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INVESTIGATION OF THE BRAIN SUBSTRATES OF ANXIOUS DEPRESSION

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Abstract

Major depressive disorder (MDD) exhibits extensive comorbidity with anxiety and poorly predictable responsiveness to different types of antidepressant drugs. In particular, melancholic MDD shows great reductions in GABA, aberrant function of the hypothalamic-pituitary-adrenal (HPA) axis as well as characteristics of anxious depression.

Mice that are heterozygous for the γ2 subunit gene of $GABA_A$Rs ($γ2^{+/-}$) exhibit a modest functional deficit in $GABA_A$Rs, yet they have been shown to recapitulate behavioral, pharmacological, and cognitive alterations reminiscent of anxiety disorders. In addition, this phenotype of $γ2^{+/-}$ mice includes increased behavioral passivity under stressful conditions, as expected of an animal model of mood disorders. Conditional knockdown of the γ2 subunit in the embryonic telencephalon was shown to result in anxious-depressive behavior similar to that of $γ2^{+/-}$ mice that carry the mutation in the germ line. By contrast, a comparable forebrain-specific γ2 subunit deficit that was delayed to the fourth postnatal week and limited to mature neurons was without anxiety- or depressive-like consequences. These findings are consistent with evidence that the vulnerability for anxiety and mood disorders is established early in life, and that they represent developmental disorders.

In this thesis I embarked on further analyzing the brain substrate of anxious depression. In particular, I investigated putative HPA axis deficits and the behavioral and endocrine responsiveness of $γ2^{+/-}$ mice to treatment with serotonin (5-HT)- and norepinephrine (NE)-selective reuptake inhibitors. In addition, I investigated the critical developmental periods responsible for anxious depressive like behavior of $γ2^{+/-}$ mice in adulthood

We found that the baseline corticosterone concentration of adult γ2-deficient mice was elevated independent of whether the genetic lesion was induced during embryogenesis or delayed to adolescence. However, the manifestation of

anxious-depressive behavior in different γ2-deficient mouse lines was correlated with early onset HPA axis hyperactivity during postnatal development. Chronic but not subchronic treatment of $\gamma 2^{+/-}$ mice with fluoxetine or desipramine normalized the anxiety-like phenotype in the novelty suppressed feeding test. Moreover, desipramine had antidepressant-like effects in that it normalized HPA axis function and depression-related behavior of $\gamma 2^{+/-}$ mice in the forced swim, tail suspension, and sucrose consumption tests. By contrast, fluoxetine was ineffective as an antidepressant and failed to normalize HPA axis function. Collectively, these data indicate that developmental deficits in GABAergic inhibition may cause behavioral and endocrine abnormalities and selective antidepressant drug responsiveness indicative of anxious-depressive disorders such as melancholic depression, which are frequently characterized by HPA axis hyperactivity and greater efficacy of desipramine versus fluoxetine.

Towards elucidating the developmental substrate of pathological anxiety and depression, we first used a chemically inducible knockout strategy to irreversibly reduce the $GABA_AR$ γ2 subunit gene dosage at different time points of postnatal brain development. Second, we used potentiation of $GABA_AR$ function with diazepam in γ2-deficient and WT mice to disturb normal activity-dependent brain development during defined temporal windows (critical developmental periods). Reducing the function of $GABA_ARs$ by hemizygous inactivation of the γ2 gene at postnatal day (P)13/14 but not P27/28 was sufficient to induce anxious-depressive-like behavior in adulthood. Potentiation of $GABA_ARs$ by treatment of wild-type mice with diazepam from P10-21 and P29-35 had selective anxiogenic- and depressant-like effects, respectively on behavior in adulthood. In addition, diazepam treatment from P10-21 had antidepressant-like effects on behavior in adulthood independent of genotype. Collectively, the data indicate that anxiety- and depression-related behavioral traits of $\gamma 2^{+/-}$ mice are independently controlled by distinct postnatal critical periods that are sensitive to both genetic impairment and pharmacological potentiation of GABAergic transmission via γ2 subunit-containing $GABA_ARs$.

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List of Abbreviations

5-HT Serotonin

5-HT1AR Serotonin type-1A receptor

5-HTT Serotonin transporter

aBST Anterior bed nucleus of the stria terminalis

ACTH Cotricotropin (Adrenocorticotropic hormone)

BrdU Bromodeoxyuridine

BZ Benzodiazepine

CA1 CA1 region of the hippocampus

CA3 CA3 region of the hippocampus

CaMKII Calmodulin kinase II alpha gene

CB Cannabinoid

CNS Central nervous system

CCK cholecystokinin

Cort corticosteroid

CRF Corticotrophin releasing factor

CRH Corticotropin-releasing hormone

DCX Doublecortin

DES desipramine

DG Dentate gyrus region of the hippocampus

DSM-IV-TR Diagnostic and Statistical Manual of Mental Disorders IV-TR

DZP diazepam

EGFP Enhanced green fluorescence protein

Emx1 Empty spiracles gene

EPM Elevated plus maze test

FLX Fluoxetine

fMRI Functional magnetic resonance imaging

FST Forced swim test

GABA γ-aminobutyric acid

$GABA_AR$ GABA type-A receptor

$GABA_BR$ GABA type-B receptor

GAD Generalized Anxiety Disorder

GR Glucocorticoid receptor

GWAS Genome-wide association studies

HPA Hypothalamic-pituitary-adrenal axis

IPSC Inhibitory postsynaptic currents

KCC-2 Potassium-Chloride cotransporter type-2

LTD Long-term-depression

LTP Long-term-potentiation

MDD Major depressive disorder

mPFC Medial prefrontal cortex

MR Mineralcorticoid receptor

MRI Magnetic resonance imaging

MRS Magnetic resonance spectroscopy

NeuN Neuron-specific nuclear protein (Neuronal nuclei)

NKCC1 Na-K-Cl cotransporter

NSF Novelty Suppressed feeding test

OFT Open field test

PAV Parvalbumin

PET Positron emission tomography

PTSD Posttraumatic stress disorder

PVN Paraventricular nucleus of the hypothalamus

R26Y Rosa26-YFP transgenic Cre recombinase reporter mouse strain

SCT Sucrose consumption test

SPECT Single photon emission computed tomography

SSRI Selective serotonin reuptake inhibitor

TAM Tamoxifen

TCA Tricyclic antidepressant

TST Tail suspension test

WT Wildtype

Z/EG LacZ/ EGFP transgenic Cre recombinase reporter mouse strain

Chapter 1: Introduction

1.1 The concepts of major depressive disorders (MDD)

1.1.1 Overview of the demography and symptoms of MDD

The lifetime prevalence of MDD is estimated to be around 16.2-17.1% in the US population, with a median age of onset of 32 years (Kessler et al. 2003; Kessler et al. 2005). Females are twice as likely to be affected as males (Kuehner 2003). With an estimated annual cost of $83 billion in 2003 MDD ranks as the second leading disease burden in established market economies (World Health Organization. and ebrary Inc. 2001; Greenberg et al. 2003).

The diagnosis of MDD is largely symptom-based due to the absence of objective diagnostics such as biomarkers, biopsies, genetic variations and neuroimaging (reviewed by Krishnan and Nestler 2008). According to the Diagnostic and Statistical Manual of Mental Disorders IV-TR (DSM-IV-TR), a patient is diagnosed as suffering from MDD if five out of nine depression-related symptoms are present for at least two weeks (Table 1.1). Some symptoms even comprise opposing components, for example, depressed and irritable mood, insomnia and hypersomnia, weight loss and weight gain, etc. These criteria result in hundreds of possible symptom combinations. Therefore, individual MDD patients may have few if any symptom in common.

In DSM-IV-TR, MDD is further subdivided into five categories among which melancholic depression and atypical depression are the most common forms. Melancholic depression is characterized by the two core symptoms depressed mood and diminished interest or pleasure seeking (anhedonia). Indeed, patients suffering from melancholic depression or unable to experience positive moods even when good things happen. Moreover, melancholic depression also often comes with substantial weight loss, psychomotor retardation, or excessive feelings of guilt. The symptoms usually appear more severe in the mornings

(American Psychiatric Association. 2000). In addition, patients suffering from melancholic depression often show hyperactivity of hypothalamus-pituitary-adrenal (HPA) axis (Gold and Chrousos 1999). In contrast to melancholic depression, patients with atypical depression are able to experience improved mood in response to positive events and tend to show increased appetite and weight gain (American Psychiatric Association. 2000) that is associated with hypoactivity of the HPA axis (Gold and Chrousos 1999).

Table 1.1 DSM criteria for major depressive episode

DSM criteria for major depressive episode:
A. At least five of the following are present simultaneously for at least 2 weeks (symptom 1 or 2 is necessary): 1. Depressed or irritable mood 2. Markedly diminished interest or pleasure in all, or almost all, daily activities 3. Substantial weight loss or weight gain 4. Insomnia or hypersomnia nearly every day 5. Psychomotor agitation or retardation nearly every day 6. Fatigue or loss of energy nearly every day 7. Feelings of worthlessness or inappropriate guilt nearly every day 8. Diminished ability to think or concentrate nearly every day 9. Recurrent thoughts of death or suicide B. It cannot be established that an organic factor is the cause and the disturbance is not a normal reaction to the death of a loved one

Abstracted from DSM-IV-TR (American Psychiatric Association. 2000)

The symptoms of MDD severely tamper the patient's social interaction and productivity. Moreover, MDD patients suffer from suicidal ideations. The suicide rate of MDD patients is 20 times higher than in the rest of the population (Harris and Barraclough 1997). Aside from suicide, MDD is also comorbid with a number of other mental disorders as well as somatic illnesses such as cardiovascular disease, type 2 diabetes, cancer, etc. (Uzun et al. 2009).

1.1.2 Comorbidity of MDD and anxiety disorders

MDD shows high levels of comorbidity with anxiety disorders. Similar to the relationship between depressed mood and MDD, anxiety in its non-pathological form is a normal reaction to aversive life events and usually accompanied by defensive behavior and behavioral inhibition. Unlike normal anxiety, pathological

anxiety is characterized by excessive and irrational anxiety or fear. In DSM-IV-TR, anxiety disorders consist of eight subtypes, among which generalized anxiety disorder (GAD), panic disorder, post-traumatic stress disorder, social phobia and specific phobias are most prevalent (Kessler et al. 2005).

In the Sequenced Treatment Alternatives to Relieve Depression project, which is the largest antidepressant effectiveness trial ever conducted, 53% of the MDD patients recruited to the study showed high levels of anxiety symptoms (Trivedi et al. 2006). Moreover, the severity of anxiety symptoms in MDD patients is inversely correlated with the treatment remission rate (Fava et al. 2008). A US national comorbidity survey in 2001-2002 with 9090 respondents found that an average of 59.2% MDD patients met the criteria for anxiety disorders (Kessler et al. 2003). Another longitudinal study incorporating surveys from 1950, 1972 and 1992 even raised the comorbidity rate to a range of 54% to 98% depending on gender and age (Murphy et al. 2004). In contrast, for those who didn't report depression, the probability of observing anxiety disorders ranged from 3% to 10% in the same study. Correspondingly, a large subset of anxiety patients also suffers from major depression (Wittchen et al. 1994; Judd et al. 1998; Murphy et al. 2004). Around two-thirds of GAD and panic disorders patients have a lifetime history of MDD. Moreover, in 63% of the cases, the onset of GAD precedes that of MDD (Kaufman and Charney 2000).

1.1.3 Treatment of MDD is hampered by lack of effective therapy, slow remission and high rate of recurrence

Pharmacotherapy of MDD shows a marked delay of therapeutic efficacy and typically requires a minimum of six to eight weeks for basic remission (American Psychiatric Association. 2000). Only one third of MDD patients respond to the first antidepressant drug therapy (Trivedi et al. 2006). Moreover, 15-33% of the patients don't respond to two or more therapies and thus are considered treatment-resistant (American Psychiatric Association. 2000; Cain 2007; Berlim et al. 2008). The first-line antidepressant drugs currently in use are all members

of the selective serotonin reuptake inhibitors (SSRIs) such as fluoxetine (common trade name Prozac) that act by blocking the function of the serotonin transporter (5-HTT). Initial treatment with SSRIs is often accompanied by elevated levels of anxiety, which may even exacerbate depressive symptoms. Another class of antidepressant drugs is tricyclic antidepressants (TCA) such as desipramine (trade names Norpramin or Petrofane). TCAs are usually reserved for patients who don't respond to SSRIs due to their relatively more severe side effects.

Another issue is the high rate of recurrence. Although prolonged antidepressant treatment has been prescribed in order to prevent recurrence, still almost 50% of the patients who recover from the previous episode will suffer another episode later in their lives. For those who have already been through two depressive episodes, the probability to experience a third episode is 80% (Fava et al. 2006; Burcusa and Iacono 2007). The treatment of adolescent and young adults raises one more question. Clinical data suggest that some SSRIs may increase suicidal ideation and suicidal behavior of young adults (Olfson et al. 2006).

1.2 Heterogeneity of MDD

1.2.1 Genetic and environmental factors in the etiology of MDD

Numerous twin studies and genome-wide association studies (GWAS) have been carried out to evaluate the relative contribution of genetic and environmental factors to MDD. Overall, twin studies in children and adolescence have identified a heritability estimate ranging from 30% to 80% (Hewitt et al. 1992; Thapar and McGuffin 1994; Edelbrock et al. 1995; Deater-Deckard et al. 1997; Eaves et al. 1997; Gjone and Stevenson 1997). In another meta-analysis that summarized five studies using samples from communities and clinics, the heritability is estimated to be 37%, whereas the rest of the variance in the liability of MDD has been attributed to individual-specific environmental effects (Sullivan et al. 2000).

Stress is perhaps the best-validated environmental risk factor. There are numerous studies that support a causal relationship between stressful life events and MDD (Ge et al. 1994; Kendler et al. 1999; Ge et al. 2001; Turner and Lloyd 2004). In contrast, the genetic risk factors are still largely unknown. Genetic epidemiological studies of identical twins suggest that GAD, PTSD and social phobia may share very similar or even identical genetic risk factors with MDD (Kendler 1996; Kendler et al. 2007). Similar to diabetes and cancer, the genetic vulnerability for MDD is thought to involve a large number of genes, such that the contribution of a single gene variant is greatly diluted and difficult to detect. Two GWAS studies with 1022 recurrent MDD patients/1000 controls and 492 recurrent MDD patients/1052 controls respectively failed to identify any SNP that reached a genome-wide significance threshold (Muglia et al. 2010). Nonetheless, several gene clusters and SNPs including $GABA_AR$ subunits, 5-HTT, etc. have been identified from more specific association studies (Caspi et al. 2003; Yamada et al. 2003; Horiuchi et al. 2004). In particular a polymorphism of the HTT gene that results in short and long alleles is of interest because it illustrates the concept of gene × environment interaction. The presence of the short allele on its own has no measurable contribution to the risk to develop MDD. However, it results in increased susceptibility of carriers to the detrimental effects of stressful life events and thereby nevertheless increases their vulnerability to develop MDD (Caspi et al. 2003). Kendler et al (1995) proposed that genetic factors influence the risk of onset of major depression in part by altering the sensitivity of individuals to the depression-inducing effect of stressful life events.

1.2.2 Brain regions implicated in MDD

It's a general belief that the neural systems that are responsible for emotion processing or communication (Table 1.2) are impaired in patients with MDD (Ende et al. 2006). To better understand the brain structures and circuits that mediate depressive episodes, attempts focused on volumetric as well as activity changes of different substructures of the diseased brain. Magnetic resonance imaging (MRI) has been widely employed in volumetric studies, while positron

emission tomography (PET) and functional MRI (fMRI) are used for studies of activity changes.

Table 1.2 Key brain regions for emotion regulation

Brain region	Function (emotion related)
Amygdala	Emotional learning, mediating autonomic expression and the cognitive experience of emotion (Davis and Whalen 2001)
Cingulate cortex	Conditioned emotional learning, vocalizations associated with expressing internal states, assessments of motivational content and assigning emotional valence to internal and external stimuli (Devinsky et al. 1995)
Hippocampus	Dorsal hippocampus: declarative memory and spatial learning Ventral hippocampus: regulation of anxiety (Engin and Treit 2007)
Hypothalamus	Peripheral expression of emotional states (Price and Drevets 2010), rewards (Nestler and Carlezon 2006)
Insula	Experiencing and recognition of emotion of disgust, anomalous mood and willed action (Ibanez et al. 2010)
Nucleus accumbens	Natural rewards (food, sex, social interaction) and drug rewards, addiction (Nestler and Carlezon 2006)
Prefrontal cortex	Cognitive aspect of emotion (Morgane et al. 2005)
Ventral tegmental area	Natural rewards (food, sex, social interaction) and drug rewards, addiction (Nestler and Carlezon 2006)

The volume of the brain as a whole was shown to be unchanged in MDD patients in most of the published studies (Pillay et al. 1997; Bremner et al. 2000; Rosso et al. 2005) with the exceptions of two studies that reported volume reduction (Steingard et al. 2002) and expanded white matter (Salokangas et al. 2002), respectively. Volumetric reductions were consistently observed in the anterior cingulate cortex (3/3 studies) and hippocampus (16/19 studies) (reviewed by Konarski et al. 2008). There is less agreement with respect to the amygdala where four out of nine studies reported a reduced volume (Sheline et al. 1998; Siegle et al. 2003; Caetano et al. 2004; Hastings et al. 2004) and two others showed an increased volume (Frodl et al. 2002; MacMillan et al. 2003). Some studies also identified changes in basal ganglia (Greenwald et al. 1997), thalamus (Vasic et al. 2008), pituitary (MacMaster and Kusumakar 2004) and insular cortex (Takahashi et al. 2010; Soriano-Mas et al. 2011).

Compared to volume measurements more consistent results are available based on functional brain imaging studies of MDD patients. The prefrontal cortex has reproducibly been reported to be less active in MDD patients compared to normal controls, whereas the anterior cingulate cortex, hippocampus and amygdala have been reported to be hyperactive (Mayberg et al. 1999; Drevets 2000; Mayberg 2003; Milad et al. 2007). In addition over-activation of the insula was also reported (Fu et al. 2004; Gotlib et al. 2005).

1.2.3 Heterogeneity in treatment responses and MDD hypotheses

Pharmacotherapy is predominant in the treatment of MDD. Consistent with the monoamine-deficit hypotheses of major depression (Bunney and Davis 1965; Schildkraut 1965; Coppen 1967; Matussek 1969), currently used antidepressant drugs are universally designed to enhance monoamine neurotransmission, either by inhibiting the reuptake of serotonin, norepinephrine, or dopamine or activating corresponding receptors (Feighner 1999). However, 15-33% of MDD patients are resistant to currently available pharmacotherapies (Cain 2007; Berlim et al. 2008). Thus, monoamine hypothesis alone cannot explain the heterogeneity of treatment responses. Furthermore, recent research has shown that some drugs that were believed to act through monoamine systems are modulating other neural systems as well (reviewed by Luscher et al. 2011).

In contrast to the delayed therapeutic effects of traditional antidepressants, (American Psychiatric Association. 2000), more recent clinical trials revealed fast antidepressant effects of a group of NMDA receptor antagonists, which supports the glutamate hypothesis of MDD (Skolnick et al. 2009). The safety and efficacy of these drugs are still up to be evaluated by clinical trials.

A third hypothesis known as the stress hypothesis is based on evidence that stress is a major vulnerability factor in mood disorders (Kendler et al. 1999; Gold and Chrousos 2002). A subset of MDD patients shows hyperactivity of the hypothalamus-pituitary-adrenal (HPA) axis (Hatzinger 2000; Holsboer 2001).

Thus, compounds that down-regulate HPA axis activity were developed and tested as potential antidepressants in clinical trials.

Generally speaking, there is a lack of predictability of whether a patient responds to a certain drug. The risk of recurrent depression after successful remission is similarly unpredictable. Recurrence of depression occurs more often in MDD patients with comorbid anxiety disorders (Wilhelm et al. 1999). A family history of psychopathology is also linked to increased risk of recurrence (Burcusa and Iacono 2007).

In summary, MDD patients exhibit extensive heterogeneity in symptoms, volumetric and functional changes of multiple brain structures, as well as treatment response.

1.3 Mouse models of MDD

The idea of developing mouse models is to recapitulate the progression of the disease in relatively simple, genetically homogeneous animals that are raised under controlled environmental conditions and amenable to genetic and other manipulations in order to facilitate further understanding of the mechanisms and better treatment designs. Constructive, face and predictive validities are typically used to evaluate the quality of animal models.

The constructive validity of an animal model of MDD dictates that the methods utilized to induce a depressive-like state are relevant to the etiology of the human disease. Given that MDD has both genetic and environmental origins, an animal model of MDD therefore ideally would rely on either genetic, environmental, or both means for induction of the disease state.

Face validity indicates that a model recapitulates important anatomical, biochemical, neuropathological and/or behavioral features of the human disease

(reviewed by Nestler and Hyman 2010). In case of MDD face validity may be the most difficult to achieve as MDD is one of the most typically human disorders and requires verbal input from the patient that cannot be replicated in animals. Most tests used to assess face validity in MDD therefore are derived from behavioral tests that have been used to assess antidepressant drug responsiveness and therefore are primarily pharmacologically validated to assess depression related phenotypes. The most popular tests are forced swim test (FST) (Lucki 1997) and tail suspension test (TST) (Steru et al. 1985; Bilkei-Gorzo et al. 2002), which are widely accepted as suitable to validate antidepressant activity in rodents. Both these tests assess the amount of escape activity a mouse exhibits in a stressful inescapable situation. Antidepressant drugs invariably show an increase in escape behavior in these tests. By extension, a depressive-like phenotype would entail reduced escape behavior compared to a normal control.

Another class of tests aims at measuring the pleasure-seeking behavior of mice, Commonly used tests in this category include the sucrose preference test (Willner et al. 1987) or sucrose consumption test (SCT) (Monleon et al. 1995; Forbes et al. 1996). Decreased preference or consumption of sucrose is interpreted as an indication of anhedonia and depression-like behavior. Anhedonia is one of the two core symptoms of MDD and mostly seen in depression patients with melancholic features (American Psychiatric Association. 2000). Thus, measuring face validity by evaluating anhenonia-like behavior is reliable for at least a subtype of MDD. One concern with these tests is that the preference and consumption of sucrose solution may be affected not only by emotionality, but also by alterations in metabolic rate.

In addition to the tests mentioned above, novelty suppressed feeding test (Santarelli et al. 2003) and social interaction test (Krishnan et al. 2007) are often performed. Although ethologically these tests measure anxiety-like behavioral inhibition in the face of an aversive situation they are highly predictive for antidepressant activity of test compounds (Suranyi-Cadotte et al. 1990; Santarelli

et al. 2003). In the novelty suppressed feeding test (NSFT), a piece of food is placed in the center of an open field (which is aversive) and the latency for the animal to start consumption of the food is scored. The social interaction test measures the amount of time a test mouse spends exploring another mouse presented in a small wire cage within a brightly lit open field or otherwise new environment. The tradeoff in both these tests is between avoidance of an aversive environment (new environment) and exploration of a possible reward. While anxiety-related behaviors are sensitive to both antidepressants and classical anxiolytics such as benzodiazepines, depression-related behaviors should not respond to benzodiazepines.

Predictive validity requires that the model responds to drug treatment in a manner similar to human patients. That is, a drug with established antidepressant efficacy should exhibit the antidepressant-like activity in mouse behavioral tests. Moreover, since healthy people do not experience any benefit from taking antidepressants the antidepressant-like behavioral change in animals should be similarly specific for the disease state and absent from normal controls.

1.4 GABAergic neurotransmission and MDD

1.4.1 GABA and its receptors

GABA is the principal inhibitory neurotransmitter in adult brain. GABAergic neurotransmission regulates neuronal excitability through two classes of receptors, the ionotropic $GABA_A$ receptors ($GABA_ARs$) and the metabotropic $GABA_B$ receptors ($GABA_BRs$). Most $GABA_ARs$ are heteropentameric GABA-gated chloride channels that consist of two α subunits, two β subunits and one of the γ1-3, δ, ε, θ, or π subunits. The combination of different subunit isoforms and splice variants gives rise to a large number of structurally, functionally and pharmacologically distinct receptor subtypes (Olsen and Sieghart 2009). These can be further classified into synaptic $GABA_ARs$ and extrasynaptic $GABA_ARs$.

The synaptic $GABA_A$Rs are located at the synaptic membranes. These receptors are distinct from extrasynaptic $GABA_A$Rs from two aspects: 1) they are less sensitive to GABA; a larger amount of GABA is therefore required for receptor activation; 2) the currents mediated by synaptic $GABA_A$Rs are typically phasic with fast decay whereas extrasynaptic receptors are tonically active. The synaptic $GABA_A$Rs identified in brain invariably contain a γ2 subunit. The α subunit can be either α1, α2 or α3. Extrasynaptic $GABA_A$Rs mostly consist of α4βδ and α5βγ2 receptors in forebrain and α6βδ in cerebellum (for review see Farrant and Nusser 2005; Luscher et al. 2011).

The $GABA_A$Rs can be further classified by their benzodiazepine (BZ) sensitivity (Luscher et al. 2011). BZs bind to the interface of α and γ subunits and act as allosteric agonists of $GABA_A$Rs. α4- and α6-containing $GABA_A$Rs are insensitive to BZs (Wafford et al. 2004). Among the receptors that are sensitive to BZs, different receptor compositions mediate different BZ responses: α1βγ2 receptor subtype mediates sedative, anterograde amnesic, addictive and most of the anticonvulsant effects of diazepam (Rudolph et al. 1999; Crestani et al. 2000; McKernan et al. 2000; Tan et al. 2010); α2βγ2 receptors mediate the anxiolytic and anti-hyperalgesic effects (Low et al. 2000; Knabl et al. 2008); α2βγ2, α3βγ2, and α5βγ2 receptors together mediate the myorelaxant effects of diazepam (Crestani et al. 2001; Crestani et al. 2002); α5βγ2 receptors also mediate the development of tolerance to the sedative functions of diazepam (van Rijnsoever et al. 2004).

1.4.2 GABA mediated neurotransmission in immature neurons

The effects of $GABA_A$Rs are largely dependent on the equilibrium potential for Cl^- (E_{Cl^-}), which is regulated by the expression of NKCC1 and KCC2 transporters. In immature neurons, activation of $GABA_A$Rs leads to efflux of Cl^- and depolarizing currents. Meanwhile, the opening of $GABA_A$Rs may also induce shunting inhibition by reducing the membrane resistance, a mechanism that is relatively insensitive to E_{Cl} (reviewed by Ben-Ari 2002).

Maintaining normal GABA circuits is crucial for brain development. In the immature brain, GABAergic synapses are formed prior to glutamatergic synapses. Activation of GABA receptors generates depolarizing potential, which drives the maturation of glutamatergic synapses. Gradually glutamate takes over as the principle excitatory transmitter while GABA switches from excitatory to inhibitory functions. The timeline of these events differs greatly by brain regions (reviewd by Ben-Ari 2002; Clarkson and Herbison 2006; Wang and Kriegstein 2009).

1.4.3 GABAergic neurotransmission and anxiety

$GABA_A$Rs are recognized as one of the key players in the pathophysiology of anxiety (for reviews see Whiting 2006; Da Settimo et al. 2007; Nikolaus et al. 2010; Uusi-Oukari and Korpi 2010). Reduced abundance of $GABA_A$R binding sites in panic disorder patients has been shown by Positron Emission Tomography (PET) as well as Single Photon Emission Computed Tomography (SPECT) (Tokunaga et al. 1997; Malizia et al. 1998; Bremner et al. 2000). Most of the brain regions that are robustly affected, including ventral basal ganglia, orbitofrontal, superior frontal, parietal and temporal cortex, left hippocampus and precuneus, are thought to control the experience of anxiety (Davidson 2000; Davidson 2002). Moreover, the BZ site antagonist ^{11}C-flumazenil precipitates panic attacks during symptom free episodes in panic patients, whereas no effect is seen in healthy subjects (Nutt et al. 1990). Besides panic disorder, $GABA_A$R deficits are also identified in the temporal lobe of patients with generalized anxiety disorder (Tiihonen et al. 1997) and medial prefrontal cortex of patients suffering from posttraumatic stress disorder (Bremner et al. 2000). Last but not least, the involvement of GABAergic transmission in anxiety is evidenced by the therapeutic anxiolytic effects of BZs (Whiting 2006).

1.4.4 GABAergic deficits in MDD patients

As discussed in Chapter 1.1, MDD is frequently comorbid with an anxiety disorder. Some earlier studies suggest that the genes for GAD and MD are very similar or possibly even identical (Kendler, 1996, Kendler *et al.*, 2007, Kendler *et*

al., 1992, Roy *et al.*, 1995). However, given the lack of clear antidepressant efficacy of benzodiazepines as antidepressants (reviewed by Luscher et al. 2011), a possible role for GABAergic deficits in the etiology of MDD has been largely neglected. The strongest evidence in support of GABAergic deficits in MDD is based on reduced GABA levels in MDD patients (reviewed by Luscher et al. 2011). Despite some inconclusive earlier findings (Francis et al. 1989; Petty 1994), recent studies with proton magnetic resonance spectroscopy show reductions of GABA in various brain regions that are implicated in emotion regulation (Chapter 1.2) such as the anterior cingulate cortex and dorsomedial/dorsolateral prefrontal cortex (Hasler et al. 2007; Bhagwagar et al. 2008). Dramatic reduction of GABA is also seen in the occipital cortex of MDD patients (Sanacora et al. 1999; Sanacora et al. 2004).

Genetic polymorphism of $GABA_AR$ subunit genes is also implicated in MDD (reviewed by Luscher et al. 2011). Genetic associations have been identified between MDD and the polymorphism of GABRA5 gene (Oruc et al. 1997) and the gene cluster encoding GABRA1 (Yamada et al. 2003; Horiuchi et al. 2004), GABRA6 and GABRG2 (Yamada et al. 2003). Furthermore, a recent study identified a male-specific association between childhood-onset mood disorders and the polymorphism of non-coding region of GABRD gene (Feng et al. 2010). In summary, genetic polymorphism studies demonstrate that GABAergic deficit can lead to mood disorders. However, the abundance of $GABA_AR$ genetic polymorphisms is relatively low, indicating that other genetic or environmental triggers of GABAergic deficits may be more important.

As mentioned in Chapter 1.2, it is well known that currently used antidepressants are designed to enhance monoaminergic neurotransmission (Feighner 1999). However, evidence that monoaminergic transmission is impaired in patients suffering from MDD is scarce. Moreover, it is inconclusive how increased monoamine transmitters affect neuronal network activity in the diseased brain (reviewed by Luscher et al. 2011). Some evidence suggests that antidepressants

may act through mechanisms that are independent of monoamine transmitter receptors (Donati et al. 2008; Zhang and Rasenick 2010). Pharmacological therapies as well as some non-pharmacological therapies normalize cortical GABA deficits in patients (Sanacora et al. 2006). Overall, amelioration of GABAergic transmission might be the key of antidepressant effects.

1.5 Modulation of GABA$_A$Rs by stress

1.5.1 GABAergic control of HPA axis

A major subset of patients suffering from severe forms of depressive disorders show increased glucocorticoid levels in serum and malfunction of the hypothalamic-pituitary-adrenal (HPA) axis (American Psychiatric Association. 2000; Hatzinger 2000; Gold and Chrousos 2002; Brown et al. 2004; Tichomirowa et al. 2005; Hennings et al. 2009). The HPA axis consists of three major components (Figure 1.1): 1) the paraventricular nucleus (PVN) of the hypothalamus that releases corticotropin-releasing hormone (CRH); 2) the anterior lobe of the pituitary gland that releases adrenocorticotropic hormone (ACTH) upon stimulation by CRH; 3) the adrenal cortex that releases glucocorticoid (corticosterone in rodents) in response to ACTH. Glucocorticoid can in turn act on pituitary and hypothalamus as well as the frontal cortex and hippocampus to initiate negative feedback.

The release of CRH from the PVN is subject to GABAergic control via projections from the frontal cortex (Diorio et al. 1993; Akana et al. 2001) and ventral hippocampus (Cullinan et al. 1993). High densities of GABA$_A$Rs are expressed on CRH neurons in the PVN (Cullinan 2000). More than a third of synapses on CRH neurons are GABAergic (Miklos and Kovacs 2002). In the adult rat brain, application of GABA$_A$R antagonist bicuculline into the PVN activates CRH neurons, which leads to a dramatic increase of adrenal release of corticosterone

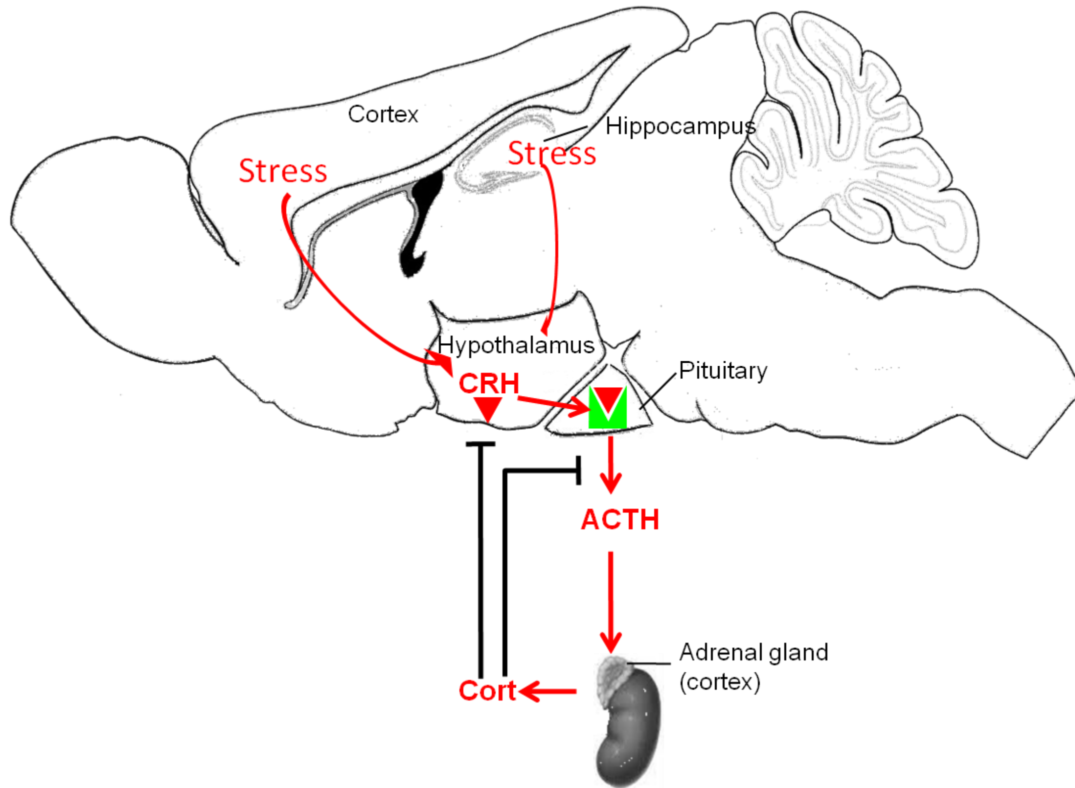

Figure 1.1 Illustration of stress activation of the HPA axis and negative feedback mediated by corticosterone. CRH, corticotropin releasing hormone; ACTH, adrenocorticotropic hormone; Cort, corticosterone. The picture of the mouse brain is adapted from www.gensat.org.

(Cort) (Borycz et al. 1992; Cole and Sawchenko 2002). On the other hand, potentiating GABAergic inhibition by muscimol microdialysis suppresses HPA axis responses to acute ether stress (Kovacs et al. 2004). Another study with young adult rats showed that both bicuculline and the $GABA_BR$ antagonist phaclofen significantly accelerate the secretion of Cort induced by acute stress (Marques de Souza and Franci 2008), indicating that GABAergic inhibition of the HPA axis is mediated by both ionotropic $GABA_ARs$ and metabotropic $GABA_BRs$.

The inhibitory signal of the prefrontal cortex to the PVN is relayed by GABAergic inhibitory neurons in the anterior bed nucleus of the stria terminalis (aBST) (Radley et al. 2009). After stress, aBST GABAergic neurons are activated by medial prefrontal cortex (mPFC) and then send the inhibitory signals to the CRH neurons in the PVN (Radley et al. 2009). Ablation of this inhibitory pathway by

lesion of the dorsal mPFC with immunotoxin leads to an approximately two-fold increase in acute stress-induced peak levels of ACTH and Cort (Radley et al. 2009). Aside from the frontal cortex, the HPA axis also receives input from the ventral hippocampus. Hippocampal neurons project to the hypothalamus via the lateral septum (Varoqueaux and Poulain 1999; Calfa et al. 2007). Blocking hippocampal neurogenesis leads to potentiated HPA axis response to stress (Schloesser et al. 2009).

1.5.2 Effects of stress on GABAergic transmission in adulthood

Effects of acute stress. Despite being regulated by GABAergic transmission, HPA axis activity can in turn affect the strength of GABAergic transmission. Cort acts on mineralocorticosterone (MR) and glucocorticosterone receptor (GR) in various brain regions to activate or repress a large number of genes (Surjit et al. 2011). Acute stress has been shown to affect $GABA_AR$ expression, extracellular GABA levels as well as the electrophysiological properties of certain neurons. However, studies addressing the effects of stress on receptor abundance have been largely inconclusive, with consequences of acute stress seemingly dependent on the type of stress protocol applied (de Groote and Linthorst 2007; Skilbeck et al. 2010). Electrophysiological studies provide more insight into the mechanisms of Cort feedback. Mimicking acute stress by application of Cort in acute dorsal hippocampal slices leads to 60% increase in the amplitude of spontaneous (s) inhibitory postsynaptic currents (IPSC) and a similar increase of miniature (m) IPSCs via activation of GRs (Maggio and Segal 2009; Hu et al. 2010). Interestingly, the frequency of sIPSCs is increased only in the dorsal hippocampus but not ventral hippocampus (Maggio and Segal 2009). Consistent with the frequency changes, acute stress enhances long-term-depression (LTD) in dorsal hippocampus and facilitates long-term-potentiation (LTP) in ventral hippocampus (Maggio and Segal 2009).

Effects of chronic stress. Chronic stress is a well-validated environmental risk factor in the etiology of MDD (Chapter 1.2.2). Consequently, chronic stress

paradigms are often used to model depressive-like symptoms in animals (Willner 2005). The changes of GABAergic transmission following chronic stress have been extensively studied in multiple brain regions of rodents. Chronic mild stress applied to adult rats has been shown to reduce extracellular GABA levels in the hippocampus as a whole (Gronli et al. 2007) and in the dentate gyrus (DG) of ventral hippocampus (Holm et al. 2011). Moreover, the reduction of GABA in ventral DG is coupled with a 60% increase in tonic GABA currents, indicating increased expression of extrasynaptic $GABA_A$Rs (Holm et al. 2011). In contrast, the sIPSC frequency is reduced by 41%, which is associated with a reduced probability of evoked GABA release (Holm et al. 2011). Interestingly, an increase in the sIPSC frequency is observed when recording from the CA1 pyramidal neurons of dorsal hippocampus (Hu et al. 2010). Different effects of chronic stress in dorsal vs. ventral hippocampus have also been reported at the cellular level. Chronic stress reduces the number of PAV positive neurons in the dorsal hippocampus (Hu et al. 2010) but not ventral hippocampus (Holm et al. 2011). Consistent with the reduced number observed in the dorsal hippocampus of rats, the overall number of PAV positive neurons in the entire hippocampus is also reduced in chronically stressed adult tree shrews (Czeh et al. 2005). The activity of another type of GABAergic interneurons, the cholecystokinin (CCK) neurons, is subject to cannabinoid (CB)-mediated depolarization-induced suppression. Chronic stress has been shown to impair this depolarization-induced suppression in the dorsal hippocampus (Hu et al. 2011) as well as the striatum (Rossi et al. 2008). This partially explains the increase of sIPSC frequency in the context of reduced PAV positive GABAergic neurons. Overall, current data indicate the differential regulations of synaptic versus extrasynaptic inhibition in ventral versus dorsal hippocampus.

In the PVN of hypothalamus, all current data point to chronic stress-induced reductions of GABAergic inhibition. The frequency of mIPSC (Joels et al. 2004; Verkuyl et al. 2004) and sIPSC (Joels et al. 2003) is reduced in response to chronic stress whereas the amplitude is unaltered (Verkuyl et al. 2004). Double-

pulse stimulation experiments show that the reduction of mIPSC is not caused by decreased releasing probability (Joels et al. 2004), indicating that the reduced GABAergic synaptic current frequency is due to reduced number of functional GABAergic synapses or enhanced tonic inhibition. Consistent with this interpretation, the amplitude of the evoked GABA currents is reduced (Joels et al. 2003; Joels et al. 2004). Semi-quantitative hybridization histochemical analyses show a reduction of the β1 and β2 subunits of $GABA_A$Rs in the medial parvocellular PVN (Cullinan 2000). However, the total number of GABAergic boutons, as labeled by GAD65 immunoreactivity, is unchanged (Flak et al. 2009).

1.5.3 Effects of early life stress

Early life stress, especially stress during adolescence, has been linked to increased vulnerability to various psychological disorders including anxiety, MDD and other mental disorders (Ge et al. 1994; Grant and Compas 1995; Ge et al. 2001; Andersen 2003; Dahl 2004; Grant et al. 2004; Turner and Lloyd 2004; Patton et al. 2007; Patton and Viner 2007). Similar to the pattern in adulthood where females are at higher risks of suffering from MDD, adolescent girls are more likely to be affected by stress and depressive moods than boys (Grant and Compas 1995; Ge et al. 2001; Patton et al. 2007). In sharp contrast to the well understanding of the behavioral consequences of stress, the mechanism behind the scene is still vague. Adolescence is marked by the onset of puberty, when the neuroendocrine system as well as other brain compartments undergoes drastic structural and functional changes. Various stress paradigms have been employed to model early life stress in rodents.

It was shown that 5-day social restraint stress reduces the food intake, body weight as well as open arm entries in the elevated plus maze test in 4-week-old but not 8-week-old mice (Stone and Quartermain 1997), suggesting that the sensitivity to chronic stress is elevated during adolescence. Similarly, mild stressors such as physical restraint stress and/or social isolation stress on rats lead to increased depressive-like behavior in the forces swim test and/or learnt

helplessness test when the stressor is applied during puberty (Leussis and Andersen 2008; Romeo 2010) but not in adulthood (Romeo 2010). The immediate effects at the molecular, cellular and circuit levels are largely unknown, whereas the long-term effects share certain similarities with that of chronic stress in adulthood. GABAergic synaptic inputs in the PVN switch from inhibition to excitation following acute pre-pubertal stress. The long-term effects of adolescent stress include alterations of hormone levels as well as $GABA_AR$ composition. Social isolation of rats for 30 days immediately after weaning induces a significant decrease of basal levels of progesterone, 3α, 5α-TH PROG, and 3α, 5α-TH DOC in the cerebral cortex and plasma (Serra et al. 2000). These neurosteroids are known to act through extrasynaptic $GABA_ARs$ whose expression level is also subject to regulation by stress (reviewed by Lambert et al. 2009). With the same stress paradigm, the immunoreactivity of α4 and δ subunit of $GABA_ARs$ is markedly increased in the granule cells of dentate gyrus and the pyramidal neurons of CA1 and CA3 of hippocampus (Serra et al. 2006). An increase of α5 subunit mRNA is seen in the PVN of rats after 3 weeks of chronic unpredictable stress, while the mRNAs encoding α1, α3, γ1 and γ2 subunits that mediate synaptic GABAergic transmission show a tendency of a small reduction (Verkuyl et al. 2004).

In addition to pubertal stress, GABAergic transmission is also affected by neonatal stress. For example, the increased neophobia and acoustic startle response seen in rats following maternal separation stress is associated with reduced expression of BZ-sensitive $GABA_ARs$ in the frontal cortex, amygdala, locus coeruleus and the n.tractus solitaries (Caldji et al. 2000). Variations in maternal care have long-lasting effects on the behavioral stress reactivity of adult animals as well as the $GABA_AR$ mRNA expression in brain regions associated with fear (Caldji et al. 2000; Caldji et al. 2003).

1.6 The cellular, cognitive and behavioral characteristics of $GABA_AR$ γ2 subunit heterozygous ($γ2^{+/-}$) mice

1.6.1 $γ2^{+/-}$ mice exhibit modest GABAergic deficits

The γ2 subunit of $GABA_ARs$ is required for postsynaptic accumulation and function of $GABA_ARs$ (Essrich et al. 1998). Mice that lack the γ2 subunit ($γ2^{-/-}$) die at birth (Gunther et al. 1995) due to the virtually complete absence of GABAergic synaptic transmission. However, mice that are heterozygous for the γ2 subunit mutation ($γ2^{+/-}$) lack an overt phenotype and were hence amenable to investigation of the role of GABAergic synaptic transmission in neuropsychiatric disorders. The $γ2^{+/-}$ mice exhibit normal $GABA_AR$ numbers as indicated by the unchanged number of SR95531 binding sites. However, they exhibit a modest reduction in the number of γ2 subunit-containing $GABA_ARs$ as indicated by the reduction (-25%) in the number of diazepam binding sites in brain extracts (Crestani et al. 1999). Further studies of $GABA_AR$ expression in situ by immunofluorescence and receptor autoradiography revealed brain region specific reductions of the γ2 subunit by 6-35%. The brain regions most affected by γ2 heterozygosity are the cingulate cortex (-25%), frontal cortex (-23%), piriform cortex (-25%), CA1 (-35%), CA3 (-28%), DG (-15%), lateral septum (-30%) and several thalamic nuclei, whereas the rest of the brain regions including the amygdala and the striatum are less affected (<10%). The magnitude of this $GABA_AR$ deficit is comparable to that seen in rodents that had been subject to maternal deprivation stress (Caldji et al. 2000; Caldji et al. 2003) or pubertal stress (Verkuyl et al. 2004). Single channel recording of cultured hippocampal neurons further shows that the receptors from $γ2^{+/-}$ neurons exhibit reduced frequency of αβγ2-mediated high conductance and increased frequency of αβ-mediated low conductance. The excitatory hippocampal circuits are largely unaltered as indicated by unaltered hippocampal long term potentiation in $γ2^{+/-}$ vs. WT mice (Crestani et al. 1999).

1.6.2 Behavioral and cognitive deficits of $\gamma2^{+/-}$ mice

Given the established role of $GABA_ARs$ as targets of anxiolytic drugs, the $\gamma2^{+/-}$ model was first analyzed with respect to anxiety related behaviors (Crestani et al. 1999). The $\gamma2^{+/-}$ mice were shown to exhibit heightened behavioral inhibition to diverse natural aversive stimuli such as new environments, open elevated spaces, and brightly lit environments (free-choice exploration test, forced exploration test, elevated plus maze test, light/dark choice test). Importantly, these behavioral abnormalities were reversed by diazepam, a prototypical anxiolytic benzodiazepine (Crestani et al. 1999). Given the rampant comorbidity of anxiety and depressive disorder a subsequent study further analyzed the behavior of γ2+/- mice with respect to depressive-like brain states. Consistent with a depressive-like phenotype, Earnheart et al showed that $\gamma2^{+/-}$ mice exhibit increased emotionality in the FST (Earnheart et al. 2007). Lastly, the behavioral abnormalities of $\gamma2^{+/-}$ mice were shown to be associated with selective cognitive deficits in fear conditioning tests such as an attentional bias for threat cues and impaired ambiguous cue discrimination (Crestani et al. 1999), which have previously been associated with anxiety and mood disorders in patients (MacLeod and Byrne 1996; Austin et al. 1999; Schatzberg et al. 2000; Rogers et al. 2004; Chan et al. 2008; Dearing and Gotlib 2009). By contrast, spatial learning and memory in the Morris water test, and hippocampus-independent fear conditioning of γ2+/- mice was unaltered, indicating that the anxiety and emotion-related impairment of γ2+/- was highly specific (Crestani et al. 1999).

1.6.3 Deficits in the maturation of adult born hippocampal neurons of γ2+/- mice

GABA-mediated activation of $GABA_ARs$ in immature neurons is depolarizing and excitatory, as described above. This excitatory function of GABA also extends to proliferating neural progenitors in the adult brain. In particular, GABAergic input to immature granule cells of the hippocampus has been shown to regulate the neuronal differentiation (Li and Pleasure 2005) and dendritic maturation (Ge et al. 2006) of these neurons in the adult brain. Interestingly, the excitatory GABA

signaling has dual effects on hippocampal neurogenesis. On one hand, the proliferation of progenitor cells is negatively regulated by GABAergic inputs (Liu et al. 2005; Tozuka et al. 2005; Wu and Castren 2009). On the other hand, GABA-mediated excitation nourishes the maturation and network integration of the postmitotic granule cells (Ge et al. 2006).

Analyses of hippocampal neurogenesis of $\gamma2^{+/-}$ mice revealed normal proliferation of neural precursor cells but reduced survival of adult-born hippocampal granule cells (Earnheart et al. 2007). This phenotype is in keeping with the γ2 subunit being essential specifically during synaptogenesis (Luscher et al. 2011). Moreover, similar deficits in neuronal maturation of hippocampal neurons are also detected in 3-week-old $\gamma2^{+/-}$ mice (Earnheart, unpublished).

1.6.4 The $\gamma2^{+/-}$ model shows selective vulnerability to mood disorders during early life

In order to delineate the developmental time course and brain regions responsible for the cellular and behavioral deficits of $\gamma2^{+/-}$ mice, the behavioral consequences of γ2 subunit deficits as well as the effects on adult hippocampal neurogenesis were analyzed in two different conditional mutant strains, utilizing the Cre-loxP system (Earnheart et al. 2007). In Emx1Cre × fγ2/+ mice, the inactivation of γ2 gene starts from embryonic day 10 and is limited to glutamatergic neurons of the telencephalon (Gorski et al. 2002; Iwasato et al. 2004; Earnheart et al. 2007). By contrast, CaMKIICre2834 × fγ2/+ mice exhibit inactivation of γ2 gene starting from the fourth postnatal week and selectively in mature glutamatergic neurons in the forebrain (Schweizer et al. 2003). Analyses of the two mutant strains show that mice with earlier onset of γ2 gene inactivation replicate the behavioral and neurogenesis deficits of the $\gamma2^{+/-}$ global knockout mice, while mice with delayed γ2 gene inactivation show normal neurogenesis and are behaviorally indistinguishable from pseudo-wildtype controls (Earnheart et al. 2007). These data suggest that either the behavioral abnormalities of $\gamma2^{+/-}$ mice are caused by a developmental GABAergic deficit or, alternatively, that they

were caused by the deficit in hippocampal neurogenesis observed in global $\gamma 2^{+/-}$ or Emx1Cre x fγ2/+ mice. One of the tasks of my thesis was to further clarify this situation.

1.7 Work presented in this study

1.7.1 $GABA_AR$ $\gamma 2^{+/-}$ mice as mouse model of anxious depression

GABAergic deficits have been widely implicated in the pathophysiology of MDD as well as various animal models of depression (Chapter 1.4 and 1.5). $\gamma 2^{+/-}$ mice recapitulate behavioral, pharmacological and cognitive alterations reminiscent of anxiety disorders (Chapter 1.6). Moreover, $\gamma 2^{+/-}$ mice show increased behavioral passivity and impaired hippocampal neurogenesis that are commonly related to depressive-like mood in animal models (Chapter 1.6).

Here we aim at further characterizing the depression-related phenotypes of $\gamma 2^{+/-}$ mice and investigating the potential causal relationship between GABAergic deficits and MDD. Since the HPA axis receives numerous GABAergic inputs and its hyperactivity is a well-replicated finding in a large subset of depression patients (Chapter 1.5), the HPA axis activity of $\gamma 2^{+/-}$ mice and the developmentally γ2-deficient mice was assessed. Behaviorally, FST and TST were used as measures of behavioral passivity under stressful conditions, while SCT was run to evaluate the pleasure-seeking behavior of mice. Each test measures different aspect of depression-related behavior (Chapter 1.3). Finally, to support the predictive validity of $\gamma 2^{+/-}$ mice as a model of depression, $\gamma 2^{+/-}$ mice were evaluated with respect to behavioral and HPA axis responses to acute and chronic treatment with different antidepressants that specifically target either serotonergic or noradrenergic transmission. It's known that some antidepressants also alleviate anxiety symptoms in patients with anxious depression. Thus we also tested for anxiolytic-like effects of antidepressant drug treatments.

1.7.2 Developmental GABAergic control of adult anxious-depressive-like behavior

The distinct behavioral profiles of Emx1Cre × fγ2/+ mice and CaMKII2834Cre × fγ2/+ mice (Chapter 1.6.4) indicate that the abnormalities seen in $\gamma2^{+/-}$ mice are most likely caused by a developmental GABAergic deficits. This is consistent with abundant evidence that the vulnerability for anxiety and mood disorders is established early in life.

Here we set out to delimit the developmental time course during which GABAergic transmission ensures normal adult anxiety- and depression-related behavioral parameters in mice. The works presented in Chapter 4 of this thesis will cover the following:

1) A chemically inducible knockout strategy was used to irreversibly reduce the $GABA_AR$ γ2 subunit gene dosage at different time points of postnatal brain development.
2) Diazepam was used to potentiate $GABA_AR$ function in γ2-deficient and WT mice to disturb normal activity-dependent brain development during defined temporal windows (critical periods) predicted to underlie normal anxiety- and depression-related behavior in adulthood.

Chapter 2: Materials and Methods

2.1 Solutions

Avertin: 2.5g 2,2,2-tribromoethanol (Sigma Aldrich, St. Louis, MO), 5 ml 2-methyl-2-butanol (Sigma Aldrich, St. Louis, MO), bring the volume to 200 ml with distilled water.

Blocking solutions: 5% normal donkey or normal goat serum, 0.1% Triton X-100 in PBS or TBS

Bromodeoxyuridine (BrdU, Sigma Aldrich, St. Louis, MO): 10 mg/ml BrdU in 0.9% sodium chloride (NaCl)

Citric acid buffer: 10mM citric acid ($C_6H_8O_7$), 0.05% Tween 20, pH 6.0. pH to 6.0 with 10 M NaOH before adding Tween-20.

Desipramine: 120 mg/L, 240 mg/L or 480 mg/L desipramine (Sandoz, Princeton, New Jersey) in tap water for ad libitum administration. 6 mg/ml desipramine in tap water for per oral (p.o.) administration. Filter solution through fluted filter paper (Whatman Inc., Florham Park, NJ).

Fluoxetine: 20 mg/L and 1 mg/ml fluoxetine (Mallinckrodt, Hazelwood, Missouri) in tap water for ad libitum and p.o. administration respectively. Filter solution through fluted filter paper (Whatman Inc., Florham Park, NJ).

Lysis buffer: 100 mM Tris-HCl pH 8.5, 5 mM EDTA, 0.2% sodium dodecyl sulfate (SDS, $NaC_{12}H_{25}SO_4$), 200 mM NaCl.

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

Phosphate Buffered Saline (PBS): 3.12 g/l $NaH_2PO_4 \cdot H_2O$, 10.99 g/l Na_2HPO_4 (anhydrous), 9.0 g/l NaCl, adjust to pH 7.4 with NaOH or HCl

Primary antibody solutions: 2% respective serum, 0.1% Triton X 100 and respective antibody in PBS or TBS

TAE: 40 mM Tris-acetate, 1 mM EDTA, adjust to pH 8.0 with NaOH

Tamoxifen: 30mg/L tamoxifen (LP Biomedicals LLC, Solon, OH) in ethanol/sunflower seed oil (1:9). Vortex briefly and sonicate with desktop sonicator until solution is clear.

Tris Buffered Saline (TBS): 8.0 g NaCl, 0.2 g KCl, 3.0 g Tris-HCl, bring the volume to 1 L and adjust to pH 7.4 with 10 M NaOH or concentrated HCl

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

2.2 Animal production and husbandry

All animal experiments were performed in accordance with National Institutes of Health guidelines. The protocols were approved by the Institutional Animal Care and Use Committee of Pennsylvania State University. $GABA_AR$ γ2 subunit heterozygous ($\gamma2^{+/-}$) mice were backcrossed to the 129X1/SvJ background (previously named 129SvJ) for more than 40 generations. Experimental $\gamma2^{+/-}$ and wild-type (WT) mice were produced as littermates by mating $\gamma2^{+/-}$ and WT mice as described (Crestani et al. 1999).

The CaMKIICre2834 × fγ2/+ and Emx1Cre × fγ2/+ mice and the fγ2/+ littermate control subjects were generated as offspring of hemizygous Cre transgenic and homozygous fγ2/fγ2 mice (Schweizer et al. 2003; Iwasato et al. 2004). All three

strains had been backcrossed into the 129X1/SvJ background for ≥ 10 generations.

CAG-CreERT2 mice (also known as CAGGCre-ERTM, Hayashi and McMahon 2002) were purchased from Jackson Laboratory (TG(CAG-CRE/ESR1)5AMC/J, JAX #004453, Jackson Laboratory, Bar Harbor, ME) and backcrossed to 129X1/SvJ mice for at least five generations. The CAG-CreERT2 × fγ2/+ mice and fγ2/+ and CAG-CreERT2 controls were littermates produced by mating of hemizygous CAG-CreERT2-transgenic and fγ2/+ mice. ROSA26-YFP (R26Y) transgenic mice used as Cre reporters (Srinivas et al. 2001) were provided by A. J. Eisch (University of Texas Southwestern Medical Center) and backcrossed to 129X1/SvJ mice for ≥ 6 generations.

Breeder mice used for the production of test mice were housed in standard shoebox cages with high fat diet and water available ad libitum. Each pregnant female was transferred to a large (gang) cage containing standard bedding supplemented with a cloth nesting square 3-5 days before delivery. The litters were left undisturbed with the dirty bedding in the corners of the cage replaced at P14 and no cage changing until the day of weaning (P21). Post-weaning mice were tagged with metal ear tags and genotyped by PCR analysis of tail biopsies (Gunther et al. 1995; Schweizer et al. 2003). Females destined for behavioral testing were then pooled by genotypes into gang cages containing 6–12 animals per cage and transferred to a separate female-only holding room. Males destined for behavioral testing were singly housed right after weaning. Both holding rooms were under a reversed 12 h:12 h light-dark cycle (dark from 12:00 - 24:00/ 13:00-1:00). The cages of behavioral test animals were changed once a week.

2.3 Genotyping

2.3.1 Preparation of tail biopsies

All mice were genotyped by 3mm-long tail biopsies. Tail snips were digested in 500 µl lysis buffer with 10 µl Proteinase K (10 mg/ml) at 55 °C for 2-5 h in a rotator until the tissues disappeared and the solution cleared. After digestion samples were spun at 13000 RPM for 5 min, isopropanol precipitated and the DNA pellet was transferred to 500 ml TE and dissolved at 55 °C for 1 h. The dissolved DNA was stored at 4 °C and used as PCR templates.

2.3.2 PCR

Primers were designed to detect the presence of the floxed locus, PGK-Neo cassette, and Cre transgene for genotyping fγ2 mice, R26Y and $\gamma 2^{+/-}$ mice, and Cre mice respectively. Another primer was designed to test the presence of wildtype allele in $\gamma 2^{+/-}$ mice by detecting the exon 8 of γ2 subunit gene. The reaction was performed in 1 X PCR buffer with 200 mM of each deoxynucleotide, 0.4 mM of each primer, 1 unit of Taq polymerase and 1 µL template. 5 µL of PCR products were mixed with 1 µl 6 × loading buffer, loaded into 1% - 2% agarose gels and run in TAE buffer at 100 V for 20 min. The band size was determined by comparison to 1kB plus DNA Ladder.

Table 2.1 PCR conditions

Strain	Locus detected	Primers	Annealing temp. (time)	Extension temp. (time)	Number of cycles	Expected size
$\gamma 2^{+/-}$	Neo gene	g2.19 g2.20	58 (45s)	65 (120s)	36	1.4 kb
$\gamma 2^{+/+}$	Exon 8 of γ2 gene	g2.19 g2.low3	58 (45s)	65 (120s)	36	2.0 kb
fγ2	loxP site	fg2 upper2 fg2 lower2	55 (30s)	72 (80s)	38	457 bp
Cre	Cre gene	Cre F Cre R	53 (30s)	72 (30s)	36	350 bp
ROSA26 - YFP	Neo gene	YFP 1 YFP 2	53 (30s)	72 (30s)	36	310 bp

Table 2.2 Primers for PCR genotyping

Primer name	Primer sequence
g2.19	5’-CATCTCCATCGCTAAGAATGTTCGGGAAGT-3’
g2.20	5’-ATGCTCCAGACTGCCTTGGGAAAAGC-3’
g2.low3	5'-GCTGACAAAATAATGCAGGGTGCCATACTC-3'
fg2 upper2	5’-GCCTGATTGTGGAAATAAAA-3’
fg2 lower2	5’-CATCCCCTTACTCTATGTC-3’
Cre F	5’-ATTTGCCTGCATTACCGGTC-3’
Cre R	5’-ATCAACGTTTTCTTTTCGG-3’
YFP1	5’-AAA GTC GCT CTG AGT TGT TAT-3’
YFP2	5’-GCGAAGAGTTTGTCCTCAACC-3’

2.4 Drug administration

Subchronic and chronic fluoxtine (20 mg/L) and desipramine (120 mg/L, 240 mg/L, 480 mg/L) were administered ad libitum in drinking water except during the sucrose consumption test when both drugs were applied p.o. at a dosage of 4 mg/kg/day and 30 mg/kg for fluoxetine and desipramine respectively. Drug administration was performed at the time specified ± 5 days. Tamoxifen (180 mg/kg/ day, i.p.) was administered on two sequential days as a suspension of 30 mg/ml in ethanol:sunflower seed oil (1:9). Diazepam (p.o., 2 mg/kg) was administered every other day for the time specified except for P10-16 treatments, where diazepam was administered daily (p.o., 1 mg/kg). Diazepam was administrated at the time specified ± 2 days for the P22-28, P29-35, and P50-56 experiments.

2.5 Behavioral assessments

The behavioral testing was performed at least 72 h after the last cage change and starting 1 h after onset of the dark phase of the light-dark cycle. Genotypes were coded such that the experimenter was unaware of the genotype. Group comparisons were between littermates.

Testing of desipramine and fluoxetine treated mice was performed at 12-14 (chronic drug treatment) or 9 weeks of age (subchronic), starting with the novelty suppressed feeding test (NSFT) and followed by the forced swim test (FST) and/or tail suspension test (TST), one week apart. Sucrose consumption test (SCT) was performed with a different set of mice starting right after chronic drug treatment at 12 weeks of age. Testing of conditional γ2 subunit deficient mice and diazepam treated mice was performed at 8 weeks of age or 4 weeks after the last injection, whichever later. The testing started with the open field test (OFT) or elevated plus maze test (EPMT) followed by the FST and/or TST, one week apart. The behavior was video recorded under red light for subsequent off-line analyses. The actual testing age is ± 5 days.

2.5.1 Open field test

Open field test was performed with the Omnitec activity box (42 × 42 cm, Omnitech Electronics Inc, Canada) or a plastic box (50 x 50 x 20 cm). Mice were put into the corner of the box and continuously tested for 15 min. The distance traveled in the first 5 min and the total 15 min as well as the time spent in the center and corners in the first 5 min were scored. Analyses of tamoxifen-injected mice and the subset of wt and $γ2^{+/-}$ injected with DZP at P14-21 were done using the Omnitec activity box and scored based on infrared beam crossing, whereas the test for DZP P29-35 and DZP P50-56 experiments were performed with the plastic box and scored with the Ethovision 7.0 video tracking system (Noldus Information Technology Inc, Leesburg, VA).

2.5.2 Elevated plus maze test

Mice were placed onto the center square of 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, and video recorded for 5 min. The number of entries and the total time spent on the closed and open arms, respectively were recorded (Crestani et al. 1999).

2.5.3 Novelty suppressed feeding test

In the NSFT (Santarelli et al. 2003), the mice were deprived of food for 18 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 nesting square (6 x 6 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 use of its forepaws.

2.5.4 Modified forced swim test

In the FST (Lucki 1997), mice were placed into a plastic bucket 19 cm in diameter and 27 cm deep and filled to 18 cm with 24-26 ºC water and videotaped for 6 min. We recorded the real time spent swimming until the first floating episode and the cumulative time spent immobile during the final 4 min, using a 5 s interval sampling method.

2.5.5 Tail suspension test

In the TST (Steru et al. 1985; Bilkei-Gorzo et al. 2002), the mice were suspended individually by their tails from a stainless steel rod (affixed with adhesive tape) that was positioned 30 cm above the floor of a test apparatus (50 x 50 x 45 cm) consisting of two 25 cm wide compartments separated by an opaque PVC board. Two mice were tested side-by-side and videotaped for 6 min. The time to first immobility and the total immobility time were recorded.

2.5.6 Sucrose consumption test

For the Sucrose Consumption Test (SCT), drug and vehicle treated mice were singly housed starting on the 24th day of drug treatment and trained to drink sucrose (10%) from the 25th to the 27th day. Water consumption from calibrated drinking tubes was then quantitated over the following three days. Finally, the mice were reexposed to sucrose for 4 days and 24 h consumption was quantitated on the last day.

2.5.7 Scoring with Ethovision 7.0 video tracking system

Ethovision 7.0 video tracking system was used to score the OFT of DZP P29-35 and P50-56 experiments and the EPMT of DZP P22-28 and P50-56. For OFT, the arena was divided into 4×4 sub-squares, with the four squares on the corners added to a cumulative zone named corners and the four in the center added to a cumulative zone named center (Figure 2.1). The tracking was started 1s after the animal was detected in the arena and lasted for 15min. Dynamic subtraction method was applied in the detection setting section to reduce background noise.

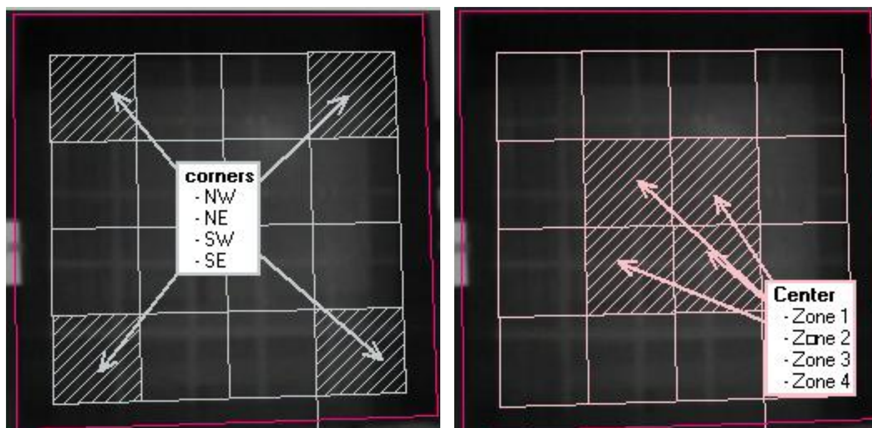

Figure 2.1 Arena settings for the OFT. Left, the corners; right, the center.

For EPMT, the cumulative zone 'closed arms' completely overlapped with the physical closed arms of the plus maze, while the open arms of the maze were divided into eight equal sized rectangles, out of which the seven distal rectangles formed the cumulative zone 'open arms' (Figure 2.2). The computer-based quantification and the manual scoring of the percentage of open arm entries showed high level of consistency (correlation coefficient 0.89, $p < 0.001$), supporting the validity of the settings.

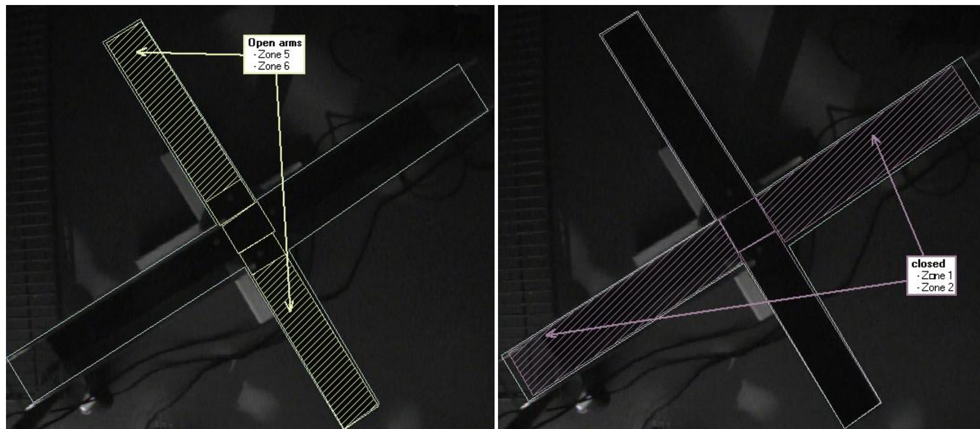

Figure 2.2 Arena settings for the EPMT. Left, the open arms; right, the closed arms.

2.6 Histology

2.6.1 BrdU injection

BrdU (10 mg/ml) was injected at a dose of 80 mg/kg four times a day at 3 hour intervals into 9 week old CAGCre-ERT2 × fγ2/+ mice and littermate controls one week after the FST was performed.

2.6.2 Transcardial perfusion

A mini-pump with variable flow rates (VWR, Radnor, PA) was calibrated to a flow rate of 10 ml/min before each perfusion session. A two-way valve allows for switching between PBS and PFA solutions. Mice were anesthetized with avertin (30 µl/g), pinned onto a dissection board, and sprayed with 70% ethanol. The chest was opened with a scissor. Then the ribs and fats that covered the heart were carefully removed until the aorta was visible. A cannula was inserted into the aorta through the left ventricle with the heart firmly held with forceps and ice-cold PBS flowing at the slowest rate. The organs began to expand and the right atrium was cut immediately. Then the PBS flow rate was adjusted and maintained at 10 ml/min for 2 min 25 sec, followed by 5 min flow of ice-cold 4% PFA at the same rate. Brain was removed, placed into 5 ml 4% PFA to allow for 12h-16h postfix at 4°C, washed with ice cold PBS for three times and then stored

in PBS with 0.05% sodium azide at 4°C. Protocol was adapted from John Earnheart, Beth Luellen (Luellen et al. 2006) and Nadia Sahir.

2.6.3 Preparation of brain sections

The fixed brain was cut approximately at Bregma 0 and Bregma -4 with the brain matrix (Electron microscopy sciences, Hatfiled, PA), glued onto the vibratome stage with the anterior part facing up, embedded in 4% UltraPure^{TM} low melting point agarose (Invitrogen, Carlsbad, CA), and then sliced into 50μm coronal sections. For storage, sections were kept in PBS with 0.05% sodium azide in 24 well cell culture dishes at 4°C.

2.6.4 Immunostaining of brain sections

For the quantification of BrdU positive cells, every 10th section was immunostained. As a basic immunostaining procedure, sections were first permeabilized with 1% Triton-X100 1 h, then incubated 1 h in blocking solution, washed 3 times 5 min in PBS and incubated in primary antibody overnight (O/N) at 4°C unless specified otherwise. On the next day sections were washed 3 times 5 min in PBS, incubated in secondary antibody for 1h and washed 3 times 5 min in PBS before proceeding to the next antibody or mounting on gelatinized slides. For fluorescence labeling of BrdU, sections were incubated in 2N HCl for 30 min at 37ºC and washed 5 min in 0.1 M sodium borate ($Na_2B_4O_7$) at room temperature followed by 3-5 times 5 min PBS washes prior to blocking. Double fluorescence labeling of BrdU/NeuN was done sequentially with BrdU labeling following that of NeuN. Permeabilization of slices with citric acid buffer is optional for NeuN staining. PBS is replaced by TBS for DCX staining to reduce background. DRAQ5 (Biostatus, UK) was applied to slices 1:1000 in PBS for 5 min after fluorescence staining of YFP and then quickly rinsed away with PBS. Protocol modified from John Clint Earnheart.

Table 2.3. Antibodies and immunostaining conditions

Marker	Antibody	Dilution	Blocking soln.	Incubation time/temp
YFP	rabbit anti-green fluorescence protein (Invitrogen, Carlsbad, CA)	1:1000	NGS/NDS	O/N
	anti-rabbit Alexa 488 (Jackson ImmunoResearch, West Grove, PA)	1:500	NGS/NDS	1h
NeuN	mouse anti-NeuN (Chemicon, Chemicon, Temecula, CA)	1:1000	NGS/NDS	O/N
	anti-mouse Alexa 488 (Jackson ImmunoResearch, West Grove, PA)	1:500	NGS/NDS	1h
BrdU	rat anti-BrdU (Accurate Chemical, Westbury, NY)	1:500	NGS/NDS	O/N
	anti-rat Cy3 (Jackson ImmunoResearch, West Grove, PA)	1:500	NGS/NDS	1h
DCX	goat anti-DCX antibody (Santa Cruz, Santa Cruz, CA)	1:1000	NDS	48h
	anti-goat Cy3 (Jackson ImmunoResearch, West Grove, PA)	1:500	NDS	1h

2.6.5 Image analysis

Fluorescence labeled brain slices were examined with confocal microscopy. Z-plane sectioning (1-2 μm steps) was performed to confirm the colocalization of BrdU/NeuN, YFP/DCX or YFP/DRAQ5 double positive cells. For BrdU/NeuN and YFP/DCX staining, pictures were taken from the dentate gyrus (DG) of hippocampus. Cells were counted if they were within the subgranule cell layer. For YFP/Draq5 double staining, pictures were taken from the DG, CA1 of hippocampus as well as frontal cortex in addition to DG.

2.7 ELISA

2.7.1 Corticosterone

Trunk blood of adult and post-weaning mice was collected from the opened body cavity after cervical dislocation 3 hours before the end of the light phase. The blood of pre-weaning mice was collected by decapitation. To avoid order of sampling effects, all animals in a cage were killed at once within <1 min. The serum was collected with Amber tubes with serum separator (BD, Franklin Lakes,

New Jersey), and Cort was quantified with the Correlate EIA Corticosterone Enzyme Immunoassay Kit (Assay Designs, Ann Arbor, Michigan).

2.7.2 Leptin

The serum was collected from trunk blood as described above. Leptin was quantified with leptin (mouse) EIA kit (#ADI-900-019A, Enzo Life Sciences, Plymouth Meeting, PA).

2.8 Statistical analysis

Statistical analyses were conducted with Minitab15 (Minitab, State College, Pennsylvania).

2.8.1 Comparison of two samples

Student's t-test, Mann-Whitney test, or X^2 goodness of fit test were used for the comparison of two samples depending on the distribution of the data. Two sample t-test is most often used to compare two group means. The assumption of this test is that the mean of the two samples is normally distributed. According to central limit theorem, the mean of a large number of independent random variables is approximately normally distributed regardless of the sample distribution. Moreover, if the population follows normal distribution, any sample from the same population follows normal distribution regardless of the sample size. Thus two-sample t-test can be performed in either of the following two situations: 1) the sampling distribution is known to be normal; 2) the sample size is large regardless of whether the population shows normal distribution or not. Generally speaking, two sample t-test is insensitive to departures from normality, even for fairly small sample sizes (for example $n > 8$) (Miller and Brown 1997). Importantly, the insensitivity to non-normality only avoids making false rejections and does not guarantee adequate statistical power to detect a difference at all. When the normality assumption doesn't hold, especially in the presence of large outliers, an alternative procedure, like data transformation followed by t-test or

nonparametric methods were used. In this thesis, Mann-Whitney test was used as a non-parametric test. Finally, the X^2 goodness of fit test was used for the analysis of FST data whenever a large proportion of mice didn't stop swimming in the entire 6 min test. In this case each mouse was assigned to one of two categories consisting of mice that (1) were swimming only or (2) that were part-time swimming and part-time floating. The data were then analyzed using the X^2 goodness of fit test.

2.8.2 ANOVA and multiple comparisons

Analyses of variance (ANOVAs) were used for analyses of genotype effects, treatment effects and genotype × treatment interactions. Experiments performed on different days were treated as blocks, and the blocking effect was treated as random factor. Most measures followed an approximate normal distribution except that the time to first immobility measurement in the forced swim test is more log normal in nature. Log transformation of the data was performed if necessary to satisfy the normality and homogeneous variance assumption. The assumptions were judged by eye based on the normality plot and residual versus fit plot. Outliers that differed from the mean by >2.5 SD were removed from the analyses in chapter 3.

Post hoc comparisons of multiple group means were done by Dunnett's or Fisher's least significant difference test. Comparisons of multiple group means can lead to inflation of the type I error (known as α, Kuehl 2000). For example, if we need to do two comparisons, and the type I error rate is 0.05 for both of them, then the probability that both comparisons are correct will be 0.95*0.95 = 0.9025. There's almost a 10% probability of making a type I error. To avoid making false conclusions, the individual p-value from each pair of comparison has to be adjusted to make sure the type I error rate of the entire experiment (also called family-wise error rate) doesn't exceed 0.05. Dunnett's test is one of the adjusting methods, which is often used to compare a reference group with the rest of groups (Kuehl 2000). In chapter 3 of this thesis, vehicle treated $\gamma2^{+/-}$ mice are

selected as the reference group and compared with vehicle treated WT mice and antidepressant treated $\gamma2^{+/-}$ mice. In chapter 4 of this thesis, CAG-CreERT2 × fγ2/+ mice serve as the reference group and are compared with CAG-CreERT2 and fγ2/+ littermates. By contrast, Fisher test doesn't control family-wise error rate. Some statisticians recommend not correcting for multiple comparisons during the analysis. Instead, the adjustment should be done while interpreting the results (Rothman 1990; Saville 1990). In the drug treatment studies, we are specifically interested in three pairs of comparisons: 1) The difference between vehicle treated WT and $\gamma2^{+/-}$ mice; 2) drug effect on WT mice; 3) drug effect on $\gamma2^{+/-}$ mice. The research interest is pre-determined prior to the experiments, thus the comparisons are considered as planed comparisons. For these reasons, we decided not to use the stringent Tukey procedure, which adjusts the p-value by the total number of possible comparisons without considering research interest. The relevant p-values from Fisher test are reported. Bonferroni correction can be made while interpreting the results. The Bonferroni adjusted p-value approximately equals to the Fisher p-value multiplied by the total number of planned comparisons.

2.8.3 Other analysis

The correlation of two variables was analyzed by simple regression, with the correlation coefficient equaling to the square root of R^2. The standard deviation used for power analysis was obtained from previous experiments. Multivariate analysis of variance (MANOVA) was performed for the analysis of center/corner distance and duration in the OFT due to the correlation of these four parameters.

Chapter 3: $GABA_A$ Receptor Deficits Cause HPA Axis Hyperactivity and Antidepressant Drug Sensitivity Reminiscent of Melancholic Forms of Depression

3.1 Increased anxiety-like behavior in $\gamma2^{+/-}$ mice is ameliorated by chronic antidepressant treatment

The behavioral phenotype of $\gamma2^{+/-}$ mice compared with WT mice includes an increased mean latency to feed in the NSFT (Figure 3.1A, vehicle treatment), as shown previously (Earnheart et al. 2007). This test is thought to have predictive validity for antidepressant drug activity in humans, although ethologically the behavioral measure is anxiety-related. Moreover, the NSFT is among the few paradigms that elicit behavioral changes selectively in response to chronic but not acute or subchronic antidepressant drug treatment (Bodnoff et al. 1988). Compared with vehicle treatment, both fluoxetine (20 mg/L in drinking water, ad libitum for 28 days) and desipramine (480 mg/L in drinking water, ad libitum for 28 days) produced anxiolytic-like effects in $\gamma2^{+/-}$ mice and effectively normalized the increased latency to feed of $\gamma2^{+/-}$ mice to WT levels (for statistics, see figure legends; a complete list of ANOVAs for all behavioral tests is provided in Table 3.1. The posthoc analysis is provided in Table 3.2). No significant treatment effects were evident in WT mice.

Analysis of genotype × treatment interactions confirmed that $\gamma2^{+/-}$ mice were sensitive to chronic treatment with desipramine, in contrast to WT mice ($p < .05$). A clear corresponding trend was also evident for fluoxetine ($p = .08$). Unlike chronic treatment, subchronic treatment with either drug for 5 days failed to reduce latency to feed in $\gamma2^{+/-}$ mice (Figure 3.1B). Moreover, although subchronic treatment with desipramine had no effect, subchronic fluoxetine further increased the latency to feed in $\gamma2^{+/-}$ mice, consistent with anxiogenic effects and elevated Cort levels reported in patients during initial treatment with fluoxetine (Gorman et al. 1987).

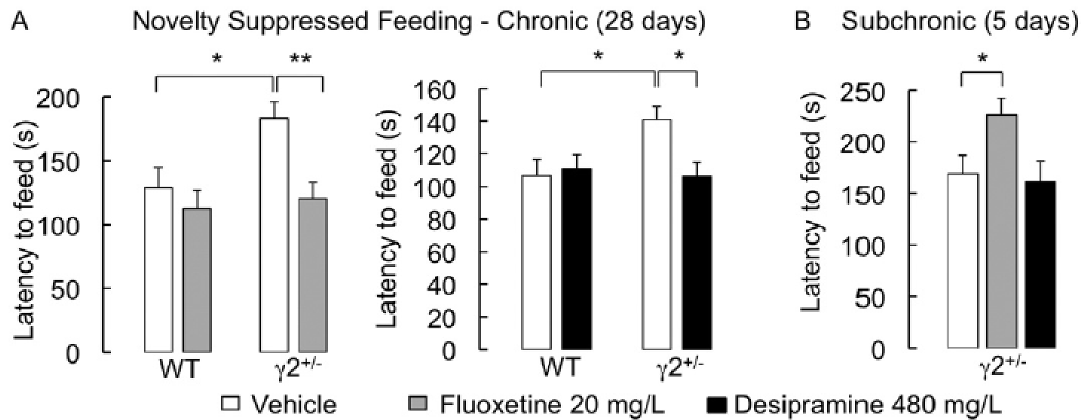

Figure 3.1 $GABA_AR$ heterozygous ($\gamma 2^{+/-}$) mice show increased anxiety-related behavior that is sensitive to chronic treatment with desipramine and fluoxetine. (A) Separate groups of mice were subject to 28-day chronic treatment with vehicle, desipramine or fluoxetine, respectively, followed by analysis in the NSFT at 12 weeks of age. Vehicle-treated $\gamma 2^{+/-}$ vs. WT mice showed increased latency to feed (n = 8–16, $p < .05$ in both experiments, analysis of variance [ANOVA], Dunnett's test), consistent with previous results (Earnheart et al. 2007). Chronic treatment of $\gamma 2^{+/-}$ mice with fluoxetine (20 mg/L in drinking water, left panel) or desipramine (480 mg/L in drinking water, right) had anxiolytic-like effects in that both drugs reduced the latency to initiate feeding compared with vehicle control subjects (fluoxetine, n = 16, $p < .01$; desipramine, n = 10–11, $p < .01$; ANOVA, Dunnett's test). By contrast, drug treatment had no effect on WT mice (n = 8–13, $p > .05$ for either drug). **(B)** Subchronic fluoxetine (5 days, 20 mg/L) vs. vehicle increased the mean latency of $\gamma 2^{+/-}$ mice to initiate feeding (n = 14–16, $p < .05$, ANOVA, Dunnett's test) and thus had an anxiogenic-like effect. By contrast, subchronic treatment of $\gamma 2^{+/-}$ mice with desipramine (480 mg/L) vs. vehicle had no effect on the mean latency to initiate feeding (n = 13–16, $p > .05$, ANOVA, Dunnett's test). *$p < .05$, **$p < .01$.

Table 3.1. ANOVA of antidepressant drug effects on behavior of $\gamma 2^{+/-}$ vs. WT mice described in Figures 3.1 – 3.4.

Test Parameter	Drug Conc. (mg/L) in Drink. Water	Group Size	Genotype Effect	Treatment Effect	Genotype x Treatment Interaction	Figure
NSF test - latency to feed	Desipramine 480	8-12	$p = 0.066$ $F(1,34) = 3.61$	$p = 0.076$ $F(1,34) = 3.36$	$p = 0.02$ $F(1,34) = 5.98$	3.1A
FS test - time to 1st immobility	Desipramine 120,240 & 480	13-29	$p = 0.044$ $F(1,140) = 4.12$	$p = 0.047$ $F(3,140) = 2.72$	$p = 0.003$ $F(3,140) = 4.97$	3.2A
FS test - immobility	Desipramine 120,240 & 480	12-29	$p < 0.001$ $F(1,134) = 12.9$	$p < 0.001$ $F(3,134) = 8.9$	$p = 0.008$ $F(3,134) = 4.09$	3.2A
TS test - immobility	Desipramine 240	10-13	$p = 0.172$ $F(1,43) = 1.93$	$p = 0.024$ $F(1,43) = 5.44$	$p = 0.303$ $F(1,43) = 1.09$	3.2B
NSF test - latency to feed	Fluoxetine 20	11-16	$p = 0.026$ $F(1,51) = 5.27$	$p = 0.004$ $F(1,51) = 9.09$	$p = 0.080$ $F(1,51) = 3.20$	3.1A
FS test - time to 1st immobility	Fluoxetine 20	12-14	$p < 0.001$ $F(1,45) = 19.6$	$p = 0.006$ $F(1,45) = 8.32$	$p = 0.034$ $F(1,45) = 4.81$	3.2C
FS test - immobility	Fluoxetine 20	12-14	$p = 0.002$ $F(1,45) = 11.3$	$p = 0.196$ $F(1,45) = 1.72$	$p = 0.385$ $F(1,45) = 0.77$	3.2C
TS test - immobility	Fluoxetine 20	12-13	$p = 0.106$ $F(1,44) = 2.73$	$p = 0.531$ $F(1,44) = 0.4$	$p = 0.306$ $F(1,44) = 1.07$	3.2D
SC test - sucrose consumption	Fluoxetine 20* Desipramine 240*	11-15	$p = 0.02$ $F(1,74) = 5.62$	$p < 0.001$ $F(2,74) = 18.5$	$p = 0.302$ $F(2,74) = 1.22$	3.4B
SC test - water consumption	Fluoxetine 20* Desipramine 240*	13-15	$p = 0.788$ $F(1,77) = 0.07$	$p = 0.008$ $F(2,77) = 5.16$	$p = 0.069$ $F(2,77) = 2.77$	3.4C
SC test - normalized sucrose consumption	Fluoxetine 20* Desipramine 240*	12-15	$p = 0.084$ $F(1,72) = 3.06$	$p < 0.001$ $F(2,72) = 34.7$	$p = 0.34$ $F(2,72) = 1.1$	3.4D

*, Antidepressant drug treatment was switched to daily p.o. injections starting on the 22th day (fluoxetine, 4 mg/kg; desipramine, 30 mg/kg)

NSF, novelty suppressed feeding; FS, forced swim; TS, tail suspension; SC, sucrose consumption

Table 3.2. Posthoc analyses of drug effects on behavior of γ2$^{+/-}$ vs. WT mice described in Figure 3.1.

Test	Behavioral Parameter	Group 1	Group 2	Group Size	Posthoc Test	Effect *p* value	Figure
NSFT	Latency to feed	γ2$^{+/-}$ vehicle	WT vehicle	8-11	Dunnett	0.035	3.1A
	Latency to feed	γ2$^{+/-}$ DES 480 chronic	γ2$^{+/-}$ vehicle	10-11	Dunnett	0.018	3.1A
	Latency to feed	WT DES 480 chronic	WT vehicle	8-10	Dunnett	0.917	3.1A
	Latency to feed	γ2$^{+/-}$ vehicle	WT vehicle	11-16	Dunnett	0.019	3.1A
	Latency to feed	γ2$^{+/-}$ FLX 20 chronic	γ2$^{+/-}$ vehicle	16	Dunnett	0.002	3.1A
	Latency to feed	WT FLX 20 chronic	WT vehicle	11-13	Dunnett	0.738	3.1A
	Latency to feed	γ2$^{+/-}$ FLX 20 subchronic	γ2$^{+/-}$ vehicle	14-16	Dunnett	0.043	3.1B
	Latency to feed	γ2$^{+/-}$ DES 480 subchronic	γ2$^{+/-}$ vehicle	13-16	Dunnett	0.999	3.1B

DES, desipramine; FLX, fluoxetine.

3.2 The γ2$^{+/-}$ mice exhibit depression-related behavior and selective antidepressant drug responses to desipramine but not fluoxetine

In the FST, γ2$^{+/-}$ mice demonstrated a phenotype indicative of behavioral despair, as suggested by the reduced mean time spent swimming until the first immobility episode and the increased time spent immobile compared with WT littermates (Figures 3.2A and 3.2C, vehicle; Table 3.3), consistent with previous results (Earnheart et al. 2007). Chronic treatment with desipramine had antidepressant-like effects in that it dose-dependently reversed the behavior of γ2$^{+/-}$ mice to that of vehicle- or desipramine-treated WT littermates. Specifically, desipramine (240 or 480 mg/L, in drinking water for 35 days) compared with vehicle treatment dose-dependently increased the time to first immobility and reduced the total time spent immobile of γ2$^{+/-}$ mice (Figure 3.2A). No behavioral effects were evident, with a low dose of desipramine at 120 mg/L (Figure 3.2A). When analyzed in the TST (Figure 3.2B), chronic desipramine (240 mg/L, 42 days) reduced the immobility of γ2$^{+/-}$ mice analogous to results from the FST. The behavior of WT mice was unaffected by chronic desipramine treatment in both the FST and TST,

similarly to the NSFT, and genotype × treatment ANOVAs of FST data indicated increased sensitivity to desipramine of $\gamma2^{+/-}$ versus WT mice ($p < .01$ for both parameters).

Chronic fluoxetine had anxiolytic-like effects in $\gamma2^{+/-}$ mice in the NSFT, similar to desipramine and as mentioned earlier (Figure 3.1A). By contrast, in the FST and TST, the behavioral effects of fluoxetine were markedly different from those of desipramine. Chronic fluoxetine compared with vehicle paradoxically reduced the latency to first immobility in WT mice in the FST and failed to ameliorate the behavioral immobility of $\gamma2^{+/-}$ mice with respect to all parameters evaluated in both the FST and TST (Figures 3.2C and 3.2D). Drug treatment had no effect on body weight (mean weights after treatment with desipramine for 6 weeks at 480 and 120 mg/L, 26.1 and 26.3 g vs. vehicle 26.8 g, $n = 16$; fluoxetine at 20 mg/L, 23.1 g vs. vehicle 23.3 g, $n = 23, 24$, $p > .05$ for all pairwise comparisons), indicating that the drug effects were not simply due to altered calorie intake or body weight. To test whether lack of a behavioral response of $\gamma2^{+/-}$ mice to fluoxetine was a peculiar feature of the 129X1/SvJ strain background of $\gamma2^{+/-}$ mice, we crossbred $\gamma2^{+/-}$ 129X1/SvJ and WT C57BL/6 mice to generate $\gamma2^{+/-}$ mice on a filial generation 1 (F1) hybrid background. Similar to $\gamma2^{+/-}$ 129X1/SvJ mice, $\gamma2^{+/-}$ (F1)C57BL/6:129X1/SvJ mice lacked antidepressant-like responses to fluoxetine in the FST, while maintaining an anxiolytic-like response in the NSFT (Figure 3.3). Thus, the absence of antidepressant-like responses of $\gamma2^{+/-}$ mice to fluoxetine is not limited to the 129X1/SvJ strain.

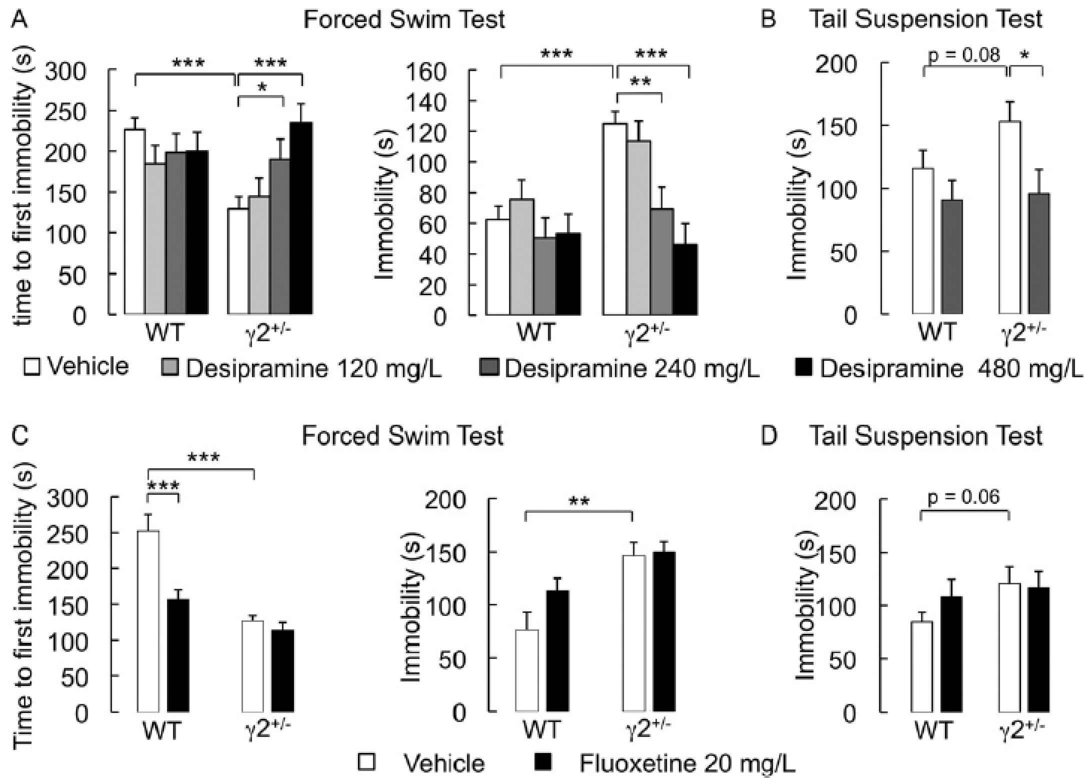

Figure 3.2 The γ2$^{+/-}$ mice show antidepressant-like responses to desipramine but not fluoxetine in the FST and TST. (A–D) Antidepressant drug- or vehicle-treated γ2$^{+/-}$ and WT mice were subjected to the FST **(A, C)** and TST **(B, D)** at 13 and 14 weeks of age, corresponding to drug treatment for 35 and 42 days, respectively. **(A)** In the FST, vehicle-treated γ2$^{+/-}$ vs. WT mice started to float sooner and spent more time immobile (n = 26–29, mean time to first immobility, $p < .001$; immobility, $p < .001$, ANOVA, Fisher test). Desipramine (240 and 480 mg/L) vs. vehicle increased the time to first immobility ($p < .05$ and $p < .001$, respectively) and reduced the total immobility of γ2$^{+/-}$ mice (n = 14–29, $p < .01$ [240 mg/mL], $p < .001$ [480 mg/mL]). No significant drug effects were observed in γ2$^{+/-}$ mice treated with 120 mg/L desipramine or with any desipramine concentration in WT mice. **(B)** In the TST, desipramine (240 mg/L) vs. vehicle reduced the immobility of γ2$^{+/-}$ mice [$t(19) = 2.26$, n = 10–11, $p < .05$] but had no significant effect on WT mice [$t(23) = .98$, $n = 13$, p = ns]. Simple comparison of vehicle-treated γ2$^{+/-}$ and WT mice revealed a tendency toward greater immobility in γ2$^{+/-}$ mice [$t(21) = 1.82$, $p = .08$]. **(C, D)** Fluoxetine (20 mg/L, 35 days) paradoxically reduced the time to first immobility of WT mice in the FST **(C)** (n = 12–14, $p < .001$, ANOVA, Fisher test) and lacked antidepressant-like effects, irrespective of test, in γ2$^{+/-}$ mice. Vehicle-treated γ2$^{+/-}$ vs. WT littermates analyzed in parallel showed the depressive-like phenotype also evident in **(A, B)** ($p < .001$). *$p < .05$, **$p < .01$, ***$p < .001$. Abbreviations as in Figure 3.1.

Table 3.3. Posthoc analyses of drug effects on behavior of γ2$^{+/-}$ vs. WT mice described in Figures 3.2.

Test	Behavioral Parameter	Group 1	Group 2	Group Size	Posthoc Test	Effect *p* value	Figure
FST	Time to first Immobility Total Immobility	γ2$^{+/-}$ vehicle	WT vehicle	28-29 26-29	Fisher Fisher	<0.001 <0.001	3.2A
	Time to first Immobility Total Immobility	γ2$^{+/-}$ DES 120	γ2$^{+/-}$ vehicle	16-29 16-29	Fisher Fisher	0.465 0.464	3.2A
	Time to first Immobility Total Immobility	γ2$^{+/-}$ DES 240	γ2$^{+/-}$ vehicle	13-29 12-29	Fisher Fisher	0.035 0.001	3.2A
	Time to first Immobility Total Immobility	γ2$^{+/-}$ DES 480	γ2$^{+/-}$ vehicle	15-29 14-29	Fisher Fisher	<0.001 <0.001	3.2A
	Time to first Immobility Total Immobility	WT DES 120	WT vehicle	16-28 16-26	Fisher Fisher	0.128 0.393	3.2A
	Time to first Immobility Total Immobility	WT DES 240	WT vehicle	16-28 15-26	Fisher Fisher	0.216 0.456	3.2A
	Time to first Immobility Total Immobility	WT DES 480	WT vehicle	16-28 16-26	Fisher Fisher	0.368 0.550	3.2A
	Time to first Immobility Total Immobility	γ2$^{+/-}$ FLX 20	γ2$^{+/-}$ vehicle	12 12	Fisher Fisher	0.642 0.769	3.2C
	Time to first Immobility Total Immobility	WT FLX 20	WT vehicle	12-14 12-14	Fisher Fisher	<0.001 0.121	3.2C
TST	Total Immobility	γ2$^{+/-}$ vehicle	WT vehicle	10-13	Dunnett	0.221	3.2B
	Total Immobility	γ2$^{+/-}$ DES 240	γ2$^{+/-}$ vehicle	10-11	Dunnett	0.071	3.2B
	Total Immobility	γ2$^{+/-}$ vehicle	WT vehicle	12-13	Dunnett	0.138	3.2D
	Total Immobility	γ2$^{+/-}$ FLX 20	γ2$^{+/-}$ vehicle	12-13	Dunnett	0.987	3.2D

DES, desipramine; FLX, fluoxetine.

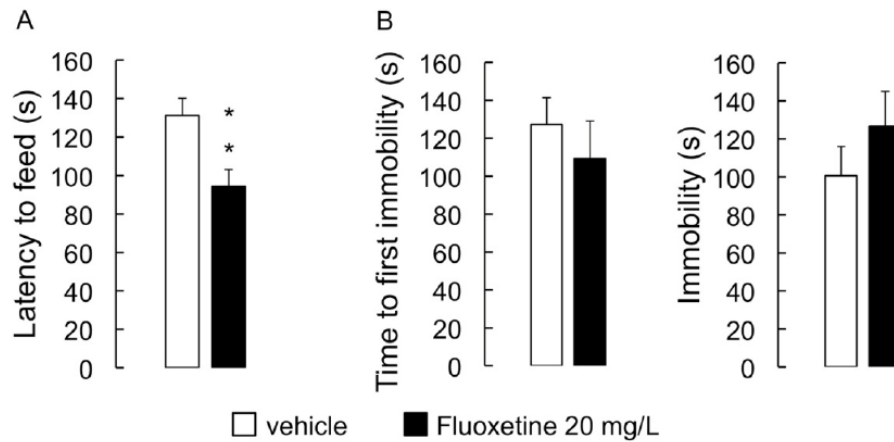

Figure 3.3. Behavioral responses of γ2$^{+/-}$ (F1)C57BL/6:129SvJ/X1 mice to chronic treatment with fluoxetine. (A) In the NSFT, fluoxetine (28 days, 20 mg/L in drinking water, ad lib) reduced the latency to feed of γ2$^{+/-}$ C57BL/6:129SvJ/X1 mice vs. untreated controls (t(22) = 2.94, $n = 12$, $p < 0.01$). **(B)** However, in the FST, fluoxetine (35 days, 20 mg/L) did not affect behavior of γ2$^{+/-}$ C57BL/6:129SvJ/X1 (F1) mice, as indicated by unaltered time to first immobility [t(30) = -0.74, n = 12, p(ns)] and total immobility times [t(30) = 1.09, p(ns)].

To substantiate the depression-related phenotype and selective responsiveness to antidepressant drugs of γ2$^{+/-}$ mice, we employed a variation of the SCT to assess anhedonia-like behavior (Figure 3.4A, Tables 3.4) (Monleon et al. 1995), a core symptom of clinical depression (Fawcett et al. 1983). Vehicle-treated γ2$^{+/-}$ mice consumed less sucrose than WT control subjects (Figure 3.4B, Table 3.4), consistent with an anhedonia-like phenotype of γ2$^{+/-}$ mice. Chronic treatment with desipramine (28+ days) increased the sucrose consumption of both γ2$^{+/-}$ and WT mice, indicating an antidepressant-like drug effect independent of genotype. By contrast, fluoxetine had an anhedonia-like depressive effect on WT but not γ2$^{+/-}$ mice, reminiscent of the genotype-specific detrimental effects of this drug in the FST. The water consumption of drug-treated γ2$^{+/-}$ and WT mice analyzed by pairwise comparison of groups was indistinguishable from vehicle control subjects. However, desipramine resulted in a genotype-independent reduction in water consumption (Figure 3.4C). Importantly, normalization of sucrose consumption to water consumption reproduced the data seen without normalization (Figure 3.4D). The data indicate that anhedonia-like behavior of

$\gamma 2^{+/-}$ mice is reversed by desipramine but not fluoxetine. Unlike in the NSFT, FST, and TST, the SCT showed antidepressant-like effects of desipramine in WT mice.

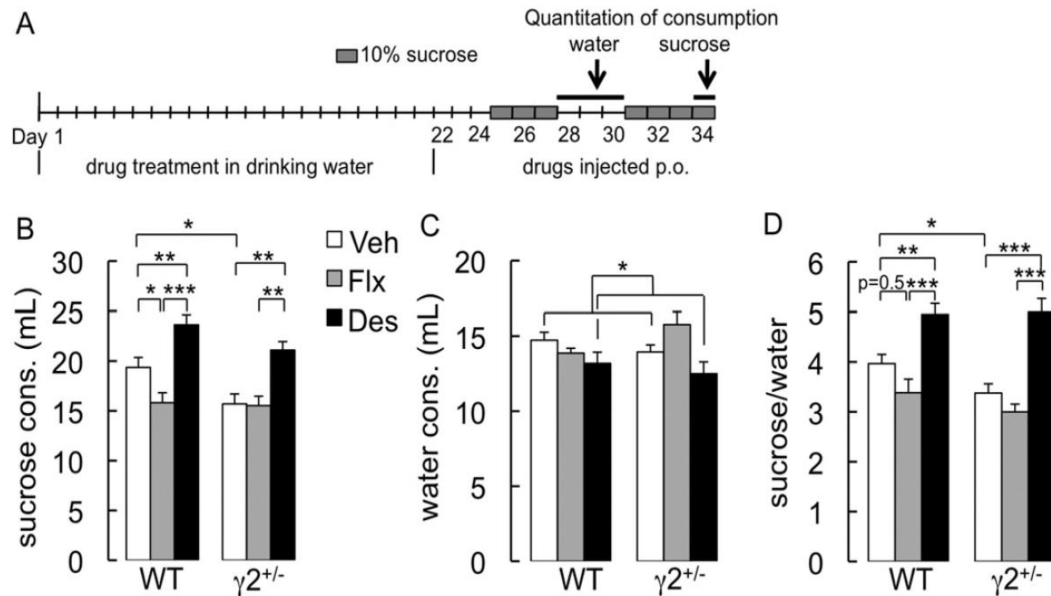

Figure 3.4 The $\gamma 2^{+/-}$ mice show antidepressant-like responses to desipramine (Des) but not fluoxetine (Flx) in the sucrose consumption test (SCT). (A) Time course of experiment: antidepressant drug treatment was initiated on Day 1 (8 weeks of age) and was switched to daily per oral (p.o.) injections starting on the 22nd day (Flx, 4 mg/kg; Des, 30 mg/kg). Vehicle (Veh)-treated mice were handled identically but injected with water. The mice were singly housed starting on the 24th day of drug treatment and trained to drink sucrose (10%) for 3 days starting on the 25th day. Water consumption (cons.) was measured over a period of 3 days from the 28th to the 30th day, and sucrose cons. was measured over 22 hours on the last day of a 4-day sucrose cons. period. **(B)** Sucrose cons.: Veh-treated $\gamma 2^{+/-}$ mice consumed less sucrose than WT littermates (n = 13–15; $p < .05$, Fisher test), indicating an anhedonia-like phenotype of $\gamma 2^{+/-}$ mice. Chronic treatment with Des had antidepressant-like effects in that it increased the sucrose cons. of both WT and $\gamma 2^{+/-}$ mice (n = 11–15; $p < .01$ for both comparisons). By contrast, Flx reduced the sucrose cons. of WT mice (n = 14–15; $p < .05$) and had no effect on $\gamma 2^{+/-}$ mice. Similar genotype and treatment effects but with lower levels of significance were evident for sucrose cons. on Days 31–33 (not shown). **(C)** Water cons. assessed by pairwise comparisons of Veh- and drug-treated groups was unaltered ($p > .05$ for all comparisons). However, Des-treated mice showed a genotype- independent reduction in water cons. ($p < .05$). **(D)** Normalization of sucrose cons. data in **(A)** for water cons. in **(B)** reproduced the anhedonia-like phenotype and accentuated the antidepressant drug effects observed without normalization. *$p < .05$, **$p < .01$, ***$p < .001$. Abbreviations as in Figure 3.1.

Table 3.4. Posthoc analyses of drug effects on behavior of γ2$^{+/-}$ vs. WT mice described in Figure 3.4.

Test	Behavioral Parameter	Group 1	Group 2	Group Size	Posthoc Test	Effect *p* value	Figure
SCT	Sucrose consumption	γ2$^{+/-}$ vehicle	WT vehicle	13-15	Fisher	0.02	3.4B
	Water consumption			13-15	Fisher	0.389	3.4C
	Sucrose/Water			13-15	Fisher	0.049	3.4D
	Sucrose consumption	γ2$^{+/-}$ DES 240	γ2$^{+/-}$ vehicle	11-13	Fisher	0.002	3.4B
	Water consumption			13	Fisher	0.126	3.4C
	Sucrose/Water			12-13	Fisher	<0.001	3.4D
	Sucrose consumption	γ2$^{+/-}$ FLX 20	γ2$^{+/-}$ vehicle	13	Fisher	0.914	3.4B
	Water consumption			13	Fisher	0.055	3.4C
	Sucrose/Water			13	Fisher	0.220	3.4D
	Sucrose consumption	γ2$^{+/-}$ FLX 20	γ2$^{+/-}$ DES 240	11-13	Fisher	0.001	3.4B
	Water consumption			13	Fisher	<0.001	3.4C
	Sucrose/Water			12-13	Fisher	<0.001	3.4D
	Sucrose consumption	WT DES 240	WT vehicle	14-15	Fisher	0.006	3.4B
	Water consumption			15	Fisher	0.08	3.4C
	Sucrose/Water			12-15	Fisher	0.002	3.4D
	Sucrose consumption	WT FLX 20	WT vehicle	14-15	Fisher	0.021	3.4B
	Water consumption			14-15	Fisher	0.337	3.4C
	Sucrose/Water			13-15	Fisher	0.051	3.4D
	Sucrose consumption	WT FLX 20	WT DES 240	14	Fisher	<0.001	3.4B
	Water consumption			14-15	Fisher	0.441	3.4C
	Sucrose/Water			12-13	Fisher	<0.001	3.4D

SCT, sucrose consumption test; FLX, fluoxetine, DES, desipramine

3.3 Leptin serum levels are reduced in γ2$^{+/-}$ mice

Leptin is a peptide hormone that regulates appetite, energy homeostasis (Elmquist et al. 1998), reproduction (Chehab 2000) and cognition (Farr et al. 2006). Reduced plasma leptin levels have been reported in rodents that were subjected to chronic stress (Lu et al. 2006) as well as MDD patients (Kraus et al. 2001; Jow et al. 2006). γ2$^{+/-}$ mice show reduced serum leptin levels compared to WT mice (Figure 3.5A). However, the significance of this change is unclear as the daily food consumption measured over 10 days and the body weight at 14 weeks of age are not affected (Figure 3.5B, C).

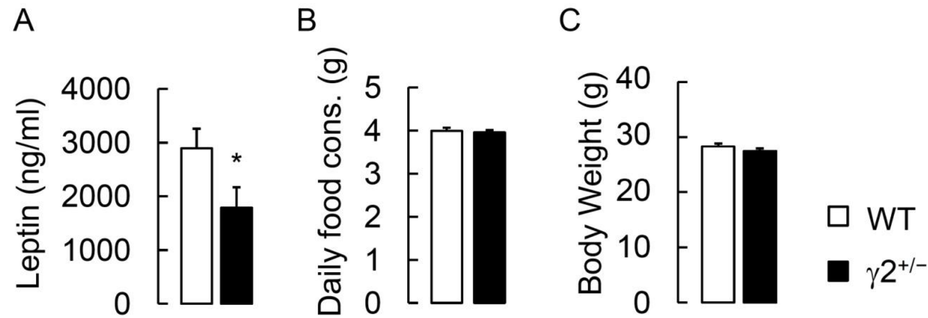

Figure 3.5. Leptin levels are reduced in γ2$^{+/-}$ mice. A) Serum leptin levels in 12-week-old females are reduced compared with WT mice (WT: 2897 ± 359.4; γ2$^{+/-}$: 1732 ± 359.4, n = 12, *p* < .05, ANOVA) **B, C)** The average daily food consumption over a period of 10 days (B) and the body weight (C) of γ2$^{+/-}$ males at 14 weeks of age is unaltered (n = 11-15, *p* > .05, two-sample t test).

3.4 Corticosterone serum levels are elevated in γ2$^{+/-}$ mice.

An HPA axis dysfunction represents the most common neuroendocrine abnormality of major depressive disorder (Tichomirowa et al. 2005). To assess whether γ2$^{+/-}$ mice exhibit altered HPA axis activity we measured serum Cort levels before and at different time points after a 5-min forced swim stressor (Figure 3.6A, Table 3.5). The baseline Cort levels of γ2$^{+/-}$ versus WT mice were elevated approximately twofold, both before the stressor and after a 60 min recovery period. By contrast, stress-induced Cort concentrations at 7.5, 12.5, and 30 min after the end of the stressor were unaltered in γ2$^{+/-}$ compared with WT mice, indicating that the response to and recovery from acute physical stress remained intact. The genotype-dependent differences in basal Cort were seen in both male and female mice (Figure 3.6B). The data indicate that γ2$^{+/-}$ mice represent an animal model of depressive disorders associated with constitutive HPA axis hyperactivity.

We previously showed that the behavioral phenotype of γ2$^{+/-}$ mice is replicated in conditional "early" γ2$^{+/-}$ mice (Emx1Cre × fγ2/+), which exhibit a $GABA_AR$ deficit induced at approximately embryonic Day 10 that is restricted to glutamatergic

neurons of the telencephalic forebrain (Earnheart et al. 2007). By contrast, the developmentally delayed forebrain-specific deficit of “late” $\gamma 2^{+/-}$ mice (CaMKIICre2834 × fγ2/+) induced between the fourth and fifth postnatal week does not affect anxiety- and depression-related behavior, suggesting selective vulnerability to GABAergic deficits during embryonic or postnatal development (Schweizer et al. 2003; Earnheart et al. 2007). Here, we examined whether differences in anxiety- and depression-related behavior of the two forebrain-specific γ2-deficient mouse lines were correlated with different deficits in HPA axis function. Baseline Cort concentrations assessed at 8 weeks of age were elevated approximately twofold in both “early” and “late” γ2-deficient mice compared with respective pseudo WT (fγ2/+) control subjects, and this was evident in both behaviorally naive animals and after recovery from stress and in both female (Figure 3.6C) and male mice (not shown). These data suggested HPA axis hyperactivity assessed in adulthood may be independent of the developmental time course of the $GABA_AR$ deficit (however, see below). The fact that Emx1Cre-mediated recombination is highly restricted to the telencephalic forebrain and absent in the hypothalamus (Iwasato et al. 2004), indicates that HPA axis hyperactivity is caused by a $GABA_AR$ deficit in the telencephalic forebrain and independent of genetic lesion of the HPA axis.

The aforementioned experiments suggested that twofold elevated baseline Cort alone is insufficient to induce the anxious depressive phenotype of “early” or global $\gamma 2^{+/-}$ mice. Alternatively, differences in behavior between “early” or global and “late” $GABA_AR$-deficient mice might reflect the different developmental time courses of HPA axis deficits. Corticosterone concentrations of global $\gamma 2^{+/-}$ mice were normal at postnatal day (P) 17 (3 days before weaning) and markedly elevated at P23 (3 days after weaning) compared with littermate WT control subjects — consistent with this latter idea — whereas “late” $GABA_AR$-deficient mice remained indistinguishable from littermate control subjects until at least P23 (Figure 3.6D). Thus, HPA axis hyperactivity that develops around the time of weaning correlates with heightened emotional behavior of γ2-deficient mice in

adulthood, whereas developmentally delayed HPA axis hyperactivity of “late” $GABA_AR$-deficient mice is without anxiety and depression-related behavioral consequences.

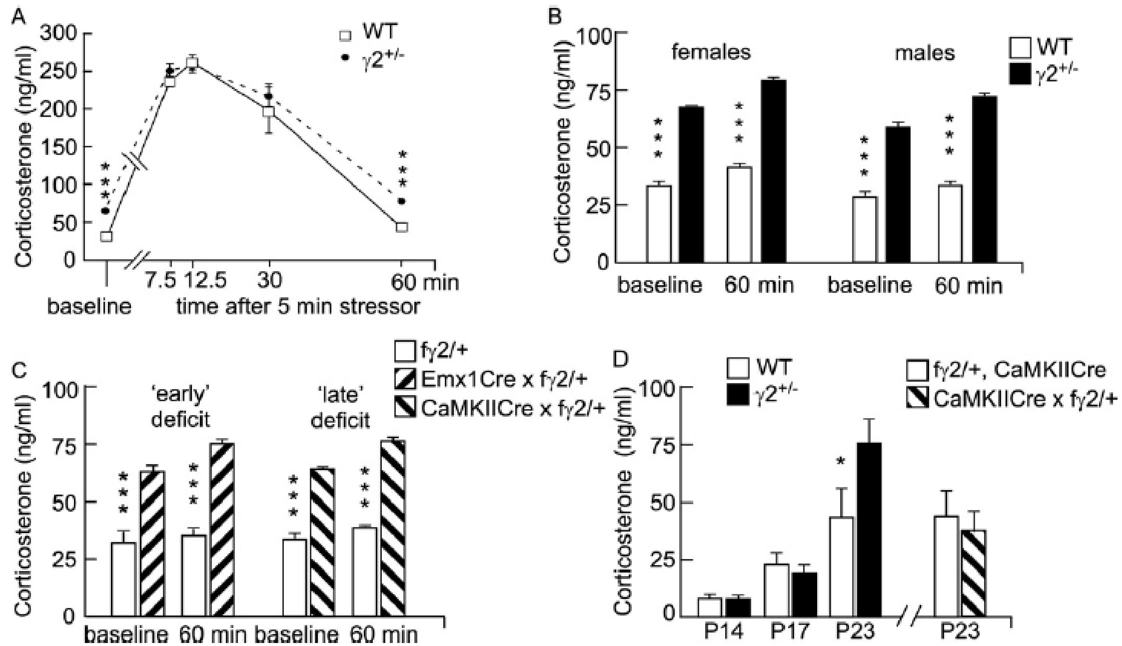

Figure 3.6 Corticosterone levels are elevated in γ2 subunit-deficient mice. (A, B) Serum corticosterone levels in 8-week-old female **(A, B)** and male **(B)** $\gamma2^{+/-}$ and WT control subjects were measured at baseline and 7.5, 12.5, 30, or 60 min after a 5-min forced swim stressor. Corticosterone levels of $\gamma2^{+/-}$ vs. WT littermates were elevated both in behaviorally naïve animals (baseline) and after 60-min recovery from a 5-min stressor, and this effect was seen in both females and males ($n = 9$, $p < .001$ for all comparisons). Stress-induced corticosterone levels 7.5, 12.5, and 30 min after the end of the stressor were not different between genotypes ($n = 3$, $p < .05$ for all comparisons). **(C)** Corticosterone concentrations at 8 weeks of age of both Emx1Cre × fγ2/+ mice with an “early” deficit and CaMKIICre2834 × fγ2/+ mice with a “late” deficit were elevated compared with respective littermate fγ2/+ control subjects, both at baseline and after recovery from stress and in both male and female mice ($n = 3$–6, $p < .001$ for all comparisons, data shown for females only). **(D)** Corticosterone concentrations of $\gamma2^{+/-}$ mice compared with WT littermates (mixed sex) were unaltered at postnatal day (P) 14 ($n = 8,12$) and P17 ($n = 6,10$; $p > .05$ in both cases) but markedly increased 3 days after weaning at P23 ($n = 8$, $p < .05$). By contrast, CaMKIICre2834 × fγ2/+ remained indistinguishable from littermate control subjects (CaMKIICre2834 and fγ2/+) until at least P23 ($n = 7,9$, $p = .95$). Values represent means ± SEMs (two-sample two-tailed *t* tests). For numerical values see Table 3.5. *$p < .05$, ***$p < .001$.

Table 3.5. Corticosterone concentrations of γ2 subunit-deficient mice.

			Time after end of a 5 min forced swim stressor			
Sex	Genotype	Baseline	7.5 min	12.5 min	30 min	60 min
Female	$\gamma 2^{+/-}$ WT	66.8 ± 1.14 33.1 ± 1.4 $n = 9$ $p < 0.001$	249.2 ± 10 237.5 ± 7 $n = 3$ $p > 0.05$	258 ± 9.4 261.3 ± 10.8 $n = 3$ $p > 0.05$	218.9 ± 16.3 197.6 ± 32.1 $n = 3$ $p > 0.05$	78.6 ± 0.6 41.1 ± 1.3 $n = 9$ $p < 0.001$
Male	$\gamma 2^{+/-}$ WT	58.1 ± 1.9 28.0 ± 1.7 $n = 9$ $p < 0.001$	nd	nd	nd	71.3 ± 0.6 32.9 ± 0.9 $n = 9$ $p < 0.001$
Female	Emx1Cre x fγ2/+ fγ2/+	62.5 ± 2.4 31.7 ± 4.4 $n = 3\text{-}5$ $p < 0.001$	nd	nd	nd	74.3 ± 1 34.9 ± 2.5 $n = 3\text{-}5$ $p < 0.001$
Male	Emx1Cre x fγ2/+ fγ2/+	53.7 ± 2.0 25.7 ± 2.0 $n = 3\text{-}5$ $p < 0.001$	nd	nd	nd	53.6 ± 1.1 28.9 ± 1.7 $n = 3\text{-}5$ $p < 0.001$
Female	CaMKIICre2834 x fγ2/+ fγ2/+	63.3 ± 0.7 33.2 ± 2.2 $n = 3\text{-}6$ $p < 0.001$	nd	nd	nd	75.7 ± 1.35 38.2 ± 0.4 $n = 3\text{-}6$ $p < 0.001$
Male	CaMKIICre2834 x fγ2/+ fγ2/+	53.4 ± 0.9 27.3 ± 1.8 $n = 3\text{-}6$ $p < 0.001$	nd	nd	nd	52.9 ± 0.7 31.2 ± 0.6 $n = 3\text{-}6$ $p < 0.001$

Serum corticosterone levels (ng/ml) of 8-week old $\gamma 2^{+/-}$, Emx1Cre x fγ2/+, and CaMKIICre2834 x fγ2/+ mice and corresponding littermate controls (WT and fγ2/+, respectively) were measured at baseline and at different time points after the end of a 5 min forced swim stressor, as indicated and illustrated in Figure 3.6. Values represent means ± SEMs, two sample two-tailed *t*-tests. nd, not determined

3.5 Antidepressant efficacy of desipramine in $\gamma 2^{+/-}$ mice is associated with normalizing effects on HPA axis function.

Remission from depressive disorders in response to antidepressant therapy is typically associated with normalization of HPA axis activity (Holsboer and Barden 1996; Ising et al. 2007). Consistent with antidepressant effects in $\gamma 2^{+/-}$ mice, chronic desipramine (28 days, 240 mg/L in drinking water) effectively reversed the increased Cort concentrations of $\gamma 2^{+/-}$ mice to WT levels, whereas it had no

effect on WT mice (Figure 3.7A). In stark contrast, fluoxetine (20 mg/L for 28 days) increased the serum Cort concentrations in WT mice and had no effect on $\gamma 2^{+/-}$ mice (Figure 3.7B). These divergent effects of desipramine versus fluoxetine on Cort levels are consistent with their different behavioral effects in the FST, TST, and SCT (Figures 3.2 and 3.4) and further suggest that fluoxetine is ineffective as an antidepressant in $\gamma 2^{+/-}$ mice. Moreover, the data indicate that the anxiolytic-like behavioral effects of fluoxetine in $\gamma 2^{+/-}$ mice in the NSFT occur despite continuously elevated Cort concentrations.

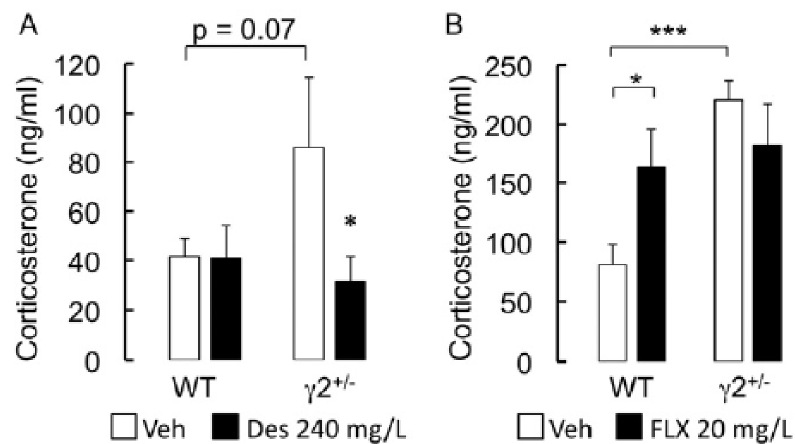

Figure 3.7 Hypothalamic-pituitary-adrenal axis function of $\gamma 2^{+/-}$ mice is normalized by Des but not Flx. (A) Desipramine (240 mg/L, 28 days) vs. Veh reduced the elevated corticosterone levels of $\gamma 2^{+/-}$ mice ($p < .05$), whereas WT mice were unaffected (n = 5–7, $p < .05$, ANOVA, Dunnett's test). **(B)** Fluoxetine (20 mg/L, 28 days) increased corticosterone levels of WT mice ($n = 9$, $p < .05$, ANOVA, Dunnett's test) and had no effect in $\gamma 2^{+/-}$ mice ($n = 9$, $P > .05$). Basal corticosterone levels were elevated in Veh-treated $\gamma 2^{+/-}$ vs. WT mice ($n = 9$, $p < .001$) as evident by a strong tendency also in **(A)** ($p = .07$). *$p < .05$, ***$p < .001$. Abbreviations as in Figures 3.1 and 3.4.

Chapter 4: Genetic and Pharmacologic Dissection of Developmental Critical Periods Underlying Anxious- and Depressive-Like Behavior

4.1 Mapping the developmental critical window for GABAergic regulation of anxious-and depressive-like behavior by tamoxifen-inducible conditional knockout of the $GABA_AR$ γ2 subunit gene

4.1.1 Characterization of CAG-CreERT2 mediated recombination.

To delimit the developmental time course underlying the previously characterized anxious-depressive phenotype of $γ2^{+/-}$ mice (Chapter 3 of this thesis) we employed a tamoxifen-inducible global knock-out strategy, using crosses between mice carrying the ubiquitously expressed and tamoxifen-inducible Cre transgene CAG-CreERT2 (Hayashi and McMahon 2002) and fγ2/+ mice (Schweizer et al. 2003). The CAG-CreERT2 transgene encodes a cytoplasmic Cre recombinase (Cre-ER^{TM}) that is fused to the hormone-binding domain of the estrogen receptor. Treatment of such mice with tamoxifen disrupts an interaction of Cre-ER^{TM} with the endogenous cytoplasmic heat shock protein 90 (Mattioni et al. 1994), thereby allowing Cre-ER^{TM} to enter the nucleus and to induce recombination of loxP site-containing target genes. The recombination profile induced by tamoxifen-mediated activation was examined by first crossing CAG-CreERT2 mice with ROSA26-YFP (R26Y) reporter mice (Srinivas et al. 2001), in which a transcriptional “stop” signal flanked by loxP sites prevents expression of a downstream gene encoding yellow fluorescence protein (YFP). Cre-mediated recombination of loxP sites was induced by injection of tamoxifen on two sequential days (postnatal day (P) 13/14 or P27/28, respectively) and the brains were analyzed four weeks later (Figure 4.1A). Immunofluorescent staining of brain sections for YFP four weeks after tamoxifen injection followed by quantitation in the dentate gyrus granule cell layer revealed similar recombination efficiency at P13/14 and P27/28, independent of the time point of tamoxifen injection (YFP-positive cells as a fraction DRAQ5 positive nuclei: P13/14, 41.7% ± 2.9%; P27/28, 42.5% ± 2.5%, n = 3, Figure 4.1B). Similar recombination

efficiencies were observed in the CA1 region of the hippocampus (46.5%) and frontal cortex (47.9%) in one mouse that showed 45% recombination in the DG. Double labeling for immature neuronal marker doublecortin (DCX) and YFP of P13/14 tamoxifen-injected mice revealed that less than 10% of DCX-positive cells were also labeled for YFP, indicating that few actively dividing stem cells had undergone Cre-induced recombination and that recombination of target genes was largely limited to postmitotic cells (Figure 4.1C).

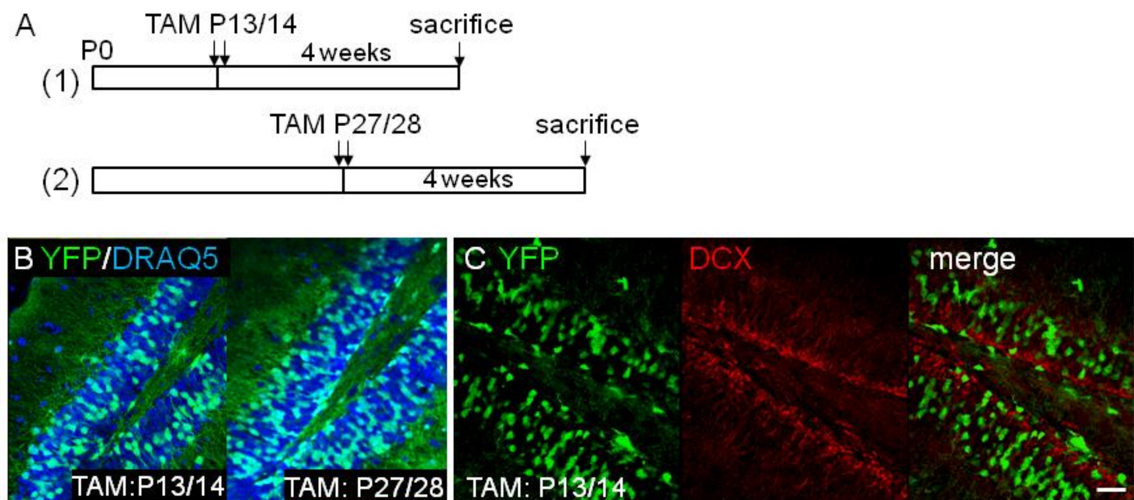

Figure 4.1. Characterization of tamoxifen and CAG-CreERT2-mediated recombination. A, B, C) Tamoxifen was injected into CAG-CreERT2 X R26Y mice on P13 and P14 (B, left panel, C) or P27 and P28 (B, right panel) to induce recombination in third or fifth postnatal week, respectively. Brains were harvested four weeks after the last injection and processed for immunostaining. **B)** Hippocampal sections through the dentate gyrus of brains harvested 4 weeks after tamoxifen injection were stained for YFP (green, indicating cells that underwent Cre-mediated recombination) and DRAQ5 (blue) to visualize cell nuclei. Note the similar staining pattern independent of the age of mice at the time of tamoxifen injection. **C)** Double staining for YFP (green) and DCX (red) revealed minimal overlap indicating a low rate of Cre-mediated recombination in actively dividing neural progenitor cells. TAM, tamoxifen; Scale bar, 50 µm.

4.1.2 Anxiety- and depression-related behavioral measures of control mice treated with tamoxifen

Our study relied principally on the EPMT and FST as behavioral tests, which are widely used to assess anxious and emotional behavioral traits in rodents. These tests were part of a larger battery of tests previously used to characterize the

anxious-depressive phenotype of γ2$^{+/-}$ mice (Chapter 3 of this thesis Crestani et al. 1999; Earnheart et al. 2007), and they reliably showed anxiolytic (EPMT) and antidepressant-like effects (FST) of diazepam and desipramine, respectively in these mice (Chapter 3 of this thesis Crestani et al. 1999). The anxiety- and depression-related behavior of fγ2/+ mice is indistinguishable from that of WT mice (Earnheart et al. 2007), in agreement with normal expression of γ2-containing $GABA_A$Rs in these mice (Schweizer et al. 2003). Recombination of floxed target genes by CAG-CreERT2-transgenic mice further relied on treatment of mice with tamoxifen. It was therefore important to address whether tamoxifen alone or unspecific Cre-activity affected baseline behavior. Tamoxifen was injected into CAG-CreERT2, fγ2/+ and WT littermates at either P13/14 or P27/28 followed by behavioral testing between 8 and 10 weeks of age. Initial evaluation indicated that tamoxifen administered at P13/14 or P27/28 had similar effects in the EPMT or FST (n = 4-13, ANOVA, $p > .5$). To increase the sensitivity for detection of behavioral alterations the data from P13/P14 and P27/P28 tamoxifen-treated mice were combined. Tamoxifen had no effect on behavior of fγ2/+ and CAG-CreERT2 mice in the EPMT (Figure 4.2A) and the FST, except for a reduction in the total time spent immobile of CAG-CreERT2 mice (Figure 4.2B). The effect of tamoxifen on CAG-CreERT2 mice in the FST was opposite to that observed in tamoxifen-treated bigenic CAG-CreERT2 x fγ2/+ mice described below (Figure 4.3) and hence did not interfere with interpretation of subsequent experiments.

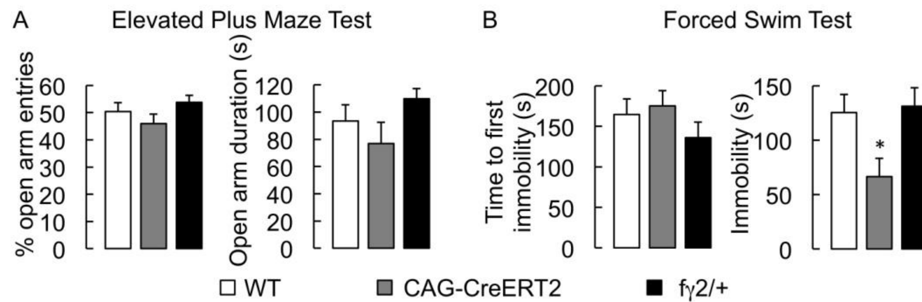

Figure 4.2. Behavioral effects of tamoxifen in the EPMT and FST independent of target gene recombination. Tamoxifen was injected into CAG-CreERT2, fγ2/+ and WT littermates at P13/14 or P27/28 to test for tamoxifen- and Cre-induced behavioral effects at 8-10 weeks of age that occurred independently of recombination of the fγ2 locus. **A)** In the EPMT, the CAG-CreERT2 and fγ2/+ mice visited and stayed in the open arms as much as WT mice (n = 14-16, ANOVA, Dunnett's test, $p > .05$ for all comparisons). **B)** In the FST, the behavior of fγ2/+ mice was indistinguishable from WT mice (n = 14-16, ANOVA, Dunnett's test, $p > .05$ for both comparisons). CAG-CreERT2 mice showed a normal latency to the first immobile episode (n = 14-16, ANOVA, Dunnett's test, $p > .05$) and an aberrant reduction in total immobility time (n = 14-16, ANOVA, Dunnett's test, $p < .05$). *$p < .05$.

4.1.3 Heterozygozity of the γ2 subunit gene induced at P13/14 but not P27/28 results in anxious-depressive-like behavior in adulthood

We first analyzed the consequences of a $GABA_A$R deficit induced at P13/14 or P27/P28 on anxiety-related behavior in the EPMT in adulthood. CAG-CreERT2 x fγ2/+ mice and CreERT2 fγ2/+ controls produced as littermates were treated with tamoxifen either at P13/14 or P27/P28 and subjected to behavioral testing between eight and ten weeks of age. In the EPMT, P13/14 tamoxifen-treated CAG-CreERT2 X fγ2/+ mice visited the open arms less often and spent less time on the open arms than fγ2/+ controls, indicating anxiety-like behavior (Figure 4.3B, for statistics see Figure legends; for a complete list of pairwise comparisons see Table 4.1). By contrast, P27/28 tamoxifen-treated CAG-CreERT2 X fγ2/+ mice analyzed identically behaved normally with respect to both parameters (Figure 4.3C). When analyzed in the FST, P13/14 tamoxifen-treated CAG-CreERT2 X fγ2/+ mice started to float sooner than fγ2/+ and CAG-CreERT2 controls (Figure 4.3D). P13/14 tamoxifen-treated CAG-CreERT2 X fγ2/+ mice also showed an increase in total immobility time but only when compared to CAG-CreERT2 mice and not when compared to fγ2/+ controls. The

aforementioned effect of P13/14 tamoxifen treatment on the total immobility of CAG-CreERT2 mice (Figure 4.2) indicates that these mice represent the more relevant control for the measure of immobility. Altered behavior of P13/14 tamoxifen-treated CAG-CreERT2 X fγ2/+ mice in the EPMT and FST was not due to a change in baseline locomotion, as indicated by the unaltered distance traveled during 15 min in the OFT (Figure 4.3E). Similar to the EPMT, the behavior of P27/28 tamoxifen-treated CAG-CreERT2 X fγ2/+ mice in the FST was indistinguishable from that of identically treated fγ2/+ and CAG-CreERT2 littermate controls (Figure 4.3F). Thus, tamoxifen-induced partial deletion of the γ2 subunit at P13/14 is sufficient to induce an anxious-depressive-like phenotype similar to that described for mice with a global ($\gamma2^{+/-}$) or embryonically-induced $GABA_A R$ deficits (Earnheart et al. 2007). By contrast, a similar reduction in the γ2 subunit gene dosage at P27/28 had no behavioral consequences that lasted to adulthood. The data suggest that postnatal development of GABAergic circuits during the third and/or fourth postnatal week is critically important for normal anxiety- and depression-related emotionality in adulthood.

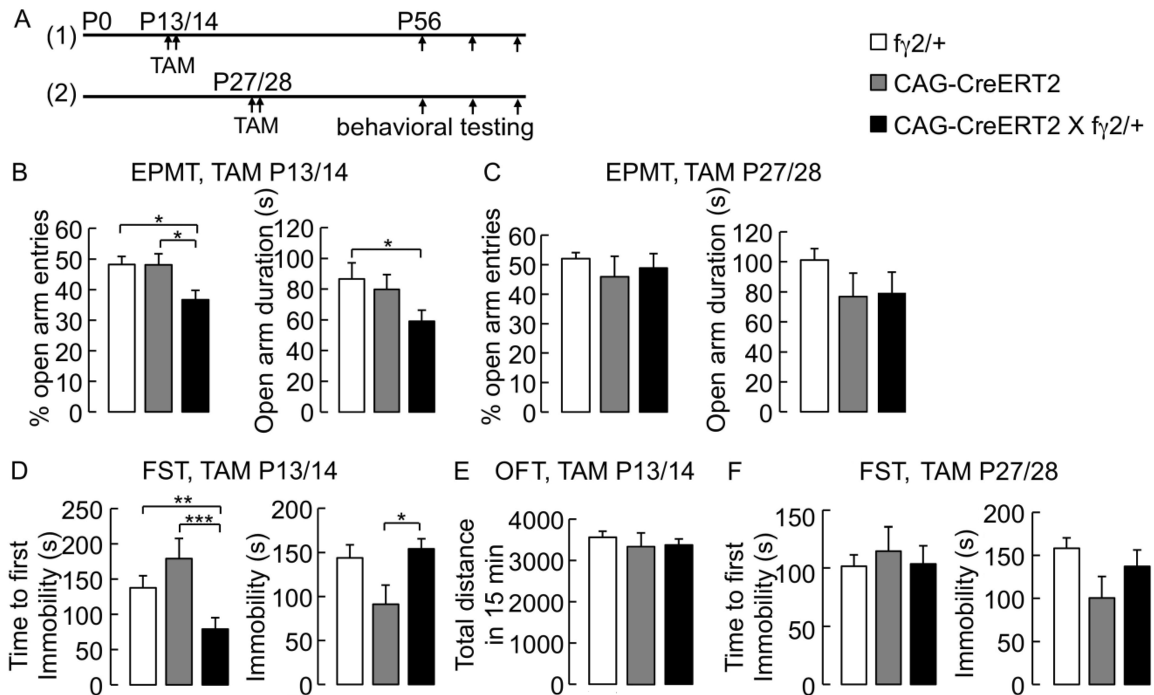

Figure 4.3. Developmental control of anxious depressive behavior induced by tamoxifen-induced Cre-mediated recombination of the fγ2 locus. A) Experimental time line. Littermates representing all three genotypes were injected with tamoxifen at P13/14 or P27/28, followed by behavioral testing with the first test starting at 8 weeks of age. **B)** In the EPMT, CAG-CreERT2 X fγ2/+ mice treated with tamoxifen at P13/14 visited the open arm less often than identically treated fγ2/+ and CAG-CreERT2 controls (% open arm entries: n = 19-32, $p < .05$ for both comparisons, ANOVA, Dunnett's test,). The P13/14 tamoxifen-treated CAG-CreERT2 X fγ2/+ mice spent less time on the open arms than identically treated fγ2/+ controls (open arm duration: n = 9-13, $p < .05$, ANOVA, Dunnett's test). **C)** CAG-CreERT2 X fγ2/+ mice treated with tamoxifen at P27/P28 were indistinguishable from identically treated fγ2/+ and CAG-CreERT2 littermate controls with respect to % open arm entries and time spent on open arms (n = 6-27, $p > .05$ for both comparisons and both parameters, ANOVA, Dunnett's test,). **D)** In the FST, P13/14 tamoxifen-treated CAG-CreERT2 X fγ2/+ mice started to float sooner than identically treated fγ2/+ and CAG-CreERT2 controls (CAG-CreERT2 X fγ2/+ vs. fγ2/+, $p < .01$; CAG-CreERT2 X fγ2/+ vs. CAG-CreERT2, $p < .001$, n = 14-20, ANOVA, Dunnett's test). P13/14 tamoxifen-treated CAG-CreERT2 X fγ2/+ mice also showed increased immobility compared to identically treated CAG-CreERT2 mice (n = 14-16, $p < .05$, ANOVA, Dunnett's test). **E)** P13/14 tamoxifen-treated CAG-CreERT2 X fγ2/+ mice travelled as much as CAG-CreERT2 and fγ2/+ littermates in the 15 min test (n = 9-13, p > .05 for both ANOVA, Dunnett's test), showing unaltered locomotion. **F)** P27/28 tamoxifen-treated CAG-CreERT2 X fγ2/+ mice were indistinguishable from identically treated fγ2/+ and CAG-CreERT2 littermates with respect to the time to first immobility and total immobility ($p > 0.05$ for both comparisons, both parameters, n = 7-27, ANOVA, Dunnett's test). TAM, tamoxifen; *$p < .05$, **$p < .01$, ***$p < .001$. All values represent group means ± s.e.

Table 4.1. One-way ANOVA analysis of genotype effects followed by comparison of CAG-CreERT2 X fγ2/+ with CAG-CreERT2 or fγ2/+ mice.

Tamoxifen Treatment	Test	Parameter	Pairwise Comparison		Group Size	Dunnett Test	Figure
P13 P14	EPM	% open arm entries	CAG-CreERT2 X fγ2/+	CAG-CreERT2	20-32	$p = 0.038$	4.3B
		Open arm duration			9	$p = 0.184$	4.3B
		% open arm entries	CAG-CreERT2 X fγ2/+	fγ2/+	19-32	$p = 0.019$	4.3B
		Open arm duration			9-13	$p = 0.050$	4.3B
	FST	Time to 1st immobility	CAG-CreERT2 X fγ2/+	CAG-CreERT2	14-16	$p < 0.001$	4.3C
		Immobility			14-16	$p = 0.026$	4.3C
		Time to 1st immobility	CAG-CreERT2 X fγ2/+	fγ2/+	14-20	$p < 0.001$	4.3C
		Immobility			14-20	$p = 0.744$	4.3C
	OFT	Total distance	CAG-CreERT2 X fγ2/+	CAG-CreERT2	7-14	$p = 0.814$	4.3F
		Total distance	CAG-CreERT2 X fγ2/+	fγ2/+	7-14	$p = 0.998$	4.3F
P27 P28	EPM	% open arm entries	CAG-CreERT2 X fγ2/+	CAG-CreERT2	6-11	$p = 0.871$	4.3D
		Open arm duration			6-11	$p = 0.994$	4.3D
		% open arm entries	CAG-CreERT2 X fγ2/+	fγ2/+	11-27	$p = 0.735$	4.3D
		Open arm duration			11-27	$p = 0.233$	4.3D
	FST	Time to 1st immobility	CAG-CreERT2 X fγ2/+	CAG-CreERT2	7-11	$p = 0.621$	4.3E
		Immobility			7-11	$p = 0.389$	4.3E
		Time to 1st immobility	CAG-CreERT2 X fγ2/+	fγ2/+	11-27	$p = 0.731$	4.3E
		Immobility			11-27	$p = 0.552$	4.3E

4.2 Mapping the developmental critical window for GABAergic regulation of anxious-and depressive-like behavior by pharmacological potentiation of $GABA_AR$ functions

4.2.1 Rational for mapping of critical periods

Analyses of γ2 knockout mice ($\gamma2^{-/-}$) indicate that the γ2 subunit is essential for the formation of 94% of BZ binding sites and for diazepam-induced behavioral effects (Gunther et al. 1995). By contrast, $\gamma2^{+/-}$ mice retain on average 75% of BZ binding sites, and their behavioral sensitivity to acute treatment with diazepam is increased rather than reduced (Crestani et al. 1999). Treatment of mice with diazepam during a critical period is thought to promote afferent input-independent neural maturation and thereby to interfere with natural activity-dependent refinement of corresponding circuits (Hensch 2004). Alternatively, in a $GABA_AR$-deficient background diazepam might help to reverse a deficit in neural

circuit maturation. We therefore explored whether results obtained by conditional gene inactivation could be corroborated by diazepam-induced disruption of critical periods controlling anxiety- and depression-related behavior. In addition we attempted to rescue the behavioral deficits of $\gamma2^{+/-}$ mice by enhancing $GABA_AR$ function with diazepam during critical periods. Young $\gamma2^{+/-}$ mice and WT littermates were subjected to one- or two-week treatment regimes consisting of daily (P10-16) or bi-daily (P14-21, P14-28, P21-28, P29-35, P50-56) injections with diazepam, followed by behavioral analyses in adulthood.

4.2.2 Diazepam treatment from P10 to P21 has anxiogenic effects on WT mice analyzed in adulthood.

Analyses of P10-16 vehicle-treated $\gamma2^{+/-}$ mice and WT littermates in the EPMT reveled an anxiety-like behavioral phenotype of $\gamma2^{+/-}$ mice as expected (Figure 4.4A, Table 4.2, 4.3 and Ref. Crestani et al. 1999). By contrast, in female mice that were vehicle-treated during the third, fourth, fifth or eighth postnatal week the anxiety-like EPMT phenotype of $\gamma2^{+/-}$ vs. WT mice was masked (Figure 4.4B, C), most likely reflecting elevated sensitivity of pubertal mice to handling (Stone and Quartermain 1997; Leussis and Andersen 2008; Romeo 2010; Shen et al. 2010). Nevertheless, daily treatment with diazepam from P10-16 had genotype-specific anxiogenic-like effects on behavior of adult WT but not $\gamma2^{+/-}$ mice, as evidenced by the fewer open arm entries and reduced time spent in the open arms of diazepam- vs. vehicle-treated WT mice (Figure 4.4A). A strong trend in the same direction was also evident for P14-28 diazepam-treated WT mice (Figure 4.4B). By contrast, diazepam had no effect on adult behavior of WT mice in the EPMT if the treatment occurred during the fourth (P22-28), fifth (P29-35), or eighth (P50-56) postnatal week (Figure 4.4C, D, E). Moreover, diazepam had no effect on behavior of $\gamma2^{+/-}$ mice in the EMPT independent of age during treatment (Figure 4.4A-E). Thus, diazepam administered between P14 and P21 and perhaps at earlier but not later developmental stages has selective anxiogenic-like effects on behavior in adulthood. Collectively the data indicate that both genetic impairment (Figure 4.3) and pharmacological potentiation (Figure 4.4) of the function of γ2-

containing $GABA_ARs$ before the fourth postnatal week leads to increased anxiety-like behavior in adulthood (Figure 5.1).

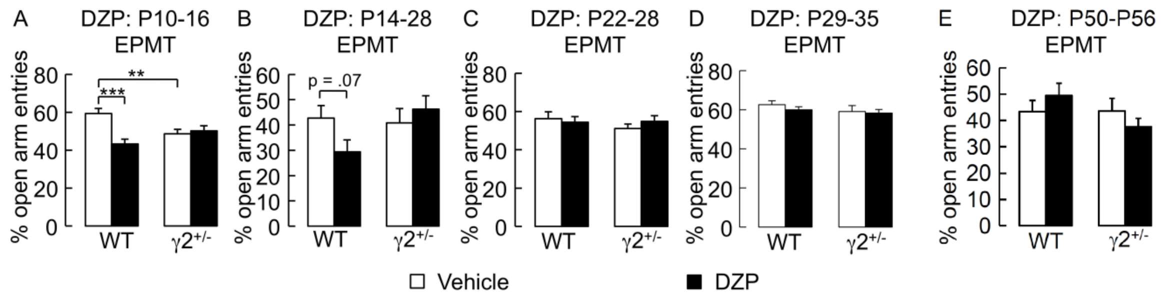

Figure 4.4. Diazepam administration from P10-21 has anxiogenic-like effects in WT mice. WT and $\gamma2^{+/-}$ mice were administered vehicle or diazepam from P10 to P16, P14 to P28 or P22 to P28, as indicated, and tested in the EPMT at eight weeks of age. **A)** The percentage of open arm entries of P10-16 vehicle-treated $\gamma2^{+/-}$ vs. WT mice was reduced as expected (n = 12-14, $p < .001$, ANOVA, Fisher test). The percentage of open arm entries in P10-16 diazepam- vs. vehicle-treated WT mice was also reduced (n = 12-13, $p < .001$, ANOVA, Fisher test). **B)** Similar to treatment at P10-16, the percentage of open arm entries of P14-28 diazepam-treated WT mice trended lower (n = 10-12, $p = .07$, ANOVA, Fisher test). **C, D, E)** In contrast to treatment at earlier ages, treatment with diazepam during the fourth (**C**), fifth (**D**) or eighth (**E**) postnatal week had no effect on WT mice tested during the 8th, 9th, and 12th week of age, respectively (n = 8-22, $p > .05$ for all comparisons, ANOVA, Fisher test). Note that in contrast to WT mice, diazepam had no effect on $\gamma2^{+/-}$ mice independent of age at the time of treatment (p > .5, n = 8-21 for diazepam effects on $\gamma2^{+/-}$ mice independent of age at time of treatment, ANOVA, Fisher test).

Table 4.2 Two-way ANOVA analysis of treatment effects, genotype effects, and treatment × genotype interactions.

Diazepam Treatment	Test	Parameter	Group Size	Genotype Effect	Treatment Effect	Genotype x Treatment	Figure
P10-16	EPM	% open arm entries	12-14	p = 0.472	p = 0.008	p = 0.001	4.4A
	EPM	Open arm duration	10-11	p = 0.610	p = 0.137	p = 0.001	*
P14-28	EPM	% open arm entries	9-11	p = 0.062	p = 0.682	p = 0.135	4.4B
P22-28	EPM	% open arm entries	8-10	p = 0.445	p = 0.745	p = 0.370	4.4C
	EPM	Open arm duration	9-10	p = 0.909	p = 0.810	p = 0.939	*
P29-35	EPM	% open arm entries	21-22	p = 0.224	p = 0.421	p = 0.663	4.4D
	EPM	Open arm duration	10	p = 0.689	p = 0.888	p = 0.746	*
P50-56	EPM	% open arm entries	8-9	p = 0.183	p = 0.999	p = 0.172	4.4E
	EPM	Open arm duration	8-9	p = 0.759	p = 0.528	p = 0.100	*

N.S., not significant, the interaction term has a p-value greater than .5 and therefore was removed from the ANOVA model for model reduction. *, data not shown in figures.

Table 4.3. Two-way ANOVA analyses followed by pair-wise comparisons of drug effects on behavior of γ2$^{+/-}$ vs. WT mice.

Diazepam Treatment	Test	Pairwise Comparison		Behavioral Parameter	Group Size	Fisher Test	Figure
P10-16	EPM	γ2$^{+/-}$ vehicle	WT vehicle	% open arm entries	12-14	$p = 0.004$	4.4A
				Open arm duration	10-11	$p = 0.034$	*
		γ2$^{+/-}$ vehicle	γ2$^{+/-}$ DZP	% open arm entries	13-14	$p = 0.667$	4.4A
				Open arm duration	11	$p = 0.160$	*
		WT vehicle	WT DZP	% open arm entries	12-13	$p < 0.001$	4.4A
				Open arm duration	10-11	$p < 0.001$	*
P14-28	EPM	γ2$^{+/-}$ vehicle	WT vehicle	% open arm entries	11-12	$p = 0.735$	4.4B
		γ2$^{+/-}$ vehicle	γ2$^{+/-}$ DZP	% open arm entries	9-11	$p = 0.491$	4.4B
		WT vehicle	WT DZP	% open arm entries	10-12	$p = 0.070$	4.4B
P22-28	EPM	γ2$^{+/-}$ vehicle	WT vehicle	% open arm entries	10	$p = 0.216$	4.4C
				Open arm duration	10	$p = 0.783$	*
		γ2$^{+/-}$ vehicle	γ2$^{+/-}$ DZP	% open arm entries	8-10	$p = 0.388$	4.4C
				Open arm duration	9-10	$p = 0.822$	*
		WT vehicle	WT DZP	% open arm entries	8-10	$p = 0.684$	4.4C
				Open arm duration	9-10	$p = 0.908$	*
P29-35	EPM	γ2$^{+/-}$ vehicle	WT vehicle	% open arm entries	21-22	$p = 0.243$	4.4D
				Open arm duration	10	$p = 0.302$	*
		γ2$^{+/-}$ vehicle	γ2$^{+/-}$ DZP	% open arm entries	21	$p = 0.796$	4.4D
				Open arm duration	10	$p = 0.373$	*
		WT vehicle	WT DZP	% open arm entries	22	$p = 0.375$	4.4D
				Open arm duration	10	$p = 0.448$	*
P50-56	EPM	γ2+/- vehicle	WT vehicle	% open arm entries	8-9	$p = 0.978$	4.4E
				Open arm duration	8-9	$p = 0.326$	*
		γ2+/- vehicle	γ2+/- DZP	% open arm entries	8-9	$p = 0.323$	4.4E
				Open arm duration	8-9	$p = 0.453$	*
		WT vehicle	WT DZP	% open arm entries	8	$p = 0.336$	4.4E
				Open arm duration	8	$p = 0.114$	*

*, data not shown in figures.

4.2.3 Diazepam administration from P14 to P21 partially rescues the anxiety-like behavior in γ2$^{+/-}$ males.

The absence of an anxiolytic-like effect of P14-P28-diazepam treatment might reflect a confounding of drug and vehicle application-induced stress, as suggested by indistinguishable behavior of vehicle treated γ2$^{+/-}$ and WT mice. WT and γ2$^{+/-}$ male mice were treated with diazepam (2 mg/kg/every other day,

p.o.) or vehicle from P14 to P21 and tested in an Open Field for 15 min at eight weeks of age. No genotype or treatment effect was evident in the total distance travelled in the entire test (Figure 4.5 A, Table 4.4, 4.5), indicating that these mice have normal locomotor activity. By contrast, vehicle-treated $\gamma 2^{+/-}$ vs. WT mice traveled significantly less in the first 5 min of the test (Figure 4.5A). This reduced activity in the novel environment seen in $\gamma 2^{+/-}$ mice indicates an anxiety-like behavior, consistent with previous results (Crestani et al. 1999). Moreover, the reduced activity is normalized by P14-21 diazepam administration (Figure 4.5A). No genotype or treatment effects were detected in the time spent in the center or corners of the Open Field ($n = 7\text{-}12$, $p > .05$, MANOVA). In the Elevated Plus Maze test, the diazepam treated mice made fewer entries to the arms of the Plus Maze (Figure 4.5B). However, diazepam vs. vehicle revealed a tendency toward increased percentage of open arm entries and the time spent on open arms of $\gamma 2^{+/-}$ mice (Figure 4.5B, $p = .1$ and $.07$ respectively), which is consistent with the changes observed in the OFT (Fig 4.5A).

Table 4.4 Two-way ANOVA analysis of treatment effects, genotype effects, and treatment × genotype interactions.

Diazepam Treatment	Test	Parameter	Group Size	Genotype Effect	Treatment Effect	Genotype x Treatment	Figure
P14-P21	OFT	% travel in the 1st 5min	7-12	p = 0.032	p = 0.285	p = 0.026	4.5A
	OFT	Distance in the 1st 5min	7-12	p = 0.144	p = 0.359	p = 0.013	4.5A
	OFT	Total distance	7-12	p = 0.097	p = 0.600	p = 0.483	4.5A
	EPM	% open arm entries	6-10	p = 0.047	p = 0.564	p = 0.111	4.5B
	EPM	Open arm duration	6-10	p = 0.077	p = 0.356	p = 0.128	4.5B
	EPM	Total entries	6-10	p = 0.973	p = 0.008	p = 0.657	4.5B

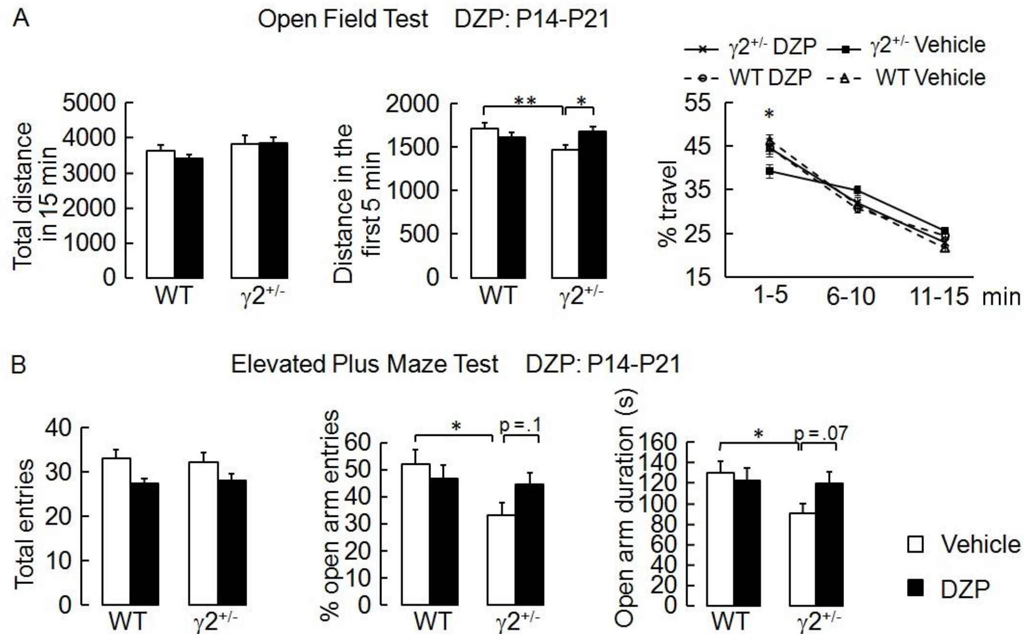

Figure 4.5 Diazepam administration from P14 to P21 partially rescues the anxiety-like behavior in γ2$^{+/-}$ males. WT and γ2$^{+/-}$ mice were administered vehicle or diazepam (DZP, 0.2mg/kg/every other day, P.O.) from P14 to P21 and tested at eight weeks of age in a 15 min Open Field test (A) or Elevated Pus Maze test (B). (A) In the open field test, mice from both genotypes and treatments traveled similar amount of distance, showing a lack of difference in locomotor activity (n = 7-12, ANOVA, Fisher test, p > .05). Vehicle treated male γ2$^{+/-}$ mice traveled less in the first five minutes than WT mice (n = 7-10, ANOVA, Fisher test, p [%travel 1-5 min] < .05, p [Distance] < .01). Diazepam vs. vehicle from P14 to P21 increased the distance traveled in the first 5 min of γ2$^{+/-}$ mice (n = 8-10, ANOVA, Fisher test, p < .05 for both parameters). (B) In the elevated plus maze test, diazepam reduced the total entries to the arms of the plus maze regardless of genotypes (n = 6-10, ANOVA, p < .01[treatment]; p>.5 [genotype; genotype× treatment]). Vehicle treated γ2$^{+/-}$ mice visited the open arm less and spent less time on the open arm than WT mice, consistent with previous studies (n = 6-10, ANOVA, Fisher test, p < .05). Diazepam vs. vehicle revealed a tendency toward increased percentage of open arm entries and open arm duration in γ2$^{+/-}$ mice (n = 8-10, ANOVA, Fisher test, p = .1 and .07 respectively).

Table 4.5. Two-way ANOVA followed by pair-wise comparisons of drug effects on behavior of γ2$^{+/-}$ vs. WT mice.

Diazepam Treatment	Test	Pairwise Comparison		Behavioral Parameter	Group Size	Fisher Test	Figure
P14-21	OFT	γ2$^{+/-}$ vehicle	WT vehicle	% travel in first 5min	7-10	p = 0.004	4.5A
				Distance in first 5min	7-10	p = 0.008	4.5A
				Total distance	7-10	p = 0.504	4.5A
		γ2$^{+/-}$ vehicle	γ2$^{+/-}$ DZP	% travel in first 5min	8-10	p = 0.021	4.5A
				Distance in first 5min	8-10	p = 0.017	4.5A
				Total distance	8-10	p = 0.901	4.5A
		WT vehicle	WT DZP	% travel in first 5min	7-12	p = 0.385	4.5A
				Distance in first 5min	7-12	p = 0.246	4.5A
				Total distance	7-12	p = 0.382	4.5A
P14-21	EPM	γ2$^{+/-}$ vehicle	WT vehicle	% open arm entries	6-10	p = 0.017	4.5B
				Open arm duration	6-10	p = 0.028	4.5B
		γ2$^{+/-}$ vehicle	γ2$^{+/-}$ DZP	% open arm entries	8-10	p = 0.110	4.5B
				Open arm duration	8-10	p = 0.074	4.5B
		WT vehicle	WT DZP	% open arm entries	6-10	p = 0.478	4.5B
				Open arm duration	6-10	p = 0.670	4.5B

4.2.4 Diazepam administration from P10 to P21 has antidepressant-like effects on mice in adulthood.

We next asked whether transient potentiation of GABA$_A$R function during specific stages of postnatal development affects depression-related behavior in the FST or TST and whether such effects are dependent on the γ2 subunit gene dosage. Analyses of baseline behavior in the FST revealed increased immobility of vehicle-treated γ2$^{+/-}$ vs. WT mice (Figure 4.6A, Table 4.6, 4.7), consistent with the depression-related phenotype of γ2$^{+/-}$ mice previously reported (Earnheart et al. 2007). By contrast, repeated vehicle administration at later ages interfered with detection of this phenotype (Figure 4.6B-D, Table 4.6, 4.7), likely due to the aforementioned increased stress sensitivity during puberty. Interestingly, diazepam vs. vehicle treatment from P10 to P16 resulted in a strong trend to delay the first immobile episode and significantly shortened the immobility time of γ2$^{+/-}$ mice ($p = .06$ and $p < .05$ respectively, Figure 4.6A). No such

antidepressant-like effect was evident in WT mice, seemingly due to high basal mobility and ceiling effects in this test, which prevented detection of further increases in mobility. To address whether P10-16 diazepam treatment also had antidepressant effects in WT mice we further subjected the same mice to a TST, which similar to the FST measures immobility of mice in an inescapable stressful situation (Figure 4.6A, right most panel). In the TST, P10-16 diazepam treatment delayed the first immobile episode of both WT and $\gamma 2^{+/-}$ mice (p [treatment] < .01, p [treatment × genotype] > .5, ANOVA, Figure 4.6A), indicating antidepressant-like effects of diazepam independent of genotype. Similar to treatment during the 2nd postnatal week, diazepam treatment in the third week (P14-21) or third plus fourth week (P14-28) had genotype-independent antidepressant-like effects on FST behavior in adulthood (P14-21: p[treatment] < .05, ANOVA; P14-28: p[treatment] = .06, ANOVA; $p < .05$, X^2 test; p[genotype × treatment] > .5 for both treatment periods, ANOVA) (Figure 4.6B, C, for X^2 analyses see Figure 4.7). Importantly, when diazepam was applied only in the fourth postnatal week, no treatment effect was observed independent of genotype (Figure 4.6D). Thus, the minimal developmental window for diazepam-induced antidepressant effects maps to the third postnatal week. While genetically reducing the function of $GABA_ARs$ has detrimental effects on both anxious and depression-related behavioral parameters, transiently potentiating GABAergic transmission in the P14-21 brain has seemingly opposing anxiogenic and antidepressant effects on behavior in adulthood.

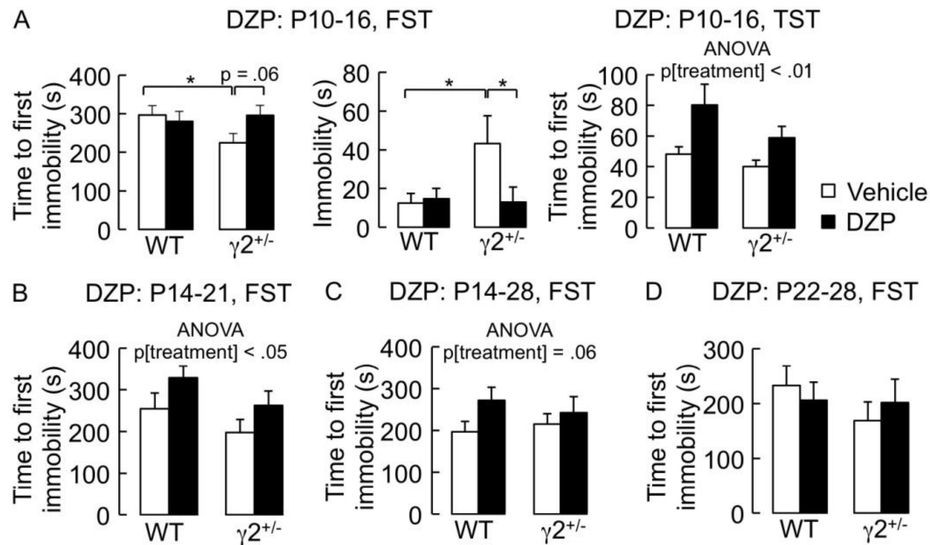

Figure 4.6. Diazepam administration from P10 to P21 has antidepressant-like effect in adulthood. WT and $\gamma2^{+/-}$ mice were treated with vehicle or diazepam during the indicated temporal windows and subjected to FSTs and TSTs between nine and eleven weeks of age**. A)** In the FST, vehicle-treated $\gamma2^{+/-}$ mice started to float sooner and spent more time immobile than identically treated WT controls, consistent with the established depressive-like phenotype of $\gamma2^{+/-}$ mice (n = 12-13, $p < .05$, ANOVA, Fisher test). P10-16 diazepam-treated $\gamma2^{+/-}$ mice showed a trend to delay the first immobility episode (n = 12-13, $p = .06$, ANOVA, Fisher test) and they spent more time immobile than vehicle-treated $\gamma2^{+/-}$ controls ($p < .05$, ANOVA, Fisher test). When analyzed in the TST at ten weeks of age, the same mice assumed an immobile position later than vehicle-treated controls, independent of genotype (n = 10-13, p < .01, ANOVA;). **B, C**) in the FST, P14-21 male or P14-28 diazepam-treated female mice analyzed at nine weeks of age assumed an immobile position later than vehicle-treated controls independent of genotype (P14-21: n = 7-12, p < .05, ANOVA; p < .01, X^2 test; P14-28: n = 9-12, p = .06, ANOVA; $p < .05$, X^2 test, Figure S2). **D)** Treatment with diazepam from P22 to P28 had no effect on time to first immobility independent of genotype (n = 9-10, ANOVA, $p = .35$). *$p < .05$.

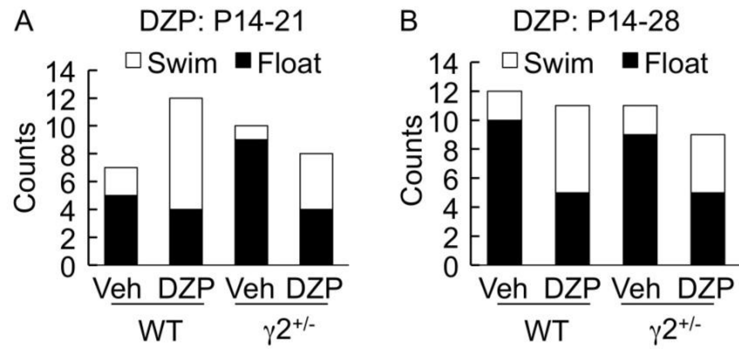

Figure 4.7. X^2 test analyses of FST described in Figure 4.6 B and 4.6C. A, B) The mice that had been tested in the FST were assigned to one of two categories consisting of mice that (1) were swimming only (Swim) or (2) that were part-time swimming and part-time floating (Float). The data were then analyzed using a X^2 goodness of fit test. Diazepam compared to vehicle increased the fraction of mice that swam during the entire test session independent of genotype and independent of whether treatment occurred in the third (**A**)(P14-21) or third and fourth (**B**)(P14-28) postnatal week (P14-21: n = 7-12, $p < .01$; P14-28: n = 9-12, $p = .06$, ANOVA; $p < .05$, X^2 test).

Table 4.6. Two-way ANOVA analysis of treatment effects, genotype effects, and treatment × genotype interactions.

Diazepam Treatment	Test	Parameter	Group Size	Genotype Effect	Treatment Effect	Genotype x Treatment	Figure
P10-16	FST	Time to 1st immobility	10-14	p = 0.266	p = 0.292	p = 0.094	4.6A
	FST	Immobility	10-14	p = 0.224	p = 0.154	p = 0.117	4.6A
	TST	Time to first immobility	10-13	p = 0.098	p = 0.005	N.S.	4.6A
	TST	Immobility	10-13	p = 0.792	p = 0.055	N.S.	*
P14-21	FST	Time to 1st immobility	7-12	p = 0.066	p = 0.042	N.S.	4.6B
P14-28	FST	Time to 1st immobility	9-12	p = 0.887	p = 0.079	N.S.	4.6C
P22-28	FST	Time to first immobility	9-10	p = 0.350	p = 0.955	p = 0.307	4.6C
	FST	Immobility	9-10	p = 0.672	p = 0.832	p = 0.719	*
P29-35	FST	Time to first immobility	11-13	p = 0.534	p = 0.234	p = 0.009	4.8A
	FST	Immobility	11-13	p = 0.049	p = 0.235	p = 0.034	4.8A
	TST	Time to first immobility	9-16	p = 0.947	p = 0.009	p = 0.001	4.8B
	TST	Immobility	11-13	p = 0.957	p = 0.300	p = 0.024	4.8C
P50-56	FST	Time to first immobility	8-9	p < 0.001	p = 0.034	p = 0.592	4.8D
	FST	Immobility	8-9	p = 0.001	p = 0.410	p = 0.593	4.8D

N.S., not significant, the interaction term has a p-value greater than .5 and therefore was removed from the ANOVA model for model reduction. *, data not shown in figures.

Table 4.7. Two-way ANOVA analyses followed by pair-wise comparisons of drug effects on behavior of $\gamma2^{+/-}$ vs. WT mice.

Diazepam Treatment	Test	Pairwise Comparison		Behavioral Parameter	Group Size	Fisher Test	Figure
P10-16	FST	$\gamma2^{+/-}$ vehicle	WT vehicle	Time to 1st immobility	12-13	$p = 0.044$	4.6A
				Immobility	12-13	$p = 0.044$	4.6A
		$\gamma2^{+/-}$ vehicle	$\gamma2^{+/-}$ DZP	Time to 1st immobility	12-13	$p = 0.060$	4.6A
				Immobility	12-13	$p = 0.040$	4.6A
		WT vehicle	WT DZP	Time to 1st immobility	11-13	$p = 0.645$	4.6A
				Immobility	11-13	$p = 0.915$	4.6A
P29-35	FST	$\gamma2^{+/-}$ vehicle	WT vehicle	Time to 1st immobility	12-13	$p = 0.139$	4.8A
				Immobility	12-13	$p = 0.901$	4.8A
		$\gamma2^{+/-}$ vehicle	$\gamma2^{+/-}$ DZP	Time to 1st immobility	12-13	$p = 0.280$	4.8A
				Immobility	12-13	$p = 0.487$	4.8A
		WT vehicle	WT DZP	Time to 1st immobility	11-13	$p = 0.009$	4.8A
				Immobility	11-13	$p = 0.023$	4.8A
	TST (Female)	$\gamma2^{+/-}$ vehicle	WT vehicle	Immobility	12-13	$p = 0.096$	4.8B
		$\gamma2^{+/-}$ vehicle	$\gamma2^{+/-}$ DZP	Immobility	12-13	$p = 0.355$	4.8B
		WT vehicle	WT DZP	Immobility	11-13	$p = 0.024$	4.8B
	TST (Male)	$\gamma2^{+/-}$ vehicle	WT vehicle	Time to 1st immobility	11-14	$p = 0.012$	4.8C
		$\gamma2^{+/-}$ vehicle	$\gamma2^{+/-}$ DZP	Time to 1st immobility	9-11	$p = 0.574$	4.8C
		WT vehicle	WT DZP	Time to 1st immobility	14-16	$p < 0.001$	4.8C

4.2.5 Diazepam administration from P29 to P35 causes a depressive-like phenotype in WT mice analyzed four to six weeks later.

Concurrent anxiogenic- and antidepressant-like effects of diazepam given from P10 to P21 together with anxious-depressive-like effects of genetic $GABA_AR$ deficits suggested to us that the developmental substrates affecting anxiety- and depression-related behaviors might be distinct. To further explore this idea we performed additional tests with mice that had been treated with diazepam during the 5th postnatal week. Interestingly, P29-35 diazepam-treated WT females tested in the FST at 11 weeks of age started to float sooner and spent more time immobile (Figure 4.8A, Tables 4.6, 4.7). No such effect was evident in $\gamma2^{+/-}$ mice consistent with their already depressive-like state at baseline. Increased immobility of P29-35 diazepam-treated WT mice was also evident in the TST, both in female (Figure 4.8B) and male mice (Figure 4.8C). Moreover, the reduced mobility seen in diazepam treated WT mice in the FST and TST was not due to

changes in baseline locomotor activity since the total distance travelled in 15 min open field test was unaltered by diazepam administration (vehicle: 4321.5 ± 205.5 cm; diazepam: 3976 ± 219 cm, n = 10, p > .05, ANOVA, Fisher test). No depressive-like long-term consequences were observed in P50-56 diazepam-treated mice (Figure 4.8D). Thus, potentiation of $GABA_AR$ function with diazepam can have anxiogenic, antidepressant or depressant effects on behavior in adulthood, depending on the exact temporal window of treatment during postnatal development.

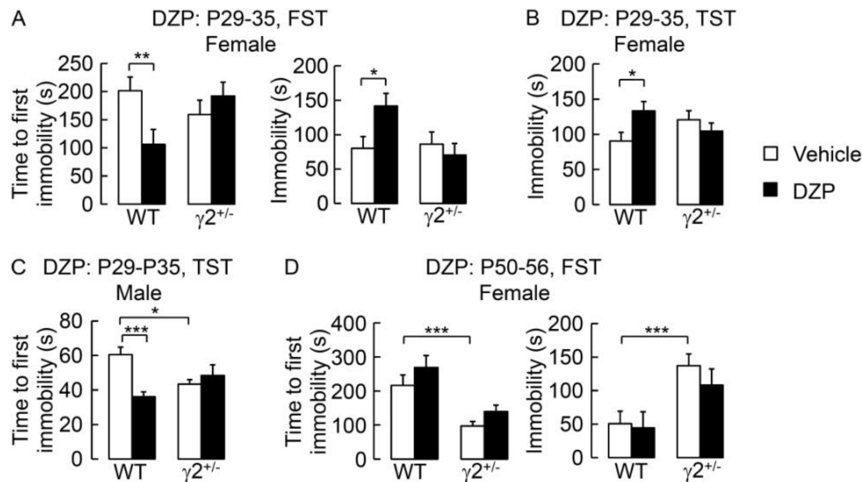

Figure 4.8. Diazepam administration from P29 to P35 causes depression-like behavior in WT mice. WT and $\gamma 2^{+/-}$ mice were administered vehicle or diazepam from P29 to P35 and tested in the FST **(A)** or TST **(B, C)** between 11 and 13 weeks of age. **(A)** Diazepam - vs. vehicle-treated WT mice started to float sooner and spent more time immobile in the FST (time to first immobility: $p < .01$; immobility, $p < .05$, n = 11-13, ANOVA, Fisher test). **(B)** In the TST, diazepam vs. vehicle increased the immobile time of WT but not $\gamma 2^{+/-}$ females (n = 11-13, $p < .05$, ANOVA, Fisher test). **(C)** In the TST, diazepam vs. vehicle reduced the latency to the first immobile episode of WT but not $\gamma 2^{+/-}$ mice (n = 14-16, $p < .001$, ANOVA, Fisher test). **(D)** WT and $\gamma 2^{+/-}$ mice were administered vehicle or diazepam from P50 to P56 and tested in the FST at 12 weeks of age. Diazepam- vs. vehicle-treated mice started to float later and spent equal amount of time immobile in the FST (time to first immobility: $p = .034$; immobility, $p = .41$, n = 8-9, ANOVA).

Chapter 5: Discussion

5.1 Overview of findings

5.1.1 $GABA_AR$ $\gamma 2^{+/-}$ mice represent a novel animal model of anxious depression

Anxiety and mood disorders represent broadly defined psychiatric syndromes that exhibit extensive comorbidity and overlapping genetic origins, yet their etiology is ill-defined and they exhibit heterogeneous and poorly predictable responsiveness to different types of anxiolytic and antidepressant drugs (Kendler 1996; Kaufman and Charney 2000; Eley et al. 2003; Murphy et al. 2004). The work presented in chapter 3 of this thesis aimed at characterizing $GABA_AR$ $\gamma 2^{+/-}$ mice in terms of endocrine and behavioral responses to antidepressant drugs and investigating the potential causative relationship between GABAergic dysfunction and depression. $GABA_AR$ $\gamma 2^{+/-}$ mice exhibit increased anxiety (Crestani et al. 1999) and emotionality (Earnheart et al. 2007). Here we confirmed these findings and further showed that the phenotype includes anhedonia and constitutively elevated serum Cort levels that are normalized by chronic treatment with the selective norepinephrine reuptake inhibitor desipramine but not the selective serotonin reuptake inhibitor fluoxetine. Furthermore, serum Cort levels are elevated in adult mice with an embryonic GABAergic defect (Emx1Cre × fγ2 mice) starting from E10 as well as late GABAergic defect (CaMKIICre2834 × fγ2 mice) starting in puberty. We also showed that the onset of aberrant Cort levels starts later in CaMKIICre2834 × fγ2/+ mice than in global knockout $\gamma 2^{+/-}$ mice, suggesting that perhaps HPA axis deficits in early life contributed to the behavioral and pharmacological phenotype of global $\gamma 2^{+/-}$ mice.

5.1.2 Developmental GABAergic control of adult anxious-depressive-like behavior

The work presented in chapter 4 of this thesis was designed to dissect the developmental substrates underlying anxious and depressive-like behavior. Genetic and pharmacological approaches were combined to weaken or potentiate GABAergic transmission, respectively at different postnatal developmental stages. CAG-CreERT2 × fγ2/+ mice showed anxious-depressive behavior in adulthood when the loss of $GABA_AR$ γ2 subunit was induced with tamoxifen at P13/14. By contrast, loss of receptors starting from P27/28 had no detectable behavioral effects. Another line of experiments was carried out to disrupt GABA homeostasis by potentiating GABAergic transmission with diazepam, an allosteric agonist of $GABA_ARs$. Diazepam administration during the second and third postnatal weeks showed prominent anxiogenic effect in WT females, while no effects or partial anxiolytic effects were seen in $γ2^{+/-}$ mice. Furthermore, mice receiving diazepam after the third postnatal week were indistinguishable from vehicle treated controls in all anxiety-related tests. Together with the observations made with the CAG-CreERT2 experiments, the results suggest that the maintenance of GABA homeostasis during the second and third postnatal weeks is critical for the normal programming of adult anxiety-related behavior. However, the temporal sensitivity to DZP in the FST and TST differed from the developmental sensitivity to diazepam in the EPMT. Diazepam showed a genotype-independent antidepressant effect when administered at P10-P16 or P14-21 and strong depressive effect on WT mice only when administered at P29-35. This represents a developmental switch from hypersensitivity to reduced GABA signaling to excessive GABA signaling in terms of the programming of depression-related behaviors. Collectively, the data indicate that GABAergic transmission-dependent anxious and depressive-like brain states are developmental programmed by multiple separate brain substrates defined by their different sensitivity to manipulation of GABAergic tone during distinct critical postnatal periods.

5.2 Selective behavioral responses to desipramine and fluoxetine with anxiolytic and/or antidepressant-like responses in $GABA_AR$ $\gamma 2^{+/-}$ mice.

The anxiolytic and/or antidepressant-like responses to desipramine and fluoxetine were seen selectively in $\gamma 2^{+/-}$ but not WT mice in NSFT, FST and TST. Moreover, the elevated Cort levels seen in $\gamma 2^{+/-}$ mice were normalized by chronic desipramine treatment. In contrast, no reduction of Cort was detected in WT mice. This indicates greater sensitivity of the $GABA_AR$-deficient brain state to the therapeutic effects of these currently used antidepressants and indicates that modest $GABA_AR$ deficits can cause deficits in serotonergic and noradrenergic transmission that are predicted based on the monoamine hypothesis of depression. Lack of behavioral responses of WT mice (129SvJ/X1 strain) to fluoxetine and desipramine seen in most tests is at odds with anxiolytic and antidepressant-like drug effects seen in other laboratories and may reflect different drug sensitivities of different inbred strains of mice (Dulawa et al. 2004; Jacobson and Cryan 2007; Sugimoto et al. 2008). Even so, our findings are consistent with a recent large META analyses of clinical studies showing that true beneficial drug effects to antidepressant medications are significant compared to placebo only in severe cases of major depressive disorder (Fournier et al. 2010). Multiple well-controlled studies also showed no positive effect of chronic imipramine, desipramine, or fluoxetine on mood in normal subjects (Mascio et al. 1964; Wittenborn et al. 1976; Gelfin et al. 1998). In our study, desipramine showed antidepressant effects in WT mice only in the sucrose consumption test (SCT), when each female mouse was singly housed and received daily p.o. administration of desipramine. Both the housing conditions (Palanza et al. 2001) and the daily manipulation and gavage feeding (Balcombe et al. 2004) that were a necessary part of the design of this experiment are well established as stressors of female mice. Thus, we cannot exclude that the antidepressant effect detected in the SCT represents a treatment × stress interaction, which would explain antidepressant effects not only in mutants but also in WT mice.

Fluoxetine differed from desipramine in that it lacked normalizing effects in $GABA_AR$ $\gamma 2^{+/-}$ mice in the FST, TST and SCT. Ethologically, the FST/TST and SCT are thought to assess depression-related behavioral despair and anhedonia, respectively, while the NSFT measures anxiety-related behavioral inhibition. Thus, desipramine showed both anxiolytic and antidepressant effects, whereas fluoxetine was merely anxiolytic. The qualitatively lesser response of $\gamma 2^{+/-}$ mice to fluoxetine than desipramine is reminiscent of severe subtypes of anxious depressive disorders including melancholic depression, which tend to be more responsive to tricyclic antidepressants (TCAs) than SSRIs (Clerc et al. 1994; Roose et al. 1994; Perry 1996; Swartz and Guadagno 1998; Parker et al. 1999; Parker et al. 2001; Bauer et al. 2002; Young et al. 2004). Importantly, there is rapidly accumulating evidence for reduced GABAergic transmission in depressed patients and these deficits are most pronounced in patients suffering from treatment resistant and melancholic subtypes of depression (Sanacora et al. 2004; Price et al. 2009; Sequeira et al. 2009; Levinson et al. 2010).

5.3 Elevated basal serum Cort levels in $GABA_AR$ γ2-deficient mice

Increased secretion of glucocorticoids and aberrant function of the hypothalamic–pituitary–adrenal (HPA) axis are well-replicated findings in a prominent subset of patients suffering from severe forms of depressive disorders, including especially melancholic depression (Brown et al. 2004; Tichomirowa et al. 2005; Hennings et al. 2009). Here it's shown that the anxious-depressive phenotype and poor responsiveness to fluoxetine of $\gamma 2^{+/-}$ mice was associated with a constitutive increase in Cort. Elevated basal activity of the HPA axis has been proposed as a characteristic of melancholic depression and, consistent with our findings in mice, linked to poor responsiveness to fluoxetine in patients (Kasckow et al. 2001; Young et al. 2004; Contreras et al. 2007). However, Cort concentrations of γ2-deficient mice are elevated even if the $GABA_AR$ deficit is delayed to adolescence, while the behavioral alterations are dependent on induction of the $GABA_AR$ deficit during development (Earnheart et al. 2007). These findings indicate that

the elevated Cort concentrations alone are insufficient to cause the behavioral changes of γ2-deficient mice and that they may represent an epiphenomenon unrelated to anxious depressive-like behavioral outcomes. However, the manifestation of altered behavior of different γ2-deficient mouse strains correlates with an earlier developmental onset of HPA axis hyperactivity (Chapter 3, Fig. 3.6D). Thus, consistent with the notion that the vulnerability to stress is elevated in the immature brain (Kendler et al. 1999; McEwen 2003; Lupien et al. 2009), HPA axis hyperactivity in young $\gamma 2^{+/-}$ mice may contribute to the perturbation of brain development that leads to anxious depressive behavior.

Cort concentrations of γ2-deficient mice were elevated even in Emx1Cre x fγ2/+ mice in which the $GABA_AR$ deficit is limited largely to the telencephalon and absent in the hypothalamus (Iwasato et al. 2004) (Schweizer et al. 2003). Therefore, the primary $GABA_AR$ deficit of γ2-deficient mice causing elevated Cort levels was extra-hypothalamic. Moreover, glucocorticoids are known to negatively affect expression of $GABA_ARs$ in the forebrain, including particularly the frontal cortex and ventral hippocampus (Orchinik et al. 2001; Caldji et al. 2004; Maggio and Segal 2009). Thus, $GABA_AR$ deficits in the telencephalic forebrain may be both a cause for, and a consequence of, HPA axis hyperactivity, a feature that may initiate a positive feedback loop that amplifies GABAergic deficits in the limbic forebrain, with HPA axis hyperactivity as an amplifying link.

The anxiolytic- and antidepressant-like behavioral effects of desipramine in the $\gamma 2^{+/-}$ model are associated with HPA axis normalizing effects, whereas the selective anxiolytic-like effects of fluoxetine occurred without reductions in Cort. Consistent with our findings in $\gamma 2^{+/-}$ mice, fluoxetine and TCAs are known to exhibit different effects on HPA axis function in animal models and patients. Fluoxetine increases Cort concentrations in animal models (Weber et al. 2006) and fails to normalize HPA axis activity in patients (Meltzer et al. 1997; Swartz and Guadagno 1998; Young et al. 2004). In contrast, desipramine and other TCAs normalize HPA axis function in animal models (Connor et al. 2000;

Santibanez et al. 2006; Weber et al. 2006) and patients (Heuser et al. 1996), and this drug effect is associated with remission from depression (Linkowski et al. 1987; Heuser et al. 1996). Reboxetine, which blocks the reuptake of norepinephrine similar to desipramine, has complex brain region-specific effects on expression of glutamic acid decarboxylase 67, a principal enzyme involved in the synthesis of GABA (Herman et al. 2003). Thus, mechanisms of norepinephrine reuptake inhibitors might involve modulation of GABAergic transmission.

5.4 The phenotype of $\gamma2^{+/-}$ mice is not associated with changes in brain concentrations of 5-HT or NE or serotonergic innervation

The monoamine hypothesis of depression suggests that altered serotonergic and/or noradrenergic transmission contributes to the etiology of mood disorders. Noradrenergic and serotonergic neurons are known to be subject to GABAergic control (Holmes et al. 2003; Aston-Jones et al. 2004), suggesting that perhaps $\gamma2^{+/-}$ mice have alterations in these neurotransmitter systems. In particular, an excess of extracellular serotonin might contribute to increased emotionality as suggested by analysis of serotonin transporter knockout mice (Holmes et al. 2003; Mathews et al. 2004). However, the total tissue levels of 5-HT, its metabolite 5-HIAA, and NE in 9-week-old $\gamma2^{+/-}$ versus WT mice in six brain areas (list areas tested) were unaltered (Shen et al. 2010). Moreover, the immunoreactivity of serotonergic axons at 3 and 9 weeks of age was unchanged except for a modest and transient yet significant reduction of serotonin axon density in the primary motor cortex of 3-week-old $\gamma2^{+/-}$ versus WT mice that was no longer evident in 9-week-old mice (Shen et al. 2010). Therefore, altered behavior of $\gamma2^{+/-}$ mice does not involve overt genotype-related changes in serotonergic or noradrenergic neurotransmitter levels or serotonergic innervation of the forebrain. However, selective changes in extracellular serotonin and/or noradrenalin cannot be excluded by these experiments.

5.5 $GABA_AR$ $\gamma 2^{+/-}$ mice exhibit features of constructive, face and predictive validity of an animal model of major depression

As discussed in Chapter 1.4, evidence supporting GABAergic deficits in human MDD includes the reduction of GABA and GABAergic neurons in MDD patients (reviewed by Luscher et al. 2011). Further evidence comes from microarray analyses of postmortem brains from depressed and healthy subjects, where a region- and subunit-specific change in $GABA_AR$ abundance was detected in MDD patients. Moreover, antidepressant therapies act in part by restoring the GABAergic malfunction in MDD patients (reviewed by Luscher et al. 2011). Collectively there is significant evidence that GABAergic deficits are associated with MDD, indicating that $\gamma 2^{+/-}$ mice have construct validity of an animal model of MDD.

Characterization of $\gamma 2^{+/-}$ mice by fear conditioning has revealed a heightened sensitivity and attentional bias towards negative associations (Crestani et al. 1999), reminiscent of corresponding cognitive deficits in melancholic depressed patients (Austin et al. 1999; Rogers et al. 2004). In addition, $\gamma 2^{+/-}$ mice exhibit selectively reduced survival of adult-born hippocampal granule cells (Earnheart et al. 2007), reminiscent of corresponding deficits in chronic stress-induced animal models of depression (Banasr and Duman 2007), which may be related to hippocampal volume reductions observed in depressed patients (Czeh and Lucassen 2007). Moreover, $\gamma 2^{+/-}$ mice show increased HPA axis activity, which is also seen in a major subset of patients suffering from severe forms of depressive disorders, including melancholic depression (American Psychiatric Association. 2000; Hatzinger 2000; Gold and Chrousos 2002; Brown et al. 2004; Tichomirowa et al. 2005; Hennings et al. 2009). In addition, $\gamma 2^{+/-}$ mice exhibit increased behavioral despair and anhedonia, which recapitulate core symptoms of human melancholic depression. Collectively, these data indicate that $\gamma 2^{+/-}$ mice have face validity of an animal model of MDD with melancholic features.

Last but not least, the endocrine and behavioral deficits of $\gamma 2^{+/-}$ mice were fully reversed by desipramine, while fluoxetine had anxiolytic like effects. Increased therapeutic efficacy of TCAs vs. fluoxetine is in keeping with data from severely depressed patients (Clerc et al. 1994; Roose et al. 1994; Perry 1996; Swartz and Guadagno 1998; Parker et al. 1999; Parker et al. 2001; Bauer et al. 2002; Young et al. 2004). These pharmacological features indicate predictive validity of $\gamma 2^{+/-}$ mice as an animal model of severe forms of MDD that are partly resistant to antidepressant drug treatment. In sum, the $GABA_AR$ $\gamma 2^{+/-}$ mouse model includes behavioral, cognitive, cellular, and endocrine dimensions as well as antidepressant drug response characteristics that may be expected of an animal model of melancholic depression.

5.6 The merits of $\gamma 2^{+/-}$ model versus stress-based models

Given that MDD has both genetic and environmental origins (discussed in Chapter 1.2), an animal model of MDD therefore ideally would rely on genetic, environmental, or both means for induction of the disease state. However, most widely accepted animal models are constructed based on the effect of stress (reviewed by Nestler and Hyman 2010).

Genetic vulnerability factors exert their effects differently from environmental factors in that genetic deficits are long-lasting whereas environmental stress tends to be temporary and thus may have reversible effects, at least if experienced in adulthood. Moreover, the depressive effects induced by specific genes might be more difficult to surpass if the drugs applied are not designed to target specific pathways. These differences might potentially affect treatment remission as well as recurrence rate. Genetic associations have been described between single nucleotide polymorphisms in a number of genes and either treatment resistant forms of MDD, the severity of depression, or the responsiveness to electroconvulsive therapy and transcranial magnetic stimulation (Anttila et al. 2007; Bocchio-Chiavetto et al. 2008; Anttila et al. 2009;

Bonvicini et al. 2010; Domschke et al. 2010; Alessia et al. 2011; Serretti et al. 2011; Winter et al. 2011). In addition, a family history of psychopathology is linked to increased risk of recurrence of MDD (reviewed by Burcusa and Iacono 2007). Even though there is currently no confirmed association between $GABA_AR$ mutations and MDD in patients it is readily conceivable that genetic defects could selectively impact $GABA_AR$ function by affecting the development of GABAergic interneurons, GABAergic synapses, or the intracellular trafficking of $GABA_AR$ to synapses. Thus, the $\gamma2^{+/-}$ model may serve as a model of any form of MDD that involves reduced GABAergic transmission as a causative step along multiple etiological sequelae leading to MDD.

One disadvantage of stress-induced rodent models is that they invariably respond to SSRIs and TCAs (Tsankova et al. 2006; Yalcin et al. 2008). This doesn't agree with the clinical observation that only one third of the patients respond to single antidepressant treatment (Trivedi et al. 2006) and 15-33% of MDD patients are treatment resistant (American Psychiatric Association. 2000; Cain 2007; Berlim et al. 2008). By contrast, the depression-related deficits in $\gamma2^{+/-}$ mice were fully rescued by the TCA desipramine but not the SSRI fluoxetine, providing a tool to investigate the molecular substrates of diverse treatment effects. Moreover, further studies about recurrence may shed light on the relationship between GABAergic transmission and recurrent depression.

5.7 CAG-CreERT2 mediated ablation of γ2 subunit

In Chapter 4, we showed that GABAergic deficits of CAG-CreERT2 × fγ2/+ mice induced by tamoxifen at P13/14 but not P27/28 results in anxious depressive-like phenotypes. This result is consistent with the established critical role of GABAergic transmission in postnatal development (Akerman and Cline 2007; Ge et al. 2007). As discussed in Chapter 1.4, development of the nervous system is a drawn out process that extends well into the postnatal period. The maturation process is mediated by excitatory GABA currents and follows a region-specific

time course (reviewd by Ben-Ari 2002; Wang and Kriegstein 2009). In the hippocampal pyramidal neurons, the transition from GABA-mediated excitation to inhibition happens in the second postnatal week (Hollrigel et al. 1998; Ganguly et al. 2001). However, the morphology and synaptic properties of GABAergic basket cell don’t reach adult levels until the end of the third postnatal week (Doischer et al. 2008). Similar maturation patterns are seen in the coherence and frequency of GABA oscillations (Lahtinen et al. 2002). In hypothalamic gonadotropin-releasing hormone (GnRH) neurons, the excitation to inhibition switch of GABA is not complete until P29 (Han et al. 2002). As for the hypothalamic CRH neurons, inhibitory GABA currents are first detected during P21-28. However, the inhibitory GABAergic actions are switched back to excitation 30 min after acute stress (Hewitt et al. 2009). Hippocampus and hypothalamus are two key regions that mediate stress response and are both implicated in the regulation of emotions (Chapter 1.5 and 1.2). Given the immature state of brain structures such as hippocampus and hypothalamus during the third postnatal week, the anxious-depressive-like phenotypes observed in the CAG-CreERT2 × fγ2/+ mice following tamoxifen injection at P13/14 are not unexpected. In contrast, at P27/28, the developing brain has reached a relatively more mature state. Tamoxifen injections at P27/28 failed to cause behavioral deficits in CAG-CreERT2 × fγ2/+ mice, indicating that mice are vulnerable to the loss of γ2 subunit approximately during the third and fourth postnatal weeks.

Further delimitation of the critical window underlying anxious depressive phenotypes is subject to technical limitations. Nuclear localization of CreERT2 was present 24 h after a single tamoxifen injection and had almost disappeared 48 h post-injection (Hayashi and McMahon 2002), indicating that the majority of Cre-mediated recombination happens within 48 h after tamoxifen administration. However, ablation of gene function is associated with a delay that is determined by the rate of protein turnover. In CaMKII2834Cre × Z/AP mice, the expression of Cre-induced reporter gene can barely be detected in the cortex at P17

(Schweizer et al. 2003). However, around 20% loss of benzodiazepine binding sites was observed in the same region at P21 when CaMKII2834Cre × fγ2/fγ2 mice are analyzed. Based on this, a conservative estimate of the delay from TAM injection to reduced surface expression of γ2 subunit is around one week. Consequently the previously defined vulnerability window should be expanded by a week (Figure 5.1A).

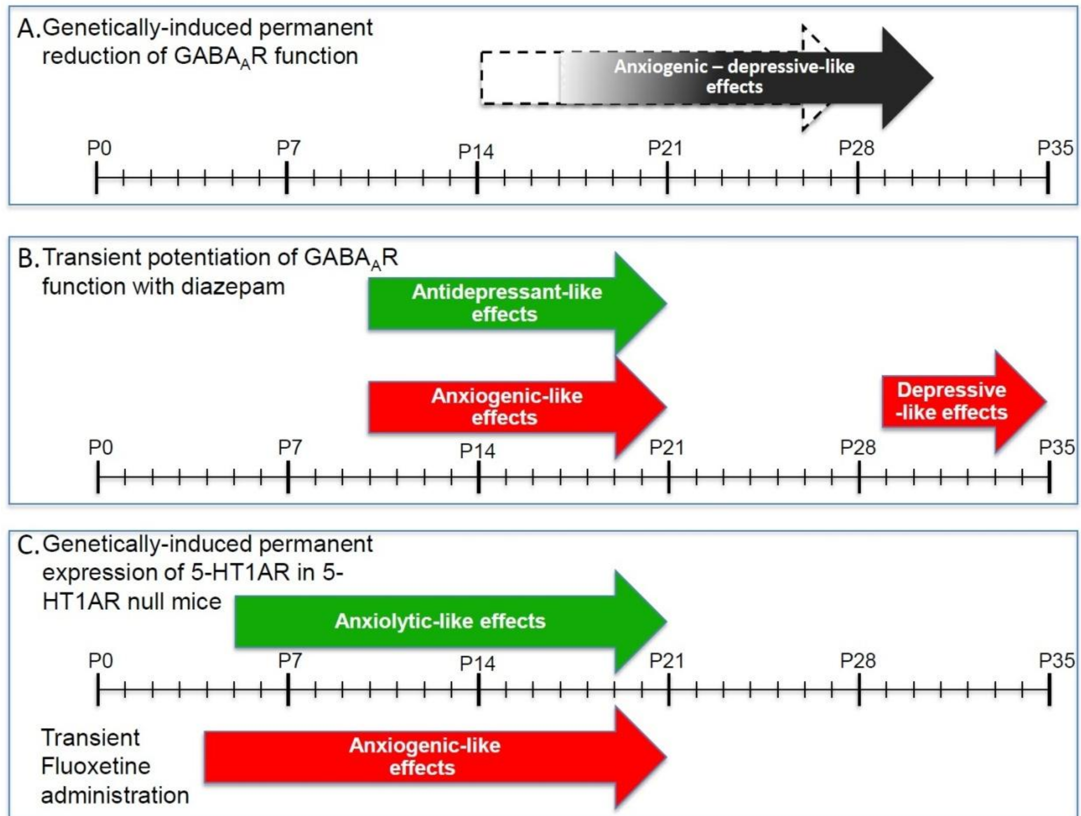

Figure 5.1. Schematic representation of developmental periods that control anxiety- and depression-related behavioral traits of the mouse in adulthood. A. A genetically-induced GABA$_A$R deficit initiated at P14 but not at P28 leads to anxious-depressive-like behavior in adulthood. **B**. Transient pharmacological potentiation of GABA$_A$R function with diazepam from P10-P21 has anxiogenic and antidepressant effects on the behavior of WT mice in adulthood. Similar treatment from P29-35 has depressive-like effects. **C.** A genetically-induced expression of 5-HT1AR starting from P5 but not P21 rescued the anxiety-like behavior seen in 5-HT1AR null mice. Fluoxetine application from P4-21 caused anxiety-like behavioral in adulthood.

Interestingly, this window overlaps with the critical period in visual cortex development (P23-28, Hensch and Fagiolini 2005). Reduced GABA level was reported in the occipital cortex of MDD patients (Sanacora et al. 1999; Sanacora

et al. 2004; Bhagwagar et al. 2008), especially in treatment resistant patients (Price et al. 2009). It has long been a mystery why deficits in occipital cortex are so often present in MDD patients. Our finding raises a possibility that the concurrence of the two deficits is caused by their similar developmental vulnerability window to GABAergic deficits.

Earnheart et al (2007) had shown that GABAergic regulation of anxiety and depression-like behavior is developmental. CaMKII2834Cre × fγ2/+ mice suffer from GABAergic deficits starting from the fourth postnatal week and are behaviorally indistinguishable from fγ2/+ controls (Earnheart et al. 2007). This seems at odds with the results from current study. However, current study differs from the CaMKII2834Cre experiments from at least three aspects. First, the onset of recombination in CAG-CreERT2 × fγ2/+ mice is quick following tamoxifen injection (Hayashi and McMahon 2002) whereas the amount of recombination in CaMKII2834Cre × fγ2/+ mice gradually increase from P17 to P34 (Schweizer et al. 2003). A sudden loss of γ2 subunit potentially produces more cellular stress. Second, In CaMKII2834Cre × fγ2/+ mice, recombination becomes detectable in multiple brain regions starting from P21 as examined with the Z/AP reporter mice (Schweizer et al. 2003). On average, recombination mediated by TAM P13/14 and CAG-CreERT2 precedes that mediated by CaMKII2834Cre by around a week. The immature brain undergoes significant amount of changes during the third and fourth postnatal week. It's possible that one week difference is important. Third, TAM/Cre mediated recombination is global whereas CaMKII2834Cre mediated recombination is restricted to forebrain mature glutamatergic neurons. Collectively, TAM P13/14 and CAG-CreERT2 induces earlier and more severe GABAergic deficits.

It's reported that less than 0.1% leaking expression of the reporter gene was detected in CAG-CreERT2 × R26R mice (Hayashi and McMahon 2002). This doesn't affect our interpretation of results since no behavioral deficits were detected when tamoxifen was injected at P27/28. Moreover, the vulnerability

window determined by our conditional knockout strategy only represents the minimum window. The time before P13/14 might be equally or more important.

5.8 Critical periods for anxiety and depression identified by diazepam administration

In Chapter 4, we showed that diazepam administration in WT mice during P10-21 and P29-35 leads to distinct behavioral consequences in adulthood (Figure 5.1B). Early exposure to diazepam has anxiogenic-like effect whereas later exposure resulted in exclusively depressive-like phenotypes. The critical period for anxiety identified by diazepam administration overlaps with the one determined using conditional activation of the 5-HT1A receptor (5-HT1AR) in 5HT1AR knockout mice (Figure 5.1C, Gross et al. 2002). In that study, transient transgene mediated expression of the 5-HT1AR beginning at P5 but not P21 rescued the anxiety-like behavior of 5-HT1AR knockout mice, indicating that critical period might be from P5 to P21. This window is also consistent with another experiment in which treatment of mice with fluoxetine during P4-21 leads to increased anxiety (Figure 5.1C, Ansorge et al. 2004). Although fluoxetine is thought to primarily have this effect through blockade or serotonin reuptake, it is noteworthy that fluoxetine and its metabolite norfluoxetine also act as potent agonists of $GABA_A$Rs (Robinson et al. 2003; Ye et al. 2008).

Diazepam administration during P29-35 leads to enhanced depressive-like phenotype in WT mice. This window coincides with early puberty in 129 X1/SvJ mice (Pinter et al. 2007). Puberty is accompanied by increased vulnerability to various mental disorders such as anxiety, depression, eating disorders and substance abuse during human adolescence (Costello et al. 2003; Patton and Viner 2007). Consistent with human studies, it has been reported that rodents show elevated sensitivity to stress and increased vulnerability to depression-like behaviors during the fifth postnatal week (Stone and Quartermain 1997; Romeo 2010). Collectively current evidence supports that puberty represents a critical

period for the programming of depression. It is rather striking that four diazepam injections were sufficient to produce a long-lasting depressive effect that couldn't be reproduced by analogous treatment before the fifth postnatal week or in the eighth postnatal week. Further testing of treatment effect in the sixth and seventh postnatal week is necessary to delimit the length of the critical period.

It is also noteworthy that the vulnerability window for anxiety (P10-21) precedes that for depression (P29-35). Based on well over a hundred parameters, the brain of a mouse at birth corresponds to that of a human embryo in mid-gestation (Clancy et al. 2001). The time of eye opening at P11 in mice corresponds to the 160th day of gestation in humans. Based on the critical developmental period regulating ocular dominance plasticity, the visual cortex of a 23 to 33-day-old mouse corresponds to that of a six-year-old human (Fagiolini and Hensch 2000; Webber and Wood 2005). Thus, critical periods for anxiety- and depression-related behavior in humans are predicted to span the time from the end of the 2nd trimester of embryonic development (anxiogenic and antidepressant effects of diazepam) to the typical start of school age (depressive effects of diazepam). Importantly, this sequence is consistent with the age of onset of the two disorders in human (median age of onset: anxiety, 11 years old, MDD, 30 years old, Kessler et al. 2005).

5.9 Genotype dependent/independent effect of diazepam

Some of the developmental effects induced by diazepam were genotype-dependent. In particular, anxiogenic effects of P10-16 and P14-28 diazepam treatment were seen in WT but not $\gamma 2^{+/-}$ mice, suggesting resistance of already $GABA_AR$-deficient circuits to further developmental GABAergic disturbances. Conversely, diazepam did not show anxiolytic effects in $\gamma 2^{+/-}$ mice, which may indicate that the relevant circuits in $\gamma 2^{+/-}$ mice were irreversibly impaired already at earlier time points of development. The baseline behavior of P14-P35-vehicle-treated $\gamma 2^{+/-}$ and WT mice in the EPMT was indistinguishable under the test

conditions (Figure 4B-D), indicating that test-specific ceiling effects cannot explain the absence of diazepam effects in $\gamma 2^{+/-}$ mice. In contrast to the anxiogenic-like effects of diazepam, the antidepressant-like drug effects in the FST and TST were evident independent of genotype in most tests, supporting the idea that the circuits underlying anxiogenic and antidepressant effects of diazepam are distinct even though their sensitivity to diazepam overlapped temporally.

5.10 Imperfect overlapping in sensitive periods determined by gene knockout and pharmacological potentiation

Tamoxifen/Cre-mediated ablation of gene function is expected to be associated with an additional delay of several days determined by the rate of protein turnover. While Cre-mediated gene ablation is permanent, diazepam treatment affected receptor function transiently. Moreover, the end of drug exposure is imprecise due to the time needed for the drug to wash out. Drug withdrawal-induced stress (Ashton 1994) might in effect extend the temporal window during which circuits are disturbed and contribute to behavioral consequences in adulthood. The gene knockout strategy and pharmacological manipulation used in this study are targeted at very similar but not identical subsets of $GABA_A$Rs. In particular, the $GABA_A$R impairment induced by hemizygous inactivation of the γ2 gene is inhomogeneous even in global $\gamma 2^{+/-}$ mice, which showed significantly greater reductions of the γ2 subunit in hippocampus (-28 to -35%) and cortical areas (-25%) than in the amygdala (<-10%) (Crestani et al. 1999; Earnheart et al. 2007). By contrast, diazepam is expected to potentiate the function of all γ2-containing $GABA_A$Rs [94% of diazepam sensitive receptors (Gunther et al. 1995)], as well as γ1- and γ3-containing receptors (6%). Thus, imperfect overlap in sensitive periods determined by gene knockout and pharmacological potentiation may reflect (i) different sensitivity of neural circuits to $GABA_A$R impairment vs. potentiation, (ii) different consequences of permanent vs. transient manipulations of GABAergic circuits, and (iii) imperfect spatiotemporal overlap of the circuits

manipulated. However, one noteworthy point is that the critical periods mapped by potentiation (diazepam experiments) or partial ablation (Tamoxifen/Cre experiments) of GABAergic transmission do not necessarily overlap since mice may exhibit selective tolerance to one condition over the other at different developmental stages.

5.11 Technical limitations for the interpretation of results

5.11.1 Effects of laboratory stress

Routine handling of mice such as lifting the mice and changing cages leads to significant changes in serum or plasma concentration of corticosterone, glucose, growth hormone or prolactin, heart rate, blood pressure and behavior. These changes usually differ from baseline by 20-100% and last at least 30 min or longer (reviewed by Balcombe et al. 2004). Pre-pubertal rats take twice as much time to recover from acute stress compared with adults (Romeo 2010). Moreover, in our hands weaning of mice at P20 induced an increase of serum corticosterone levels that lasts for at least 48 h post weaning (Table 5.1). It has been reported that GABAergic transmission in part controls the timing of puberty (reviewed by Terasawa 2005). Infusion of GABAergic antagonist bicuculline causes precocious puberty in monkeys (Keen et al. 1999). WT 129 X1/SvJ mice normally start puberty at P29 (Pinter et al. 2007), whereas $\gamma2^{+/-}$ mice may reach puberty at a different age due to GABAergic deficits. Given the heightened stress sensitivity during peri-puberty and puberty (reviewed by Romeo 2010), it's possible that weaning-induced stress affects $\gamma2^{+/-}$ and WT mice differently. Due to handling-induced stress associated with drug treatment by bi-daily gavage (Balcombe et al. 2004), the difference between WT and $\gamma2^{+/-}$ mice might represent a genotype × stress interaction. Gavage feeding of vehicle during P14-28, P22-28, P29-35, or P50-56 was potent enough to mask the genotype differences between WT and $\gamma2^{+/-}$ mice. Diazepam might only induce depression related phenotypes if paired with stress. Furthermore, although the time course and peak levels of Cort measured following a forced swim stressor in adulthood

was unaffected in $\gamma2^{+/-}$ mice, we cannot exclude the possibility that young $\gamma2^{+/-}$ mice are more sensitive to stress than WT littermates.

Table 5.1 Corticosterone serum levels post weaning

Hours post weaning	Genotype	CORT (ng/ml)
24 h	WT	171.7 ± 16.8
	$\gamma2^{+/-}$	183.6 ± 18.2
48 h	WT	96.7 ± 16.2
	$\gamma2^{+/-}$	98 ± 13.6
72 h	WT	43.0 ± 13
	$\gamma2^{+/-}$	75.7 ± 11

5.11.2 Limitations of the FST and TST

In Chapter 4, the testing of depression-like behavior relies on FST and TST. The advantage of FST and TST is that they have high predictive validity for antidepressant drug responses. However, their relevance to depression and other mood disorders is not validated. In addition, FST is very sensitive to water temperature. In vivo dialysis experiments in adult rats show that swimming at 35 ºC leads to increased hippocampal GABA while swimming at 25 ºC leads to decreased hippocampal GABA (de Groote and Linthorst 2007). The sensory system might also affect the results of FST. Therefore additional more elaborate testing (i.e. the Sucrose Preference test, Social Defeat, Learned Helplessness) will be essential to confirm that P29-35 diazepam results in depressive like brain states.

5.12 Outlook

5.12.1 Identifying the molecular substrates of MDD and treatment remission

$\gamma2^{+/-}$ mice show behavioral, cognitive, cellular and endocrine deficits that are characteristic of melancholic depression and provide a valuable tool for the investigation of GABAergic contribution to the progression and maintenance of depressive brain states. Recent advances in DNA, RNA and protein quantification technologies make it possible to study genome-wide transcriptional

regulation. Comparing the gene expression profiles in WT and $\gamma 2^{+/-}$ mice in brain regions that are implicated in MDD may shed light on the possible molecular substrates that are involved in MDD. The interaction between GABAergic transmission and other pathways might also be identified. The selective antidepressant effect from desipramine but not fluoxetine also enables us to further explore the molecular substrates involved in treatment remission. Moreover, given the lack of objective diagnosis of MDD, $\gamma 2^{+/-}$ mice can also be used to explore possible biomarkers which dictate depressive brain states.

5.12.2 Exploring the role of HPA axis hyperactivity in depression

We've shown that baseline corticosterone concentrations in adult γ2-deficient mice were elevated independent of whether the genetic lesion was induced during embryogenesis or delayed to adolescence. However, the manifestation of anxious-depressive behavior in different γ2-deficient mouse lines was correlated with early onset HPA axis hyperactivity during postnatal development (Chapter 3). When tamoxifen was injected at P13/14, the onset of GABAergic deficits in CAG-CreERT2 × fγ2 mice precedes puberty, the critical developmental stage for HPA axis maturation. Moreover, the critical period of depression previously identified by diazepam administration also overlaps with puberty. Future experiments will need to address the functionality of HPA axis in CAG-CreERT2 × fγ2 mice with tamoxifen injection at P13/14 and WT mice with diazepam administration from P29-35. If a hyperactivity of HPA axis is present, we can further test if administration of CRF receptor antagonist during the critical period has antidepressant effect or not.

5.12.3 Investigating the etiology of MDD

Critical periods regulating anxiety- and depression-related behavior in mice may help understand the developmental etiology of anxious depression in patients. In particular, MDD is associated with loss of GABAergic interneurons (Rajkowska et al. 2007) and reduced brain concentrations of GABA (Sanacora et al. 1999; Hasler et al. 2007). The anxious-depressive like behavioral, endocrine and

pharmacological phenotypes of $\gamma 2^{+/-}$ mice indicate that reduced GABAergic transmission through $GABA_A$Rs may be causal for MDD (Chapter 3). It's possible that the immature brain regions are more likely to be affected by diazepam due to the critical role of depolarizing GABA in brain maturation. However, other brain structures might also be affected given the general instability of the young brain and the enormous network connections. Further experiments should aim at identifying the key brain regions that are affected by diazepam as well as the changes in gene expression and the electrophysiological properties of the neurons.

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Professional Experience:

2005 - 2011 Graduate Assistant, The Pennsylvania State University
2006 Teaching Assistant, The Pennsylvania State University
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Publications:

1. **Shen Q**, Lal R, Luellen BA, Earnheart JC, Andrews AM, Luscher B. gamma-Aminobutyric acid-type A receptor deficits cause hypothalamic-pituitary-adrenal axis hyperactivity and antidepressant drug sensitivity reminiscent of melancholic forms of depression. Biol Psychiatry. 2010 Sep 15;68(6):512-20.
2. Hao G, Wang D, Gu J, **Shen Q**, Gross SS, Wang Y. Neutral loss of isocyanic acid in peptide CID spectra: a novel diagnostic marker for mass spectrometric identification of protein citrullination. J Am Soc Mass Spectrom. 2009 Apr;20(4):723-7.
3. Li P, Wang D, Yao H, Doret P, Hao G, **Shen Q**, Qiu H, Zhang X, Wang Y, Chen G, Wang Y. Coordination of PAD4 and HDAC2 in the regulation of p53-target gene expression. Oncogene. 2010 May 27;29(21):3153-62
4. Luscher B, **Shen Q**. Reply to Commentary by Markou and Geyer, on GABA(A)R deficient mice as an animal model of melancholic depression. Biol Psychiatry. 2010. Correspondence.
5. Luscher B, **Shen Q**, Sahir N. The GABAergic deficit hypothesis of major depressive disorder. Mol. Psychiatry. 2011 (16) 383-406. Review

Fellowships and Awards:

2005 Homer F. Braddock Fellowship, The Pennsylvania State University.
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