The Pennsylvania State University

The Graduate School

Eberly College of Science

AN INVESTIGATION OF THE MOLECULAR AND CELLULAR SUBSTRATES OF ANXIETY AND DEPRESSION-LIKE DISORDERS

A Thesis in

Biochemistry, Microbiology, and Molecular Biology

By

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Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Doctor of Philosophy

May 2007

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Abstract

In humans, heightened trait anxiety is a vulnerability factor for diverse psychiatric disorders, especially generalized anxiety disorder (GAD) and major depression. Besides multiple genetic factors, environmental factors such as early life stress are believed to increase vulnerability for anxiety and mood disorders. Early life stress has been shown to modulate the stress response in adulthood through changes in gene expression and changes in neurotransmitter systems. Stress, including especially early life stress, is also a potent inhibitor of adult hippocampal neurogenesis, a mechanism that has been implicated in the etiology of depression. Conversely, deficits in neurotransmission, including neurotransmission by γ-aminobutyric acid (GABA), in the cortex and hippocampus are implicated in the etiology of anxiety and depressive disorders. GABA type A ($GABA_A$) receptors are key control elements of anxiety states based on the anxiolytic properties of benzodiazepines (BZs), which act as allosteric $GABA_A$ receptor agonists.

Heterozygous deletion of the γ2 subunit of $GABA_A$ receptors results in modest but significant deficits in BZ binding sites as well as altered channel conductance, deficits in trafficking and clustering of $GABA_A$ receptors at postsynaptic sites. However, there is no change in the number of GABA binding sites indicating that the number of $GABA_A$ receptors was unaltered in these mice. This subtle deficit in $GABA_A$ receptors is associated with behavioral, pharmacological and cognitive alterations indicative of elevated trait anxiety. The $\gamma 2^{+/-}$ mice exhibit neophobia and marked behavioral avoidance to natural or learned stressors. This phenotype is

reversed to wildtype (wt) levels following BZ treatment reflecting sensitivity of pathologically anxious patients to anxiolytics. Altered behavior of $\gamma 2^{+/-}$ mice is associated with cognitive deficits including enhanced 1-s trace conditioning and impaired ambiguous cue conditioning but unimpaired spatial memory.

In the present thesis, cell-type-specific and developmentally controlled inactivation of the $\gamma 2$ subunit gene was used to further analyze the mechanism underlying anxiety-like behavior of $\gamma 2^{+/-}$ mice. Recombination of a 'floxed' $\gamma 2$ subunit (f$\gamma 2$) allele in either of two different Cre-expressing mouse lines results in a reduction of $GABA_A$ receptors specifically in glutamatergic neurons of the forebrain. In adulthood, Emx1Cre x f$\gamma 2$/+ and CaMKIICre2834 x f$\gamma 2$/+ mice exhibited comparable deficits in BZ binding in the forebrain. Specifically, both mouse lines showed substantial reductions in BZ binding in the forebrain, including the cortex, CA1 region of the hippocampus and striatum, with less severe deficits in the amygdala and the dentate gyrus. However, in Emx1Cre mice Cre-mediated recombination occurs in immature glutamatergic neurons of the embryonic and adult forebrain while in CaMKIICre2834 mice recombination is developmentally delayed to mature neurons of the adult brain.

The $GABA_A$ receptor deficits in immature glutamatergic neurons of the embryonic forebrain resulted in heightened anxiety-like behavior similar of that seen previously in $\gamma 2^{+/-}$ mice. This included increased risk assessment behavior and neophobia in paradigms without an implicit threat and increased avoidance to naturally aversive

stimuli. In addition, Emx1Cre x fγ2/+ mice and $\gamma 2^{+/-}$ mice showed increased behavioral inhibition in paradigms with predictive validity for antidepressant drug effects in humans. By contrast, the developmentally delayed $GABA_A$ receptor deficit of CaMKIICre x fγ2/+ mice was without behavioral effect. Thus, the first three weeks of life represent a $GABA_A$ receptor dependent critical period in the establishment of normal emotionality of mice.

In addition to behavioral deficits, γ2 subunit inactivation in immature forebrain glutamatergic neurons was associated with significant deficits in adult hippocampal neurogenesis. Decreases in adult born neurons in the adult hippocampus were associated with normal cell proliferation, but a reduction in the number of cells expressing mature neuronal markers. Reductions in neurogenesis were characterized by normal proliferation and initial neuronal differientiation, indicating a selective vulnerability of maturing neurons to modest functional deficits in $GABA_A$ receptors. By contrast, a developmentally delayed $GABA_A$ receptor deficit in mature glutamatergic neurons of the forebrain did not affect adult hippocampal neurogenesis. Therefore, normal emotionality and adult hippocampal neurogenesis have in common that they are dependent on developmental $GABA_A$ receptor in immature glutamatergic neurons. Thus, $GABA_A$ receptor deficits in immature neurons of the developing and/or adult brain are implicated in the etiology of heightened negative emotionality, which in humans is associated with generalized anxiety disorder and major depression.

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5-HT	Serotonin
$5\text{-HT}_{1A}R$	Serotonin type-1A receptor
5-HTT	Serotonin transporter
ACTH	Cotricotropin (Adrenocorticotropic hormone)
BrdU	Bromodeoxyuridine
BZ	Benzodiazepine
CA1	CA1 region of the hippocampus
CA3	CA3 region of the hippocampus
CaMKIIα	Calmodulin kinase II α gene
CNS	Central nervous system
CRF	Corticotrophin releasing factor
CRH	Corticotropin-releasing hormone
CRH1R	Corticotropin-releasing hormone type-1 receptor
CRH2R	Corticotropin-releasing hormone type-2 receptor
CS	Conditioned stimulus
DCX	Doublecortin
DG	Dentate gyrus region of the hippocampus
EGFP	Enhanced green fluorescence protein
Emx1	Empty spiracles gene
EPM	Elevated plus maze test
FCE	Free choice exploration test
FST	Forced swim test
GABA	γ aminobutyric acid
$GABA_AR$	GABA type-A receptor
GAD	Gereralized Anxiety Disorder
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GR	Glucocorticoid recpetor
HPA	Hypothalamic-pituitary-adrenal axis
KCC-2	Potassium-Chloride cotransporter type-2

LDC	Light-dark choice test
MR	Mineralcorticoid receptor
MRI	Magnetic resonance imaging
MRS	Magnetic resonance spectroscopy
NeuN	Neuron-specific nuclear protein (Neuronal nuclei)
NMDAR	Glutamate receptor, (N-methyl D-aspartate 1)
NSF	Novelty Suppressed feeding test
PAV	Parvalbumin
PET	Positron emission tomography
SGZ	Sub granular zone
SPECT	Single photon emission computed tomography
STG	Superior temporal gyrus
SVZ	Sub ventricular zone
US	Unconditioned stimulus
wt	Wildtype
Z/EG	LacZ/ EGFP transgenic Cre recombinase reporter mouse strain

Acknowledgments

This Thesis, as any act of scholarly work, is as much a log of a journey as it is a simple record of experiments carried out. It is a work of toil and joy, frustration and accomplishment. It is impossible to reach the end of any journey without the help of others. Therefore I would like to express my gratitude to the following people:

To Dr. Bernhard Luscher, my advisor, thank you for pointing me in the right direction when I could not see which way to turn. Without your debth of experience and drive to learn more this thesis would not have been possible.

To Dr. Cheng Fang, Xu Yuan and Shoko Masuda, thank you for constant encouragement and support to celebrate the successes and help me through the failiors.

To Sue Lingenfelter, thank you for finding a way to help me find what I needed,; mice, skills or just a push along the way.

To Drs. Beth Leullen and Nadia Sadir, thanks for all of the instruction and reassurance that it was worth taking each step of the journey.

To Drs. Cheryl Keller and Melisa Aldred, I give my appreciation for showing me around the lab and helping me start thing off right.

Thanks to Ashley Stull for not giving up with frustrating experiments.

Thank you to the rest of the Luscher Lab members both present and past. Claude Schweizer, Michelle Martin, Dinese Fidelle, Scott DiLoreto, Bradley Moatz and Qiuying Sheng for all of the technical help and moral aupport.

My appreciation goes to collaborators Drs. Florence Crestani, Takuji Iwasato and Shigeyoshi Itohara and to Drs. Doug Cavener, Anne Andrews and Byron Jones for materials and advice.

Finally thank you so much to Mom, Dad, John and Jen Biddle, Jason Monnell and Christine McGuinness and Jason and Robyn Mercer. You are the best cheerleaders anyone could ever hope for, be it on the trail, over a glass of wine or on the phone. I am forever greatful.

Chapter 1 Introduction

1.1 The Concept of Anxiety and Mood Disorders

1.1.1 Non-pathological anxiety relates to fear

Environmental experience and genetic makeup establish personality and temperament during the development of an individual. Children that show more inhibition in response to unfamiliar social and nonsocial stimuli exhibit this behavioral response throughout adolescence to adulthood (Chambers et al., 2004). These inhibited patterns of coping with aversive situations have strong cognitive and biological parallels with fear response (Fox et al., 2005). Fear is a natural physiological response initiated by an aversion to a potentially threatening and clearly identifiable stimulus. A more generalized weariness over threatening aspects of environment that is often associated with apprehension about future events is called anxiety. While anxiety has many of the same features as fear, it lasts for a longer period of time, may not be associated with an eliciting stimulus, and may lack clear adaptive significance (Charney and Deutch, 1996). Non-pathological forms of anxiety, which relate to individual differences in temperament and personality, can be subdivided into state vs. trait anxiety (Spielber et al., 1970). Whereas state anxiety refers to a situational acute level of anxiety, trait anxiety describes a long-term personality trait of an individual to show an increased anxiety response. Trait anxiety, or neuroticism, is a risk factor for major depression and generalized anxiety disorder (GAD) and is a strong predictor for comorbidity between depression and anxiety disorders (Andrews, 1996; Bienvenu et al., 2001; Hettema et al., 2004).

1.1.2 Pathological anxiety disorders show varying symptoms

Excessive trait anxiety is characterized by increased behavioral inhibition and by cognitive dysfunction including i) a tendency to interpret ambiguous situations as threatening, ii) an attentional bias for the preferential processing of threat cues, and iii) a bias for explicit memory of threat (McNally, 1996). While mild anxiety can be a

protective trait, the range of pathological anxiety disorders has disabling characteristics. Substantial evidence suggests that elevated trait anxiety represents a general vulnerability factor for anxiety and mood disorders (Andrews, 1996; Akiskal, 1998). Patients diagnosed with GAD suffer from intrusive worry about everyday life circumstances and social competence. They are chronically anxious and fearful of some ineffable future cataclysm and exhibit autonomic hyperarousal. Panic disorder, in contrast, is characterized by paroxysmal anxiety attacks with a sudden unanticipated onset. However, there is overlap between types of anxiety disorders. For example, GAD can lead to panic attacks as a person becomes more concerned about a specific fixation. In each case a patient accepts that the anxiety is irrational, but they simply cannot avoid expressing it. In obsessive-compulsive disorder (OCD) a patient has recurring, and disturbing thoughts or obsessions, which lead to anxiety. These obsessions are partially alleviated by repetitive behaviors or mental acts called compulsions, which occupy more than one hour a day and cause enough disturbance to significantly impact life. Likewise social and specific phobias lead to avoidance behavior that can interfere with a job or family relationship. This in turn can trigger anxiety about the disorder itself, thereby compounding the problem. Anxiety disorders can be associated with triggers. An example of this is post-traumatic stress disorder (PTSD), in which an experienced traumatic event leads to severe psychological consequences. The effects can be long lasting, causing the person to relive the event or affecting the person's ability to cope with responses associated with trauma. This leads to hyperarousal characterized by difficulty concentrating or falling and staying asleep, irritability, and hypervigilance (Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision, 2000). Together the different types of anxiety disorders constitute one of the most frequently occurring classes of psychiatric illnesses, which affect about 40 milllion American adults age 18 (about 18%) and over in a given year (Kessler et al., 2005). Anxiety disorders have a greater lifetime prevalence in women than in men and treatment costs are estimated as high as $44 billion a year in the United States alone (Kessler et al., 1994; Greenberg et al., 1999). In addition, comorbidity between anxiety and other mood disorders put a significant proportion of

the population at risk for other life threatening disorders like depression. (DSM-IV-TR, 2000; Bystritsky et al., 2006)

1.1.3 Anxiety and mood disorders show high levels of comorbidity

Like anxiety, depression can be a normal coping mechanism to singular traumatic events. Pathological depression, however, represents a distinct change from baseline behavior. Depression is characterized by symptoms that include disinterest in pleasurable stimuli, irritability, feeling of helplessness or worthlessness, decreased ability to concentrate, thoughts of suicide and depressed mood. Physical symptoms of depression can include decreased or increased appetite, weight, or sleep schedule (DSM-IV-TR, 2000). Similar to anxiety disorders, women are twice as likely as men to experience an episode of major depression (Sloan and Kornstein, 2003). While patients who suffer from anxiety disorders are likely to suffer from depression, 50-60% of individuals with major depressive disorder report a lifetime history of one or more episodes of pathological anxiety (Kaufman and Charney, 2000; Murphy et al., 2004). Furthermore, 42% of patients with GAD experience at least one major depressive episode during their lifetime (Brawman-Mintzer et al., 1993). Genetic evidence suggests that comorbidity with depressive illness is pronounced among patients suffering from GAD (Chambers et al., 2004) and the twin studies suggest that the genetic substrates of major depression and GAD are closely interrelated (Kendler et al., 1992; Roy et al., 1995). GAD almost invariably (90%) has comorbidity with another type of psychiatric disorder (Wittchen et al., 1994) such as other anxiety disorders and depressive disorders (Carter et al., 2001). Several large twin studies show that anxiety and mood disorders and even eating disorders have common genetic origins (Eley and Plomin, 1997; Eley et al., 2003). It has been known for sometime that similar genes underlie not only anxiety and mood disorders but also non-pathological neuroticism (Jardine et al., 1984) and there is substantial, though not complete, overlap between genetic factors that influence individual variation in neuroticism and those that increase risk for both GAD and major depression (Hettema et al., 2006; Kendler et al., 2006).

1.2 Brain systems in anxiety disorders

Genetic evidence and comorbidity studies suggest that anxiety and mood disorders share similar etiologies that are linked to trait anxiety (Andrews, 1996; Chambers et al., 2004; Hettema et al., 2006). However, these disorders have distinct differences in symptomology and responsiveness to treatment.

1.2.1 BZs control anxiety through $GABA_ARs$

Traditionally, the most standard pharmacologic treatments for anxiety disorders use benzodiazepines (BZs; (Kapczinski et al., 1994), which potentiate the function of a major subset of γ-aminobutyric acid receptors ($GABA_ARs$). They act rapidly and potently but tend to loose efficacy over time with a marked risk for the development of tolerance and dependence (Steppuhn and Turski, 1993; File and Fernandes, 1994; Marin et al., 1996). GABA is a primary inhibitory neurotransmitter that is ubiquitous throughout the brain. By enhancing $GABA_AR$ function, BZ site ligands such as diazepam act as anxiolytics, whereas ligands that reduce $GABA_AR$ function (inverse agonists) induce fear (Mohler et al., 1996). Patients with panic disorders display an altered BZ pharmacology. For example, the BZ-antagonist flumazenil has no behavioral effect in healthy individuals but can induce panic attacks during symptom free episodes in panic disorder patients who perceive this drug as an inverse agonist of $GABA_ARs$ (Nutt et al., 1990). Thus, $GABA_ARs$ are key control elements in the regulation of anxiety.

1.2.2 Brain imaging indicates anxiety disorders are characterized by distinct differences in the forebrain

Emotions including anxiety and depression are regulated by limbic areas of the forebrain, which influence endocrine and autonomic nervous system function. In patients suffering from panic attacks, positron emission tomography (PET) studies show decreased BZ binding in the parahippocampal-hippocampal area, orbitofrontal cortex, and insula (Malizia et al., 1998; Malizia, 2002). However, decreases in $GABA_AR$ binding in patients with panic and post-traumatic stress disorders was most

reduced in the prefrontal cortex (Bremner et al. 2000). In contrast, a reduction of $GABA_A$Rs in patients with GAD appears to be more localized to the temporal lobe (Tiihonen et al., 1997). Brain volume analyses of pediatric GAD patients by magnetic resonance imaging (MRI) revealed significantly larger superior temporal gyrus (STG) and amygdala volumes and enhanced right>left asymmetry in STG volumes (De Bellis et al., 2002). Thus, although the brain substrates for panic disorder and GAD appear to be distinct, abnormalities in the prefrontal cortex, superior temporal gyrus and amygdala are implicated in multiple types of anxiety disorders, thereby supporting the idea that impaired cognitive control over threat-related information may have a key role in both of these anxiety disorders (Figure 1.1A).

1.2.3 GABAergic system is altered in cognitive brain areas during major depression

Brain regions implicated in the pathology of depressive disorders comprise two neural systems: 1) a ventral system, including the amygdala, insula, ventral striatum, ventral anterior cingulate gyrus, and prefrontal cortex, are utilized in identification of the emotional significance of a stimulus, production of affective states, and automatic regulation of emotional responses; and 2) a dorsal system, including the hippocampus, dorsal anterior cingulate gyrus, and prefrontal cortex, for the conscious and effortful regulation of affective states and subsequent behavior (Figure 1.1A; Kapczinski et al., 1994; Phillips et al., 2003). Evidence in support of this notion includes brain-imaging studies that show differences in blood flow or associated measures in these regions in depressed patients (Drevets, 2001; Liotti and Mayberg, 2001). In postmortem studies, atrophy or loss of neurons in the prefrontal cortex and hippocampus of patients suffering from depression and anxiety disorders occurs particularly in those patients who have suffered an early childhood trauma (Vythilingam et al., 2002). This loss of neurons in the prefrontal cortex corresponds to a loss of a selective subpopulation of GABAergic neurons expressing calbindin, whereas parvalbumin-positive neurons appear to be present in normal numbers (Figure 1.2; Rajkowska et al., 2007). Consistent with a deficit in GABAergic neurons, magnetic resonance spectroscopy (MRS) studies revealed reduced

concentrations of GABA in the occipital cortex of patients suffering from depression (Sanacora et al., 1999). This deficit was normalized by treatment with the clinical antidepressants, selective serotonin reuptake inhibitors (SSRIs; Sanacora et al., 2002; Bhagwagar et al., 2004). Occipital GABA levels are also reduced in non-medicated patients with panic disorders and in subjects with mood altering premenstrual dysphoric disorder (Goddard et al., 2001; Epperson et al., 2002; Chang et al., 2003). These similarities in the pathology of anxiety and depression disorders along with the effectiveness of BZ for anxiety disorders implicate GABA in the pathology of both anxiety and depression (Bodnoff et al., 1988).

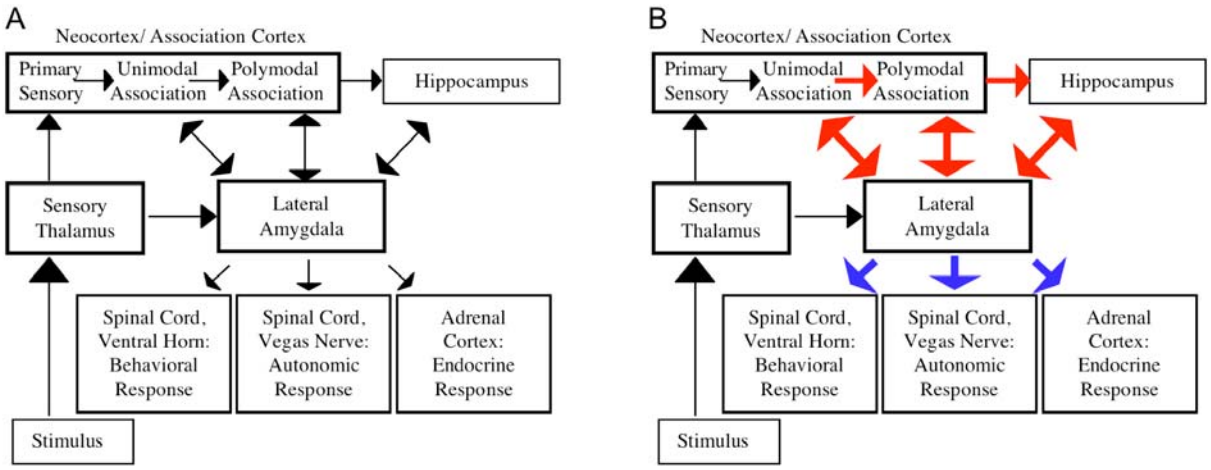

Figure 1.1 Schematic diagram of a proposed fear circuit implicated in trait anxiety of $\gamma 2^{+/-}$ mice

A. Pathological anxiety and depressive disorders are associated with cortical regions and the hippocampus, which are involved in the processing and consolidation of threat cues respectively. Cognitive control over threat related information leads to enhanced processing in the amygdala and autonomic, behavioral and endocrine responses such as freezing, changes facial expression, or alterations in homeostasis **B**. Hyperactivity due to a deficit in GABAergic inhibition and/or deficits in GABAergic neurons or the hippocampus and cortex are highlighted in red. Resultant increases in secondary activity are seen in the amygdala and downstream targets are pictured in blue. Adapted from (LeDoux, 1995; Davis et al., 2003).

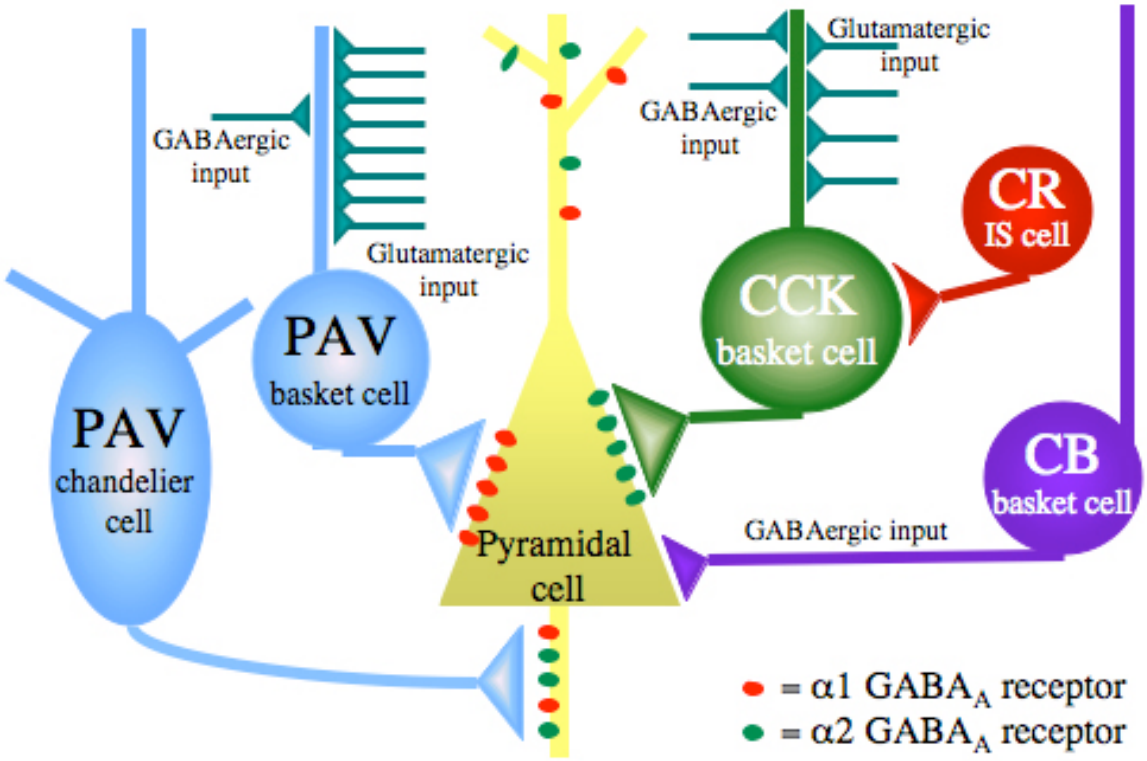

Figure 1.2 Diverse types of interneurons mediate the activity of pyramidal cells in the mammalian cortex

Pyramidal cells of the mammalian cortex comprise 70-80% of the neuronal population. These cells mediate the majority of exicitatory activity through interconnectivity in the cortex and projections to the rest of the contral nervous system (CNS). Their activity is modulated by a diverse network mostly inhibitory interneurons (comprising 20-30%). These interneurons can be characterized on the basis of morphology and by the presence of intracellular markers such as parvalbumin (PAV, blue), calbindin (CB, purple), calretenin (CR, red) and cholecystokinin (CCK, brown). Interneurons can modulate the firing pattern of pyramidal cells either directly or indirectly, as is the case with local interneuron-selective inhibitory cells expressing CR (CR IS cell). Major differences between PAV- and CCK-containing basket cells are their connectivity features and receptor expression patterns. For example, axon-targetting Chandeleir cells are in a complex position to override all the complex dendritic integration and somatic gain setting by 'editing' the action potential output of pyramidal cells. Each axon terminal synapsing on the interneurons (glutamatergic or GABAergic) corresponds to 1000 synapses, reflecting true differences in the relative weight of excitatory and inhibitory inputs. Knowing that anxiolytic effects of benzodiazepines are mediated solely by a2-

subunit-containing $GABA_A$ receptors, it is important to note that synapses formed by CCK-positive basket cells on pyramidal cells operate mostly via a2-subunit-containing $GABA_A$ receptors, whereas PV-positive basket-cell synapses contain largely α1 subunits suggesting the importance of both populations of neurons in behavioral response. (adapted from Freund, 2003; Markram et al., 2004)

1.2.4 BZs ineffective for treatment of depression

The idea that clinical anxiety and mood disorders might have distinct differences in their underlying substrates is highlighted by the effectiveness of BZs for treatment. (reviewed in McNaughton and Gray, 2000). BZ use is ineffective for the long-term treatment of depressive illness, panic disorder and PTSD and they do not affect simple phobia or active avoidance behavior in laboratory animal experiments (TK et al., 1995; File et al., 1998; Davidson, 2004). An association between reduced $GABA_AR$ function and deficits in mood-related behavior seems counterintuitive, given that benzodiazepines as a class of drugs are known to lack antidepressant drug efficacy (Schatzberg and Cole, 1978; Tiller et al., 1989). A possible explanation is that depression may involve deficits in GABAergic transmission mainly during development. GABAergic transmission in the immature brain, unlike in adulthood, is mainly excitatory (Cherubini et al., 1991; Represa and Ben-Ari, 2005). Thus the inability of BZs to ameliorate depressive symptoms may reflect the fact that GABAergic transmission in adulthood is no longer excitatory and cannot reverse a defect in emotional circuitry that occurred during development.

1.2.5 Monoamine systems mediate behavioral inhibition through connections to the forebrain

Selective serotonin reuptake inhibitors (SSRIs) have largely replaced BZs. because they exhibit less tolerance and therefore less of a risk for dependence (Otto et al., 1993; Gorman, 2002; Allgulander et al., 2003; Kapczinski et al., 2003). In contrast to BZs, SSRIs and other antidepressant drugs exhibit a slow onset of therapeutic efficacy. However, acute administration of SSRIs immediately increases the amount of serotonin (5-HT) at the synapse (Smith et al., 2000; Bystritsky, 2006). The increase in serotonin, therefore, does not correlate with the observation of antidepressant effects, suggesting SSRIs alleviate depressive symptoms by some other downstream mechanism

The ascending reticular activating system consists of a diffuse network of neurons in the brainstem and basal forebrain that mediates arousal and behavioral inhibition, both of which represent important aspects of anxiety-related behavior. Forebrain projections originating from noradrenergic neurons in the locus coeruleus and serotonergic neurons in the raphe nuclei, together with cholinergic neurons, provide the neural substrate for the processing of sensory stimuli in the neocortex as a function of arousal level (Hebb, 1955; Robbins and Everitt, 1995). Iontophoresis in animals indicates noradrenaline and acetylcholine increase signal to noise ratios of neuronal activity (Sillito, 1987), while serotonin has been shown to reduce them (Waterhouse et al., 1986). Functional imaging studies show that SSRI treatment can dampen excitability in the brain (Duman et al., 1997). Reductions in 5-HT_{1A} receptors (5-HT_{1A}Rs) have been shown in the limbic systems and in the frontal, temporal and limbic cortex of patients with major depression (Meltzer and Maes, 1995; Sargent et al., 2000). Serotonergic neurons in the raphe nucleus are inhibited by iontophoretically applied GABA, indicating that the serotonin system is under GABAergic control. BZs, which increase punished responding, likewise reduce the firing of serotonergic neurons (Laurant et al., 1983). Conversely, evidence from mouse models indicate loss of 5-HT_{1A}R function is associated with profound changes in $GABA_A$R expression in the amygdala and prefrontal cortex, although these alterations in BZ sensitivity and GABA uptake were dependant on strain background (Sibille et al., 2000; Pattij et al., 2002; reviewed in Toth, 2003; Bruening et al., 2006). These examples of crosstalk between the GABA and serotonin systems in the brain suggest that normal emotionality is dependent on a balance of neuronal activity involving different neurotransmitter systems throughout the brain.

1.3 Measuring anxiety / mood disorders in rodents

Neuropharmacological and neuroanatomical parallels between human and rodent emotional states make rodents an excellent experimental system that is amenable to genetic dissection of quantitative behavioral traits such as mood, personality and intelligence (Flint and Corley, 1996). While it is impossible to

replicate all aspects of human behavioral disorders, animal models allow the experimental examination of simple interactions between brain systems and circuits and environmental stimuli that can be representative of more complex phenomena (Gottesman and Gould, 2003). An animal that is to be considered a good model of clinical conditions should reflect behavioral and physiological human response (face validity), have a biological rational underlying a human condition (construct validity) and be sensitive to pharmacological treatments that are known to be effective in humans (Clement et al., 2002).

1.3.1 Unlearned fear can determine face validity

Face validity of anxiety-like behavior in rodents is often based on the innate (unlearned) tendency to avoid naturally aversive stimuli such as novel environments (Open Field Test), elevated and open spaces (Elevated Plus maze), or brightly illuminated areas (Light-Dark Choice test; Crawley, 1985; Lister, 1987; Misslin et al., 1989). These tests rely competing motivations between aversion to a potential threat (novel space or objects or both) and an ambiguous or a positive stimulus such as exploratory drive (Griebel et al., 1993) or food (Novelty Suppressed Feeding; Bodnoff et al., 1988). In these tests, the anxiety-related response is reflected in increased behavioral inhibition, a phenomenon attributed mainly to stimulus processing in the hippocampal formation in both animals (Blanchard and Blanchard, 1988; Gray and McNaughton, 1996; Rogan and LeDoux, 1996) and humans (Knight, 1996).

1.3.2 Learned fear can further assess face validity

Training an animal in a learned fear paradigm to associate a conditioned stimulus (CS; tone, light, new context) with a unconditioned stimulus (US; puff of air, mild foot shock) causes the animal to exhibit freezing or a startle response as a characteristic sign of fear in the presence of the CS only (Bolles and Grossen, 1970; Blanchard and Blanchard, 1988; Davis, 1992; LeDoux, 1995; Fendt, 1998). If the CS is an explicit signal such as light or a tone that is temporally overlapping with a US (delay conditioning), then learning of the fear response is dependent on the amygdala but

largely independent of the hippocampus (Phillips and LeDoux, 1992). Fear-associated input to the basolateral amygdala is mediated by two pathways which are independently sufficient to mediate conditioning to simple CS: the thalamo-cortico-amygdala and the direct thalamo-amygdala projections (Romanski and LeDoux, 1992; Quirk et al., 1997; Davis, 1998; Fendt, 1998; Fendt and Fanselow, 1999).

However, if the CS in fear conditioning represents a complex stimulus such as a specific context (context conditioning) or if a short time interval separates the CS and US (trace conditioning), then learning of the fear response is dependent on both the amygdala and hippocampus (Kim and Fanselow, 1992; Phillips and LeDoux, 1992). Trace but not delay conditioning is impaired by hippocampectomy or hippocampus CA1 region-specific ablation of NMDA-type glutamate receptors (McEchron et al., 1998; Huerta et al., 2000). Hippocampal and cortical lesion interfere with an animal's ability to differentiate between a CS cue (paired with US) and an ambiguous cue (non-paired) or stimulus (stimulus generalization) indicating that thalamic projections to the amygdala, which are left intact when the cortex is damaged, are unable to discriminate between the CS and the ambiguous stimulus (Jarrell et al., 1987; Holland and Gallagher, 1999). The lateral nucleus of the amygdala receives inputs from both the sensory cortex as well as from the sensory thalamus, and thus likely represents the sensory interface for both cortical and thalamic sensory systems (LeDoux, 1992). Context conditioning models the hypervigilence and generalized distress normally associated with anxiety disorders because it is not triggered by an explicit cue (Grillon, 2002). Matching physiological state similarities between human patients and animal models using variants of Pavlovian conditioning discussed here determine face validity of animal models.

1.3.3 Pharmacological responses can assess predictive validity

Pharmacological analysis of animal behavior has contributed to the identification of neural systems and neurotransmitters involved in the expression of anxiety and depression (Gordon and Hen, 2004a). Behavioral inhibition in mice and in humans is reduced by treatment with anxiolytic drugs such as BZs and SSRIs, establishing criteria for predictive validity (Borsini et al., 2002; Grillon, 2002). After treatment with

anxiolytic drugs mice show decreased behavioral inhibition on free choice exploration (Griebel et al., 1993), light dark choice test (Misslin et al., 1989) and elevated plus maze test (Lister, 1987). Chronic but not acute treatment with antidepressants leads to decreased behavioral inhibition in the novelty suppressed feeding test once again suggesting some commonality between anxiety and depression (Suranyi-Cadotte et al., 1990; Santarelli et al., 2003). The forced swim test (FST) is based on the fact that animals placed in short term inescapable situations tend to adopt an immobile stance (Porsolt et al., 1977; Porsolt et al., 1978). 'Learned helplessness' reflects a lowered mood state associated with depression (Cryan et al., 2005c). Escape behaviors (swimming) are increased significantly in comparison to defeat behaviors (floating) when the animals are treated with chronic levels of the SSRI fluoxetine (Dulawa et al., 2004). The FST models predictive validity for depression due to its sensitivity for the efficacy of multiple types of antidepressants. In addition, drugs with anti-anxiety activity such as BZs do not show activity in the FST (Cryan et al., 2005a). It has been suggested that antidepressants delay a shift from an active coping strategy involving swimming and searching for an escape route to a passive strategy of immobility and energy conservation (Cryan et al., 2005a). Nevertheless, any inference about the mood state of a rodent with respect to 'learned helplessness,' or 'behavioral despair' remains highly controversial and depression related behavioral assays are conservatively valid only as screens for antidepressants (Gardier and Bourin, 2001)..

1.4 Mouse Models of Anxiety

1.4.1 Mouse models of anxiety disorders must reflect construct validity

Mouse models that have similar underlying biological rational to human mood disorders are said to have construct validity. There are many models for both anxiety and depression that have varying degrees of construct validity which have been review in depth elsewhere (Clement et al., 2002). While the plurality of mechanisms implicated using mouse models reflects the polygenetic nature of anxiety and mood

disorders, GABAergic, monoaminergic and cholinergic systems are widely recognized as major contributors to the etiology of these diseases (Bloom, 1995).

1.4.2 $GABA_AR$ subunit composition predicts BZ effect

Anxiety reducing effects of BZs make $GABA_ARs$ an obvious candidate for modulations of anxiety disorders. $GABA_ARs$ are heteropentomeric ligand gated ion channels composed of $\alpha 1–6$, $\beta 1–3$, $\gamma 1–3$, δ, ε, π, and θ subunits (Moss and Smart, 2001; Luscher and Keller, 2004). BZs (specifically diazepam) sensitive $GABA_ARs$ are composed of α, β, and $\gamma 2$ subunits that can be distinguished by the presence of different α subunits ($\alpha 1$, $\alpha 2$, $\alpha 3$ or $\alpha 5$). By modifying the drug binding site with an in vivo knock-in substitution of an arginine for a conserved histidine, the anxiolytic activity of diazepam was assigned to $\alpha 2$ subunit containing $GABA_ARs$ while the sedative, myorelaxant and amnesic drug effects were mediated by different combination of $GABA_AR$ subtypes containing different subunits (Rudolph et al., 1999; Low et al., 2000; McKernan et al., 2000; Crestani et al., 2002; reviewed in Mohler et al., 2002). Expression of $\alpha 2\beta 2\gamma 2$ subtype of $GABA_ARs$ is limited to the limbic system, providing still more evidence for the role of corresponding brain regions in anxiety-like disorders. These receptors are thought to provide an off switch for neuronal firing due to their concentration at axon initial segment synapses of neurons. This off switch function on mossy fiber terminals might make these receptors gatekeepers for information flow from the dentate gyrus to the CA3 and CA1 regions (reviewed in Keller et al., 2004).

1.4.3 $\gamma 2^{+/-}$ mice model trait anxiety

In the brain the majority of $GABA_ARs$ are composed of 2α, 2β and a $\gamma 2$ subunit with subunit type conferring specific characteristics of inhibition (discussed above and reviewed in Lüscher, 2002). BZs act as allosteric agonists of the $\gamma 2$ subunit containing $GABA_ARs$ (Mohler et al., 2002). Mice that are heterozygous for the $\gamma 2$ subunit gene ($\gamma 2^{+/-}$) of $GABA_ARs$ show a subtle reduction in postsynaptic $GABA_ARs$ and provide a genetically defined model of trait anxiety that reproduces molecular, pharmacological, behavioral and cognitive abnormalities of human anxiety disorders

(Crestani et al., 1999). This has been documented by five lines of evidence: I) The $GABA_AR$ dysfunction in $\gamma2^{+/-}$ mice can be visualized by decreased BZ binding and reduced postsynaptic receptor clustering. This deficit is most pronounced in hippocampus and neocortex (Reiman et al., 1984; Nordahl et al., 1990; Schlegel et al., 1994; Kaschka et al., 1995). These brain areas known to be affected in human anxiety disorders (Tiihonen et al., 1997; Malizia et al., 1998). II) The $GABA_AR$ deficit in $\gamma2^{+/-}$ mice results in enhanced emotional reactivity to naturally aversive stimuli, as demonstrated by the increased aversion to novelty (Free Choice Exploration test, Open Field test), unprotected space (Elevated Plus Maze), and brightly illuminated areas (Light Dark test). Importantly, the deficits seen in the Free Choice Exploration test represent increased risk assessment behavior and neophobia that is independent of the inherent stress associated with a brightly lit or exposed space (Griebel et al., 1993). These anxiety-related responses are thought to be mediated by the septo-hippocampal system in both animals and humans (Gray and McNaughton, 1996). III) Behavioral inhibition of $\gamma2^{+/-}$ mice is reversed to wildtype (wt) levels by treatment with diazepam, similar to the human condition (O'Boyte et al., 1986; Glue et al., 1995). IV) $\gamma2^{+/-}$ mice appear to perceive an ambiguous threat stimulus to be as threatening as a fully predicting stimulus. In contrast, context conditioning and learning and memory in the Morris water maze test are unaltered in $\gamma2^{+/-}$ mice, indicating that sensory processing, implicit forms of learning and spatial learning and memory are normal in $\gamma2^{+/-}$ mice. These features are reminiscent of anxiety states in humans that are characterized not only by harm avoidance behavior but also by a bias for interpreting ambiguous scenarios as threatening, an attention bias favoring the selective processing of threat cues, and a bias for explicit memory of threat (McNally, 1996). V) $\gamma2^{+/-}$ mice exhibit increased responsiveness in the passive avoidance paradigm indicating a heightened emotional memory for negative associations, a feature reminiscent of the bias for explicit memory of threats in humans (McNally, 1996). The locally limited $GABA_AR$ deficit of $\gamma2^{+/-}$ mice is consistent with hippocampal and cortical hyperactivity, a feature proposed to be causal for anxiety disorders in humans (Figure 1.1B; McNaughton, 1997). Importantly, the anxiety-like phenotype of $\gamma2^{+/-}$ mice is based on a comparatively

modest molecular deficit that may be more representative of human behavioral traits than homozygous loss of function mutations typically used in animal models of anxiety.

1.4.4 5-HT and GABA act in concert to modulate behavioral inhibition

One of fourteen classes of 5-HT receptors, the 5-HT_{1A}R are G-protein coupled receptors expressed as autoreceptors by serotonergic neurons in the raphe nucleus of the brainstem and postsynaptic receptors by non-serotonergic neurons of forebrain regions including the hippocampus, septum and cortex. As is the case with the presynaptic receptor, 5-HT_{1A}R activation in the forebrain decreases the firing rate of the postsynaptic cell by hyperpolarizing the cell membrane (Schmitz et al., 1998). 5-HT_{1A}R agonists are generally anxiolytic (Davidson et al., 1999) and the behavioral effects of the SSRI fluoxetine are dependent on 5-HT_{1A}R expression (Santarelli et al., 2001). Moreover, 5-$HT_{1A}R^{-/-}$ mice exhibit increased behavioral inhibition in a number of tests based on challenge with a threatening context, (Heisler et al., 1998; Parks et al., 1998; Ramboz et al., 1998; Gross et al., 2000; Klemenhagen et al., 2006). While, endocytic down regulation of presynaptic 5-HT_{1A}Rs in the raphe nucleus may be a prerequisite for the anxiolytic effects of SSRIs and 5-HT_{1A}R agonists (Riad et al., 2004), selective expression of the 5-HT_{1A}R in the forebrain but not in the raphe nucleus is sufficient to rescue the anxiety-like phenotype of 5-HT_{1A}R knockout mice (Gross et al., 2002). Moreover, rescue of normal behavior is dependent on expression of 5-HT_{1A}Rs during the first three weeks of postnatal development, indicating that the behavioral deficit observed in global 5-$HT_{1A}R^{-/-}$ mice is due to a developmental deficit in the forebrain. Thus, postsynaptic 5-HT_{1A}Rs in the forebrain are essential for developmental refinement of the circuitry underlying normal levels of anxiety behavior (reviewed in Gordon and Hen, 2004b). 5-HT_{1A}R in the hippocampus mediate hyperpolarization of both the excitatory pyramidal and granule neurons, which are the principal elements of the hippocampal neural circuitry, and inhibitory GABAergic interneurons that modulate the function of the pyramidal and granule cells (Gulyas et al., 1999). Serotonin can also excite neurons, in the case of pyramidal cells presumably through 5-HT_4Rs

(Andrade, 1991), and in the case of interneurons through 5-HT_2 and 5-HT_3Rs (Ropert and Guy, 1991; Shen and Andrade, 1998; Lee et al., 1999). Similar to 5-HT_{1A}R-mediated hyperpolarization of pyramidal cells, depolarization of interneurons is predicted to increase GABAergic inhibition and to reduce excitability of pyramidal cells and thus performing an anxiolytic function. 5-$HT_{2/3}$ agonists administered systemically or into the hippocampus are generally anxiogenic (Whitton and Curzon, 1990; Griebel, 1995). 5-HT_{1A}Rs and 5-HT_3Rs have also been identified on GABA terminals that innervate CA1 pyramidal cells (Katsurabayashi et al., 2003). Serotonin acting at these two receptor subtypes is therefore predicted to up- and down-regulate GABA release, respectively and to modulate excitability of pyramidal cells indirectly.

1.4.5 Hypothalamic-pituitary-adrenal axis activity balances physiological responses to stress

CRH is involved in regulation of the hypothalamic-pituitary-adrenal (HPA) axis, which has been implicated in depressive and anxious disorders (reviewed in Nestler et al., 2002). Predictably, the stress hormones CRH and urocortin are both anxiogenic in tests of behavioral inhibition towards to naturally aversive stimuli (Moreau et al., 1997). Both hormones can act through CRH1R and CRH2R receptors. Whereas CRH1R knockout mice tend to be less fearful than controls (Smith et al., 1998; Timpl et al., 1998; Contarino et al., 1999) the opposite is seen in CRH2R knockout mice (Bale et al., 2000; Coste et al., 2000; Kishimoto et al., 2000). CRH1R receptors in limbic system are essential for the anxiogenic response to CRH as shown in forebrain-specific CRH1R knockout mice in which regulation of the HPA was left intact (Muller et al., 2003). Infusion of CRH1R antisense oligonucleotides indicates that the anxiogenic response of CRH is at least in part mediated by neurons in the amygdala (Liebsch et al., 1995). Other evidence suggests that the anxiogenic effect of CRH involves increased signaling through 5-HT_{1A}Rs in the hippocampus (Kagamiishi et al., 2003). CRH-containing neurons of the hypothalamic paraventricular nucleus (PVN) receive and integrate inputs from inhibitory pathways from the hippocampus, excitatory pathways from the amygdala and inputs are from

ascending monoamine pathways. CRH release into the hypophyseal portal system activates the corticotrophs of the anterior pituitary to release corticotrophin (ACTH). ACTH reaches the primary adrenal cortex via the bloodstream, where it stimulates the release of cortisol (in humans) or corticosterone (in humans rats and mice; (reviewed in Herman et al., 2003). Mineraocorticoid receptors (MR) bind to cortisol with high affinity and mediate the initial appraisal and onset of the stress response while glucocorticoid receptors (GR) bind cortisol with less affinity, terminate stress activity and mediate stress recovery (de Kloet et al., 2005). MR and GR are co-expressed in hippocampal pyramidal cells, the amygdaloid and lateral septal nuclei, and some cortical areas (Patel et al., 2000). Monoamergic and peptidergic inputs to the hippocampus enhance the expression of MR in particular (Maccari et al., 1992; Yau et al., 1994; Gesing et al., 2001; Lai et al., 2003) indicating not only that these brain systems are interrelated but that their complete effects are inseparable from one another. GR mRNA expression and hormone binding activity are increased by treatment with desipramine (a trycyclic compound) or peroxitine (an SSRI; Pariante et al., 2001) suggesting that this system could also be involved in clinical treatment for mood disorder (de Kloet et al., 2005). While global GR deletion mice die during perinatal development (Schmid et al., 1995), deletion of forebrain GR expression result in a time and deletion dependent increase in despair and anhedonia reversible with treatment of mice with the tricyclic antidepressant imipramine (Boyle et al., 2005). Conversely, overexpression of GRs in the forebrain results in an increased anxious phenotype on the EPM, and hypersensitivity to desipramine but not fluoxetine (Wei et al., 2004). Thus, the HPA axis interacts with the serotonergic system in order to modulate the stress response.

1.5 Developmental aspect of anxiety and mood disorders

1.5.1 Maternal Stress alters brain function at the genetic level

As noted above, an individual sets up a pattern of behavioral responses that will apply in one way or another until adulthood. During development genetic programming prescribes the manner of behavioral responses and is itself modified

by these interactions. In the rodent model, natural and individual differences in maternal care influence the stress response of adult offspring (Liu et al., 1997; Caldji et al., 1998). The offspring of poor maternal caregivers (low licking and grooming/low arched back nursing) exhibit higher pituitary-adrenal responses and show increased behavioral inhibition in an open field than those of good maternal caregivers and this effect is reversed by cross-fostering (Francis et al., 1999). Maternal care alters $GABA_AR$ binding (BZ autoradiography) as well as the expression of the $GABA_AR$ subunit $\alpha 1$ in the medial prefrontal cortex, hippocampus and amygdala and $\gamma 2$ subunit in the amygdala (Caldji et al., 1998; Caldji et al., 2004). Once again $GABA_AR$ subtype deficits seen in rats receiving poor maternal care can be rescued by cross fostering to good mothers. In addition, subtype specific $GABA_AR$ deficits and enhanced neophobia which normally make BALB/c mice a model for physiological anxiety (Belzung, 2001), were reversed by cross fostering to less anxious C57BL/6 mothers (Caldji et al., 2004). Maternal separation also leads to increased anxiety and altered HPA axis formation that can be rescued by SSRI treatment (Plotsky and Meaney, 1993; Huot et al., 2001). Differences in developmentally established phenotypes extend to the epigenetic level. Reduced maternal care increases methylation of the GR gene promoter in the hippocampus of offspring during the first postnatal week. Methylation is associated with increased histone acetylation and transcription factor (nerve growth factor inducible protein 1) binding. Infusion of histone deacetylase inhibitor not only blocked methylation and transcription factor binding, but also reduced HPA response to stress. The methylation effects were dependant on the rearing mother (Weaver et al., 2004). These effects are reversible in the adult brain as indicated by infusion of a precursor to S-adenosyl methionine into the left ventricle of adult rats (Weaver et al., 2005). This suggests that although difference in maternal care result in fairly stable epigenetic changes, deficits in adulthood caused by developmental stress might not be permanent. Nevertheless, this phenotypic plasticity emphasizes the importance of a developmental model in establishing a pattern of behavioral response to stressful or potentially threatening situations.

1.5.2 Stress-related changes to the developing brain modulate stress response in the adult

There may be a specific critical window during development in which a normal stress response is established. In a model of prenatal stress, SSRIs were able to normalize the stress response when pups were treated during postnatal weeks 1-3 and not during postnatal weeks 4-6 (Ishiwata et al., 2005). This suggests that the first three weeks of life are critical for formation of normal stress response. In contrast, early postnatal SSRI treatment (P4-P21) in the absence of a prenatal stressor resulted in heightened neophobia (Open Field Test and EPM) and decreased stress avoidance (Shock Avoidance) mimicking the effect of serotonin transporter (5-HTT) deletion (Ansorge et al., 2004). Interestingly, in a behavioral test used to assay pharmacological models of depression (NSF test), there was a treatment by genotype effect. 5-HTT$^{+/+}$ mice show no response to developmental SSRI treatment while 5-HTT$^{+/-}$ and 5-HTT$^{-/-}$ mice show increased behavioral inhibition (Ansorge et al., 2004). This modulation of the 5-HTT effect on the serotonergic system suggests that in mice the first three weeks of life provide a critical period for the establishment of neuronal circuits involved in anxiety and depression-like behavior. By extension, in humans who carry one or more copies of the short allele of the 5-HTT linked promoter polymorphism had more depressive symptoms, diagnosed depression and suicidal behavior after a significant early life stress than people carrying only long copies of the allele (Caspi et al., 2003; Kendler et al., 2005). Furthermore, healthy individuals with one or more copies of the short 5-HTT allele show more neuronal activity in the amygdala in response to fearful stimuli than those homozygous for the long allele (Hariri et al., 2002; Hariri and Weinberger, 2003; Hariri et al., 2005; Heinz et al., 2006). Thus, this genetic linkage to depression associated with environmental stress correlates with changes in circuitry, which set up interactions with risk factors for anxiety disorders and depression that dramatically influence the difference between normal behavior and pathology.

1.6 Neurogenesis and anxiety and mood disorders

1.6.1 Adult neurogenesis contributes functional neurons to the forebrain

Throughout development, the process of neuronal proliferation and maturation provides plasticity for the formation of circuits that build systems encoding for behavioral response (Figure 1.3; Ming and Song, 2005; Leonardo and Hen, 2006). The experience driven generation of neuronal circuits is restricted to distinct time periods during development in most brain regions (Hensch, 2004; Knudsen, 2004). However, neurons continue to differentiate in at least two specific brain regions. In the subventricular/periventricular zone (SVZ) from they migrate to the olfactory bulb along the rostral migratory stream. Additionally neurons migrate into the granular cell layer from the subgranular zone (SGZ) and become incorporated into the dentate gyrus (Lois and Alvarez-Buylla, 1994; Gould and Gross, 2002; van Praag et al., 2002). While a significant proportion of these neurons seem to die, many integrate into functional circuits (Dayer et al., 2003; reviewed in Kempermann et al., 2004). Within the adult hippocampus, these neurons include glutamatergic granule cells whose mossy fiber projections innervate the CA3 region (Song et al., 2002; van Praag et al., 2002) and a significant portion (approx. 16%) of GABAergic parvalbumin-positive basket cells (Liu et al., 2003). The incorporation of new neurons into the hippocampus results in behavioral changes relating to learning and memory as well as emotional regulation (Gould et al., 1997; Gould et al., 1998; Tanapat et al., 1998; Gould et al., 1999; van Praag et al., 1999; Farmer et al., 2004).

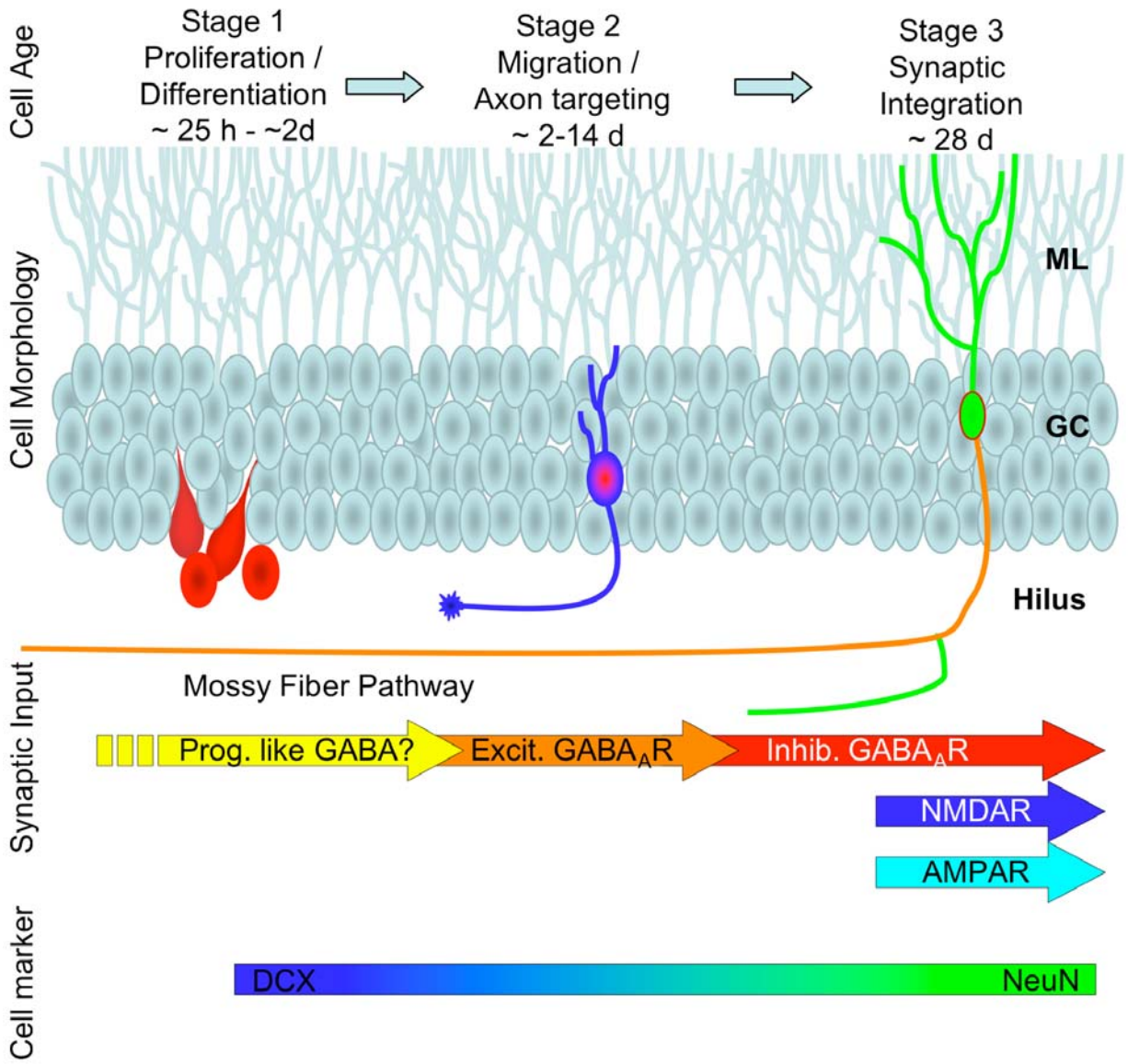

Figure 1.3 Generation of new neurons in the dentate gyrus (DG) of the hippocampus from neural stem cells in the subgranular zone (SGZ)

Neurogenesis in the DG is composed of three, targetable developmental steps. Step 1. Proliferation and Differentiation: Stem cells (not shown) with their cell bodies located within the SGZ in the DG give rise to transient amplifying cells (red circles), which in tern divide into immature neurons (red cells). Shortly after neuronal proliferation these cells begin to express the immature neuronal marker Doublecortin (DCX) Stage 2. Migration: Immature neurons (blue) migrate a short distance into the granule cell layer and extend axonal projections along mossy fiber pathways to the CA3 pyramidal cell layer. They send their dendrites in the opposite direction toward the molecular layer. At this stage spontaneous and evoked synaptic input on immature neurons comes only via depolarizing GABAergic neurotransmission. Stage

3. Synaptic integration: New granule neurons (green) receive inputs from the entorhinal cortex and send outputs to the CA3 and hilus regions visa the mossy fiber pathway. These cells signal via fast inhibitory GABAergic neurotransmission and fast excitatory glutamatergic neurotransmission. Fully mature neurons can be identified by expression of the neuronal specific nuclear protein NeuN. DG, dentate gyrus region; ML, molecular cell layer; GL, granular cell layer. (Adapted from Ming and Song, 2005; Adapted from Lledo et al., 2006)

1.6.2 Neurogenesis is affected by stress

Neurogenesis and neuronal circuit formation alter behavior and are in turn altered by stressful experience from early development throughout life. Developmental stress modifies substrates of emotional response as noted above, and also results in decreased hippocampal neurogenesis (Czeh et al., 2001; Czeh et al., 2002; Coe et al., 2003; Mirescu et al., 2004). This negative effect of stress on neurogenesis extends to adulthood (Montaron et al., 1999). Neurogenesis deficits from stress-induced activation of the HPA axis are blocked by antagonists of CRH1Rs and reversed by chronic treatment with diverse antidepressant drugs (tricyclic antidepressants and SSRIs; (Duman et al., 2001b; Duman et al., 2001a; Malberg and Duman, 2003; Alonso et al., 2004). Non-drug based antidepressant treatments such as increased physical exercise (Churchill et al., 2002; Cotman and Berchtold, 2002) or electroconvulsive therapy (Madsen et al., 2000; Scott et al., 2000; Hellsten et al., 2002) as well as most drug based antidepressant treatments increase neurogenesis (Gould and Tanapat, 1999; Coyle and Duman, 2003; Castren, 2004). Consistent with this idea, stress affects neuron growth and dendritic remodeling, and decreased levels of neurotrophins in the hippocampus are associated with chronic stress (Duman et al., 1997; McEwen, 1999). Dendritic remodeling can be reversed or prevented by antidepressant treatment (Wood et al., 2004; McEwen and Olie, 2005) suggesting that anxiety and mood disorders not only interfere with the proliferation of new neurons but also their maturation and incorporation into brain circuits. Furthermore, the antidepressant behavioral effects of the SSRI fluoxetine are dependent on adult hippocampal neurogenesis (Santarelli et al., 2003). Fluoxetine affects a specific class of actively proliferating neuronal progeniter cells (as opposed to quiescent stem cell) which then divide and mature into neurons as they migrate into the granular cell layer (Encinas et al., 2006). Interestingly, antidepressants increase neuronal cell death as well as neurogenesis suggesting that their effects may be dependent on increasing neuron turnover and hence network plasticity in the hippocampus (Sairanen et al., 2005).

1.7 Essential Functions of GABA in Postnatal Development

1.7.1 GABAergic transmission switches from excitatory to inhibitory during development

GABA plays an essential role in the maturation and refinement of neural networks during postnatal brain development (Figure 1.3 and 1.4; reviewed in Ben-Ari, 2002; Owens and Kriegstein, 2002). GABAergic neurons develop mostly before glutamatergic neurons and initially provide most of the excitatory drive in the nervous system owing to a high intracellular chloride concentration. GABAergic transmission undergoes a functional switch from being excitatory in immature neurons to inhibitory in adult neurons. This switch is due to transcriptional induction of the potassium-chloride co-transporter KCC2, which causes a change in the chloride equilibrium membrane potential during neuronal maturation. Interestingly, this KCC2-mediated change in the effect of GABA is dependent on $GABA_AR$ function itself (Dolmetsch et al., 2001; Ganguly et al., 2001; Hubner et al., 2001). This suggests that a deficit in $GABA_ARs$ or GABA might cause a deficit in neural maturation or delay in the switch from excitation to inhibition by GABA. Indeed, activation of $GABA_ARs$ promotes the differentiation and inhibits the proliferation of neural progenitors in the developing cortex (Antonopoulos et al., 1997). Mice that are deficient in the small isoform of glutamic acid decarboxylase (GAD65, the last enzyme in the synthesis of GABA) exhibit a delay in the critical period that determines ocular dominance, an experience-dependent refinement of the visual cortex during development (Hensch et al., 1998). Potentiation of $GABA_ARs$ function with diazepam has the opposite effect accelerating critical period resolution. Therefore, excitatory vs. inhibitory switch for $GABA_ARs$ provide plasticity for new neurons of the subgranular zone to extend axonal processes to the appropriate target areas and integrate into hippocampal circuitry into the perforant pathway of the hippocampus (Gould and Gross, 2002; van Praag et al., 2002).

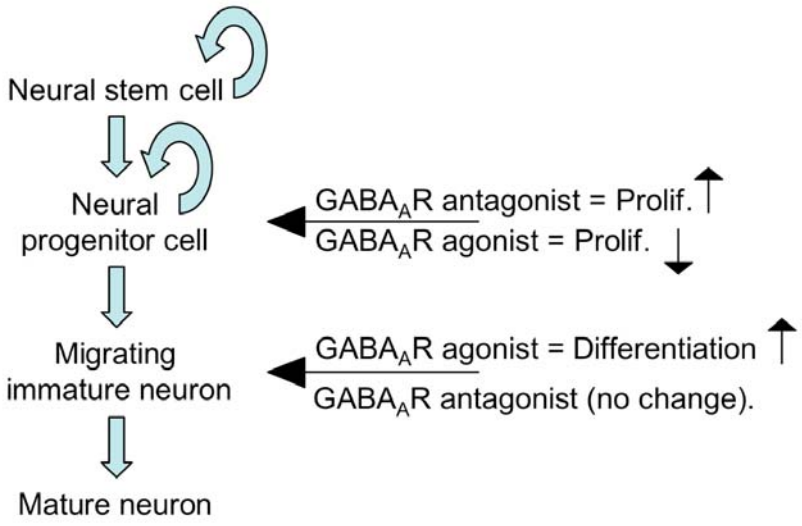

Figure 1.4 Pharmacological modulation of proliferating and differentiation neurons in the adult subgranular zone (SGZ) of the dentate gyrus (DG)

GABAergic control over maturation of newly born cells in the adult brain is illustrated by pharmacological modulation of GABAergic neurotransmision. GABAergic excitation via a $GABA_AR$ agonist decreases the number of labeled proliferating cells and increases the number of cells surviving to neuronal fates. By contrast treatment with a GABAergic antagonist does not result in a detectible increase in differentiated neurons. It has been proposed that GABAergic activation decreases the number of neuronal progenitor cells by accelerating their differentiation. By contrast $GABA_AR$ antagonist inhibits cell differentiation and results in increase transiently amplifying cells (Tozuka et al., 2005; Ge et al., 2006; Adapted from Borta and Hoglinger, 2007)

1.7.2 GABA modulates neurogenesis

Irrespective of when they are born newborn neurons pass through the same sequence of developmental milestones; initial activation by GABAergic extrasynaptic inputs, activation of inhibitory GABAergic synaptic inputs and finally activation by glutamatergic inputs (Figure 1.3; Esposito et al., 2005; Overstreet Wadiche et al., 2005; Ge et al., 2006; Karten et al., 2006). The process by which adult-born neurons differentiate, migrate and integrate into functional synaptic circuits is regulated in large part by GABAergic depolarization (Figure 1.4; Lledo et al., 2006; Ge et al., 2007). While in the SVZ newly born cells migrate long distances along the rostral migratory stream and their division is controlled by GABA releasing neuroblasts (Liu et al., 2005). New neurons in the SGZ migrate into the inter granular layer where their proliferation is controlled by GABAergic activity in local mature circuitry (Figure 1.4; Tozuka et al., 2005; Wang et al., 2005; Ge et al., 2006). Much like neurons developing in neonates, GABAergic transmission of immature cells in the adult dentate gyrus is depolarizing to the cell due to a high internal Cl- concentration (Ben-Ari, 2002; Tozuka et al., 2005). Interestingly, when targeted mutations are introduced to reduce intracellular Cl- concentrations in immature neurons, GABA causes hyperpolarization instead of depolarization. These modified cells exhibit significant deficits in their dendritic development and delayed formation of GABAergic and glutamatergic synapses in the brain (Ge et al., 2006). This suggests GABAergic depolarization acts as the driving factor to integrate new neurons into existing brain circuitry. Reduced depolarizing GABA-mediated network activity seen in the normal adult brain may explain why development of granular cells takes much longer in adults than in neonates (Overstreet et al., 2004; Overstreet-Wadiche et al., 2006). After the formation of synapses new DG neurons are more easily potentiated by glutamatergic inputs, possibly easing their connectivity to existing neuronal circuits (Wang and Olsen, 2000; Snyder et al., 2001; Schmidt-Hieber et al., 2004).

1.8 Results presented in this study

Work presented in this PhD Thesis involves the further characterization brain substrates of anxiety and mood disorder. Heightened, neophopbia seen in $\gamma 2^{+/-}$ mice (Crestani et al., 1999) was replicated as a proof of concept that theses mice are models of trait anxiety independent of laboratory or experimenter. Furthermore, subtle deficits in $GABA_AR$ seen in $\gamma 2^{+/-}$ mice resulted in increased behavioral inhibition in two separate assays sensitive to antidepressants indicating that these mice represent a model of negative emotionality (Gamez et al., 2006). The substrate for this heightened negative emotionality was further analyzed using conditional inactivation of a 'floxed' $\gamma 2$ subunit gene by means of two different Cre-transgenes that differ in their developmental expression pattern. In particular work presented here demonstrates the following:

1) Cre induced recombination of a LoxP containing $\gamma 2$ allele is sufficient to induce a cell-type specific and developmentally controlled $GABA_AR$ deficit in forebrain glutamatergic neurons of a mouse
2) $GABA_AR$ deficits in immature glutamatergic neurons during embryonic stages of development results in neophoba and increased behavioral inhibition in behavioral assays sensitive to the clinical effects of antidepressants
3) The behavioral phenotype seen in mice with conditional or global $GABA_AR$ deficits in immature glutamatergic neurons of the forebrain was coincident with deficits in neurogenesis characterized by normal stem cell proliferation and cell-type specification.
4) There is no change in the total binding of $5\text{-}HT_{1A}R$ agonist due to subtle deficits in $GABA_ARs$

Chapter 2 Materials and methods

2.1 Solutions

Anesthetic: ketamine/xylazine/acepromazine injected110/20/3 mg/kg. For 10 ml; 1 mL ketamine, 0.9 ml xylazine, 0.27 ml Acepromazine, (Henry Schein, Melville, NY) 7.83 ml ddH_2O, filter sterilize and inject at 11 ml/kg.

β-Gal Stain: 0.5 mg/ml X-Gal, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$ in embryo wash

Blocking solutions: PBS or TBS, 5% respective serum and 0.01% Triton X 100

Citric acid buffer: 10mM citric acid ($C_6H_8O_7$), 0.05% Tween 20, pH 6.0; mix 1.92 g $C_6H_8O_7$ (anhydrous) to 1000 ml distilled H_2O and dissolved. The pH was adjusted with 10 M NaOH to 6.0 and then 0.5 ml of Tween 20 was added and mixed well.

Cryoprotectant: For 1 l, 200g sucrose ($C_{12}H_{22}O_{11}$), 200 mg sodium azide (NaN_3), 300 ml ethylene glycol ($C_2H_6O_2$) and 500 ml 0.05M phosphate buffer at pH 7.4 and filter

0.05M Phosphate Buffer: For 500 ml; 1.31 g sodium phosphate monobasic monohydrate ($NaH_2PO_4 \bullet H_2O$), 5.75 g sodium phosphate dibasic anhydrous (Na_2HPO_4).

Embryo Wash: 2 mM magnesium chloride ($MgCl_2$) and 0.02 % NP40 in PBS pH 7.4

3.33% Gelatin solution; For 1500 ml, 1.5 g gelatin was added to 1450 ml ddH_2O and heated while stirring until it was completely dissolved. In a separate beaker, 0.15 g chromium potassium sulfate ($CrK(SO_4)_2 \cdot 12\ H_2O$) was added to 50 ml dd$H_2O$ and

stirred without heating until it was completely disolved. Solutions were combined immediately prior to use and excess bubbles removed.

Glial Medium: Minimum Essential Medium with Earle's salts (MEM), 1% Penicillin-streptomycin, 1% Glutamax 1, 10% Fetal Bovine Serum, 0.6% glucose ($C_6H_{12}O_6$)

HBSS; 1X Hank's balanced salt solution, 0.1M HEPES buffer, 1% Penicillin-streptomycin

High Fidelity PCR Buffer: 600 mM Tris-SO_4 (pH8.9), 180 mM ammonium sulfate ($[NH_4]_2[SO_4]$)

Lysis buffer: 100 mM Tris-HCl pH 8.5, 5 mM diaminoethanetetraacetic acid (EDTA, $C_{10}H_{16}N_2O_8$), 0.2% sodium dodecyl sulfate (SDS, $NaC_{12}H_{25}SO_4$), 200 mM sodium chloride (NaCl), 100 µg/ml proteinase K (Prot. K)

Mounting Solution: 1:1 glycerol ($C_3H_5(OH)_3$): 0.1 M sodium bicarbonate ($NaHCO_3$)

4% Paraformaldehyde (PAF): 40 g paraformaldehyde ($(CH_2O)n$) to 400 ml hot ddH_2O was heated, while stirring, to ~90˚C for 40-45 min and 10N sodium hydroxide (NaOH) added dropwise until solution clears. Paraformaldehyde solution was filtered into 0.15 M phosphate buffer, cooled to 4˚C and titrated to pH 7.4 with NaOH or HCl.

0.15M Phosphate Buffer: 3.93 g $NaH_2PO_4•H_2O$, 17.24 g Na_2HPO_4 (anhydrous), Brought to volume with 600 ml ddH_2O

PCR reaction buffer: 500 mM potassium chloride (KCl), 15 mM $MgCl_2$, 100 mM Tris-HCl, pH 9.0

Phosphate Buffered Saline (1 X PBS): 3.12 g/l $NaH_2PO_4·H_2O$, 10.99 g/l Na_2HPO_4 (anhydrous), 9.0 g/l NaCl, solution pH 7.4

Primary antibody solutions: PBS or TBS, 2% respective serum, 0.01% Triton X 100 and respective antibody

TAE: 40 mM Tris-acetate, 1 mM EDTA, solution pH 7.0

Tail Fixative for LacZ tail staining (genotyping): 4% Paraformaldhyde in 2X PBS, pH 7.4, 0.02% NP40

Tris Buffered Saline (TBS): 8.0 g/l NaCl, 0.2 g/l KCl, 3.0 g/l Tris, pH to 7.4 with NaOH or HCl

TE: 10 mM Tris-HCl pH 8.0, 0.5 mM EDTA pH 8.0

2.1.1 Microscope slides

Gelatin subbed slides: 25 x 75 mm micro slides (VWR cat no 48312-002) were immersed in 2 l hot tap H_2O containing 20 g Powdered detergent (Alcanox, New York, New York) and rinced thoroughly in cold tap H_2O. Then the slides were dipped in 80% ethanol and allowed to dry overnight. Slides were then immersed in gelatin solution by occasionally dunking for a total of 60 s. Excess gelatin was drained and slides were placed diagonally and allowed to dry overnight. Gelatinised slides were stored under desiccation at 4˚C.

Poly-D-Lysine coated slides: 25 x 75 mm micro slides (VWR cat no 48312-002) were immersed for 5 sec in 0.01% (w/v) poly-D-lisine hydrobromide and air dried. Slides were stored at 4˚C.

2.2 Animal production and husbandry

All animal experiments were approved by the Institutional Animal Care and Use Committee of Penn State University and were performed in accordance with relevant guidelines and regulations. Heterozygous ($\gamma2^{+/-}$) and wild type littermate control mice were produced by crossing $\gamma2^{+/-}$ and wt 129SvJ mice as described (Crestani et al., 1999). The $\gamma2^{+/-}$ mice had been backcrossed onto a 129SvJ background for > 40 generations. Conditional heterozygous knock-out mice and f$\gamma2$/+ control littermates were generated by mating Emx1Cre (Iwasato et al., 2000) or CaMKIICre2834 hemizygous mice with f$\gamma2$/f$\gamma2$ or f$\gamma2$/+ mice (Schweizer et al., 2003), all of which had been backcrossed into the 129SvJ background for ≥ 6 generations. Breeder mice were housed in standard shoebox cages with standard chow and water available *ad libitum.* Mouse litters for behavioral testing were produced one litter per large (gang) cage containing standard bedding supplemented with cloth nesting squares. The litters were left undisturbed without change of bedding until the day of weaning (P21), at which time they were genotyped by PCR analysis of tail biopsies (Günther et al., 1995; Schweizer et al., 2003) and tagged with metal ear tags. Females destined for behavioral testing were then separated by genotype and pooled into gang cages containing 8–12 animals per cage and transferred to a separate female-only holding room under a reversed light dark cycle (dark from 12:00 - 24:00). Mice were housed in the same room in which they were tested and traffic in that room was kept to a minimum especially during the dark cycle. The cages of behavioral test animals were changed once a week. Mice were placed in clean cages no less than 72h prior to testing to prevent interference associated with exposure to the novel environment of a clean cage. Testing occurred during the first 6h of the dark cycle and mice were tested in a random order and placed into clean cages following each test. Genotypes were coded such that the experimenter doing the further testing and data analyses was unaware of the genotype. Z/EG mice (Novak et al., 2000) were purchased from Jackson Laboratories (Bar Harbor, Maine).

2.3 Genotyping

2.3.1 Preparation of tail biopsies

All mice were genotyped by tail biopsies approximately 5 mm in length taken with scissors flamed to prevent cross contamination and cautherize wound. Tail snips were placed in eppendorf 1.5 ml tubes and incubated in 500 µl lysis buffer and 5 µl Proteinase K (10 mg/ml) rotating at 55˚C for at least 4 h. After digestion samples were spun 14000 RPM for 10 min, isopropanol precipitated and DNA pellet was transferred to 500 ml TE and dissolved at 55˚C for 1 h. Tail DNA was stored at 4˚C until use and kept at -20˚C if needed. See below for detection of Z/AP and Z/EG transgenes by LacZ staining (Laird et al., 1991).

2.3.2 PCR

PCR reactions were used to detect the presence of the floxed locus, PGK-Neo cassette and Cre transgene. Reaction were carried out in 1 X PCR reaction buffer with 200 mM of each deoxynucleotide, 0.4 mM of each primer and 1 ml of Taq polymerase. Eppendorf thermocyclers and 500 ml reaction tubes were used. The PCR products were analyzed using 1% - 2% agarose minigels run in TAE at 40 – 60 V. PCR products were loaded into gels in 1 X DNA loading buffer and band size was determined by comparison to 1kB plus Ladder DNA Standard.

Table 2.1 Parameters of PCR reactions

Locus detected	primers used	Annealing Temp (time)	Elongation temp (time)	Number of cycles
neo gene	g2.19, g2.20	57˚C (35 s)	65˚C (2 min)	36
upstream loxP site	fg2 upper 2, fg2 lower 2	55˚C (30 s)	72˚C (80 s)	38
Cre gene	Cre up (Tsein), Cre low (Tsein)	55˚C (40 s)	65˚C (2 min)	36

Table 2.2 Primers for PCR Genotyping

Name	Sequence
γ2.19	5'-CATCTCCATCGCTAAGAATGTTCGGGAAGT-3'
γ2.20	5'-ATGCTCCAGACTGCCTTGGGAAAAGC-3'
fγ2upper2	5'-GCCTGATTGTGGAAATAAAA-3'
fγ2lower2	5'-CATCCCCTTACTCTATGTC-3'
Cre up (Tsein)	5'-AGATGTTCGCGATTATC-3'
Cre low (Tsein)	5'-AGCTACACCAGAGACGG-3'

2.3.3 Detecting Z/AP and Z/EG transgenes

Z/AP and Z/EG tail snips (5 mm in length taken as above) were stained with X-Gal to detect the presence of a Lac Z gene. Tails were trnansfered to a 96 well plate and fixed 30 min at 4˚C in 200 ml 4% paraformaldhyde, 0.02% NP40. Tails were washed 3 times 10 min embryo wash and incubated overnight at 37˚C in 150 ml XGAL stain. Dark Blue tails indicated the presence of a LacZ gene.

2.4 RTPCR from Cell Culture

2.4.1 Cell Culture

Cortical hemispheres are isolated from E14-E17 mice and transferred to a 50 ml centrifuge tube containing 6 ml HBSS. 1.5 ml each of 2.5% trypsin and 1% DNAse are added and tubes are incubated 37°C for 15 min swirling periodically. Solution is triterated with a fire polished transfer pipette, filtered through a 72mm into 3 ml FBS and centrifuged at 800-900 rpm for 5-6 min. Supernatant is removed and pellet resuspended in 20 ml glial medium which is plated onto 75 cm^2 flasks or 30 x 10 mm culture dishes (Corning Life Sciences, Acton, MA). Cells were incubated in an atmosphere of 5% CO_2 and washed in 1X PBS and fresh media is added every 2-3 days. At near confluency cells are rinsed in 1X PBS and then with 1.0 ml 0.05% trypsin 0.53mM EDTA. After aspiration cells are incubated with 1.0 ml 0.05% trypsin 0.53 mM EDTA for 2-3 min, resuspended in 30 ml glial media and split into 3 flasks. Neuronal Cultures were carried out according to Alldred et al. (Alldred et al., 2005). Breifly, Cortical hemispheres were collected in PBS containing 5.5 mM glucose and incubated for 15 min at RT in 1X PBS containing with papain (0.5 mg/ml) and DNase I (10 g/ml) (both from Sigma, St. Louis, MO) and 1 mg/ml bovine serum albumin (Fraction V, Sigma) and 10mM glucose. The cells were triturated with a fire-polished Pasteur pipette and plated on poly-L-lysine-coated 30 x 10 mm culture dishes at 4 X 10^4 cells per square centimeter MEM (Invitroge) containing 10% v/v fetal bovine serum (FBS) (Invitrogen) in an atmosphere of 10% CO_2. After 60 min, the medium was replaced with fresh MEM containing 10% v/v FBS and cells were incubated for 5-10 days.

2.4.2 RTPCR

RTPCR was performed using TRIzol® Plus RNA Purification System (Invitrogen, Carlsbad, CA.) and all reagents and protocols provided by the manufacturer were used. Brief descriptions of protocols are provided here.

2.4.3 RNA isolation

Cells were lysed directly in a culture dish by adding 1 ml of TRIzol® Reagent to a 3.5 cm diameter dish, and passing the cell lysate several times through a pipette. Homogenized samples were incubated for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. Add 0.2 ml of chloroform per 1 ml of TRIzol® Reagent. Sample tuber were shaken vigorously by hand for 15 seconds and incubated at 15 to 30°C for 2 to 3 minutes. Samples were centrifuged at 10,000 x g for 15 minutes at room temperature. Following centrifugation, the mixture separated into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase containing RNA. Aqueous phase was transferred to a fresh tube and precipitated by mixing with 0.5 ml of isopropyl alcohol per 1 ml of TRIzol® Reagent used for the initial homogenization isopropyl. Samples were incubated at room temperature for 10 minutes and centrifuged at 10000 × g for 10 minutes at room temperature, the supernatant was removed and the pellet was washed once with at least 1 ml of 75% ethanol per 1 ml of TRIzol® Reagent used for the initial homogenization. Samples were mixed by vortexing, centrifuged at 7,500 × g for 5 minutes at room temperature air dried and resuspended in DEPC treated ddH_2O.

2.4.4 First-Strand Synthesis Using Oligo (dT)

RNA/primer mixtures for first strand synthesis contained up to 5 µg total RNA and 50 ng of control RNA. In addition RNA primer mixtures consisted of 10 mM dNTP mix and 0.5 µg (1 µl) Oligo $(dT)_{12-18}$ brought to 10 µl in DEPC-treated H_2O. These samples were incubated at 65° C for 5 min, then placed on ice for at least 1 min. 10 µL of cDNA synthesis mix was added to each RNA/primer mixture such that each reaction contained 2 µl 10X RT buffer , 4 µl 25 mM $MgCl_2$, 2 µl 0.1 M DTT, 1 µl RNaseOUT™ (40 U/µl) and 1 µl Superscript III RT (200 U/µL). Samples were incubated 50 min at 50°C. A second control was prepared without reverse transcriptase (RT). Reactions were terminated at 85°C for 15 min and chill on ice. Samples were collected by centrifugation, 1 µl of RNase H was added to each tube

and each tube was incubated for 20 min at 37°C. cDNA synthesis was stored at -20°C for used in PCR.

2.4.5 PCR amplification

50 ml samples were brought to a final concentration of 1X High Fidelity PCR buffer, 0.2 mM dNTP, 2 mM $MgSO_4$, and 0.2 mM of each primer (upper and lower). 0.2 ml Taq polymerase and 2 µl of cDNA per reaction were used. PCR was then carried out using 40 cycle where each cycle consisted of denaturing at 94º C for 30 sec, annealing at 58º C for 30 sec and extending at 64º C for 60 sec. After the PCR, the DNA products were analyzed using a 1-2% agarose gel run in TAE at 40-60V.

Table 2.3 Primers for RTPCR

Name	Sequence
Upper (for γ2 RTPCR)	5'-GTGGAGTATGGCACCCTGCATTATTTTGCT-3'
Lower (for γ2 RTPCR)	5'-CAAAAGGCGGTAGGGAAGAAGATCCGAGCAT-3'
GAPDH (upper RTPCR)	5'-GTGGCAAAGTGGAGATTGTTGCC-3'
GAPDH (lower RTPCR)	5'-GATGATGACCCGTTTGGCTCC-3'

2.5 Behavioral assessments

Behavioral testing was performed at the age of 8–10 weeks, at least 72 h after the last cage change and during the first 6 h of the dark phase. Each mouse was used in one test only, unless indicated otherwise. The behavior was video recorded in the holding room under red light for subsequent off-line quantification.

2.5.1 Free Choice Exploration

In a compartment containing six units (10 x 10 x 20 cm each), mice were placed individually into one segment comprising three interconnected units. After 24 h of familiarization, the remaining three novel units were made accessible and the retractions from entering the novel segment, the number of visits and the total time spent in familiar and novel units were recorded over the duration of 5 min (Crestani et al., 1999).

2.5.2 Light Dark Choice

In a chamber containing a dark and a lit box (20 x 20 x 15 cm) connected by a tunnel (5 x 8 x 10 cm), mice were tested for 5 min following placement into the lit box (illumination 700 lux). The total time spent in the lit area and the number of transitions between compartments was recorded (Crestani et al., 1999).

2.5.3 Elevated Plus Maze

On an elevated crossbar (30 cm per arm x 5 cm wide x 40 cm tall) with two walled (20 cm, transparent) and two open arms, mutant mice and littermate controls were placed onto the center square and video recorded for 5 min. The number of entries and the total time spent on closed and open arms were recorded (Crestani et al., 1999).

2.5.4 Novelty suppressed feeding test

Mice were deprived of food for 24 h preceding placement into the corner of a plastic box (50 x 50 x 20 cm) containing 3 cm of bedding and a pellet of food placed on a white paper platform (8 x 8 x 0.5 cm) in the center of the cage. Each test lasted 5 min and the measure of interest (chewing) was scored when the mouse was sitting on its haunches and biting with the use of its forepaws (Santarelli et al., 2003).

2.5.5 Modified forced swim test

Mice that had been tested in the novelty suppressed feeding test a week earlier were placed into a plastic bucket 19 cm in diameter and 27 cm deep and filled to 18

cm with 22 - 25°C water and videotaped for 6 min. The real time spent swimming to the first floating episode and the cumulative time spent immobile during the final 4 min. A sampling method was used in which the predominant behavior over a 5 s interval were recorded (Lucki, 1997; Cryan and Lucki, 2000; Cryan et al., 2002).

2.6 Autradiography

2.6.1 Preparation of brain sections for autoradiography

Brains of euthanized mice (CO_2) were removed quickly, frozen with powdered dry ice, mounted onto a cork support with OCT (Tissue-Tek, Torrance, CA) and stored at -80˚C in an airtight container. 12 mm coronal or parastgital sections were prepared and mounted on Poly-D-Lysine coated slides. Sections were dried under a stream of room temperature air for 30 min and stored for at least 3 days at -80˚C.

2.6.2 Labeling sections with [^{3}H] flumazenil

Sections stored at -80˚C were dried under a stream of room temperature air from a hair dryer, preincubated with two 10 min washes in 50 mM Tris HCl, pH 7.5 rinsed in ice cold ddH_2O and dried again under a stream of room temperature air. The sections were then incubated on an aluminum block on ice with 400 ml per slide with 5 nM [^{3}H] flumazenil (Ro 15-1788; 87 Ci/mol) in 50 mM Tris HCl (pH 7.5). After 60 min incubation, the slides were washed three times for 20 sec in 50 mM tris HCl (pH 7.5), rinsed in ddH_2O and dried under a stream of room temperature air for at least 30 min. When sections were dry they were exposed to ^{3}H-Hyperfilm (Amersham Biosciences, Arlington Heights, IL) for 7-10 days at room temperature (for an image for publication) or ^{3}H-phosphoscreen for 24-36 h at RT (Günther et al., 1995; Schweizer et al., 2003).

2.6.3 Labeling sections with 8-Hydroxy-[^{3}H] DPAT

Sections stored at -80˚C were dried under a stream of room temperature air from a hair dryer, preincubated with two 10 min washes in 50 mM Tris HCl, pH 7.5 rinsed in ice cold ddH_2O and dried again under a stream of room temperature air. The sections were then placed in a box and incubated at room temperature with 400 μl per slide with 1.5 nM [^{3}H]8-OH-DPAT in 50 mM Tris/HCl, 180 mM NaCl, 5 nM KCl, 2.5 mM calcium chloride ($CaCl_2$) 1.2 mM $MgCl_2$, and 10 μM pargyline (Sigma Adlrich, St. Louis, MO) pH 7.4. After 60 min incubation the slides were washed three times 20 sec in 50 mM tris HCl (pH 7.5), rinsed in ddH_2O and dried under a stream of room temperature air for at least 30 min. When sections were dry they were exposed to ^{3}H-phosphoscreen (Amersham Biosciences) for 24-36 h at room temperature. To determine nonspecific binding adjacent brain sections were incubated in the above incubation buffer in the presence of 1μM serotonin (Watanabe et al., 1993).

2.6.4 Image analysis

Coronal and parasagital sections were analysed by either outlining the area of interest (hippocampus, amygdala, globus pallidus) or overlaying the area of interest with a 0.5 x 0.5 mm box (cortex, striatum, thalamus). The density of BZ binding sites was quantified in three sections per brain (*n* = 10-12 brains per genotype) using a Cyclone storage phosphor imaging system with Optiquant software (Perkin-Elmer, Shelton, CT) and [^{3}H] microscale autoradiography standards (Amersham Biosciences). To compare across experiments multiple sections from the same brains were exposed in different experiments and pixel intensity per area was normalized.

2.7 Histology

2.7.1 BrdU injection

For quantification of neuronal survival, eight week-old mice were administered BrdU (4 x 80 mg/kg i.p. at 2 h intervals, in saline at 8 mg/ml adjusted to pH 7.4 with NaOH). For acute labeling of replicating cells, mice were injected with a single dose of BrdU of 200 mg/kg at a concentration of 20 mg/ml and the brains were harvested 24 h later.

2.7.2 Transcardial perfusion

Peristaltic pump and tubing was calibrated to a flow rate of 10 ml/min before each days perfusing session using ice cold PBS. Tubing was set up with a two-way valve that allows flow to switch between PBS and 4% PAF flow. The mouse was anesthetized with ketamine/xylazine/acepromazine (110/20/3 mg/kg), pinned onto a dissection board on its back and rinsed with ethanol. An incision was made from abdomen towards neck and then through the ribs with sharp scissors and extra care taken to avoid cutting the heart or lungs. Top part of the ribs and chest were held with hemostats and the heart was completely exposed and free of tissue. PBS flow was started with a very slow drip of PBS. With the heart held firmly with forceps, the cannula was slowly inserted into left ventricle and into aorta. Once animal's organs started to expand, right atrium was cut and the PBS flow rate was adjusted to 10 ml/min for 2 min 25 sec. By this time the animal's liver became clear and the PBS flowing out of the mouse was free of blood. Following PBS, flow was switched to 4% PAF for 10 min at 10 ml/min and the mouse was unpinned from the dissection board. At this time the animal's limbs straightened and toes curled and eventually it became completely stiff. Brain was removed, placed into 40 ml 4% PAF postfix for 12 h at 4˚C (in 50 ml conical tubes) and then equilibrated sequentially for approximately 12 hours each in 20% and 30% sucrose in PBS. Brains were stored until use in -80˚C. Protocol was adapted from W.E.Lyons and Laura Mamounas as well as Beth Luellen (Luellen et al., 2006).

2.7.3 Preparation of brain sections for immunohistochemistry

Serial coronal sections (35 μm) through the hippocampus were taken with a microtome (Microm, Walldorf, Germany). Breifly brains were kept as cold as possible using dry ice and sections were taken starting at Bregma: 0.50 mm and ending at Bregma -4.60 mm approximately. Coordinates were determined by eye comparison to Paxinos and Franklin The Mouse Brain in Stereotaxic Coordinates 2nd edition. Sections were placed in 24 well plates (2 brains per plate divided such that each well contained every 12th section) containing 1 ml cryoprotectant per well and stored at 20˚C until use.

2.7.4 Immunostaining

Sections were incubated in 12 well plates (Corning Multiwell 353043) and transferred from one 2 ml wash or incubation to the next using 15mm Netwell™ Insert with 74 µm Mesh Size Polyester Membrane (Corning #3477). For the basic immunostaining procedure sections were washed 5 times, 5 min each wash in PBS to rinse away cryoprotectant, incubated 1 h in blocking solution, washed 3 times 5 min in PBS in incubated in primary antibody overnight at 4˚C. On the next day sections were washed 3 times 5 min in PBS, incubated in secondary antibody for 1h and washed at least 3 times 5 min in PBS before mounting using paint brushes and mounting solution on gelatinized slides. Antibody solutions and specific staining are indicated below and in Table 2.

For fluorescent double labeling using BrdU antibody all sections were labeled sequentially. Following labeling with the first primary antibody sections were fixed in 4% paraformaldehyde at room temperature for 20 min and washed at least 4 times 5 min in PBS. Then sections were incubated in 2N HCl for 30 min at 37ºC and washed 5 min in 0.1M sodium borate ($Na_2B_4O_7$) at room temperature followed by 3-5 times 5 min in PBS. Sections were labeled stained and mounted normally as described above. Protocol modified from ChengJi Zhou (personal communication, Univ. of California, Davis).

For fluorescent labeling with NeuN antibody sections were incubated in citric acid buffer 90˚C for 30 min and allowed to cool to room temperature while shaking (approx 20 min). Sections were then washed ≥ 4 times 5 min in PBS labeled as indicated above. Protocol modified from IHCworld.com

Table 2.4 Antibodies and immunostaining conditions

Antibody	Dilution	Blocking Solution	Incubation Time	Antigen Presentation/ Changes to Protocol
Rat monoclonal anti-BrdU (Accurate Chemical, Westbury, NY)	1 to 500	Donkey Serum	Overnight	2M HCl incubation 37˚C (30 min)
Mouse monoclonal anti-NeuN (Chemicon, Chemicon, Temecula, CA)	1 to 1000	Donkey Serum	Overnight	Citric acid incubation 90˚C (30 min)
Mouse monoclonal anti-PAV (Sigma, St. Louis, MO)	1 to 1000	Donkey Serum	Overnight	none
Guinea Pig polyclonal anti-DCX (Chemicon, Temecula, CA)	1 to 9000	Goat Serum	48 h	TBS for soln. to reduce background
Donkey anti-Rat Cy3 (Jackson Immuno. Research, West Grove, PA)	1 to 500	Donkey Serum	1 h	none
Donkey anti-Mouse FITC (Jackson Immuno. Research, West Grove, PA)	1 to 500	Donkey Serum	1 h	none
Donkey anti-Mouse FITC (Jackson Immuno. Research West Grove, PA)	1 to 500	Donkey Serum	1 h	none
Goat anti-Geunea Pig FITC (Molecular Probes, Eugene, OR)	1 to 400	Goat Serum	1 h	TBS for soln. to reduce background
Goat anti-Rat Cy3 (Jackson Immuno. Research, West Grove, PA)	1 to 500	Donkey Serum	1 h	none

2.7.5 Image Analysis

For quantification of BrdU-positive cells, sections were coded such that the experimenter was blind to the genotype. Every twelfth section through the hippocampus was quantified using optical sectioning by confocal microscopy. BrdU-positive cells were counted if they were within the subgranule cell layer, i. e. within one cell width of the granule cell layer or within the granular cell layer of the dentate gyrus. Z-plane sectioning (1 μm steps) was performed to confirm colocalization of BrdU/NeuN or BrdU/DCX double positive cells.

2.8 Statistical analysis

The criteria for choice of either parametric or non-parametric two-mean comparison tests was a total number of values (*N)* of 30 (Snedecor and Cochran, 1989; Conover, 1999). Accordingly, t-tests for either pooled (when n1 = n2 ± 2) or separate variance (when n1 and n2 were unequal) were chosen for N ≥ 30. Non-parametric Mann-Whitney tests were used for N ≤ 30). In the LDC test (Fig. 4B), a 2 x 2 genotype-area analysis of variance with area as a within subject factor was used because the preference for the dark area varies with the degree of aversion to the lit area. An unweighted solution was used because of inequality of n1 = 9 and n2 = 22 (*N* = 31). The investigators conducting the behavioral experiments, autoradiography tests and immunohistochemical analyses were blind to the genotype of the animals/tissue.

Chapter 3 Results

3.1 Conditional knockdown of the γ2 subunit of $GABA_A$Rs

3.1.1 Predicted expression pattern of Cre-expressing lines

The Emx1Cre and CaMKIICre2834 mouse lines express Cre under the control of two different cell type-specific gene promoters and have been used to spatially and temporally limit Cre expression and hence γ2 gene recombination in fγ2/+ or fγ2/fγ2 mice. The first Cre-expressing line, Emx1Cre, takes advantage of the promoter region for the homeobox domain gene *Emx1* by the insertion of a Cre recombinase gene into the *Emx1* locus (Iwasato et al., 2000). Recombination in this line begins at embryonic day 10 (E10) and is limited to glutamatergic neurons and glia of the forebrain (Gorski et al., 2002; Iwasato et al., 2004). Therefore, recombination of an fγ2 allele is expected to result in a functional reduction of the γ2 subunit exclusively in forebrain glutamatergic neurons due to lack of expression of the γ2 subunit gene in glia (see below, and Fraser et al., 1995). Consistent with this prediction, mice with an Emx1Cre driven homozygous deletion of the γ2 subunit gene (Emx1Cre x fγ2/fγ2) appear normal at birth, show retarded growth and, develop severe motor deficits and die before the fourth postnatal week (Schweizer, 2003).

In the second Cre-expressing line, CaMKIICre2834, the expression of Cre recombinase is driven by an 8.5 kb region derived from the CaMKIIα gene (Mayford et al., 1996; Schweizer et al., 2003). Recombination is first detected in the hippocampus at postnatal day 17 (P17) and gradually becomes more widespread throughout the forebrain reaching maximal levels by P34. As predicted by the developmental time course of Cre-mediated recombination induced by CaMKIICre2834 (Schweizer et al 2003), CaMKIICre2834 x fγ2/fγ2 mice develop normally until the fourth postnatal week and die with an epilepsy-like phenotype during the fifth postnatal week (average life expectancy 30.5 ± 8.6 days, n = 11, s.d.). Expression of the CaMKIIα gene is limited mostly to glutamatergic neurons and absent from GABAergic neurons of the forebrain (Benson et al., 1992; Jones et

al., 1994). Cre-recombinase in CaMKIICre2834Cre mice is expressed in specifically in forebrain glutamatergic neurons (Schweizer, 2003; Schweizer et al., 2003). Therefore, CaMKIICre2834 and Emx1Cre transgenes differ in their developmental recombination patterns but are predicted to ultimately result in a comparable $GABA_AR$ deficit in adulthood.

3.1.2 Functional reduction of BZ binding $GABA_ARs$

The γ2 subunit is essential for the formation of BZ binding site (Günther et al., 1995). Loss of γ2-containing $GABA_ARs$ in conditional γ2 knockout mice was directly visualized and quantified using [^{3}H] flumazenil phosphorimage analysis. Previously, it was determined that there is no loss of BZ binding sites due to the floxed allele of the γ2 subunit (Schweizer et al., 2003). Schweizer et al (2003) were able to show a significant reduction in forebrain BZ binding in Emx1Cre x fγ2/fγ2 mice when assayed at P14 and CaMKIICre2834 x fγ2/fγ2 mice starting at P21 and reaching maximal levels by the fifth postnatal week (Schweizer, 2003; Schweizer et al., 2003).

It is predicted that BZ binding deficits in Emx1Cre x fγ2/+ and CaMKIICre2834 x fγ2/+ mice will be spatially similar but more subltle than those seen in Cre-expressing fγ2/fγ2 mice. Confirming this hypothesis, ten-week-old conditional γ2 knockout mice had a similar reduction in [^{3}H] flumazenil binding, irrespective of whether recombination of the fγ2 locus was induced in the embryo by Emx1Cre or during adolescence by CaMKIICre2834 (Figure 3.1). In both Emx1Cre x fγ2/+ and CaMKIICre2834 x fγ2/+ mice, reductions in BZ sites were limited to the forebrain and most pronounced in the striatum (Emx1Cre x fγ2/+, -31.9%; CaMKIICre2834 x fγ2/+, -25.3%, compared to respective fγ2/+ littermate controls), hippocampus (CA1, -24.8%, -26.5%), and neocortex (-19.3%, -11.4%), with less of a reduction in the dentate gyrus (DG, -11.5%, -13.5%) and the amygdala (-13.1%, -11.5%) and no significant deficits in thalamus, globus pallidus and cerebellum. Given the relatively modest reduction in BZ binding in the striatum of the global $\gamma2^{+/-}$ mice (Crestani et al., 1999) and the low amount of recombination induced by Emx1Cre and

CaMKIICre2834 in the striatum of reporter strains (Iwasato et al., 2000; Schweizer et al., 2003; Iwasato et al., 2004), it is surprising to see such a robust reduction in BZ binding in Emx1Cre x fγ2/+ and CaMKIICre2834 x fγ2/+. This reduction is most likely an indirect result of altered gene expression (see Discussion). Importantly, the deficits seen in Emx1Cre x fγ2/+ and CaMKIICre2834 x fγ2/+ mice are comparable regardless of the time point of onset of γ2 subunit containing $GABA_AR$ deficits.

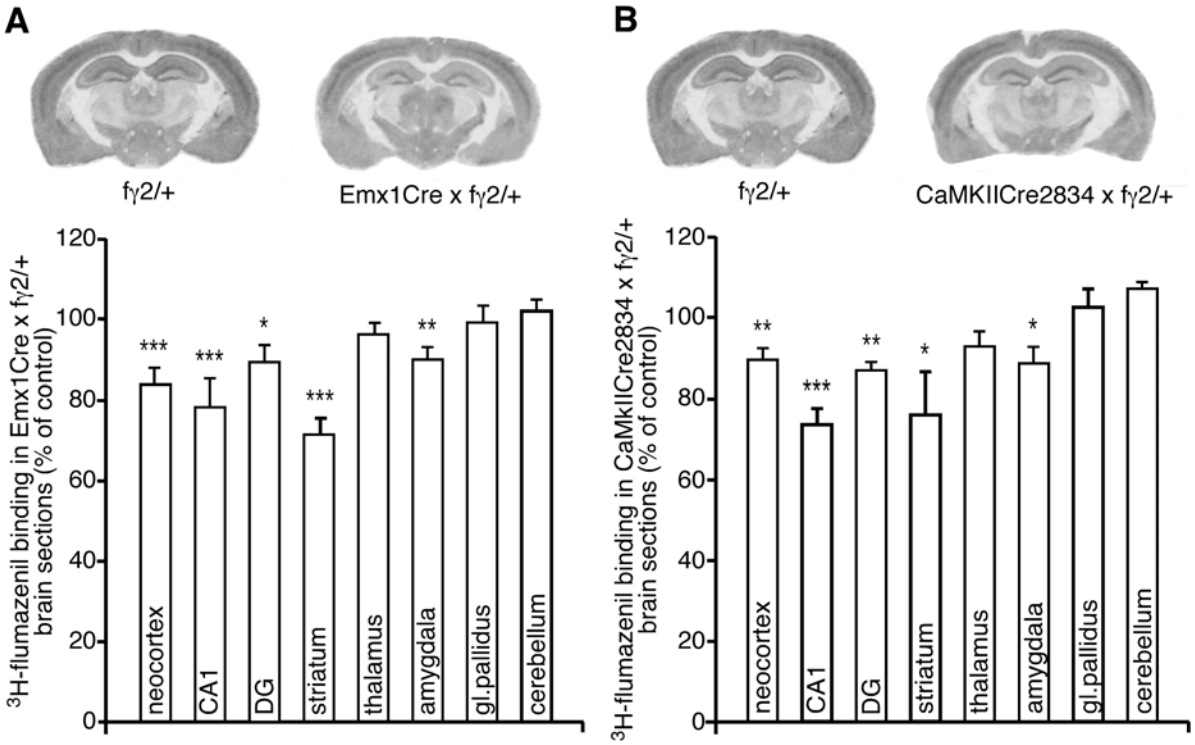

Figure 3.1 Quantification of $GABA_AR$ deficits in Emx1Cre x fγ2/+ and CaMKIICre2834 x fγ2/+ mice by [^{3}H]flumazenil autoradiography and phosphorimage analysis

A, Representative autoradiographs of brain sections from an eight week-old Emx1Cre x fγ2/+ mouse and a fγ2/+ littermate control, together with the density of BZ binding sites determined in select brain regions. The density of [^{3}H] flumazenil binding sites in Emx1Cre x fγ2/+ was reduced in neocortex and CA1 region of hippocampus [t(38) = 4.6 and 4.4, respectively, t-test) as well as in dentate gyrus (U = 21), striatum (U = 13) and amygdala (U = 10, Mann Whitney). BZ sites were unchanged in thalamus, globus pallidus and cerebellum (U > 26). **B**, Representative sections of CaMKIICre2834 x fγ2/+ and control brains and results of quantification of [^{3}H] flumazenil binding sites as in (A). The density of [^{3}H] flumazenil sites in CaMKIICre2834 x fγ2/+ mice was reduced in neocortex and CA1 region of hippocampus [t(38) = 3.0, P < 0.01 and t(38) = 7.5), and in the dentate gyrus, striatum and amygdala (U = 19, 8.0 and 8.0, respectively) compared to littermate controls. BZ site densities in thalamus, globus pallidus and cerebellum were unaltered (U > 31). Results represent means SEM (n = 9 –20 per genotype; *p 0.05, **p 0.01, ***p 0.001, t-test or Mann–Whitney test as indicated).

3.1.3 Cell-type specificity of γ2 subunit expression

The similarities of the $GABA_AR$ deficits in CamKIICre2834 x fγ2/+ and Emx1Cre x fγ2/+ mice compared to controls is consistent with the notion that the γ2 subunit is only expressed in neurons and absent in glial cells. However, $GABA_ARs$ have been detected acutely in dissociated astrocytes suggesting that the γ2 subunit might be expressed in low levels in non-neural cells (MacVicar et al., 1989; Fraser et al., 1996; Israel et al., 2003). In order to confirm that the γ2 subunit was absent in astrocytes, primary astrocyte cell cultures were labeled with γ2 antiserum (Fritschy and Mohler, 1995) and compared. Immunostained $\gamma2^{-/-}$ coverslips confirmed there was no overt difference between the signal from astrocytes containing the γ2 subunit and those that do not, indicating the fluorescent signal seen in astrocytes is due to background staining (Figure 3.2A-B). This is consistent with previous results from in situ hybridization experiments (Laurie et al., 1992a; Laurie et al., 1992b; Persohn et al., 1992; Wisden et al., 1992). To confirm these results, RT PCR analyses were performed to compare the γ2 subunit mRNA expression levels of cultured wildtype (wt) astrocytes and neurons. No γ2 subunit mRNA was detected in astrocytes. By comparison γ2 subunit mRNA was readily detected in neurons. Similar mRNA levels were detected for the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in both samples, confirming that the RNA had been transcribed into cDNA in both samples (Figure 3.2C). Therefore, $GABA_AR$ deficits in Emx1Cre x fγ2/+ mice are limited primarily to the glutamatergic neurons of the forebrain.

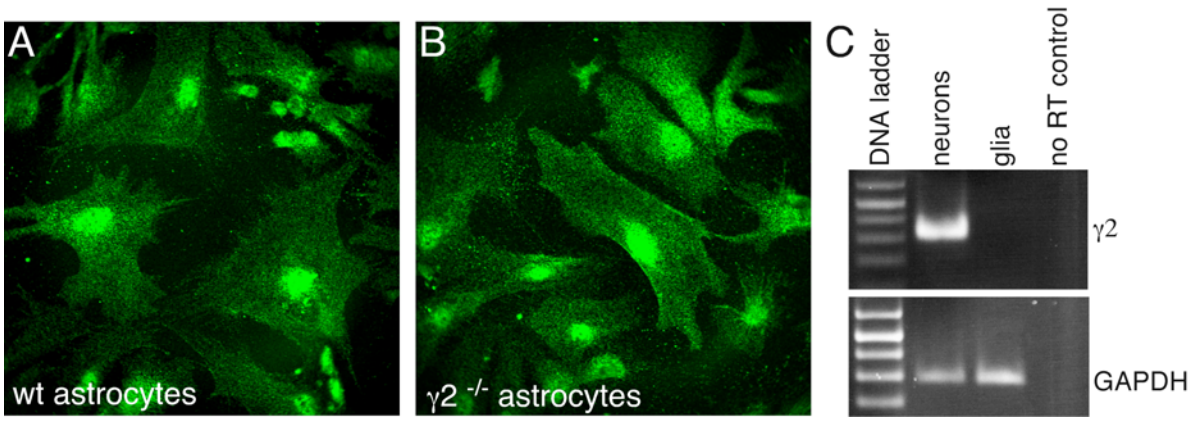

Figure 3.2 The γ2 subunit is absent from cultured astrocytes

A, B, Astrocytes were cultured from embryonic neocortex of E18 wt and $\gamma2^{-/-}$ embryos. Cultures were subjected to immunofluorescent staining with antisera selective for the γ2 subunit (Fritschy and Mohler, 1995). Note the week presumably unspecific staining for the γ2 subunit that is preserved in the absence of the γ2 subunit in $\gamma2^{-/-}$ neurons. **C,** Reverse transcriptase PCR analyses of RNA prepared from cultured cortical neurons and astrocytes. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) a protein involved in glycolysis was used as a positive control while a reaction involving no reverse transcriptase was used as a negative control. The data confirm previous evidence from *in situ* hybridization data that the γ2 subunit is selectively expressed in neurons (Laurie et al., 1992a; Laurie et al., 1992b; Persohn et al., 1992; Wisden et al., 1992).

3.1.4 Cell-type specificity of Cre recombination

Mouse lines, which express an easily detectable reporter gene after a Cre-induced recombination event, allow for the characterization of recombination patterns in Cre-expressing transgenic lines. Z/EG transgenic mice express enhanced green fluorescence protein (EGFP) after a recombination event (Novak et al., 2000). The Z/EG transgene consists of a pCAGGs promoter directing the expression of a LoxP flanked βgeo (lacZ/neomycin-resistance) fusion gene and three SV40 polyadenylation sequences. This sequence is followed the coding sequence of the EGFP protein and a rabbit β-globulin polyadenylation sequence. Thus, before Cre-induced recombination the βgeo reporter is expressed while after recombination EGFP is expressed. The EGFP reporter becomes visible already at the morula stage (≤ E2.5) depending on the Cre-expressing line that Z/EG mice are mated to. Mice hemizygous for Z/EG and either Cre-expressing mouse line were used to determine regional and cell-type specificity of Cre recombination. Emx1Cre x Z/EG and CaMKIICre2834 x Z/EG brain sections were both stained for the internueronal marker parvalbunim (PAV). In both mouse lines there was no colocalization between PAV positive cells and EGFP positive cells indicating that recombination does not occur in the significant population of PAV expressing interneurons (Figure 3.3; Freund, 2003; Markram et al., 2004).

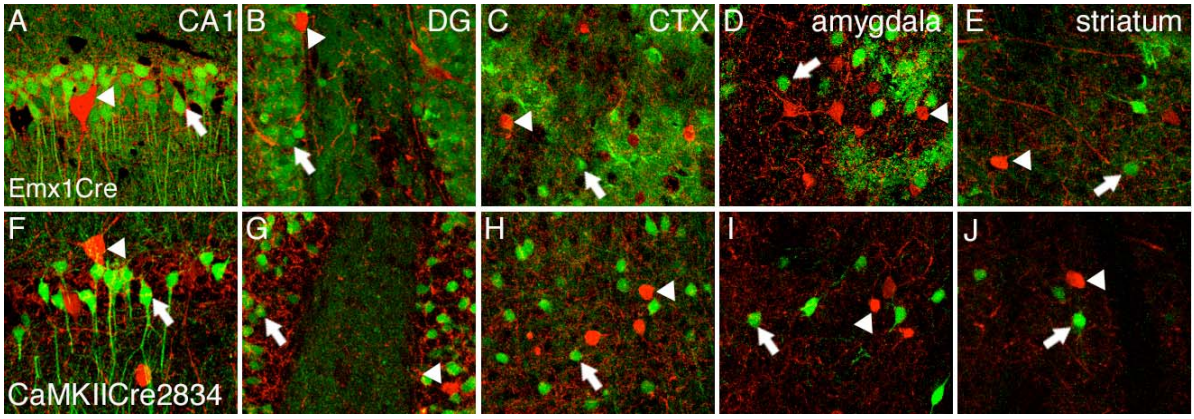

Figure 3.3 Lack of Cre-induced recombination in PAV-positive interneurons, a major subpopulation of GABAergic interneurons

Brain sections of Emx1Cre x Z/EG (**A-E**) and CaMKIICre2834 x Z/EG mice (**F-J**) were stained for PAV and tested for colocalization of PAV and Cre-mediated GFP expression. Representative images are shown for the hippocampal CA1 region (A, F), dentate gyrus (B, G), neocortex (C, H), amygdala (D, I) and striatum (E, J). Arrows point to GFP-positive cells that were subject to Cre-induced recombination, arrowheads point to PAV-positive interneurons. Images represent stacks of confocal images. Note the lack of overlap between PAV- and GFP-positive cell populations. Consistent with published results, GFP-positive cells in Emx1Cre x Z/EG sections suggest recombination in both glia and glutamatergic neurons, while Cre-induced expression of GFP in CaMKIICre2834 x Z/EG brain sections is selective for glutamatergic neurons, as expected.

Both Cre transgenes differ in their expression pattern with regard to the development of the animal and it is hypothesized that they will differ in developmental onset of expression with respect to the maturation of individual cells. To test this, Emx1Cre x Z/EG and CaMKIICre2834 x Z/EG brain sections were immunofluorescently labeled with an antibody to doublecortin (DCX) a microtubule binding protein expressed transiently in immature neurons for 2-3 weeks following the last division of neural progenitors (Brown et al., 2003). Mice were transcardially perfused at 6 weeks of age. At this developmental time point recombination is complete in both Cre-expressing mouse lines. New neurons are born in two brain regions in adults; the subgranular zone (SGZ) of the dentate gyrus (DG) and the subventricular zone (SVZ, see Introduction; Lois et al., 1994; Gould and Gross, 2002; van Praag et al., 2002). In the SGZ of Emx1Cre x Z/EG sections 75 ± 2.15% of DCX expressing cells also expressed EGFP indicating that adult-born immature neurons undergo Cre induced recombination in this mouse strain (Figure 3.4A-A'', n = 3). In contrast only 2 ± 0.66% of DCX expressing cells also expressed EGFP in the SGZ of CaMKIICre2834 x Z/EG sections (Figure 3.4B-B'', n = 3). By comparison to the SGZ, there were double-labeled DCX/EGFP positive cells in the SVZ of Emx1Cre x Z/EG brains, but they only contributed to 7.4 ± 0.5% of the overall population of DCX positive cells (Figure 3.5B-C, n = 3). No DCX/EGFP positive cells were identified in the SVZ of CaMKIICre2834 x Z/EG brains (Figure 3.5D-E, n = 3). This indicates that $GABA_AR$ deficits induced by the Emx1Cre transgene would extend to immature neurons of the SGZ and SVZ while CaMKIICre2834 induced deficits would be selectively in mature neurons.

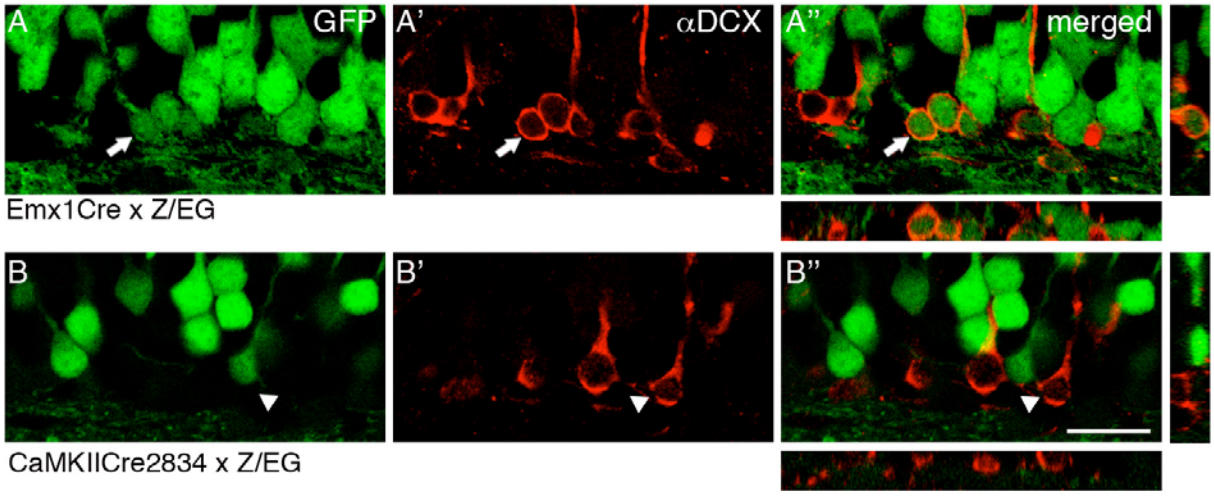

Figure 3.4 Differential Emx1Cre- and CaMKIICre2834-mediated recombination in immature neurons of the subgranular zone

Coronal brain sections of six week-old Emx1Cre x Z/EG (**A-A'**') and CaMKIICre2834 x Z/EG mice (**B-B"**) through the hippocampus (A-A", B-B") were stained with antibody for DCX (red) and analyzed for colocalization with Cre-induced GFP (green) by confocal microscopy. Note the recombination in immature (DCX-positive, arrow) granule cells in the subgranule cell layer of Emx1Cre x Z/EG mice (A-A"), whereas DCX-positive cells in corresponding sections of CaMKIICre2834 x Z/EG mice lack GFP (B-B", arrowhead). Merged green and red images (A", B") include orthogonal views of X-Z and Y-Z planes to confirm colocalization of GFP and DCX in sections of Emx1Cre x Z/EG mice. GFP-positive cells are more abundant in Emx1Cre x Z/EG than CaMKIICre2834 x Z/EG mice, consistent with Cre-mediated recombination extending to glia in Emx1Cre x Z/EG but not in CaMKIICre2834 x Z/EG mice. Scale bar, 20 μm.

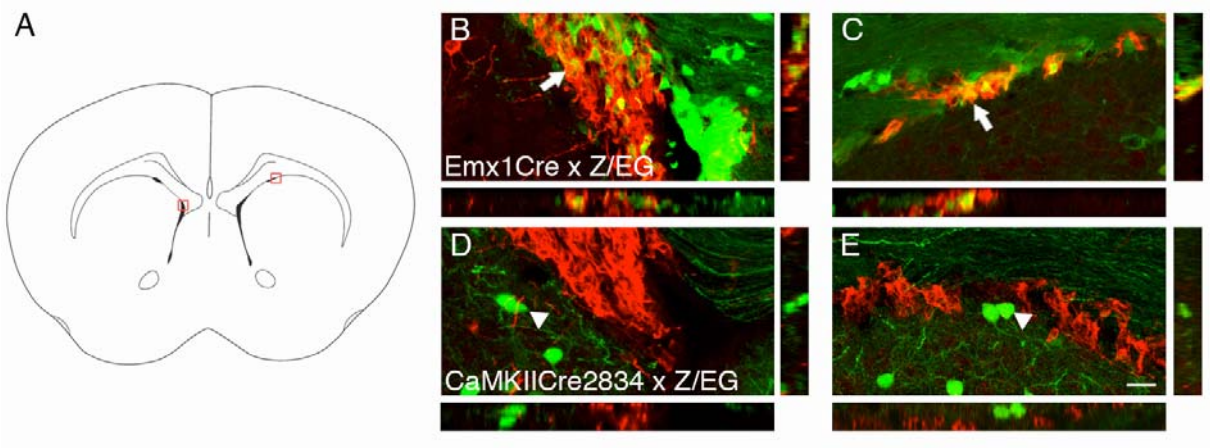

Figure 3.5 Emx1Cre- and CaMKIICre2834-mediated recombination in the subventricular zone

Coronal brain sections through the lateral ventricles of six week-old Emx1Cre x Z/EG (**B-C**) and CaMKIICre2834 x Z/EG mice (**D-E**) were stained with antibody for DCX (red) and analyzed for colocalization with Cre-induced EGFP (green) by confocal microscopy. **A,** Schematic drawing indicating the location (red squares) of medial (B, D) and dorsal (C, E) sections of the periventricular zone. DCX/GFP-double positive neurons were evident in the subventricular zone of Emx1Cre x Z/EG mice (B, C, arrows) but absent in corresponding sections of CaMKIICre2834 x Z/EG mice (D, E). Images include orthogonal views of X-Z and Y-Z planes to confirm colocalization of GFP and DCX in sections of Emx1Cre x Z/EG mice. Once again, GFP-positive cells are more abundant in Emx1Cre x Z/EG than CaMKIICre2834 x Z/EG mice, consistent with Cre-mediated recombination extending to glia in Emx1Cre x Z/EG but not in CaMKIICre2834 x Z/EG mice. Scale bar, 20 μm.

3.2 Mapping the brain substrates of anxiety

3.2.1 Standardization for behavioral analysis

Standardization of behavioral testing methodology is paramount towards establishing gene x environment interactions. Individual differences in husbandry, testing conditions or even experimenters themselves can affect the outcomes of an experiment (Wahlsten et al., 2003). While the $\gamma 2^{+/-}$ mouse has been extensively characterized and shows the behavioral hallmarks of General Anxiety Disorder (GAD; Crestani et al., 1999) as a proof of concept it was necessary to replicate these experiments in separate lab locations. Dimensions and materials for testing apparatuses as well as husbandry conditions were matched as closely as possible to those used in the previous work conducted at the University of Zurich (Crestani et al., 1999). Early experiments suggested that background stress levels masked the effects of genotype differences with respect to behavior (data not shown). To account for this, extensive precautions were taken regarding animal husbandry to limit stressful conditions not only in adults but also during postnatal development (see Materials and Methods). Because fighting stress associated with establishing of a social hierarchy in each cage was seen in early tests with male mice (data not shown) and anxiety disorders are more prevalent in women than in men, analysis was performed exclusively on female mice. Mice heterozygous for the $\gamma 2$ subunit were compared to wt littermates at 8 weeks of age on the Free Choice Exploration (FCE) test (Figure 3.6). As expected, $\gamma 2^{+/-}$ mice made more retractions before entering a novel unit and spent less time in the novel compartment compared to wt controls. There was no difference in genotype for familiar units visited indicating the deficit was not due to locomotor deficits. Thus, replication of earlier experiments showing enhanced neophobia due to deficits in $GABA_A$Rs not only demonstrates standardization across laboratories but also the reliability of phenotype.

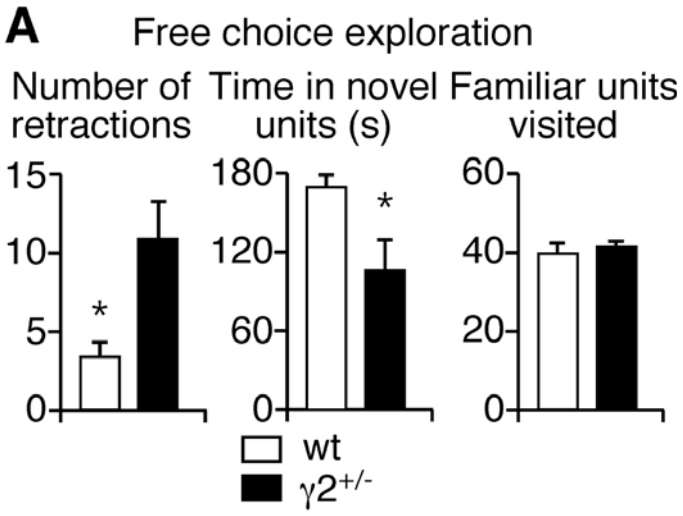

Figure 3.6 Enhanced neophobia in $\gamma 2^{+/-}$ mice can be replicated

When tested in the Free Choice Exploration test, $\gamma 2^{+/-}$ mice (n = 14) spent less time in the novel units (U = 51, * P < 0.05, Mann-Whitney) and showed more retractions when entering novel units than wt littermate controls (n = 16) during 5 min following the removal of the partition (U = 53, * P < 0.05). However, the mice showed normal locomotion as indicated by the unchanged number of familiar units visited during the test period. Data represent means ± SEM.

3.2.2 Forebrain deletion of $GABA_A$Rs leads to enhanced neophobia

In order to determine the behavioral effects of forebrain glutamatergic cell-specific deficits in $GABA_A$Rs, mice were tested for their reactivity to novel and adverse environments. Emx1Cre x fγ2/+ mice and fγ2/+ littermate controls (8 weeks of age) were tested in the FCE, Light Dark Choice (LDC), and Elevated Plus Maze (EPM) tests (Crestani et al., 1999). Compared to fγ2/+ littermates, Emx1Cre x fγ2/+ appeared neophobic similar to global $\gamma 2^{+/-}$ mice (see above and Crestani et al., 1999). In the FCE test, Emx1Cre x fγ2/+ mice made more retractions before entering a novel unit and spent less time in the novel compartment compared to fγ2/+ controls (Figure 3.7A). In the LDC test, the mean time of exposure to the lit area was diminished (Figure 3.7B). Furthermore, in the EPM test, Emx1Cre x fγ2/+ mice made fewer entries into the open arms compared to controls (Figure 3.7C). Once again the lack of genotype-related differences in the mean number of familiar units visited in the FCE test, transitions in the LDC test and closed arm entries in the EPM indicates a retained overall interest for the test situation and unaltered levels of locomotion of Emx1Cre x fγ2/+ mice. Consistent with normal expression of the γ2 subunit from the fγ2 locus, anxiety-related behavior of fγ2/+ mice was indistinguishable from that of wt (+/+) and Emx1Cre mice, as indicated by unchanged behavior in the FCE and LDC tests (Figure 3.8). Thus, a forebrain-specific deficit in $GABA_A$Rs that is most pronounced in the striatum, hippocampus (CA1 and DG) and neocortex induced selectively in the Emx1 lineage of cells is sufficient to reproduce the behavioral responses characteristic of trait anxiety previously seen in global $\gamma 2^{+/-}$ mice.

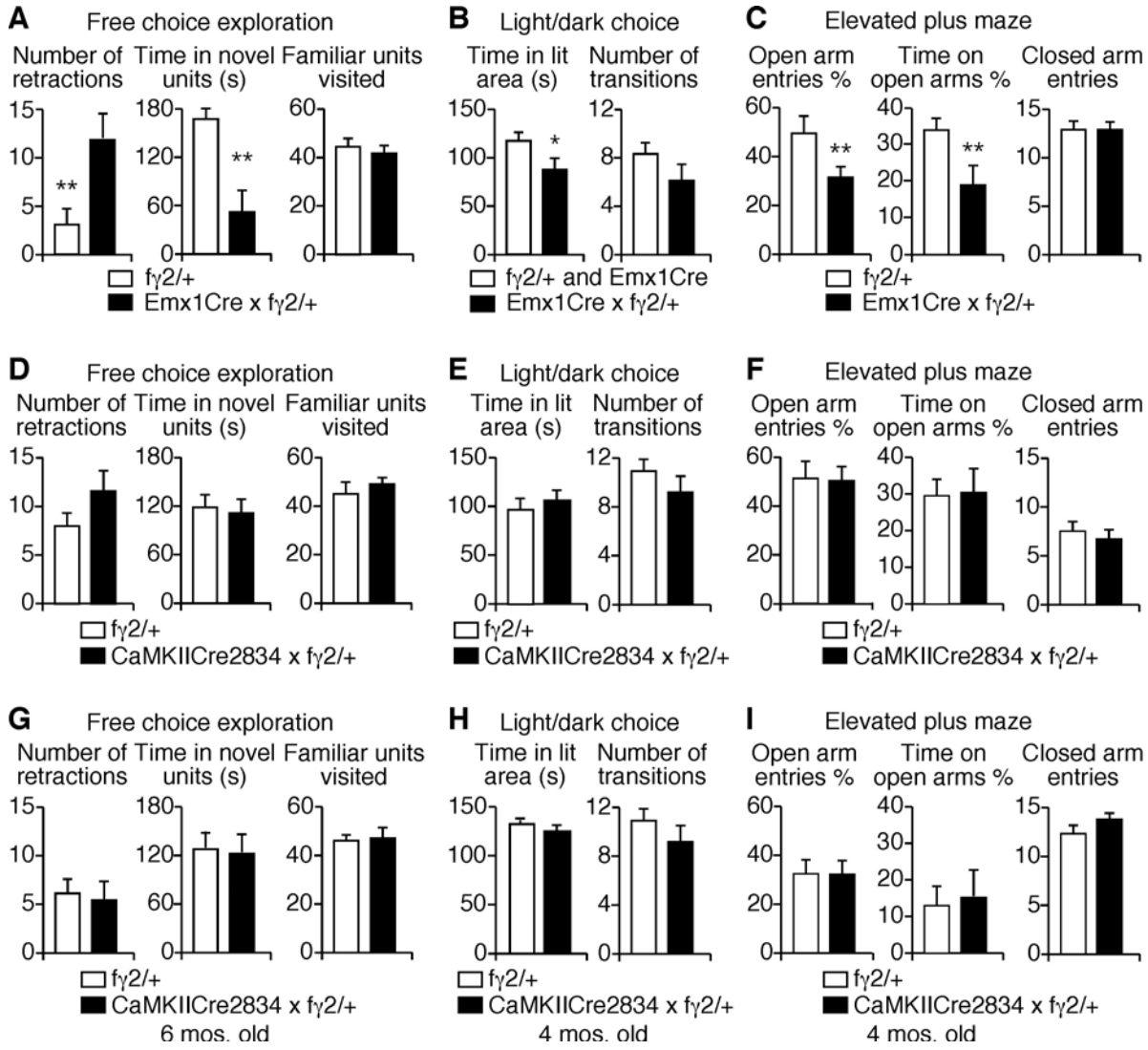

Figure 3.7 $GABA_A$Rs act during development to control trait anxiety

A-C. Heightened anxiety-related behavior of Emx1Cre x fγ2/+ mice. In the free choice exploration test (A), Emx1Cre x fγ2/+ mice made more retractions from entering a novel unit following the removal of the partition ($U = 10.5$, $P < 0.01$, n = 8-10 per genotype) and, once they entered, they spent less time in the novel compartment ($U = 8$, $P < 0.01$) than fγ2/+ littermate controls. The mean number of familiar units visited was similar in the two groups ($U = 28.5$, ns). In the light-dark choice test (B), the mean time spent in the lit area was significantly lower in Emx1Cre x fγ2/+ (n = 9) compared to fγ2/+ littermate control mice (n = 22) ($P < 0.05$, Least Significant Difference test after unweighted mean genotype x area ANOVA with area as within subject factor, $F(1, 29) = 4.0$, $P = 0.05$). The group difference was not significant for the mean number of light-dark transitions [$t(13.17) = 1.8$, $P < 0.1$, t-test with separate variances]. In the elevated plus-maze (C), the mean

proportion of both entries and time spent on the open arms over 5 min were decreased in Emx1Cre x fγ2/+ compared to littermate control mice (U = 19.5 and 20, respectively, P < 0.01, n = 10-14 per genotype). **D-F**. The anxiety-related behavior of CaMKIICre2834 x fγ2/+ mice and littermate fγ2/+ controls was tested under the same conditions. In the free choice exploration test (D), CaMKIICre2834 x fγ2/+ (n = 22) mice behaved as controls (n = 30) with respect to mean number of retractions [t(36.99) = 1.5] and mean time spent in the novel compartment [t(45.58) = 0.3]. In the light-dark choice test (E), CaMKIICre2834 x fγ2/+ mice were indistinguishable from controls for the mean time spent in the lit area and the mean number of light-dark transitions (U > 34, n = 10 per genotype). In the elevated plus-maze (F), CaMKIICre2834 x fγ2/+ mice did not differ from controls with respect to the mean proportion of entries and time spent on the open arms and the number of closed arm entries [t(34) ≤ 0.7, n = 18 per genotype]. All experiments were performed with females reared in group-housed cages. Data are representative of two to four experiments each. Values shown represent group means ± SEM. **G-I,** The anxiety-related behaviour of 4-6-months-old CaMKIICre2834 x fγ2/+ mice was assessed as in (D-F). In the free choice exploration test (G), six months-old CaMKIICre2834 x fγ2/+ (n = 9) were indistinguishable from fγ2/+ littermate controls (n = 16) with respect to the number of retractions from entering a novel unit, the total time spent in the novel units, and the number of familiar units visited (for the three variables, U > 60). Behavior in the light-dark choice test (H) of CaMKIICre2834 x fγ2/+ mice (n = 7) assessed at 4 months of age mice did not differ from fγ2/+ (n = 21) with respect to both the total time spent in the lit area and the number of light-dark transitions (U > 45). Similarly, behavior in the elevated plus-maze (I) of four month-old CaMKIICre2834 x fγ2/+ mice (n = 7) was indistinguishable from fγ2/+ littermates (n = 21) with respect to the number of open and closed arm entries and time spent on the open arms (U > 38). Data represent means ± SEM.

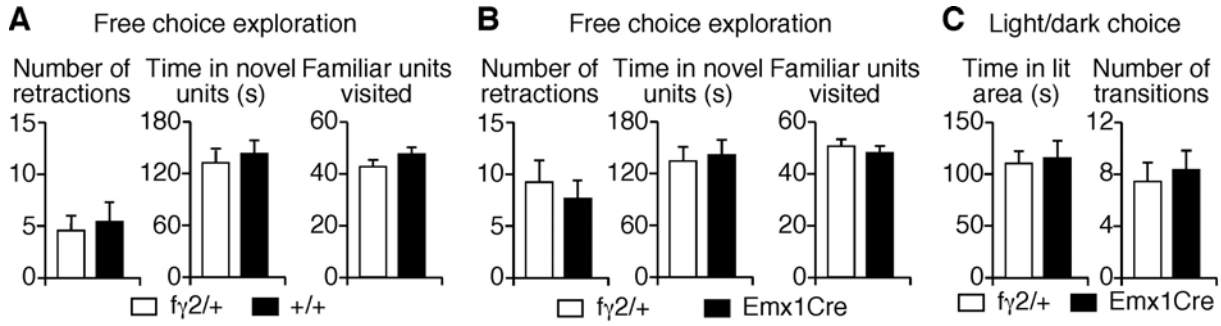

Figure 3.8 Normal anxiety-related behavior seen in pseudo-wt mice

A. In the free choice exploration test fγ2/+ mice were indistinguishable from wt littermates (+/+) with respect to the number of retractions from entering a novel unit, the total time spent in the novel units, and the number of familiar units visited [for the three variables, $t(36) < 2.00$, Student t-test, $n = 19$ per group]. **B**. In the same test Emx1Cre mice were indistinguishable from fγ2/+ littermates [for the three variables, $t(36) < 1.00$, $n = 18$-20]. **C**. In the light-dark choice test, Emx1Cre mice did not differ from fγ2/+ with respect to total time spent in the lit area and the number of light-dark transitions [$t(37) < 1.00$, $n = 19$-20]. Data represent means ± SEM.

At ten weeks of age, CaMKIICre2834 x fγ2/+ mice exhibit a regional $GABA_AR$ deficit similar to that of Emx1Cre x fγ2/+ mice. However, recombination induced by CaMKIICre2834 is delayed from the first detection at P17 to maximal adult levels by the fifth postnatal week (Schweizer et al., 2003). This developmentally delayed deficit in $GABA_ARs$ was not associated with an anxiety-like phenotype as indicated by the absence of a behavioral difference between CaMKIICre2834 x fγ2 mice and fγ2/+ controls analyzed at eight weeks of age in FCE, LDC or EPM (Figure 3.7D-F). To determine if the anxiety-like phenotype is dependant on the specific developmental time point for onset of $GABA_AR$ deficit and not on the overall length of exposure to deficit anxiety-related behavior of CaMKIICre2834 x fγ2/+ mice at four to six months of age was assessed. Similar to results obtained with mice tested at eight weeks of age, these older CaMKIICre2834 x fγ2/+ mice were indistinguishable from fγ2/+ littermates in the FCE, LDC and EPM tests (Figure 3.7G-I). The data suggest that the anxiety-related behavior of CaMKIICre2834 x fγ2/+ mice is insensitive to adult-specific $GABA_AR$ deficits and stable throughout the lifespan of the animal.

3.3 $GABA_AR$ deficit results in heightened behavioral inhibition in behavioral assays used to determine antidepressant efficacy

Anxiety disorders and major depressive disorders not only have a high comorbidity but also share in contributing genetic factors (Chambers et al., 2004; Kessler et al., 2005). It is hypothesisized that since a developmental deficit in the γ2 subunit gives rise to an anxiety like phenotype it might also lead to behavioral characteristics of depression. However, it is impossible to know if a rodent is actually 'feeling' depressed. Therefore in order to assess depression-like behavior rodents are tested for a clinically relevant drug response in specific paradigms that elevate stress levels or mediate so-called 'learned helplessness' (Cryan et al., 2005c). The forced swim (FS) test (Lucki, 1997; Dulawa et al., 2004) was used to assess both of these behaviors in $\gamma2^{+/-}$, Emx1Cre x fγ2/+ and CaMKIICre2834 x fγ2/+ mice. Nine-week-old global $\gamma2^{+/-}$ mice more rapidly developed an immobile posture compared to

wt as indicated by the reduced mean time spent swimming until the first immobility episode. In addition $\gamma 2^{+/-}$ mice spent more time immobile over the duration of the test compared to wt (Figure 3.9A). Emx1Cre x fγ2/+ were indistinguishable from controls with respect to time spent to first immobility but accumulated more time in an immobile posture than controls, similar to global $\gamma 2^{+/-}$ mice (Figure 3.9B). In contrast, CaMKIICre2834 x fγ2/+ mice were indistinguishable from fγ2/+ littermate controls (Figure 3.9C). This decreased transition time from active (swimming) to passive (floating) response indicates that developmental deficits in $GABA_A$Rs increase behavioral inhibition in pharmacological models of depression-like behavior (Cryan et al., 2005b).

The same mice were further tested in the NSF paradigm, a conflict-based test that elicits competing motivations; the drive to eat and the fear of entering an open/novel and therefore aversive area. Latency to begin eating in this test is sensitive to chronic but not acute antidepressant drug treatments as well as anxiolytic drugs (Suranyi-Cadotte et al., 1990; Santarelli et al., 2003). Both global $\gamma 2^{+/-}$ and Emx1Cre x fγ2/+ mice exhibited a longer delay in initiating feeding when forced into a novel environment than the respective control mice, whereas CaMKIICre2834 x fγ2/+ mice did not differ from controls (Figure 3.9D-F). The data indicate that a global or Emx1 cell lineage-specific $GABA_A$R deficit that is present throughout development, but not a $GABA_A$R deficit induced in brain of adolescent mice, results in greater avoidance responses to stress, reminiscent of a depressive-like behavior.

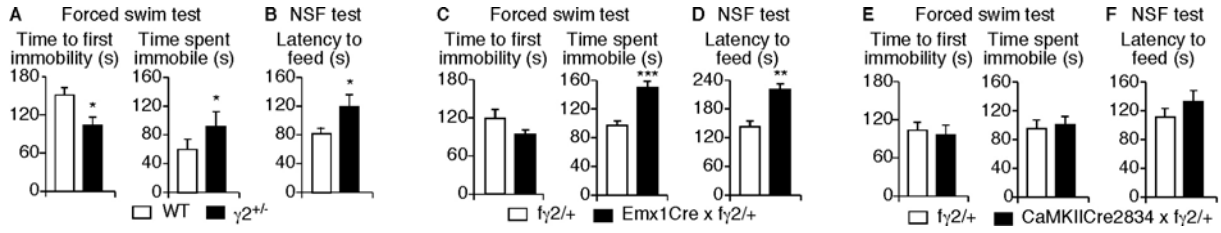

Figure 3.9 A developmental deficit in γ2 subunit-containing $GABA_A$Rs results in behavior associated with a depressive-like behavioral state

The three different mouse lines ($\gamma2^{+/-}$, Emx1Cre x fγ2/+, and CaMKIICre2834 x fγ2/+) together with their respective littermate controls (wt and fγ2/+ mice, respectively) were subjected to the modified forced swim (Lucki, 1997) (**A, C and E**) and novelty-suppressed feeding (Santarelli et al., 2003) **(B, D and F**) tests. A. In the forced swim test, $\gamma2^{+/-}$ mice started floating significantly earlier and spent more time immobile than wt controls (U = 13.0 and 20.0, respectively, $P < 0.05$, n = 9 per genotype). C. Tested under the same conditions, Emx1Cre x fγ2/+ mice did not differ significantly from fγ2/+ with respect to mean time to first immobility (U = 26) but exhibited an increase in the mean time spent immobile compared to controls (U = 2.5, n = 6-14, $P < 0.001$). E. CaMKIICre2834 x fγ2/+ mice were indistinguishable from fγ2/+ littermates for both parameters. B. In the novelty-suppressed feeding test, $\gamma2^{+/-}$ mice showed a heightened mean latency to initiate feeding compared to wt (U = 19, $P < 0.05$, n = 9 per genotype). D. Likewise the mean latency to initiate feeding was significantly increased in Emx1Cre x fγ2/+ compared to controls (U = 3.0, n = 6-14, $P < 0.001$). F. In the novelty-suppressed feeding test, CaMKIICre2834 x fγ2/+ mice were indistinguishable from fγ2/+ littermate controls (U > 33, n = 8-12 per genotype). Values represent group means ± SEM.

3.4 $GABA_AR$ deficit interaction with 5-$HT_{1A}R$ deficits

$GABA_AR$ deficits due to heterozygous expression of the $\gamma 2$ subunit result in an anxiety and depression-like phenotype similar to homozygous deletion of the 5-$HT_{1A}R$ (Heisler et al., 1998; Parks et al., 1998; Ramboz et al., 1998; Gross et al., 2000). Similarly, the later phenotype is mediated by a forebrain specfic deletion of 5-$HT_{1A}R$ during early postnatal development (Gross et al., 2002). These similarities in the development-dependent behavioral consequences of the $GABA_AR$ $\gamma 2^{+/-}$ and $5\text{–}HT_{1A}R^{-/-}$ deficits suggest possible functional interactions between the forebrain GABAergic system and postsynaptic 5-$HT_{1A}Rs$. To determine whether the $\gamma 2^{+/-}$ $GABA_AR$ deficit results in reduced expression of 5-$HT_{1A}Rs$, binding levels of the 1A subtype-specific agonist [^{3}H] 8-hydoxy-*N,N*-dipropyl-2-amino-1,2,3,4-tetrahydronaphthalene were compared in $\gamma 2^{+/-}$ and wt mice (n≥10 mice per genotype unless noted; Watanabe et al., 1993; Li et al., 2000). Brain regions were selected based on three factors. First, most of the brain regions assayed have been shown to play a role in emotional behavior. Second, the neocortex, CA1, CA3 and DG regions of the hippocampus, thalamus, striatum and globus paladus showed a reduction in BZ binding site containing $GABA_AR$ in the $\gamma 2^{+/-}$ mice (Crestani et al., 1999). By contrast the cerebellum and interpeduncular nucleus of the $\gamma 2^{+/-}$ mice did not show deficits in $GABA_AR$ and were quantified as possible control. Finally, the raphe nucleus and substanitia nigra were selected based on their importance in the serotonergic system (see Introduction) and the robust expression of 5-$HT_{1A}Rs$ seen by autoradiographs. Surprisingly, there was no difference in 5-$HT_{1A}R$ agonist binding in any brain region analyzed: neocortex (students t-test, t = 0.84), CA1 (t = 0.62), CA3 (t = 0.27), dentate gyrus (*U* = 63.0), striatum (t = 0.26), thalamus (t = 0.41), amygdala (t = 0.57), globus pallidus (t = 1.29), interpeduncular nucleus (t = 0.24), raphe nucleus (t = 1.56), substantia nigra (t = 0.55) and cerebellum (n = 4 per genotype, *U* = 3.0, Figure 3.10). Thus, anxiety and depression-like behavior seen in $\gamma 2^{+/-}$ mice is not mediated by changes in the expression of 5-$HT_{1A}Rs$ (see Discussion).

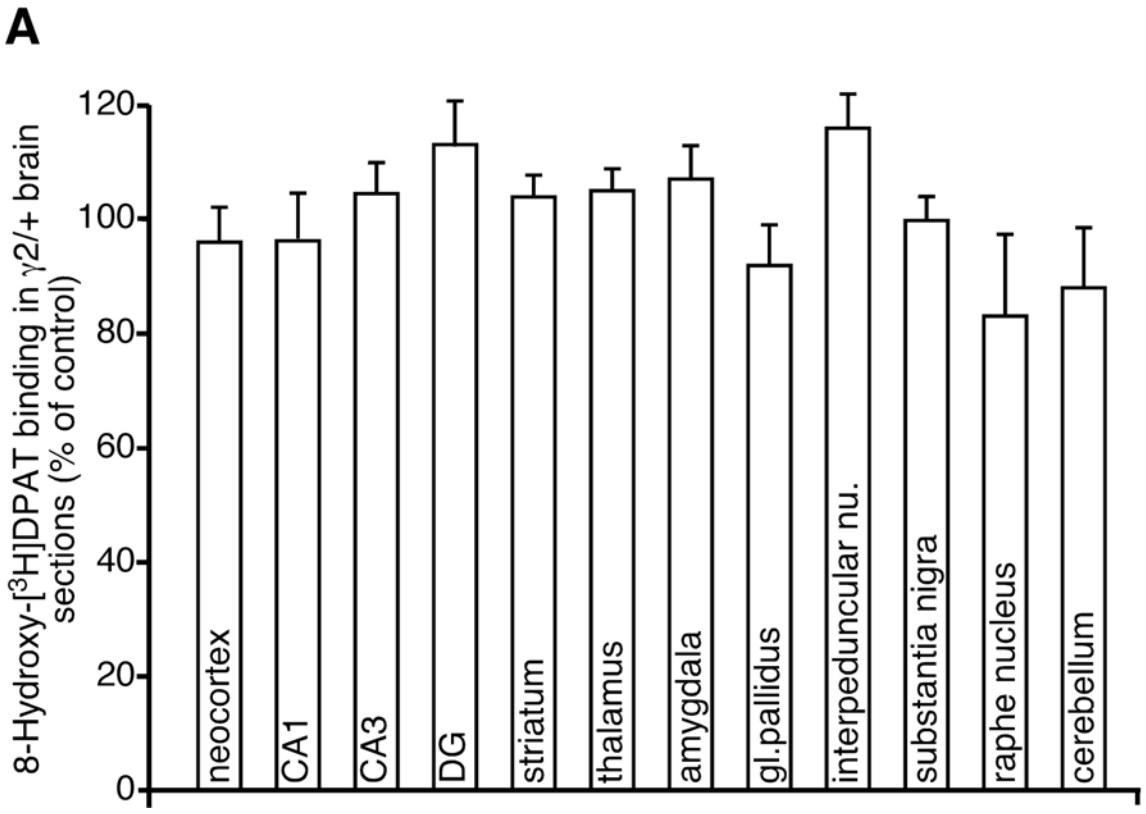

Figure 3.10 Quantification of 5-HT$_{1A}$R binding in $\gamma 2^{+/-}$ mice by [^{3}H] 8-OH-DPAT autoradiography and phosphoimage analysis

The density of [^{3}H] 8-OH-DPAT binding sites in $\gamma 2^{+/-}$ was unchanged compared to wt littermate controls in any brain region analyzed. The unchanged regions in $\gamma 2^{+/-}$ mice included; neocortex (93.5 ± 5.9% of wt) CA1 (93.7 ± 8.1%), CA3 (101.9 ± 5.3%), dentate gyrus (110.2 ± 7.5%), striatum (101.2 ± 3.7%), amygdala (104.3 ± 5.7%), interpeduncular nucleus (113.5 ± 5.5%), thalamus (102.0 ± 3.6%), globus pallidus (89.4 ± 7.4%), substantia nigra (97.3 ± 4.1%), raphe nucleus (80.9 ± 13.9%) and cerebellum (85.7 ± 10.3%, $U > 81$ in dentate gyrus, $U > 13$ in cerebellum and $t(22) \geq 0.24$ for all other values , ns, $n \geq 10$ per genotype with the exception of cerebellum where n = 4 per genotype). Results represent means ± SEM.

3.5 $GABA_AR$s control adult hippocampal neurogenesis

3.5.1 $GABA_AR$ deficit leads to reduced survival of adult-born neurons

Adult hippocampal neurogenesis has been implicated in the stress response, with increases in stress deceasing neurogenesis and antidepressant treatments causing increases in neurogenesis (Duman et al., 2001b; Duman et al., 2001a; Malberg and Duman, 2003; Duman, 2004). Furthermore, GABAergic transmission controls the proliferation of neural progenitor cells and the integration of immature neurons into established neural networks (Liu et al., 2005; Tozuka et al., 2005; Ge et al., 2006). In order to determine whether heterozygous deletion of the γ2 subunit affects the survival of newly born adult hippocampal neurons, mice were injected with the thymidine analog 5-bromo, 2-deoxyuridine (BrdU) at 8 weeks of age and transcardially perfused 4 weeks later. There was no significant reduction in the total number of BrdU positive cells in $\gamma2^{+/-}$ (532.8 ± 111.1) compared to wt mice (604 ± 103.3, *n* = 5 per genotype, *U* = 11, ns). However, the number of neurons per-hippocampus that were doubly positive for BrdU and the pan-neural marker NeuN was drastically reduced in $\gamma2^{+/-}$ (218.4 ± 34.0) compared to wt mice (398 ± 65.6, -35.4%, *U* = 3.5, *P* < 0.05) indicating a deficit in the differentiation and/or survival of adult-born hippocampal neurons (Figure 3.11B). Furthermore, comparison of Emx1Cre x fγ2/+ and littermate control mice revealed a deficit in BrdU/NeuN double positive cells (-41.5%) in Emx1Cre x fγ2/+ mice that was virtually identical to that of global $\gamma2^{+/-}$ mice (Emx1Cre x fγ2/+, 223.2 ± 51.7; fγ2/+, 381.6 ± 18.7, *n* = 5 per genotype, *U* = 2.0, *P* < 0.05). There was no change in the number of BrdU positive cells in Emx1Cre x fγ2/+ mice (Emx1Cre x fγ2/+, 528.0 ± 82.0; fγ2/+, 640.8 ± 42.6). However, the numbers of BrdU positive cells (926.4 ± 50.7) and BrdU/NeuN positive neurons (487.2 ± 43.7) in the dentate gyrus of CaMKIICre2834 x fγ2/+ mice were not different from littermate fγ2/+ controls (BrdU, 816.0 ± 60.7, *n* = 5 per genotype, *U* = 12.5, ns; BrdU/NeuN, 494.4 ± 25.0, *U* = 12.0, ns). Thus, mouse lines that show developmental $GABA_AR$ deficits in glutamatergic neurons of the forebrain show not only an anxious and depression-like phenotype but also show deficits in adult-born cells adopting neuronal fates in the dentate gyrus.

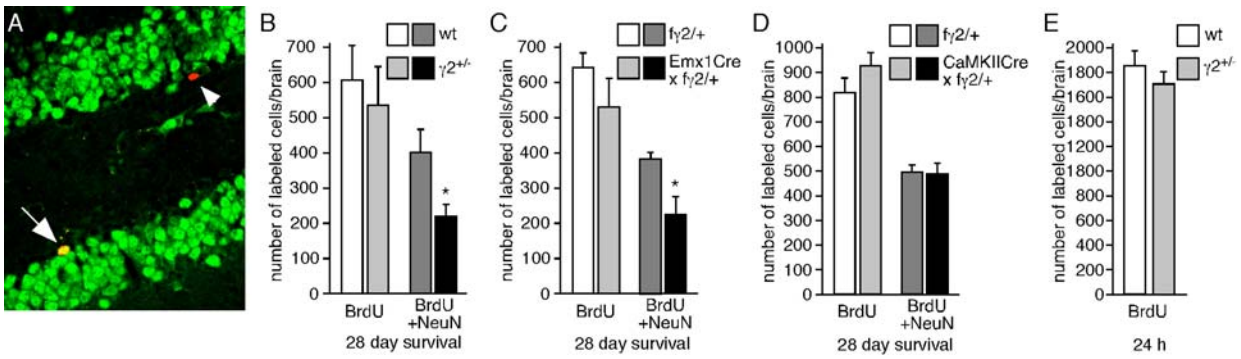

Figure 3.11 A developmental but not adult deficit in γ2 subunit-containing $GABA_A$Rs leads to a reduction in adult hippocampal neurogenesis

Hippocampal brain sections of mice labeled at 8 weeks of age and harvested 4 weeks later were stained using antibodies specific for BrdU (red) and the pan-neural marker NeuN (green). **A**. A representative confocal image of a section through the dentate gyrus shows a BrdU-positive neuron (arrow) and an adult-born non-neural cell (arrowhead). **B-D**. Quantification of total BrdU-positive cells and BrdU/NeuN doubly positive neurons in the subgranule and granule cell layer of serial hippocampal sections of $\gamma2^{+/-}$ (A), Emx1Cre x fγ2/+ (B), and CaMKIICre2834 x fγ2/+ (C) mice compared to littermate controls (n = 5-6 mice per genotype in each experiment) revealed a profound reduction in BrdU/NeuN doubly positive neurons relative to littermate controls in $\gamma2^{+/-}$ (54.9 ± 8.5% of wt, U = 3.5, P < 0.05) and Emx1Cre x fγ2 mice (58.5 ± 13.5% of fγ2/+, U = 2.0, P < 0.05), which suffer from a $GABA_A$R deficit throughout brain development. In contrast, the proportion of adult-born neurons in the hippocampus of CaMKIICre2834 x fγ2 mice was not different from that of control littermates (U = 12.0, s). The number of total BrdU-positive cells did not differ significantly from controls in any of the mutant mouse lines analyzed ($\gamma2^{+/-}$, 88.2 ± 18.4% of wt; Emx1Cre x fγ2/+, 82.4 ± 12.8% of fγ2/+ and CaMKIICre2834 x fγ2/+, 113.5 ± 6.2% of fγ2/+; U > 6.5 for all three groups). **D**. The number of proliferating cells in the subgranule and granule cell layers of mice labeled with BrdU 24 h before harvesting of the brain was not different in $\gamma2^{+/-}$ compared to wt mice ($\gamma2^{+/-}$, 92.2 ± 5.1% of wt, n = 5 per genotype, U = 9.5, ns). Data indicate means ± SEM.

3.5.2 Adult Neuronal Proliferation Unaffected by $GABA_AR$s

Deficits seen in adult-born mature neurons in mice with subtle $GABA_AR$ deficits raise questions about when these cells are lost. Unaltered levels of BrdU labeled cells 4 weeks after BrdU injection in both $\gamma2^{+/-}$ and Emx1Cre x fγ2/+ compard to controls suggested that proliferation might be unaffected in these mouse lines. To directly compare the proliferation of replicating hippocampal cells of $\gamma2^{+/-}$ and wt littermates, the mice were injected with a single large dose of BrdU and the brains were harvested 24 h later. Unaltered numbers of BrdU labeled cells in the SGZ of the DG of $\gamma2^{+/-}$ (1704 ± 94.9) compared to wt mice (1848 ± 125.0, *n* = 5 per genotype, *U* = 9.5, ns) indicate that deficits in $GABA_AR$s do not affect cell divisions of neural stem and progenitor cells in the hippocampus but most likely affect cell at subsequent postmitotic stages of differentiation into mature neurons and/or their integration into the neural network (Figure 3.11C).

3.6 $GABA_AR$ deficit results in a cell-specific deficit in replicating cells

To further determine the stage at which maturation deficits in $GABA_AR$s affect new neurons in the hippocampus, mice were injected with BrdU at three weeks of age. At this time point there are a greater population of replicating neural progenitors leading to a greater number of labeled cells, which facilitates subsequent characterization of adult-born cells. Once again mice were injected with high concentrations of BrdU (200 mg/kg) and brains were harvested 24 hours later and again there were unaltered numbers of BrdU labeled cells in the subgranular zone of the dentate gyrus in $\gamma2^{+/-}$ (3381.6 ± 331.5) compared to wt mice (3885.6 ± 260.9, n = 5 per genotype, *U* = 7.0, Figure 3.12D). In order to determine whether $\gamma2^{+/-}$ mice showed deficits in early neuronal maturation BrdU colocalization with the immature neuronal marker doublecortin (DCX) was examined. DCX is expressed selectively in postmitotic immature neurons within a day and for maximally 4-weeks after the last cell division (Brown et al., 2003), Mice were injected with the metabolically with BrdU (4 times every 2h, 80mg/kg) and brains were harvested 48h later. Again there was no difference in the number of DCX/BrdU double positive cells between $\gamma2^{+/-}$ (4442.0

± 519.5) and wt mice (4015.2 ± 227.0, n ≥ 5 per genotype, *U* = 13.0, Figure 3.12E). The elevated numbers of BrdU positive cells in brains harvested at 48 hours compared to those harvested at 24h reflect the difference in the injection time course used for medium and long-term (4 injections of 80 mg/kg 2h apart) versus short-term survival (1 injection of 200 mg/kg). Multiple injections labeled cells born over an 8h period while a single injection labeled cells in a narrow time window only. Finally, 3-week-old $\gamma 2^{+/-}$ mice injected with BrdU for a long-term (4 week) survival paradigm showed deficits in BrdU/NeuN cells reminiscent of 8-week-old $\gamma 2^{+/-}$ mice (Figure 3.12F, $\gamma 2^{+/-}$ = 1248.0 ± 123.8, wt = 1779 ± 134.2, n = 4 per genotype, P = 0.028, *U* = 0.0). This indicates that $\gamma 2^{+/-}$ mice develop a deficit in newly born neurons after they proliferate as they mature and form postsynaptic connections (see Discussion). Therefore, $GABA_A$Rs are necessary for the long-term survival of adult-born neurons in the SGZ.

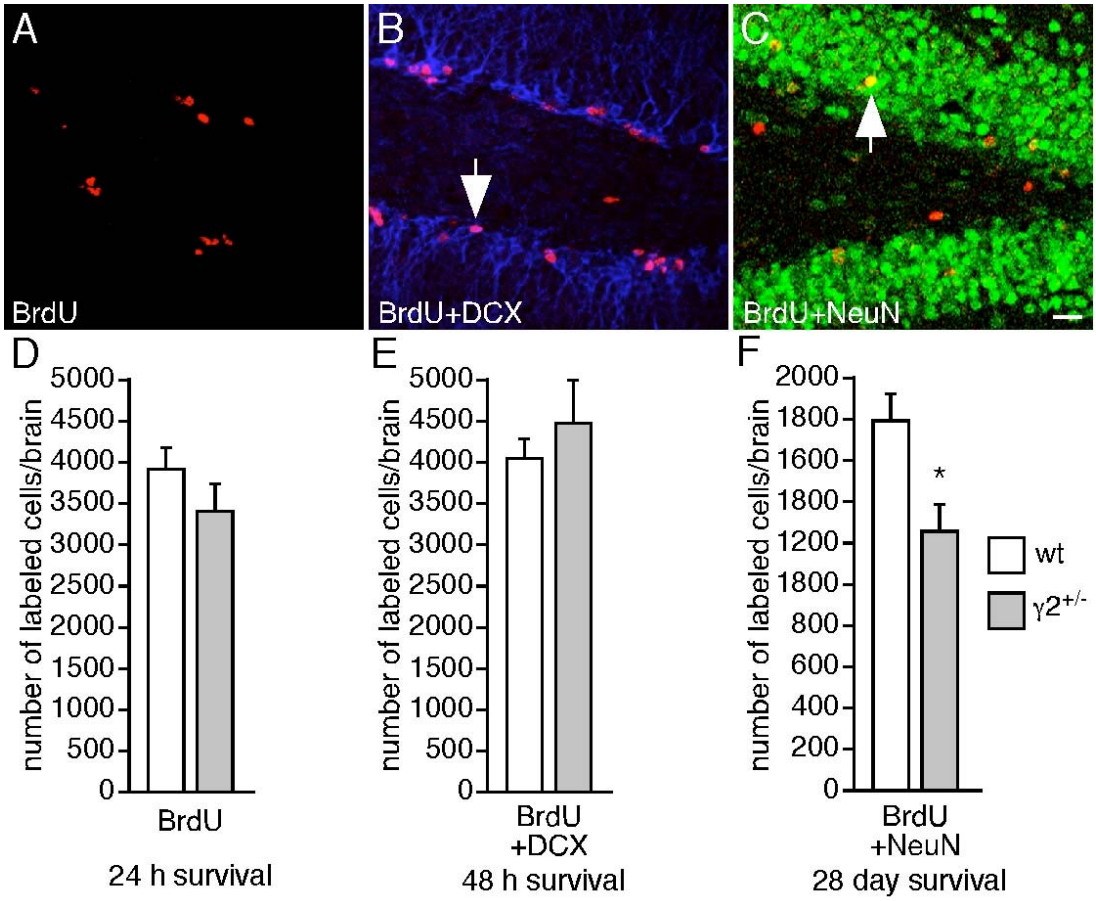

Figure 3.12 Quantification of BrdU positive cells in the SGZ of the DG using three different survival paradigms

An immature neuronal deficit in $GABA_AR$ results in deficits in maturation of adolescent born neurons. **A-C**. Representative images showing BrdU labeled brain sections from three week old mice euthanized 24h (A), 48h (B) and 28d (C) after BrdU injection. Arrow indicates a neuron that is doubly positive for BrdU in combination with the immature neuronal marker DCX (B) or the mature neuronal marken NeuN (C). Scale bar, 20μm. **D**. To determine the number of proliferating cells P21 mice were injected with once a high concentration BrdU (200m mg/kg) and brains were harvested after 24 h. There was no significant difference in the number of BrdU positive cells of $\gamma2^{+/-}$ mice compared to wt littermate controls (87.03 ± 8.5% of wt, n = 5 per genotype, U = 7.0, ns). **E**. To label immature neurons, progenitor cells of P21 mice were metabolically labeled (4 times 80 mg/kg every 2h) and brains were harvested after 48h. There was no difference in BrdU/DCX positive cells in $\gamma2^{+/-}$ micecompared to wt control (104.85 ± 5.5% of wt, n = 6 per genotype, U = 13, ns) **F**. By comparison there was a significant deficit in BedU/NeuN positive cells of P21 $\gamma2^{+/-}$ mice labeled under the same conditions and sacrificed at 28d (70.15 ± 7.0% of wt; n = 4 per genotpye, $P < 0.05$, U = 0). Data indicate means ± SEM.

Chapter 4 Discussion

4.1 Overview of findings

Work presented here was designed to elucidate molecular and cellular substrates necessary for the development of normal emotionality in the mouse model. Towards this end, the Cre-loxP system was employed to limit expression of the γ2 subunit of $GABA_A$Rs in glutamatergic neurons of the forebrain. Emx1Cre x fγ2/+ mice exhibit Cre-mediated recombination in immature neurons of the embryonic and adult brain while in CaMKIICre2834 x fγ2/+ mice recombination becomes first evident in three-week-old mice selectively in mature neurons (This Thesis and Gorski et al., 2002; Schweizer et al., 2003; Iwasato et al., 2004). It was shown that a developmental $GABA_A$R deficit in forebrain glutamatergic neurons results in enhanced neophobia. This is reminiscent of an anxiety-like phenotype seen in global $\gamma2^{+/-}$ mice. However, a developmentally delayed $GABA_A$R deficit was without behavioral effect. Furthermore, the manifestation of the anxiety-like phenotype due to developmental deficits in $GABA_A$Rs are associated with increased behavioral inhibition in situations known to be sensitive to antidepressant drug treatment. This same developmental $GABA_A$R deficit leads to a deficit in adult hippocampal neurogenesis, specifically, the maturation of adult-born neurons in the SGZ of the DG. Heretozygous inactivation of the γ2 subunit of $GABA_A$Rs did not result in detectable deficits in 5-HT_{1A}Rs suggesting that deficits seen in both neurogenesis and behavior due to a modest reduction of $GABA_A$Rs occurs independently of detectable changes in the expression of 5-HT_{1A}Rs.

4.2 Cre-mediated recombination of an fγ2 allele in the forebrain

When either an Emx1Cre or a CaMKIICre2834 mouse is mated to a mouse that is homozygous for the floxed γ2 allele, the resultant Cre-expressing fγ2/+ offspring exhibit modest deficits in BZ binding of $GABA_A$Rs in the forebrain (Figure 3.1). In adult mice this deficit in $GABA_A$Rs is comparable, regardless of which Cre-expressing mouse line is used. By comparison, $\gamma 2^{+/-}$ mouse brains show the greatest reduction of BZ binding in the frontal cortex (–23%), CA1 region of the hippocampus (–35%), and the thalamus (–30%) and more moderate decreases in the striatum (–6%), globus pallidus (–13%), DG (–15%) and the amygdala (Crestani et al., 1999). Although regional reductions of BZ binding in the CA1 and cortex of conditional and global γ2 subunit knockout mice are comparable, normal BZ binding seen in the thalamus and globus paladus of conditional γ2 knockout mice indicate a more forebrain specific $GABA_A$R deficit. However, Cre-mediated recombination leads to an unexpectedly high reduction of BZ binding sites in the striatum of CaMKIICre2834 x fγ2/+ and Emx1Cre x fγ2/+ mice. Reductions in BZ binding in the striatum of these conditional knockout mice might not be due to loss of gene function in the striatum but instead indicate downregulation of $GABA_A$Rs in the striatum due to an imbalance of neural activity that is unique to conditional γ2 subunit heterozygous mice. Generally, changes in expression of BZ binding sites in $\gamma 2^{+/-}$ mice are likely to reflect a combination of loss of gene function, reduced neurogenesis, and adaptive changes in transcription, translation and stability of receptor subunit(s), which may be subject to regulation by regional alterations in GABAergic activity. The observed reduction of BZ binding sites in the striatum, which was much less pronounced in global $\gamma 2^{+/-}$ mice (Crestani et al., 1999) is likely to be the result of such an adaptation.

4.3 Cell-type-specific recombination in Cre-transgenic lines

4.3.1 The γ2 subunit is necessary for postsynaptic localization of $GABA_A$Rs in neurons

Phasic and tonic inhibition correlate with functionally and structurally different subtypes of $GABA_A$Rs at postsynaptic and extrasynaptic sites respectively (Fritschy and Brunig, 2003; Mody and Pearce, 2004). The γ2 subunit of $GABA_A$Rs is essential for BZ-binding of $GABA_A$Rs, normal $GABA_A$R channel function, and normal formation of postsynaptic receptor clusters during synaptogenesis (Günther et al., 1995; Essrich et al., 1998; Crestani et al., 1999; Lorez et al., 2000). Multiple experimental approaches suggest that the vast majority of postsynaptic $GABA_A$Rs contain a γ2 subunit that is essential for the localization of α1 and α2 receptors during synaptogenesis (Benke et al., 1996; Essrich et al., 1998; Luscher and Keller, 2004). Thus, the importance of the γ2 subunit for normal neuronal function cannot be underestimated. There are also multiple lines of evidence that astrocytes respond to GABA via BZ sensitive $GABA_A$Rs, indicating that astrocytes express γ1, γ2 or γ3 subunits (MacVicar et al., 1989; Fraser et al., 1995; Israel et al., 2003). Although the γ2 subunit could be expressed at very low levels or in other non-neuronal cell types, immunofluorescence and RTPCR did not detect it's expression in cultured astrocytes (Figure 3.2). Along with *in situ* hybridization experiments, this strongly supports the notion that the γ2 subunit is absent in astrocytes and selectively expressed in neurons. CaMKIICre2834 expressed in fγ2/fγ2 mice resulted in a distinct loss of postsynaptic $GABA_A$Rs on dendrites of pyramidal cells of the forebrain (Schweizer, 2003; Schweizer et al., 2003). Thus, based on the expression pattern of Emx1Cre, CaMKIICre2834, and the γ2 subunit, Cre-induced recombination of an fγ2 allele results in a functional deficits in postsynaptic $GABA_A$Rs in neurons of the forebrain.

4.3.2 Recombination does not occur in PAV positive interneurons

In both Emx1Cre x Z/EG and CaMKIICre2834 x Z/EG mice, Cre-mediated expression of EGFP is absent in PAV-expressing cells (Figure 3.3), a major population of GABAergic interneurons including multiple types of basket and axo-axonic cells (Kosaka et al., 1987; Markram et al., 2004). PAV is calcium binding protein that is expressed in approximately half of all interneurons including most if not all fast spiking interneurons, a cell type indispensable for basic cortical processing (Figure 1.1; Chow et al., 1999; Freund, 2003; Markram et al., 2004). It cannot be ruled out at present that Cre-recombinase is expressed in other populations of non-glutamatergic neurons of Emx1Cre x Z/EG mice, given and the plurality of interneuron subtypes (Markram et al., 2004). However, as mentioned above, the expression pattern of αCaMKII is limited to glutamatergic neurons and the expression pattern of Emx1 is limited to glutamatergic neurons and glia cells (Benson et al., 1992; Jones et al., 1994; Gorski et al., 2002; Iwasato et al., 2004). Therefore, Cre-mediated recombination seen here is consistent with published results suggesting that recombination of an fγ2 allele is limited to glutamatergic neurons in both Emx1Cre x fγ2/+ and CaMKIICre2834 x fγ2/+ mice.

4.3.3 Cre-Mediated recombination in immature neurons of the SVZ suggest that Cre is expressed in glutamatergic neurons

As mentioned in the introduction, in the adult brain neurogenesis is limited to the SGZ of the DG and the SVZ of lateral ventricles. The majority of DCX positive cells in the DG also expressed EGFP in Emx1Cre x Z/EG mice and although recombination in immature neurons extended to newly replicating cells in the SVZ, Cre-mediated recombination did not occur in the vast majority of DCX positive cells in the rostral migratory stream (Figures 3.4 and 3.5). Most of the cells migrating to the olfactory bulbs along the rostral migratory stream are glomerular and granule inhibitory interneurons (Ge et al., 2006; Lledo et al., 2006). EGFP/DCX positive cells in the rostral migratory stream of Emx1Cre x Z/EG mice exhibit non-neural morphology and most likely represent glial cells types, supporting the idea that

recombination of the γ2 subunit in Emx1Cre x fγ2/+ mice occurs in glutamatergic cells.

4.3.4 Differential developmental specificity of Cre-recombination used to assess the functionality of $GABA_AR$s

The first three weeks of postnatal mouse development represent a critical period for the formation of many neuronal circuits (Knudsen, 2004). Conditional inactivation of the γ2 subunit gene using the CaMKIICre2834 and Emx1Cre lines results in comparable forebrain-specific $GABA_AR$ deficits. However, whereas Emx1Cre x fγ2/+ suffer from a deficit already in the embryo and throughout postnatal development, the receptor deficit of CaMKIICre2834 x fγ2/+ mice is delayed to after the critical postnatal period of brain maturation. At the cellular level, the deficit in Emx1Cre x fγ2/+ neurons precedes synapse formation, whereas CaMKIICre2834 x fγ2/+ neurons exhibit a partial loss of the γ2 subunit only after synaptogenesis is already completed. The γ2 subunit is necessary for the formation of fast inhibitory GABAergic synapses (Essrich et al., 1998; Crestani et al., 1999; Li et al., 2005b). Thus, a loss of the γ2 subunit during development in immature neurons of Emx1Cre x fγ2/+ mice is expected to alter synaptogenesis during critical periods of neuronal circuit formation. In contrast, CaMKIICre2834 x fγ2/+ mice are expected to show a $GABA_AR$ deficit only after the initial formation of neuronal circuits. Whereas homozygous deletion of the γ2 subunit indicates that this subunit is required for the maintenance of $GABA_AR$ localization at synapses (Schweizer et al., 2003), heterozygous expression of the γ2 subunit results in a much more subtle loss of inhibitory function. Global $\gamma2^{+/-}$ mice do show at most a 28% reduction in postsynaptic $GABA_AR$ clusters and reduced single channel conductance, but unaltered numbers of $GABA_ARs$ based on measurements of GABA binding sites (Günther et al., 1995; Essrich et al., 1998; Crestani et al., 1999; Lorez et al., 2000). The detrimental behavioral and neurogenic consequences of this deficit during development illustrate the importance of normal $GABA_AR$ expression for neural circuit formation and neurogenesis. Conversely, unaltered behavior and neurogenesis in response to $GABA_AR$ deficit in fully differentiated

neurons in the adolescent brain illustrates the stability and inertness to insult of existing circuits and neurons in the mature brain.

4.4 GABAergic control over neuronal differentiation and integration

4.4.1 Deficits in neurogenesis are due to changes in synaptic GABAergic transmission

It is well established that GABAergic transmission controls neuronal proliferation in the embryo (LoTurco et al., 1995; Haydar et al., 2000). In addition, electrophysiological and pharmacological evidence indicate that GABAergic input via $GABA_A$Rs controls the rate by which new neurons mature and integrate into established neural networks (Liu et al., 2005; Tozuka et al., 2005; Ge et al., 2006; Ge et al., 2007). The role of GABAergic depolarizing currents in neuronal proliferation (Tozuka et al., 2005) lead to the prediction that embryonic deficits in $\gamma 2$ subunit-containing $GABA_A$Rs in global $\gamma 2^{+/-}$ and Emx1Cre x f$\gamma 2$/+ mice might affect the proliferation of neural progenitor cells. However, there was no significant change in the number of proliferating cells in the DG of $\gamma 2^{+/-}$ mice. This suggests mitosis of progenitor cells was not affected and deficits in $\gamma 2$ subunit containing $GABA_A$Rs resulted in impaired maturation and/or differentiation of new cells after their initial division. In agreement with unaltered proliferation of neuronal progenitor cells, $\gamma 2^{-/-}$ mice are born without overt developmental brain abnormalities and the $\gamma 2$ subunit is largely dispensable for proper expression of non-synaptic receptors (Günther et al., 1995). However, the $\gamma 2^{-/-}$ mice die shortly after birth, once the $\gamma 2$ subunit becomes essential for GABAergic synapse formation (Essrich et al., 1998; Li et al., 2005a). Therefore more subtle developmental deficits in $GABA_A$Rs seen in $\gamma 2^{+/-}$ and Emx1Cre x f$\gamma 2$/+ mice are predicted to primarily or exclusively affect late stages of differentiation, at a time when GABAergic input to immature granule cells switches from slow excitation to fast inhibition, and these neurons are expected to integrate into neural networks (Overstreet Wadiche et al., 2005; Song et al., 2005; Lledo et al., 2006; Overstreet-Wadiche et al., 2006; Overstreet-Wadiche and Westbrook, 2006).

4.4.2 $GABA_AR$ deficit leads to a deficit in neuronal maturation

Newborn neurons in the adult brain, as in neonates, follow a stereotypical integration process: first receiving tonic GABA activation, then GABA mediated phasic, synaptic inputs and finally glutamate-mediated synaptic inputs (Ben-Ari, 2002; Owens and Kriegstein, 2002; Wang et al., 2002; Liu et al., 2005; Wadiche and Jahr, 2005; Ge et al., 2006). Newborn neurons extend axonal projections along the mossy fiber pathway to their target CA3 pyramidal cell layer within 4 to 10 days after division (Hastings and Gould, 1999) while within 2 weeks their dendrites grow in the opposite direction to the molecular layer where they increase in complexity over months (van Praag et al., 2002). Only after neurons form connections with their target cells do they start firing action potentials, most likely to avoid defective activation of existing cognitive processes in mature networks (Ming and Song, 2005). During the initial 48 hours following cell division, there is no deficit in newborn neurons in the dentate gyrus of $\gamma 2^{+/-}$ mice. At this point DCX/BrdU positive cells are committed to a neuronal fate (Brown et al., 2003), have started migration to target brain regions (des Portes et al., 1998; Gleeson et al., 1998), and are depolarized by nonsynaptic GABAergic transmission (Ben-Ari, 2002; Liu et al., 2005). Targeted mutations, which cause GABA to have hyperpolarizing rather than depolarizing effects on DCX-positive immature neurons, result in deficits in dendritic development and delayed synapse formation (Ge et al., 2006). In contrast, proliferating neurons in the adult DG of $\gamma 2^{+/-}$ mice matured normally through initial stages of migration and there was no reduction in DCX-BrdU positive cells compared to wt. Thus, the $\gamma 2$ subunit is not only dispensable for neuronal proliferation (Günther et al., 1995) but it is most likely not necessary for initial stages of cell fate specification. Therefore, the reduction in BrdU/NeuN positive cells is not likely to be due to an increase in cells adopting non-neuronal fates. Future experiments using different BrdU lbeling paradeigms and other types of Cre-epressing mouselines will determine at what stage during maturation of adult-born neurons the $\gamma 2$ subunit is essential for GABAergic synaptic formation and innervation (see Overview; (Essrich et al., 1998; Li et al., 2005a; Fang et al., 2006).

4.5 Establishing the neuronal substrates of behavioral inhibition

4.5.1 Early prenatal period is critical for establishing normal emotionality

Neuronal circuits are shaped by experience during early postnatal life (Knudsen, 2004). Control of the timing, duration, and closure of heightened levels of brain plasticity in early life affect the formation of mature circuits for the visual, motor, and auditory system and even multimodal functions such as communication (birdsong and human language) and emotionality (reviewed in Hensch, 2004). Genetic mutation and promoter polymorphism in the serotonergic system as well as maternal effects on the HPA axis and GABAergic system all reflect heightened sensitivity of developing brain systems and circuits in establishing emotionality (Gross et al., 2002; Caldji et al., 2003; Caspi et al., 2003; Caldji et al., 2004; Weaver et al., 2004). Here, moderate early postnatal stress associated with animal husbandry affected neophobia in adults and utmost care was taken to ensure environmental stability before weaning animals to be used in behavioral tests (data not shown, see materials and methods for husbandry conditions). Thus, practical experience predicts that the first three weeks of postnatal life are critical to establish normal anxiety and depression-like behavior.

4.5.2 Developmental deficit in $GABA_A$Rs leads to altered emotionality

Using conditional $\gamma 2$ knockout mice, it was shown that a developmental $GABA_A$R deficit in forebrain glutamatergic neurons results in enhanced neophobia reminiscent of anxiety-like phenotype seen in global $\gamma 2^{+/-}$ mice (Figure 3.7). The same developmental deficit lead to enhanced behavioral inhibition reminiscent of pharmacological models of depression (Figure 3.8). Conversely, a developmentally delayed $GABA_A$R deficit was without behavioral effect independent of testing at eight weeks or four to six months of age. This indicates that lack of behavioral consequences in mice that suffered a delayed $GABA_A$R deficit was not simply due to insufficient time for deficit to infect neuronal circuits. Furthermore, the behavioral effects seen in $\gamma 2^{+/-}$ and Emx1Cre x fγ2/+ suggest that $GABA_A$R deficit in immature neurons during the first three weeks of development alters the formation of circuits

involved in mood state. As discussed in the introduction, mice that are deficient GAD65 exhibit a delay in the critical period that determines ocular dominance (Hensch et al., 1998). A developmental deficit in postsynaptic $GABA_AR$s could alter the formation of a functional synapse by interfering with the switch from GABA mediated tonic excitation to phasic inhibition. Alternatively, deficits in synaptic inhibition at early stages in neuronal development could affect the pruning of unnecessary neuronal connections associated with a critical period (reviewed in Hensch, 2004), which then could lead hyperactive circuitry in the forebrain and downstream targets. In either case, the developmental reduction in $GABA_AR$ seen in $\gamma2^{+/-}$ and Emx1Cre x fγ2/+ mice results in an alteration in a critical period for the establishment for normal emotionality similar to that seen due to a developmental deletion of postsynaptic 5-$HT_{1A}R$ (Gross et al., 2002; Leonardo and Hen, 2006).

4.5.3 $GABA_AR$ deficit could affect mossy fiber pathway

Alterations in behavioral neuronal circuitry could be a more direct effect of $GABA_AR$ deficits in maturing granular cells. Unlike pyramidal neurons, which contain $\alpha2\beta\gamma2$ subunit-containing $GABA_AR$s on dendrites, soma and axon initial segment, granule cells contain similar or identical $GABA_AR$s at mossy fiber terminals (Bergersen et al., 2003). Tonic inhibition by ambient GABA via these receptors has been proposed to inhibit action potential-dependent neurotransmitter release of mossy fiber terminals onto CA3 pyramidal cells (Ruiz et al., 2003). Behaviorally, mossy fiber-CA3 synapses are believed to hold a gatekeeper function for information flow from the dentate gyrus to the CA3-CA1 region of the hippocampus and to play a role in the memory of sequences of events (Nakazawa et al., 2003). $GABA_AR$-deficient hyperactive mossy fiber-CA3 synapses might explain facilitated trace conditioning of $\gamma2^{+/-}$ mice (Crestani et al., 1999). Although it remains to be seen whether mossy fiber-CA3 synapses are functionally impaired in $\gamma2^{+/-}$ mice, it is possible that generation of this population of neurons is reduced. Compared to their embryonic counterparts, adult-born granular cells have a prolonged period of maturation, integration into neural networks and synaptogenesis, suggesting that they may be especially vulnerable to $\gamma2$ subunit deficits (Overstreet Wadiche et al.,

2005; Ge et al., 2006; Overstreet-Wadiche et al., 2006). Thus, $GABA_AR$ deficit in Emx1Cre x fγ2/+ and $\gamma2^{+/-}$ could affect mossy fiber granular cells by altering their connectivity, limiting their maturation in neurogenesis or both. Deficits in this specific granular cell population could lead to the cognitive deficits seen in $\gamma2^{+/-}$ mice (Crestani et al., 1999) and even to the heightened anxiety-like behavior presented here (Becker and Wojtowicz, 2007).

4.6 Behavioral deficits seen in $\gamma2^{+/-}$ and Emx1Cre x fγ2/+ are the result of $GABA_AR$ reduction

Heightened anxiety and depression like behavior seen in $\gamma2^{+/-}$ and Emx1Cre x fγ2/+ may depend $GABA_AR$ deficits exclusively during development. It has long been known that the majority of synaptic formation as well as HPA axis formation occur during the first few weeks after birth (Crain et al., 1973; Sapolsky and Meaney, 1986; Kendell et al., 2005). Furthermore, GABA modulates or is modulated by almost all neurotransmitters or neuromodulators in the CNS including extensive interrelation with other modulators of mood state including noradrenergic, dopaminergic and serotonegic transmission (Brambilla et al., 2003). Moreover, a developmental $GABA_AR$ deficit affects the balance of transmission during the establishment mature circuits involved with emotionality. Due to extensive crosstalk between the GABAergic and serotonergic system (Sibille et al., 2000; Allain et al., 2005; Li et al., 2005b), developmental deficits in $GABA_ARs$ are predicted to alter anxiety and depression-like behavior in part through effects on of 5-HT receptors. However, there was surprisingly no change of $5\text{-}HT_{1A}R$ levels in $\gamma2^{+/-}$ mice (Figure 3.9). Receptor autoradiography in whole unfixed brain sections using [^{3}H] 8 OH-DPAT detects expression of both intracellular and extracellular receptors. It is likely that only functional extracellular $5\text{-}HT_{1A}R$ espression is altered by the subtle forebrain specific $GABA_AR$ deficit. Such differences are likely to be beyond the level of detection of these autoradiographs. Alternatively, not all monoaminergic (including other subtypes of 5-HT receptors) receptors are addressed here leaving open the option that these might be more dramatically affected. Nevertheless, if the changes in to $5\text{-}HT_{1A}R$ are this small, then it could suggest that the mood related effects seen

in $\gamma 2^{+/-}$ and Emx1Cre x fγ2/+ mice are due primarily to $GABA_AR$ deficits. More experiments with different serotonin receptor subtypes and even other neural systems (noradrenergic system) will be needed to determine if this is the case.

4.7 Measuring enhanced behavioral inhibition as a component of anxiety and mood disorders

4.7.1 Neophobia in $\gamma 2^{+/-}$ mice is a replicable measure of altered emotionality

It is unrealistic if not impossible to measure all of the aspects of human anxiety and mood disorders in the mouse model due to the complexity of the human brain. Additionally, advancement in understanding of the brains complexity results in an ever-changing array of symptoms associated with mood disorder. However, well-defined and measurable components that make up more complex disorders can be linked to distal genes and are of some use when dissecting complex phenotypes (Gottesman and Gould, 2003; Hasler et al., 2004). These components are coherent with the human disorder and in the context of affective disorders range from failure to suppress cortisol levels after dexamethasone challenge to increased 'neuroticism' (Leonardo and Hen, 2006). Most importantly these simple components have been shown to be reproducible across labs making them reliable for the mapping of genetic effects (Wahlsten et al., 2003). In both Switzerland (Crestani et al., 1999) and now here in this Thesis in the United States enhanced neophobia seen in the $\gamma 2^{+/-}$ mouse is a replicable component of anxiety (Figure 3.6). The robust effect is shown to be independent of lab setting or experimenter indicative of the reliability of $\gamma 2^{+/-}$ mice as a model of chronic trait anxiety (Lister, 1990; Crestani et al., 1999; McNaughton, 1999).

4.7.2 Forebrain specific deficits in both anxiety and depression due to $GABA_AR$ deficits reflect trait anxiety

$GABA_AR$ reduction in the forebrain of global and conditional $\gamma 2$ subunit knockout mice is most pronounced in brain regions known to be affected by anxiety and mood disorders in the human population. Support for an essential role of the hippocampus

in anxiety-like behavior include (but are not limited to) several lines of evidence. First, anxiety-like behavior is associated with alterations of hippocampal firing in humans (Gray and McNaughton, 2000). Second, alterations in anxiety-like behavior can be induced by ventral hippocampal lesions (Kjelstrup et al., 2002; Bannerman et al., 2003; Bannerman et al., 2004). Finally, forebrain-specific deletion of 5-HT_{1A}R (Gross et al., 2002), or corticotropin releasing receptor hormone-1 (CRH1R) deletion (Muller et al., 2003) lead to heightened anxiety-like behavior. Thus, the conclusion that forebrain-specific reduction of $\gamma 2$-containing $GABA_A$Rs leads to anxiety-like behavior is not unexpected given that the deficit is very prominent in the CA1 region and DG of the hippocampus.

As mentioned in the Introduction, the cortex and the hippocampus are affected in human populations suffering from GAD and depression (Tiihonen et al., 1997; Phillips et al., 2003). Interestingly, phenotypic-factor analyses to examine co-morbidity of internalizing disorders indicates that 'anxiety-misery' factor based GAD and major depression more strongly correlate with each other than they do with 'fear' factor based panic and phobia disorders (Krueger, 1999; Vollebergh et al., 2001). In support of selective alterations in anxiety-related but not simple fear-related neural circuits, $\gamma 2^{+/-}$ mice interpret partial fear stimuli to be as threatening as a fully conditioning fear stimulus in an ambiguous cue discrimination test and retain negative associations to a fear stimulus in 1-s trace conditioning, but they do not show increased fear responses in simple delay conditioning or context conditioning (Crestani et al., 1999). Furthermore, both Emx1Cre x f$\gamma 2$/+ and $\gamma 2^{+/-}$ mice show increased risk assessment behavior and increased neophobia in the free choice exploration test that is devoid of intrinsic stress as well as increased behavioral inhibition in forced swim test and novelty suppressed feeding, which are sensitive to the effects of antidepressants (Figures 3.7 and 3.8). Thus, developmental reduction of $GABA_A$Rs results in a condition that reflects 'anxiety-misery' and trait anxiety or neuroticism in humans. Interestingly, although genetic risk factors indexed by the personality trait anxiety contribute substantially to risk for both major depression and GAD, much of the genetic covariance between the two disorders are independent of

trait anxiety (Kendler et al., 2006). In addition, genetic risk factors not shared with neuroticism are important for major depression and GAD (Hettema et al., 2006). This suggests that Emx1Cre x fγ2/+ and $\gamma 2^{+/-}$ mice represent a pharmacological model of trait anxiety for a large yet specific human population.

4.7.3 Developmental effects of $GABA_AR$ deficit indicative of trait anxiety

Increased neuroticism during childhood represents a life long risk factor for the development of pathological anxiety disorders, depressive disorders or both (Chambers et al., 2004; Hettema et al., 2006; Kendler et al., 2006). Both the behavioral phenotype seen in Emx1Cre x fγ2/+ and $\gamma 2^{+/-}$ mice, and the behavioral inhibition seen in human anxiety disorders (Chambers et al., 2004) are dependant on alterations during central nervous system development to mediate an effect in the adult. Patients suffering from depression and anxiety disorders show atrophy or loss of neurons in the prefrontal cortex and hippocampus (Gurvits et al., 1996; Sheline et al., 1996; Shah et al., 1998). This is particularly severe in those patients who have suffered an early childhood trauma (Vythilingam et al., 2002). Likewise, Emx1Cre x fγ2/+ and $\gamma 2^{+/-}$ mice show deficits in the maturation of adult-born neurons in the SGZ of the hippocampus. Although there is not an overt difference in the size of Emx1Cre x fγ2/+ and $\gamma 2^{+/-}$ brains, future experiments will determine if the reduction in neurogenesis seen in these mice results in a more subtle change in hippocampal or cortical volume due to a reduction of the number of mature neurons in each of these brain structures. It is interesting to note, however, that 5-$HT_{1A}R^{-/-}$ mice do not show deficits in neurogenesis further implicating them as models of state anxiety. Therefore, 5-HT_{1A}R deficits alone are insufficient to reproduce all of the behavioral and neurogenesis deficits associated with GAD and major depression suggesting that the serotonergic system acts in concert with $GABA_A$Rs to produce a phenotype.

4.7.4 $GABA_AR$ deficit-induced changes in behavior could be caused by maturational deficits during development or adulthood

Deficits in neurogenesis and deficits in behavior seen in the $\gamma 2^{+/-}$ and Emx1Cre x fγ2/+ mice have in common that they are dependant on a developmental deficit in $GABA_ARs$. In both mouse lines $\gamma 2$ subunit containing $GABA_ARs$ are limited during the development of the organism as well the development of individual neurons as they migrate and incorporate into the DG. The $GABA_AR$ deficit of the CaMKIICre2834 x fγ2/+ mice was comparable in magnitude and regional distribution to that of the Emx1Cre x fγ2/+ mice but was limited to mature neurons that had already undergone synapse formation (Schweizer et al., 2003). Enhanced behavioral inhibition could be the result of a $GABA_AR$ deficit specifically during the first three weeks of postnatal development or the result of a receptor deficit specifically in immature neurons. Future experiments in which inducible recombination is limited to progenitor cells will help to resolve this issue.

4.8 Deficits in neurogenesis are coincident with behavioral deficits

Deficits in neurogenesis are implicated in the etiology of mood disorders based on the neurogenesis-promoting effect of diverse antidepressant therapies, the detrimental effect of stress on neurogenesis, and the hippocampal atrophy that is notable in depressed patients (Duman, 2004). These results raise the question whether the deficits in neurogenesis of Emx1Cre x fγ2/+ and $\gamma 2^{+/-}$ mice are causal for the behavioral changes observed or if they are simply a side effect seen in relation to heightened behavioral inhibition. Studies in rodents showing that early life stress induced in pups by maternal deprivation results in reduced expression of $GABA_ARs$ and increased anxiety related behavior in adulthood (Caldji et al., 2000, 2003; Caldji et al., 2004), as well as reduced neurogenesis (Mirescu et al., 2004) provide evidence that there is a functional link between neurogenesis and normal emotionality. However, the inhibitory effect of stress on neurogenesis is due to reduced proliferation rather than maturation of adult-born neurons (Gould et al., 1998; Malberg and Duman, 2003). Nevertheless, the evidence presented here

suggests that developmental deficits in $GABA_A$Rs are not merely an inconsequential side effect of stress but instead may represent an important step in a succession of events that link early life stress to heightened anxiety and emotional behavior and deficits in neurogenesis in adulthood.

The association between neuronal proliferation and changes in behavior appear to be only part of the picture. Elimination of adult neuronal proliferation by irradiation blocks the affects of antidepressants, however, it does not result in enhanced behavioral inhibition in and of itself (Santarelli et al., 2003). Deficits in neural proliferation induced in adult rats by drug-induced serotonergic denervation or serotonin depletion are insufficient to alter emotional behavior (Henn and Vollmayr, 2004; Rosenbrock et al., 2005; Ueda et al., 2005), suggesting that impaired neurogenesis alone is not causal for altered behavior. However, given the generally low fraction of adult-generated neurons that survive to maturity even in control animals of the later studies, it is unclear whether reduced proliferation of progenitors has resulted in reduced production of adult-born mature neurons. The slow onset of therapeutic efficacy of antidepressants parallels this long timescale of maturation adult-born neurons. By comparison, a developmental reduction of $GABA_A$Rs results in a deficit in the maturation adult-born neurons that is coincident with heightened behavioral inhibition associated with anxiety and depression-like behavior. Normal emotionality therefore, could be dependant the maturation and integration of new neurons into the hippocampus and independent of proliferation in particular. However, antidepressants increase neuronal cell death as well as neurogenesis suggesting that antidepressant action may be dependant on neuronal turnover and not neurogenesis per se (Sairanen et al., 2005). If this is the case than the balance between new neurons integrating into the hippocampus to replace older cells could maintain normal emotional circuitry.

4.9 Outlook

The present study demonstrates that induction of a subtle $GABA_AR$ deficit in immature forebrain glutamatergic neurons of the embryo results in deficits in the maturation of adult-born hippocampal neurons associated with heightened behavioral inhibition to naturally aversive stimuli. In contrast, a $GABA_AR$ deficit induced in mature glutamatergic neurons of the adult brain did not result in behavioral deficits or a reduction in neurogenesis.

Increased neophobia and behavioral inhibition in paradigms that are responsive to the effects of antidepressants indicate the $\gamma2^{+/-}$ mice represent an animal model negative emotionality, a personality trait that is common to both generalized anxiety disorder and major depression (Gamez et al., 2006). As a model of negative emotionality $\gamma2^{+/-}$ mice are expected to respond to the behavioral effects of antidepressants in addition to their demonstrated behavioral sensitivity to BZ (Crestani et al., 1999). As described in the Introduction, SSRIs are commonly used antidepressants, which are thought to act in part by reversing deficits in serotonergic neurotransmission by increasing 5-HT at the synapse. However, behavioral deficits seen in $\gamma2^{+/-}$ mice are not associated with a detectable deficit in $5\text{-}HT_{1A}R$. Furthermore, unpublished experiments from Beth Luellen, a postdoctoral researcher in Bernhard Luscher's lab, indicate that there was no reduction in 5-HT or serotonergic axons in $\gamma2^{+/-}$ mice. These data suggests there is at most a very limited reduction in serotonergic neurotransmission in $\gamma2^{+/-}$ mice. In addition, SSRIs are dependant on adult hippocampal neurogenesis for behavioral effect (Santarelli et al., 2003) and those effects may be mediated by increasing neuronal turnover (Sairanen et al., 2005). Therefore, $\gamma2^{+/-}$ mice are also not expected to be responsive to SSRIs due to a deficit in maturation of new neurons. Preliminary behavioral assessment indicated $\gamma2^{+/-}$ mice are responsive to oral treatment the tricyclic compound desipramine, but they are unresponsive to oral dosage with the SSRI fluoxetine (data not shown, oral dose 18 mg/kg fluoxetine and 120mg/kg desipramine, test performed by Qiuying Shen and Clint Earnheart). Future experiments will confirm

this behavioral data and delineate possible antidepressant-mediated effect on neurogenesis in $\gamma 2^{+/-}$ mice, but this preliminary data suggests deficits in $GABA_AR$ may be associated with treatment resistant forms of mood disorder.

Additionally, future experiments proposed here in which drug-inducible Cre-mediated recombination of the $\gamma 2$ subunit is limited to progenitor cells will address multiple interrelated questions. First, as mentioned above limitation of a $GABA_AR$ deficit to immature neuron of the adult forebrain above will determine if trait anxiety seen in $\gamma 2^{+/-}$ mice is the result of a deficit specifically during the first three weeks of postnatal development or a receptor deficit specifically in immature neurons. Second, it has been suggested that cognitive deficits in $\gamma 2^{+/-}$ mice is resultant from hippocampal and cortical hyperexcitability due to decreased inhibition via the hippocampal mossy fiber pathway. Mossy fiber granular cells are one of the few cell populations that continue to differentiate in the adult brain. Therefore, $GABA_AR$ deficit specifically in immature neurons of the adult brain should limit inhibition of the CA3 by mossy fiber cells, and if the hypothesis is true, result in cognitive deficits. Third, it has been proposed and discussed here and elsewhere that the first three weeks of postnatal life represent a critical period for the establishment of neuronal circuitry (Gross et al., 2002). As demonstrated using Emx1Cre x f$\gamma 2$/+ mice, subtle deficits in $GABA_AR$ during early postnatal development interfere with this critical period formation. Drug controlled inactivation of the $\gamma 2$ subunit, could narrow down the time window for critical period formation. Deficits in behavior and neuronal maturation due to developmental inactivation of the $\gamma 2$ subunit in forebrain glutamatergic neurons are likely to involve a complex interplay of multiple factors including all of those all mentioned above.

The results of this thesis suggest that a subtle deficit in $GABA_AR$ in immature neurons of the developing forebrain could serve as the common brain substrate for anxiety and depression-like disorders and deficits in adult neurogenesis. Future experiments, which limit $GABA_AR$ deficit even further or probe pharmacological consequences of $GABA_AR$ deficit will clarify brain substrates involved in not only

behavioral inhibition but also brain development. Characterization of these brain substrates using specifically regulated $GABA_A$ receptor deficits in the mouse model will give direction to future studies of the brain systems involved in generalized anxiety disorder and major depression in humans.

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