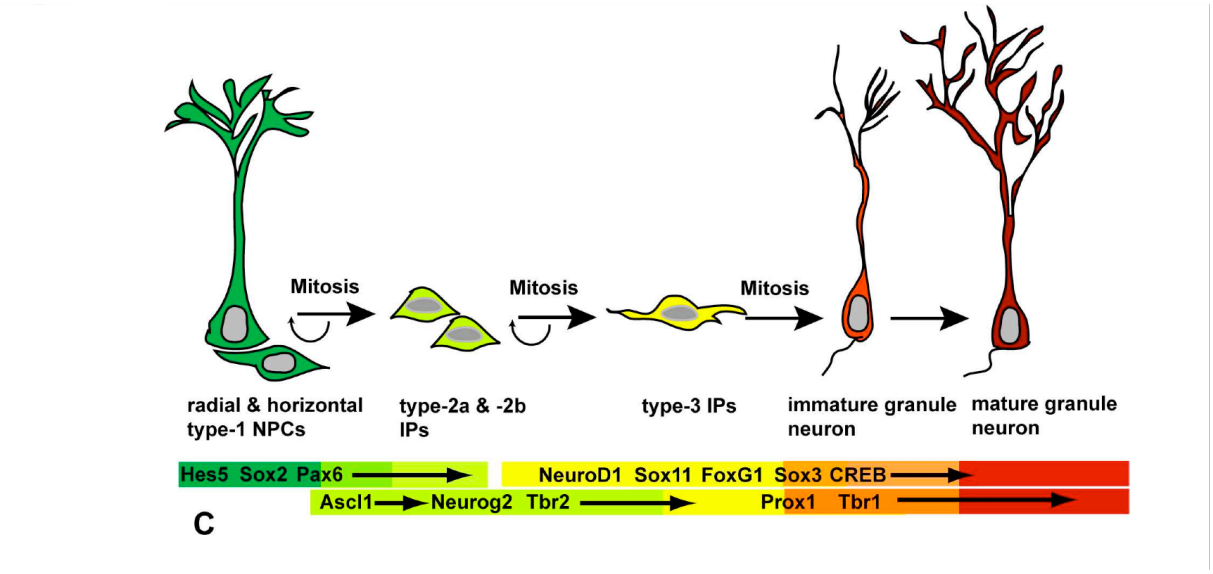
been observed in Type 2/3 cells (Hodge et al., 2008; Roybon et al., 2009). Furthermore, during embryonic cortical development Tbr2 was shown to be down-regulated as cells exit the cell cycle and differentiate, and Tbr2 down-regulation was observed to coincide with upregulation of Tbr1 (Hevner et al., 2001). It has been observed that this expression pattern is recapitulated in the adult SGZ, where Tbr1 is expressed in newly generated post-mitotic neurons (Hodge et al., 2008; Roybon et al., 2009).

Hes5, as mentioned earlier, is a bHLH transcription factor that is downstream of canonical Notch signaling. During embryonic development Hes5 was observed in neural progenitor cells. In the adult SGZ, Hes5 has been shown to be expressed in Type 1 cells (Lugert et al., 2010). Mash1 (also called Ascl1), as discussed earlier, is a pro-neural bHLH transcription factor expressed in progenitor cells of the embryonic cortex. In addition, Mash1 expression has been observed in Type 2a cells in the SGZ (Kim et al., 2007). In the SGZ, the transition from Type 2a to Type 2b progenitors coincided with marked downregulation of Mash1, and upregulation of another bHLH transcription factor, Neurogenin2 (Roybon et al., 2009).

As intermediate progenitor cells become committed to a neuronal lineage in the Type 2b and Type 3 stage, the expression of the bHLH transcription factor, NeuroD1 is upregulated (Hodge et al., 2008; Roybon et al., 2009). NeuroD1 is coexpressed with Tbr2 in many Type 3 cells, which closely parallels that seen in the embryonic cortex where Tbr2 and NeuroD are coexpressed in the terminally dividing cells (Englund et al., 2005).

In summary, the sequence of transcription factors expressed during development of the SGZ progenitor cells closely recapitulates many aspects of embryonic neurogenesis. Specifically, there appears to be a sequence of expression of the transcription factors, Pax6 → Neurogenin2 → Tbr2 → NeuroD1 → Tbr1 as progenitors differentiate into post-mitotic neurons in the adult hippocampus and the developing cortex (see Figure 6, Kempermann et al., 2004).

Figure 6.



In closing, it is of interest to bring attention to the transcription factors involved in embryonic corticogenesis that have been reported to be connected to autism. The implicated transcription factors, that I am aware of, include Pax6 (Davis et al., 2008; Graziano et al., 2007; Szatmari et al., 2007; Maekawa et al., 2009; Osumi et al., 2010), Dlx genes (Hamilton et al., 2005; Liu et al., 2009; Nakashima et al., 2010), Arx (Brooks-Kayal, 2010), Tbr1 (Bedogni et al., 2010), Engrailed2 (Benayed et al., 2005), HoxA1 (Conciatori et al., 2004), and NPAS1 (will be discussed here). All of these are critical regulators of brain development, and some continue to be expressed in the adult brain, emphasizing the importance of these processes in the etiology of autism.

CHAPTER FOUR

Transient Enlargement in Brain Size and Increased Neuron Density in Mice Lacking the Neuronal PAS Domain 1 Transcription Factor

4.1 Summary

The genomes of vertebrate organisms encode two related transcription factors designated neuronal PAS domain protein 1 (NPAS1) and NPAS3. Both of these regulatory proteins are selectively expressed in the central nervous system. Despite sharing extensive sequence identity in their respective bHLH DNA binding domains, and in their directly repeated PAS-A and PAS-B domains located immediately C-terminal to the bHLH domains, the NPAS1 and NPAS3 proteins appear to have evolved diametrically opposed biological roles in directing formation of the vertebrate brain. Mice bearing targeted disruption in both alleles of the *NPAS3* gene suffer from impaired neurogenesis (Pieper et al., 2005). By contrast, as documented in this report, mice lacking the NPAS1 transcription factor exhibit precisely the opposite phenotype. Measurements of new neuron formation in NPAS1-deficient animals ranging from five to ten weeks of age revealed a 31.5% increase in bromodeoxyuridine (BrdU) incorporation into newborn, hippocampal neurons compared with wild type littermates. It was likewise observed that the brain weight of postnatal NPAS1-deficient mice was 32% greater than wild type littermates, and that neuron density was 62% greater in NPAS1-deficient animals compared with wild type littermates. Difference in brain weight as a function of genotype disappeared by three months of age, and the difference in neuron density was reduced to a 37% increase in NPAS1-deficient animals compared to wild type littermates. Differences in both brain size and neuron density as a function of genotype disappeared in animals older than two years of age. Finally, after performing a comprehensive battery of behavioral assays, NPAS1-deficient animals were observed to be hypersensitive to both acoustic and tactile stimulation, relative to wild type littermates. It may be notable that transient, postnatal increase in brain size and neuron density

in NPAS1-deficient mice, coupled with selective hypersensitivity to acoustic and tactile stimulation, mimic anatomical and behavioral features associated with autism.

4.2 Introduction

Neuronal PAS domain 1 (NPAS1) was discovered in 1997 as a brain-specific transcription factor first detected at embryonic day 15 of the developing mouse embryo (Zhou et al., 1997). Its expression was observed to peak at around postnatal day 3, and *in situ* hybridization assays in adult mice revealed expression localized to the cortex, hippocampus, thalamus, hyopothalamus and superior colliculus. Subsequent immunohistochemical staining assays employing antibodies to NPAS1 revealed an expression pattern largely restricted to inhibitory neurons in the aforementioned brain regions, as well as localized expression in the subgranular region of the dentate gyrus (Erbel-Sieler et al., 2004). More recently, a highly related transcription factor designated neuronal PAS domain 3 (NPAS3), was also reported to be expressed selectively in the rodent brain. The bHLH DNA binding domains of NPAS1 and NPAS3 are 76.5% identical in amino acid sequence, and the PAS-A and PAS-B domains of the two proteins are 98.0% and 47.7% identical respectively (Brunskill et al., 1999). The mammalian NPAS1 and NPAS3 transcription factors appear to be evolutionary counterparts of the product of the *Trachealess* gene of *Drosophila melanogaster* (Zhou et al., 1997, Brunskill et al., 1999). Aside from sharing substantive relatedness in amino acid sequence, the fly and mammalian transcription factors may regulate common biological pathways, including components of the fibroblast growth factor (FGF) signaling pathway (Pieper et al., 2005). Despite the high levels of amino acid sequence homology in their bHLH, PAS-A and PAS-B domains, as well as their utilization of the aryl hydrocarbon nuclear translocator (ARNT) as an obligatory, heterodimeric partner, the NPAS1 and NPAS3 proteins appear to be endowed with diametrically opposing functional properties. Three polypeptide segments within the NPAS1 transcription factor have been shown to have repressive activity (Teh et al., 2006). As such, NPAS1 is considered to be a transcriptional repressor. By contrast, the carboxyl terminal domain of the NPAS3 transcription factor contains a glycine-rich domain that has been reported to function as a transcriptional

activation domain (Pickard et al., 2006). A second major difference between NPAS1 and NPAS3 is the size of their encoding genes. The 11 exons of the *NPAS1* gene are arranged within a 20-kb segment of mouse chromosome 7 (human chromosome 19), whereas the equivalent exons of the NPAS3 gene span nearly a megabase of mouse chromosome 12 (human chromosome 14). A translocation between chromosomes 9 and 14 that disrupts the NPAS3 gene has been reported in a family suffering from schizophrenia (Kamnasaran et al., 2003), and NPAS3-deficient mice have been reported to have behavioral abnormalities reminiscent of schizophrenia (Erbel-Sieler et al., 2004), as well as a distinct deficit in hippocampal neurogenesis (Pieper et al., 2005).

Here we report the results of both behavioral and morphological studies of NPAS1-deficient mice. In contrast to NPAS3-deficient mice which suffer from impaired hippocampal neurogenesis, animals missing the NPAS1 transcription factor display a diametrically opposed phenotype. NPAS1-deficient animals are born with larger than normal brains which are packed with an over-abundance of neurons. Measurements of hippocampal neurogenesis via bromodeoxyuridine (BrdU) incorporation reveal a significant enhancement of neuron birth in animals between 5 and 10 weeks of age. The enlarged brain size observed shortly after birth, as well as the over-abundance of neurons, dissipate with age. Finally, NPAS1-deficient animals exhibited enhanced sensitivity to acoustic and tactile stimulation. The spectrum of morphological and behavioral abnormalities displayed by NPAS1-deficient mice bear resemblance to abnormalities observed in a substantive proportion of autistic children, raising the possibility that enhanced levels of neuron birth and density may constitute a cardinal feature of autistic disease.

4.3 Materials and Methods:

Approval for the animal experiments described herein was obtained by the University of Texas Southwestern Medical Center Institutional Animal Care and Use Committee. The targeting construct used to disrupt the NPAS1 gene placed the β-galactosidase gene of *Escherichia coli* in frame with the N-terminal 41 aa of the truncated NPAS1 polypeptide.

Mice. *NPAS1*$^{+/-}$ females (F_1) mated to *NPAS1*$^{+/-}$ males (F_1) produced C57Bl/6J strain litters that were fully back-crossed. Animals were group housed until time of testing. Genotypes were established by PCR analysis, as described (Erbel-Sieler et al., 2004).

Immunohistochemistry. Brain tissue was prepared by perfusion with PBS and 4% paraformaldehyde fixation, and immersed in 4%PFA overnight. Individual brain samples were sunk in 30% sucrose before freezing and sectioning on a sliding microtome into 40 μm free floating sections in PBS. Immunohistochemical staining was performed with primary antibodies against the following antigens: NeuN (1:2000, Millipore), Gad-67 (1:1000, Chemicon), S100-β (1:1000, Santa Cruz Bio.), BrdU (1:100, Roche). All primary antibodies were diluted in 5%BSA/T-PBS. Biotinylated secondary antibodies (diluted in 3%BSA/T-PBS) were used in conjunction with ABC reagent and DAB, for chromagenic detection of the marker.

Stereology. Immunohistochemically stained sections of 40 μm thickness were analyzed using the Stereologer 2000 program (the unbiased guide to stereology). For each brain sample, 10 sections were analyzed. Cells were counted in a specified area in several random positions throughout each section. The same procedure was employed for each cell type analyzed.

Quantification of Basal in Vivo BrdUrd-Labeled Proliferating Neural Precursor Cells. Basal levels of neural precursor cell proliferation in the dentate gyrus granular layer (GL) subgranular zone (SGZ) were quantified in brains of 5-10 week old mice through immunohistochemical detection of incorporation of the thymidine analog BrdUrd (Sigma–Aldrich) in dividing cells in the brain. Mice were housed individually in standard cages with *ad libitum* access to food and water and were injected i.p. once daily with BrdUrd (50 μg/g of body weight) for 12 days, according to established methods (Kempermann et al., 1997). On day 13, mice were anesthetized and transcardially perfused with 4% paraformaldehyde in PBS at pH 7.4. Brains were dissected, immersed in 4% paraformaldehyde overnight at 4°C, and then sliced coronally on a sliding microtome into 40 μm-thick free-floating sections that were subsequently processed for

immunohistochemical staining with a mouse monoclonal antibody to BrdUrd (1:100, Roche). Omission of primary antibodies served as a negative control (data not shown). Unmasking of BrdUrd antigen was achieved through incubating tissue sections for 2 h in 50% formamide/2XSSC at 65°C, followed by 5 min wash in 2XSSC and subsequent incubation for 30 min in 2 M HCl at 37°C. Diaminobenzidine was used as a chromogen. Images were analyzed with a Nikon Eclipse 90i motorized research microscope with Plan Apo lenses coupled with Metamorph Image Acquisition software (Nikon). Sectioning, immunohistochemistry, and counting of immunopositive cells were executed by separate investigators in a double-blinded manner. Immunopositive cells in the dentate gyrus GL and adjacent SGZ, defined as a two-cell-body-wide zone of the hilus along the base of the GL, were counted in both hemispheres of every third section (~10 –15 sections per animal) progressing posteriorly from the point where the supra-pyramidal and infra-pyramidal blades are joined at the crest region with the dentate gyrus oriented horizontally beneath the corpus callosum.

Behavioral Test Battery. Animals (aged 10-12 weeks) were tested in a battery of behavioral testing paradigms. A general health check and neurologic exam were first taken. Next, the following tests were performed on the animals: elevated plus maze, open field, acoustic startle response, pre-pulse inhibition, fear conditioning, Morris water maze, air puff response, and social preference.

Acoustic Startle Response. Mice were tested for simple auditory response in SR-Lab Systems test chambers (San Diego Instruments). After 5 min of acclimatization in the chamber (background noise, 70 dB), a session of four sets of trials was presented. Each set contained nine 40-msec stimuli (70, 74, 78, 82, 86, 90, 100, 110, and 120 dB) presented in pseudorandom order. The intertrial interval averaged 15 sec and ranged from 10 to 20 sec. The subject's response was recorded over 65 msec starting at the onset of the stimulus, and the maximum startle amplitude (Vmax) was determined.

Open-Field Activity. Animals were placed in a novel open-field box (MED Associates, St. Albans, VT; 28x28 cm) and tracked by an array of photocell beams for the duration of 12 min. Activity was then analyzed for distance traveled, stereotypic counts, and thigmotaxis.

Rotarod. Mice were placed on a 3.8-cm rotating rod (CR-1 Rotamex system, Columbus Instruments, Columbus, OH). The rotation speed started at 5 rpm and increased by 10 rpm per min (maximum speed, 75 rpm). Latency to fall and speed at fall were measured. If an animal fell in <10 sec, it was placed back on the rod for a maximum of five times. Immediately after falling, the mouse received an electrical shock (0.2 mA, 1 sec). Each mouse was subjected to four trials a day, with at least 1 h recovery time between trials, for 3 consecutive days.

Prepulse Inhibition. Mice were tested in SR-Lab Systems chambers (San Diego Instruments). Background-noise level in each chamber was 70 dB. After 5 min acclimatization in the chamber, a session was presented in 64 trials. Each session consisted of five trial types: a plain acoustic startle pulse (40 msec, 120 dB), three different acoustic prepulses (20 msec, either 74, 78, or 86 dB) followed 100 msec later by an acoustic startle stimulus (40 msec, 120 dB), and trials with no stimulus presented to measure baseline movements. The subject's response was recorded over 1 sec starting at the onset of the stimulus, and the maximum startle amplitude (Vmax) was determined. Sessions were split into four blocks of trials. Block 1 contained six plain acoustic startle pulses. Blocks 2 and 3 contained 26 trials each. In each block the five different trial types were presented in pseudorandom order with an intertrial interval averaging 15 sec (range, 8–23 sec). Block 4 contained six plain acoustic startle pulses. After testing, average values of Vmax per animal were calculated for the five different trial types within each block. The % prepulse inhibition (%PPI) was then defined as 100 ´ [(average of plain startle pulses block 2 + 3) – (prepulse plus startle pulse)] / average of plain startle pulses block 2 + 3.

Cued and Contextual Fear. Part 1 (pretraining) of the CCF test involved a 10-min exploration of the test chamber (Med Associates). In part 2 (training), 24 h later, the mice were placed in the same test chamber and allowed to explore for 3 min. The conditioned stimulus, 30 sec of 80 dB white noise, followed and ended with the unconditioned stimulus, a 1-sec 0.5-mA shock. The conditioned stimulus–unconditioned stimulus pairing was repeated for a total of three times, making the entire test duration 6 min. In part 3 (testing), the mice were first returned to the identical test chamber 24 h after the training for a 3-min assessment of contextual learning. Two hours thereafter the animals were assessed for cued learning in

a novel environment, a test chamber identical to the one used in parts 2 and 3, except it was painted black (versus unpainted aluminum), scented with 0.1% acetic acid (versus ethanol) and contained a smooth red floor (versus shock grid). The animals were allowed to explore the novel test chamber for 3 min followed by a 3-min period in the presence of the acoustic stimulus (cued). During the training and test phases the animals were monitored by video and displayed on a computer (FREEZEFRAME, Actimetrics, Wilmette, IL). Data analysis was carried out in two ways: the analysis of motion indices from the FREEZEFRAME program, and the scoring of freezing behavior by direct observation (10-sec intervals) in which freezing was defined as total immobilization except for respiratory movements.

Social Preference. In a 3-chamber box, the mice were subjected to two trials. In the first 10-minute trial, one side had a stopper under a wire cup, and the other side had an unfamiliar mouse under a wire cup. In the second 10-minute trial, one side had the mouse that was met in the first trial, and the other side had a new unfamiliar mouse. A video camera above was used to track the movement and interaction with the mice or the stopper.

4.4 Results:

Postnatal NPAS1-Deficient Mice Have Enlarged Brains.

Litters of pups born from a crossing of male and female mice heterozygous at the NPAS1 locus were sacrificed fourteen days after birth. Variation in brain tissue size was recognized in individual mice and quantified by weight. As shown in Figure 1, the variation in brain size and weight was subtle, but readily assigned to genotype. Animals bearing a targeted disruption (with a β-galactosidase in-frame insertion after amino acid 41) on both alleles of the NPAS1 gene displayed visibly larger brains than heterozygous and wild type littermates (Figure 1A), and weighed roughly 32% more (Figure 1B). Despite this difference in overall brain size, no obvious differences in morphological organization of the brains were observed in sectioned material as studied by light microscopy. The relative sizes of cortex, cerebellum and other major features of the brain were proportionally similar in tissue samples evaluated from animals of all

three genotypes, and there were no measurable differences in organ weights (liver, lungs, hearts) among the animals.

NPAS1-Deficient Mice Contain an Increased Number of Neurons in the Cortex.

Upon observing the histology of sectioned brain tissue, there appeared to be an increase in the density of cells in the cortex of NPAS1-deficient mice. In order to analyze this observation more thoroughly, stereologic analyses were performed. To measure the number of neurons in the cortex, NeuN, a marker for mature neurons was used for immunohistochemistry, and NeuN+ cells were subsequently counted in the cortex using the Stereologer 2000 program. The postnatal day 14 (P14) NPAS1-deficient mice, whose brains weighed more, had a corresponding increase in the number of neurons in the cortex. At P14, NPAS1-deficient mice had 62% more neurons in the cortex than wild type mice (Figure 1C). Stereology was also used to analyze the cortex of mice that were sacrificed at around 12 weeks old. The number of NeuN+ cells was increased in NPAS1-deficient mice at this age as well, however, to the lesser degree of 37% more neurons in NPAS1-deficent mice compared to wild type littermates (Figure 2A). Immunohistochemical staining, and stereology for Gad-67 was also performed in the adult animals. GAD67 is one of the major isoforms of glutamic acid decarboxylase, the enzyme that synthesizes GABA, and it serves as a marker of inhibitory interneurons. There was an increase in Gad-67+ inhibitory neurons as well, and the increase was at roughly the same ratio as the increase in the NeuN+ neurons (Figure 2B). Furthermore, stereology analyzing the number of S100-β+ cells was used to measure the number of astrocytes and oligodendrocytes in the cortex. In contrast to the increased number of both total (NeuN+) and inhibitory (Gad67+) neurons, there was no increase observed in the number of astrocytes or oligodendrocytes (Figure 2C). These data suggest there is a significant and selective increase in the number of both excitatory and inhibitory neurons in the cortex of NPAS1-deficient mice.

NPAS1-Deficient Mice Exhibit Increased Hippocampal Neurogenesis.

Neurogenesis arises in the adult mammalian brain predominantly from neural precursor cells residing

in the hippocampal dentate gyrus SGZ and the subventricular zone (SVZ) of the lateral ventricle. These regions provide cells throughout adult life to the dentate gyrus and the olfactory bulb, respectively (Gage, 2000). We used previously established methods (Kempermann et al., 1997) to quantify neural precursor cell proliferation, through immunohistochemical detection of incorporation of the thymidine analog bromodeoxyuridine (BrdU). BrdU injections were given to 5-10 week old mice for 12 days, and they were sacrificed on the 13th day. BrdU+ cells were then counted in the SGZ of the dentate gyrus of the hippocampus. This assay measures the combined proliferation and survival of newborn neurons in the hippocampus. There was a 31.5% increase in hippocampal neurogenesis in NPAS1-deficient mice compared to wild type mice (Figure 3). Although there was increased neurogenesis in the 5-10 week old mice, this could not account for the increased neuron numbers, or the enlarged brain size seen in the younger (P14) NPAS1-deficient mice. Increased neurogenesis would have had to occur either embryonically, or early postnatally, for there to be such a substantial increase in the neuron number at the P14 time point.

Increase in Brain Weight and Neuron Density are Transient Phenotypes.

Although the postnatal day 14 NPAS1-deficient mice were observed to have an increased brain weight and size, when the adult brain tissues (animals ~12 weeks old) were analyzed, there were no differences in size or weight observed between the brain tissues of NPAS1-deficient mice and their wild type littermates (Figure 4B). Considering this, it was of interest to perform further investigations of the older animals. As discussed earlier, stereology was employed to analyze the neuron number in the mice at ~12 weeks, and there was still a substantial increase in neuron number (37% increase) in NPAS1-deficient mice (Figure 4E), albeit at a lesser degree than the P14 mice (62% increase). However, when investigations of 2-3 year old mice were performed, there were no differences observed in brain weight, brain size or neuron number in NPAS1-deficient mice compared to wild type littermates (Figures 4C and 4F). Therefore, the enlarged brain size, and the increase in neuron number, appear to be transient phenotypes which are most pronounced at a young age, and slowly normalize as the mice age.

Interestingly, this closely mimics what occurs in the brain tissue of a substantial fraction of autistic children (Aylward et al., 2002; Courchesne et al., 2001).

NPAS1-Deficient Mice are Hypersensitive to Acoustic Stimuli.

The acoustic startle response test is normally used to test the hearing of mice. A normal increase in the startle response of mice occurs with increasing decibel levels of noise. When this test was performed, it was observed that NPAS1-deficient mice displayed a trend of having an increased startle response to the noise, which is most prominent at the four loudest decibel levels (Figure 5A). Statistical analysis of the differences in startle response between the NPAS1-deficient mice and the wild type littermates was performed using 2-way ANOVA resulting in a p-value of 0.0029. These data suggested that NPAS1-deficient mice are hypersensitive to acoustic stimuli.

NPAS1-Deficient Mice are Hypersensitive to Tactile Stimuli.

To further characterize the sensory abnormalities of NPAS1-deficient mice, we employed the air puff test. The air puff test is run in the same chamber as the startle response test and the PPI test. The air puff is blown on the mice in conjunction with noises, similar to the PPI test, but with air after the pre-pulses instead of noise. With no noise before the air, or a very low decibel noise preceding the air, NPAS1-deficient mice startled significantly more than the wild type mice (Figure 5B). If the preceding noise level was louder, NPAS1-deficient mice did not startle more than wild type mice in response to the air puff. The louder noises may startle NPAS1-deficient mice to a degree that they are desensitized to the air puff. This may be because they are more sensitive to the louder noises than the wild type mice (Figure 5A). However, if the noise levels are low or not present, NPAS1-deficient mice are more sensitive to the air puff than the wild type mice. Therefore, NPAS1-deficient mice may be hypersensitive to both tactile stimuli and acoustic stimuli.

NPAS1-Deficient Mice Do Not Display Other Behavioral Abnormalities

A battery of behavioral tests were performed on NPAS1-deficient mice to assess for neurologic, psychiatric, and social impairments. The additional behavioral tests included the elevated plus maze, the open field test, rotarod, fear conditioning, Morris water maze, PPI, and the 3-chamber social interaction test. No abnormalities were observed in NPAS1-deficient mice relative to wild type littermates in any of these tests. The only two tests in which NPAS1-deficient mice displayed clear phenotypes were the acoustic startle and air puff response tests, as were discussed above.

Attempts towards the identification of genes that may be regulated by NPAS1.

Knowing that NPAS1 expression in the brain is highest in the early post-natal days, it was of interest to determine if there were genes that NPAS1 may regulate at this time. DNA microarrays were performed on post-natal day 3 mouse brains, comparing NPAS1-deficient mice to wild type littermates, in order to find genes exhibiting differential expression between the two genotypes. Microarray analysis with Partek and GeneSpring software showed that there were several genes with significant differences in expression between the NPAS1-deficient mice and wild type littermates ($p < 0.05$ by ANOVA). Two of the most interesting genes that were differentially expressed were Ube3a and Pwcr1. Both of these genes appear to be expressed at a significantly lower level in NPAS1-deficient mice compared to wild type littermates. In humans, it is known that a loss of Ube3a expression can lead to Angelman syndrome, an imprinted gene disorder. Ube3a is maternally expressed, and imprinted on the paternal allele, so Angelman syndrome can be caused by paternal disomy, or mutations or deletions in Ube3a on the maternal allele. Pwcr1 (Prader-Willi control region) is in the same region of the genome, however, it is expressed from the paternal allele, and silenced on the maternal allele. Prader-Willi is also an imprinted gene disorder, being caused by maternal disomy in this region, or deletions in the paternal allele. Ube3a is a ubiquitin E3 ligase, and Pwcr1, or Snord116, is a small nucleolar RNA. Although the cardinal symptoms of these disorders are different, both can display autistic behaviors. We were able to confirm the lower expression of Ube3a in NPAS1-deficient mice by QPCR. However, Pwcr1 is a very small RNA that cannot be easily assayed by QPCR. The same differences in Ube3a expression were not seen in an RNA-Seq

experiment. Western blots and immunohistochemistry were also performed for Ube3a, and were inconclusive. The changes were not obvious, if there were any at all. Therefore, we decided not to pursue this hypothesis any further.

Having observed these two imprinted genes change in expression level as a function of genotype at the NPAS1 locus, we decided to look for other imprinted genes that may have significantly different expression levels in NPAS1-deficient mice. We found 7 more imprinted genes whose expression varied significantly as a function of NPAS1 genotype. Interestingly, expression of all of these genes changed in a direction that would suggest too much imprinting and lowered expression, or higher expression if the anti-sense transcript is the allele that is methylated. Excess methylation and lowered expression of the anti-sense transcript would lead to increased expression of the imprinted gene (Table 3).

Table3

Imprinted Genes

Gene	Imprint	Regulation in NP1 -/-
Ube3a	paternal	↓
Pwcr1	maternal	↓
Necdin	maternal	↓
L3mbt	maternal	↓
Impact	maternal	↓
Kcnq1	overlying transcript	↑
Osbpl5	overlying transcript	↑
Kcnq1ot1	maternal	↓

Many of these genes were confirmed by QPCR. However, after reading the data from Gregg et al. about imprinting in the brain (Gregg et al., 2010), it was clear that the trend of an abnormal number of imprinted genes changing in the NPAS1-deficient mice may not be any more than one would expect to see by chance. In other words, any knock out mouse could have had this many imprinted genes on its list of differential gene expression.

Other genes of interest obtained from the microarray data were related to apoptosis and cell survival or proliferation (Table 4).

Table 4.

Growth and Apoptosis Related Genes

Gene	Description	Regulation in NP1 -/-
Mapk8	JNK, c-Jun N-terminal kinase	↓
Gsk3b	Glycogen synthase kinase 3-β	↓
Unc5c	Unc-5 homolog C	↓
Spry2	Sprouty homolog 2	↓
Frag	FGF receptor activating protein	↑
Nenf	Neuron derived neurotrophic factor	↑
Naip	NLR family, apoptosis inhibitory protein	↑
Sipa	Signal-induced proliferation associated gene	↑
Pa2g4	Proliferation-associated 2G4	↑

One gene that is particularly interesting from this list is Gsk3β. As noted in the introduction, low activity of Gsk3β can prevent phosphorylation of β-catenin, and this stabilizes β-catenin, which can lead to increased cell proliferation. This pathway is implicated in causing the macrocephaly seen in both the PTEN-deficient mice, and the transgenic β-catenin mice (Kwon et al., 2006; Chenn and Walsh et al., 2002). According to the microarray, the expression of Gsk3β was significantly lower in NPAS1-deficient mice. QPCR confirmed this, but not consistently. Also, Western blots on solubilized brain tissue, and immunohistochemistry on brain slices were inconclusive. If we strongly felt that this was the mechanism for the increased neuron number and increased brain size, it would be important to check β-catenin levels in NPAS1-deficient mice, or phosphorylation levels of β-catenin. The other genes in Table 4 were not

pursued further. In summary, we are not certain of any genes that are regulated by NPAS1, since we do not have concrete and consistent evidence to support any of these findings.

4.5 Discussion and Conclusions:

We have observed that young, post-natal NPAS1-deficient mice have an over abundance of neurons in the cortex and an enlarged brain size. These abnormalities normalize partially within 1-2 months, and fully by 1–3 years. This transient brain enlargement mimics what apparently occurs in autistic children who have enlarged brains (Courchesne et al., 2011). We have also observed that NPAS1-deficient mice appear to be hypersensitive to acoustic and tactile stimuli. Many autistic children are hypersensitive to auditory and tactile stimuli as well (Kanner, 1943; Baron-Cohen et al., 2009; Kern et al., 2006; Leekam et al., 2007). According to our observations, it may be possible that the NPAS1-deficient mice are more sensitive to acoustic stimuli than to tactile stimuli, since they do not startle more to the air puff when louder noises precede the air puff. Alternatively, it could be that NPAS1-deficient mice are unable to process two sensory inputs at the same time (they may have difficulty with parallel processing of multiple stimuli), a phenomenon that has also been observed in autistic children (Foss-Feig et al., 2010).

It is also interesting to note that although the paralogous NPAS1 and NPAS3 transcription factors seem to have evolved from the *Drosophila* gene *Trachealess*, they appear to have adopted opposing functions in the brain. It is thought that NPAS3 is a transcriptional activator (Pickard et al., 2006), while NPAS1 is a repressor (Teh et al., 2006). NPAS3-deficient mice were observed to have decreased body weight, impaired PPI, noticeable “darting” behavior, impaired nesting behavior, decreased hippocampal neurogenesis, electrophysiological dysfunctions, and severe attenuation of dendritic branching and spine density (Erbel-Sieler et al., 2004; Pieper et al., 2005). In addition, it has been reported that disruptions in the NPAS3 gene are connected to schizophrenia, learning disabilities and mental illness (Kamnasaran et al., 2003; Macintyre et al., 2010; Pickard et al., 2009; Pickard et al., 2005; Pickard et al., 2006). Moreover, NPAS3-deficient mice displayed schizophrenic-like phenotypes. By contrast, NPAS1-deficient mice displayed phenotypes similar to those seen in autistic children, such as the increased brain size and

increased number of neurons (Kanner, 1943; Courchesne et al., 2001; Aylward et al., 2002), and hypersensitivity to acoustic and tactile stimuli (Baron-Cohen et al., 2009; Kern et al., 2006; Leekam et al., 2007).

The opposing functions of NPAS1 and NPAS3 are intriguing because of the two opposing phenotypes of autism and shizophrenia observed in NPAS1-deficient mice and NPAS3-deficient mice, respectively. The hypothesis that schizophrenia and autism display diametrically opposite sets of phenotypes and conditions was previously described by Crespi and colleaugues (Crespi, 2010). The theory is based on well-documented pathologic and genomic evidence. For example, whole brain size is reduced in schizophrenia from birth onwards (Cannon et al., 2002; McIntosh et al., 2006; Tamminga and Holcomb, 2005). Moises and colleagues also noted that a considerable range of birth deficiencies, including low birth weight, late maturation, and small brain size are found in schizophrenic patients (Moises et al., 2002). It has been suggested that reduced brain size is due in part to slow brain maturation in individuals with psychosis (Crow, 1996; James et al., 1999; Saugstad, 1998). On the other hand, in autism, increased cortical thickness has been observed (Courchesne et al., 2001; Hardan et al., 2006; Piven et al., 1995), and an increase in head and brain size is one of the most consistent anatomical findings in autistic children (DiCicco-Bloom et al., 2006; Aylward et al., 2002; Sparks et al., 2002; Herbert et al., 2003). Concordant with this difference in brain size, autism has been associated with loss of function in genes that act as negative regulators of growth-signaling pathways such as the PI3K, Akt and mTOR pathways (Cusco et al., 2009; Hoeffer et al., 2008; Kwon et al., 2006), whereas schizophrenia has been associated with reduced function or activity of genes that up-regulate PI3K, Akt and other growth-related pathways (Emamian et al., 2004; Kalkman, 2006; Stopkova et al., 2004). In addition, copy number variations (CNVs) such as *duplications* in 1q21.1 and 16p13.1 have been associated to autistic individuals with increased head size, brain volume, or cortical thickness (Ullmann et al., 2007), whereas reduced brain size has been associated with schizophrenia-linked CNVs at 1q21.1 and *deletions* of 16p13.1 (Hannes et al., 2009; Ullmann et al., 2007). Furthermore, data from several CNV studies have found that autism and schizophrenia are associated with reciprocal variants, such that at four loci,

deletions predispose to one disorder, whereas duplications predispose to the other (Crespi et al., 2010). Moreover, it has been hypothesized that autistic behavior may result from hypo-development of the social brain, with a deficiency in social phenotypes such as theory of mind, language, sense of self in relation to others, and social interaction (Baron-Cohen et al., 2009; Hill and Frith, 2003). In contrast, schizophrenic and psychotic behavior such as hallucinations, paranoia, megalomania, and ascription of causal purpose to inanimate objects may be interpreted as a hyper-development of the social brain (Crespi and Badcock, 2008).

In closing, NPAS1 and NPAS3 appear to exert opposing functions in brain development, as well as in adult neurogenesis. NPAS3 expression is first seen in the embryonic mouse telencephalon and neural tube at around E9.5, and becomes restricted to the neopallial layer of the cortex at around E15, which is precisely when NPAS1 expression is first seen in the developing embryonic brain. It is therefore possible that NPAS3 may act to increase neurogenesis, whereas NPAS1 may act to slow down neurogenesis, as has been seen in the adult hippocampus. Mutations and translocations in NPAS3 have been shown to predispose individuals to schizophrenia and other psychiatric disorders (Kamnasaran et al., 2003; Pickard et al., 2005, 2006, 2009; Macintyre et al., 2010). Given the phenotypes that have been observed in NPAS1-deficient mice, one might predict that mutations in NPAS1 could predispose individuals to autism. Indeed, John Rubenstein and Steve Hamilton have found variations in NPAS1 in autistic children (Figure S1). However, these variations have not been published yet.

The opposite functions of NPAS1 and NPAS3 observed in the mice, and their respective associations with autism and schizophrenia recapitulate the diametric opposition of phenotypes seen in the two disorders. Hopefully the results we have presented here will make some contribution to studies in the fields of autism and schizophrenia. It seems unlikely that these disorders will be “curable”, since the problems seem to occur during development of the brain. Once the developmental disruption occurs, it is difficult to envision treatments that could repair abnormal brain morphology and wiring. Decreasing neurogenesis would not appear to be a good treatment for autism, even if increased neurogenesis is the “cause”. Therefore, there is much more research that needs to be done before we can understand either

of these disorders. We close with the hope that studies of the NPAS1 and NPAS3 transcription factors will further our understanding of the causes of autism and schizophrenia, and help us to understand the brain as a whole.

Figures:

Figure 1. NPAS1-deficient mice have a larger brain size, and an increased number of neurons in the cortex compared to heterozygous and wild type littermates at post-natal day 14.

Post-natal day 14 (P14) brains of NPAS1-deficient mice are significantly larger than heterozygous NPAS1 and wild type littermates (A). The brain weight of the NPAS1-deficient mice is significantly larger at P14 as well (B) ($n \geq 7$ for each genotype, * = $p < 0.01$). There is also an increased number of neurons in the cortex of NPAS1-deficient mice at P14, as seen through stereology for NeuN (C) ($n = 4$ for each genotype, * = $p < 0.001$).

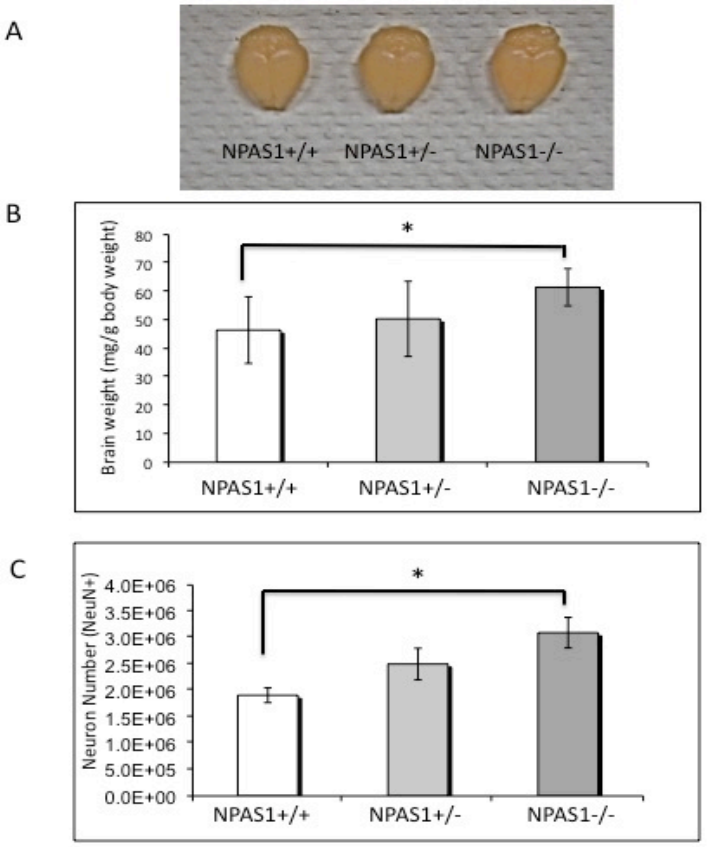


Figure 2. NPAS1-deficient mice have an over-abundance of neurons in the cortex.

The number of neurons in NPAS1-deficient mice is increased, as seen through stereology counting NeuN+ neurons in the cortex (A). GABA inhibitory neurons (Gad-67+) are also increased in the NPAS1-deficient mice, and they are increased at the same ratio as the NeuN+ neurons (B). There is no increase in S100-β+ cells (a marker for astrocytes and oligodendrocytes) (C). Therefore, it is thought that there may be a generalized increase in all neurons, but not necessarily all cell types. ($n \geq 10$ for each genotype, * = $p \leq 0.003$)

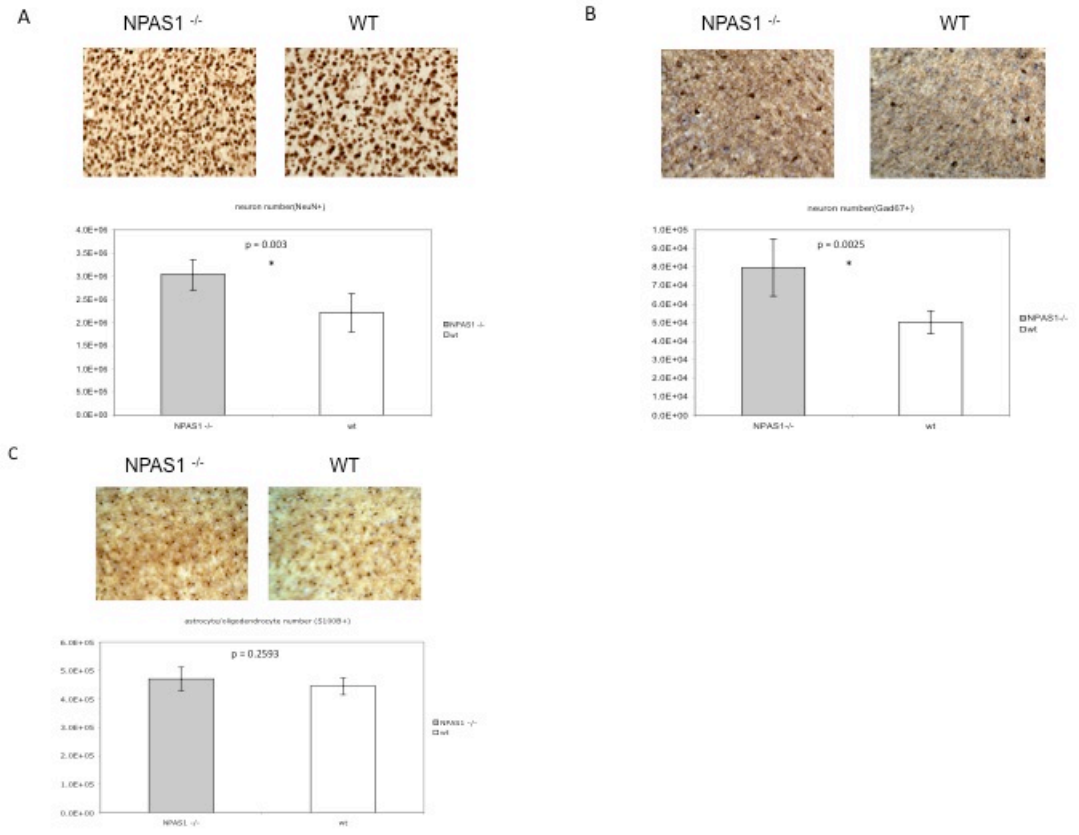


Figure 3. NPAS1-deficient mice have increased hippocampal neurogenesis.

There is an increase in neurogenesis in NPAS1-deficient mice in the SGZ of the hippocampus. This was measured via BrdU labeling. BrdU injections were given to 5-10 week old mice for 12 days, and they

were sacrificed on the 13th day. Then BrdU+ cells were counted in the SGZ. This is a measure of proliferation and survival of newborn neurons in the hippocampus. (n ≥ 22 for each genotype, * = p = 0.007).

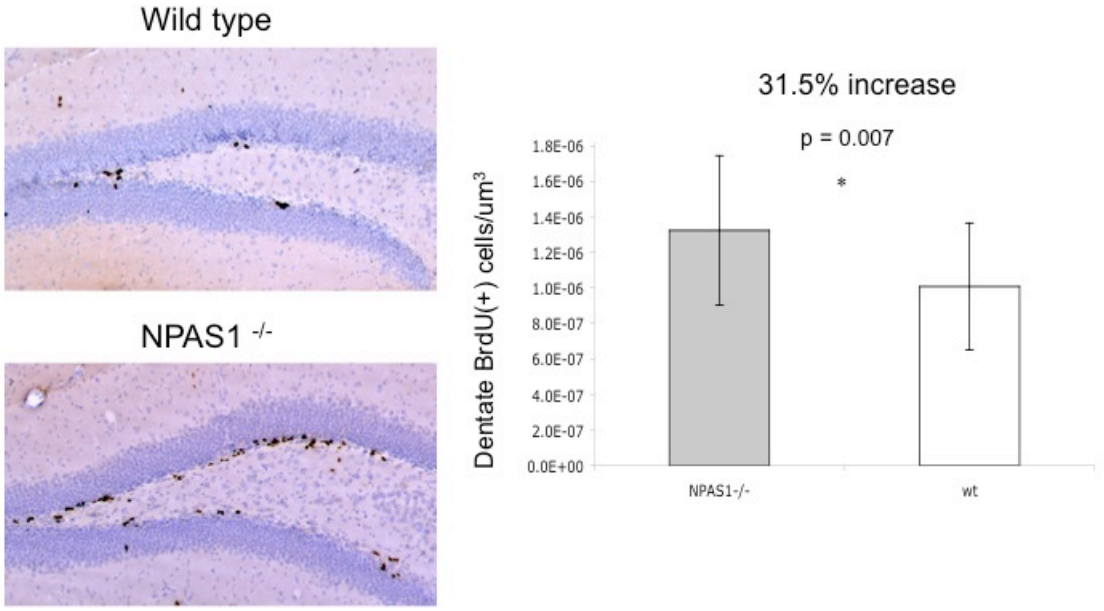


Figure 4. The enlarged brains and increased neuron numbers in the NPAS1-deficient mice are transient phenotypes.

NPAS1-deficient mice have larger brains than normal mice at post-natal day 14 (A) (n ≥ 7 for each genotype, * = p < 0.01). However, this enlarged brain phenotype normalizes as the mice age (B-C) (n ≥ 5 for each genotype). NPAS1-deficient mice have an increased number of neurons (as seen through stereology counting NeuN+ neurons in the cortex). At post-natal day 14, there is a substantial increase (62% more neurons) (D). In the adult mice (which are ~12 weeks old), there is a 37% increase in neuron number (E) (n ≥ 10 for each genotype, * = p ≤ 0.003). In older mice (2-3 yrs), there is no increase in

neuron number (F) (n =5 per genotype). Therefore, the increase in brain size and increase in neuron number in the cortex are transient phenotypes, which are most pronounced at a young age, and slowly normalize as the mice age.

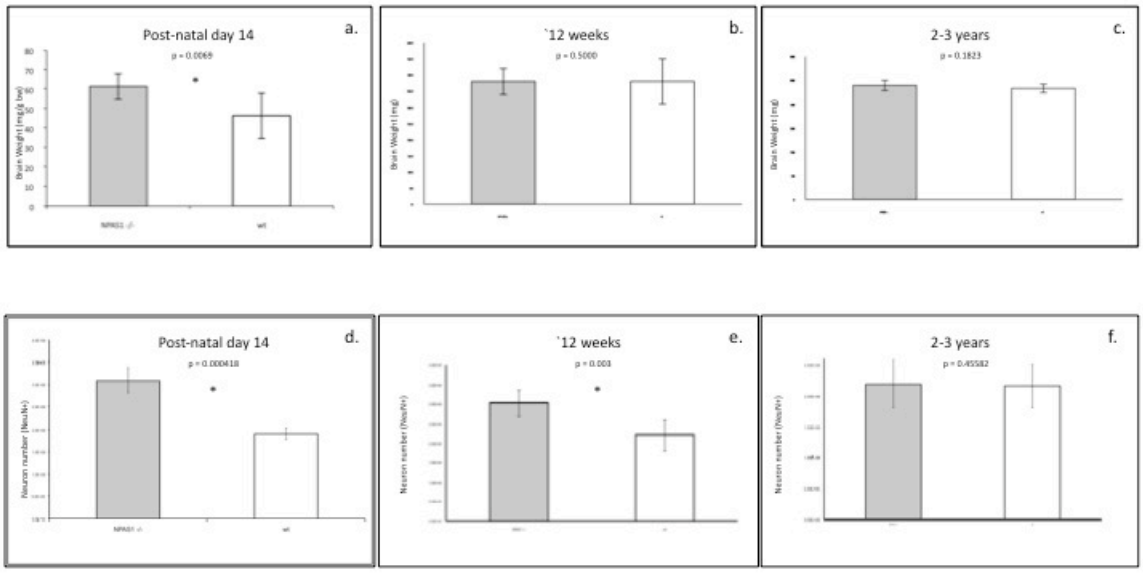


Figure 5. NPAS1-deficient mice are hypersensitive to acoustic and tactile stimuli.

NPAS1-deficient mice startle more than normal mice in a test for acoustic startle response. They are hypersensitive to acoustic stimuli, especially at louder dB levels. 2-way ANOVA shows a significance of p = 0.0029 (A) (n > 22 per genotype).

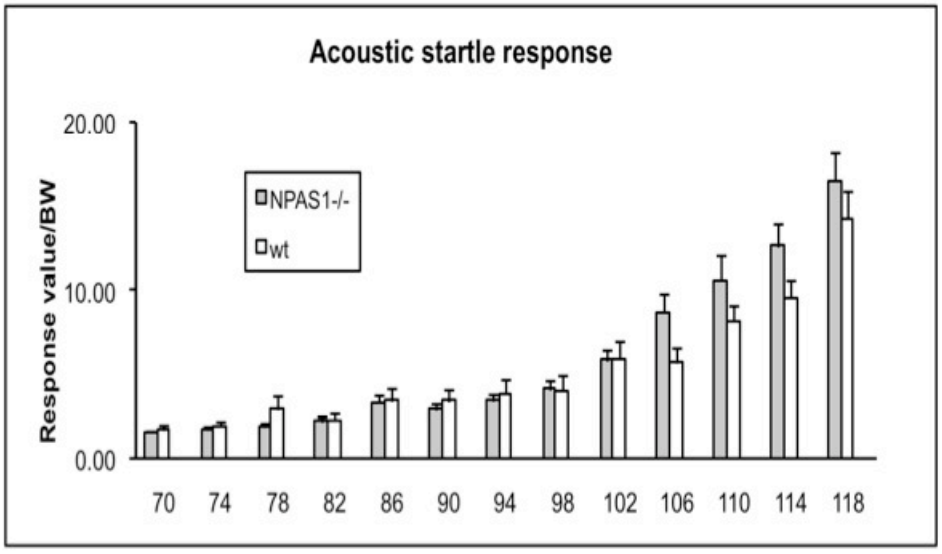

Acoustic startle response
Response value/BW
20.00
10.00
0.00
NPAS1-/-
wt
70
74
78
82
86
90
94
98
102
106
110
114
118

Figure 5B. NPAS1-deficent mice startle more than normal mice in response to an air puff, if the air puff is not preceded by a loud noise. When no noise or a soft noise is present before the air puff, the NPAS1-deficient mice startle significantly more. However, if a louder noise is present before the air puff, NPAS1-deficient mice seem to be desensitized to the air puff, or they are so startled by the noise that they do not respond to the air puff as much. Therefore, they seem to be sensitive to tactile stimuli, but loud acoustic stimuli can override the sensitivity to the air puff (B) ($n \geq 9$ per genotype, * = $p < 0.05$).

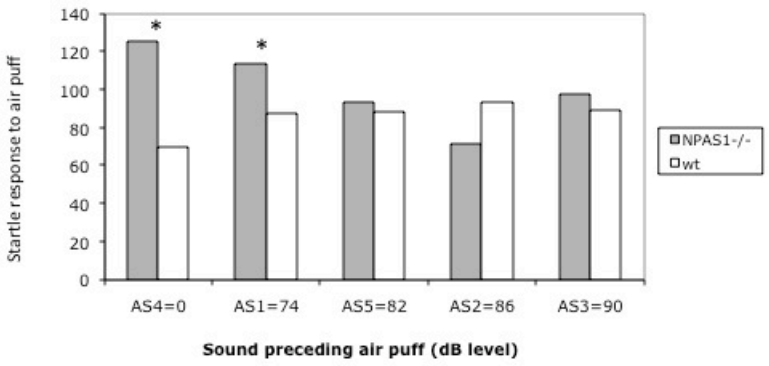
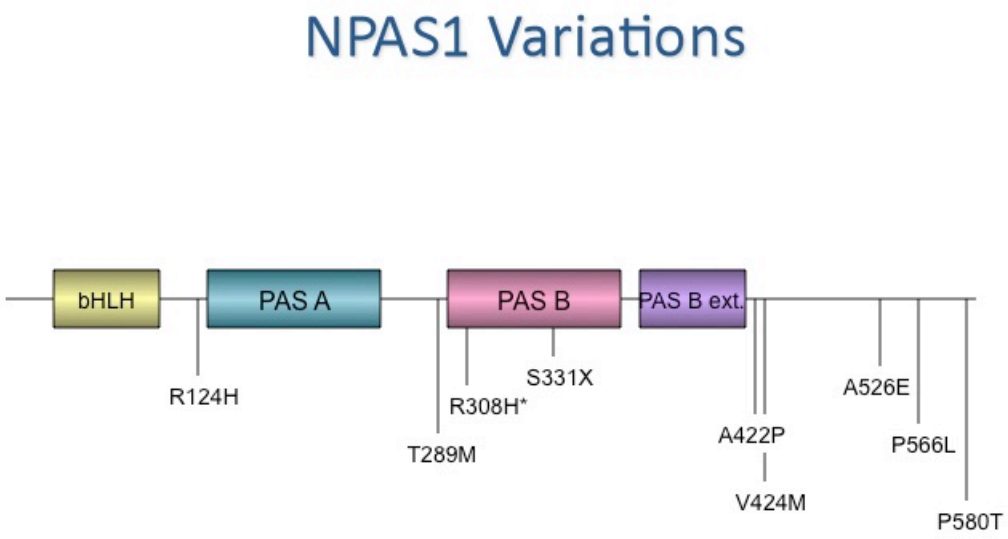


Figure S1. Variations in NPAS1 found in autistic children.



* R308H - found in 3 patients – one family had a mother carrying the variation, 2 affected siblings carrying the variation, and 1 unaffected sibling did not carry the variation

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