BEYOND NEURONS: THE ROLE OF THE OLIGODENDROCYTE-SPECIFIC GENE CNP1 IN MAJOR DEPRESSIVE DISORDER

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Altered oligodendrocyte structure and function is implicated in major mental illnesses, including low density and reduced expression of oligodendrocyte-specific gene transcripts in major depressive disorder (MDD). These features are also observed in the unpredictable chronic mild stress (UCMS) and chronic corticosterone (CORT) rodent models of the illness; however, whether oligodendrocyte changes are a causal component of MDD is not known. The oligodendrocyte-specific gene 2’-3’-cyclic nucleotide-3’-phosphodiesterase (CNP1) is a key component of axoglial communication and has previously been implicated in psychiatric disorders. A recent study in our lab found decreased levels of CNP1 in the amygdala (a central region of mood regulation) of human post-mortem MDD subjects and mice exposed to UCMS.

In an attempt to determine whether altered CNP1 (or disrupted oligodendrocyte integrity) represents a causal factor in MDD, we investigated MDD-related features in mice lacking CNP1 (CNP1\textsuperscript{KO} mice). In terms of technical development, we: 1) initiated use of a novel Z-score normalization procedure to counteract variability and extract a more comprehensive and translational view of our behavioral analyses, and 2) propose that the fear conditioning paradigm can be used to assess corticolimbic dysfunctions relating to mechanisms of MDD. Together, our studies in CNP1\textsuperscript{KO} mice reveal a surprising profile of behavioral resilience that is accompanied by patterns of amygdala-
related dysfunction. In addition, we show robust upregulation of oligodendrocyte and immune related transcripts in the basolateral amygdala of CNP1<sup>KO</sup> mice. This pattern is suggestive of compensatory changes for oligodendrocyte structure/function and indicative of an association between oligodendrocytes and immune function. Together, these studies demonstrate that disruption of oligodendrocyte function (via CNP1 ablation) can impact circuits mediating emotionality in mice resulting in abnormal affective behaviors. However, downstream molecular changes combined with the observation of longterm detrimental consequences in these mice (e.g. neurodegeneration), is suggestive of a maladaptive role for CNP1 in MDD.
TABLE OF CONTENTS

1.0 INTRODUCTION .......................................................................................................................... 16

1.1 MAJOR DEPRESSIVE DISORDER ............................................................................................ 17
  1.1.1 Relevant Clinical Risk Factors for MDD ........................................................................... 19
      1.1.1.1 Sex Differences in MDD ......................................................................................... 19
      1.1.1.2 MDD and Age ........................................................................................................ 21
  1.1.2 Neurobiology of MDD ................................................................................................. 22
      1.1.2.1 Neurocircuitry of MDD ....................................................................................... 22
      1.1.2.2 Stress and Neuroendocrine Changes in MDD ..................................................... 26
      1.1.2.3 Genetics of MDD ................................................................................................. 27

1.2 USING RODENTS TO INVESTIGATE MDD ............................................................................ 28
  1.2.1 Difficulties in Using Rodents to Investigate MDD ......................................................... 28
  1.2.2 UCMS and CORT Rodents Models of Depression ......................................................... 30
      1.2.2.1 Behavioral Correlates of MDD ............................................................................. 30
      1.2.2.2 Cellular and Physiological Effects ......................................................................... 31
  1.2.3 Circuitry Underlying Fear and Emotion in Rodents ...................................................... 34
      1.2.3.1 Fear Conditioning ................................................................................................. 34
      1.2.3.2 Fear Extinction ...................................................................................................... 35
      1.2.3.3 Corticolimbic Coordination in Fear Conditioning ............................................... 37
      1.2.3.4 Effects of Stress on Fear Conditioning .................................................................. 37
  1.2.4 Using Knockout Mice ...................................................................................................... 38

1.3 THE ROLE OF GLIA IN MDD ............................................................................................... 39
  1.3.1 Glial Structure and Function .......................................................................................... 39
      1.3.1.1 Microglia ................................................................................................................ 40
      1.3.1.2 Astrocytes ............................................................................................................ 41
1.3.1.3 Oligodendrocytes ................................................................. 42
1.3.1.4 NG2 Cells ............................................................................. 45
1.3.2 Glial Alterations in MDD ......................................................... 46
1.4 CNP .............................................................................................. 50
1.4.1 Structure and Localization of CNP ...................................... 50
1.4.2 Function of CNP ................................................................. 52
1.5 SUMMARY OF INTRODUCTION AND SIGNIFICANCE OF RESEARCH. ................................................................. 54
2.0 PAPER 1: INTEGRATED BEHAVIORAL Z-SCORING INCREASES THE SENSITIVITY AND RELIABILITY OF BEHAVIORAL PHENOTYPING IN MICE: RELEVANCE TO EMOTIONALITY AND SEX ................................................................. 55
2.1 ABSTRACT .................................................................................. 56
2.2 INTRODUCTION ........................................................................... 57
2.3 METHODS .................................................................................. 59
2.4 RESULTS .................................................................................... 64
2.5 DISCUSSION ............................................................................... 80
3.0 PAPER 2: RESILIENT EMOTIONALITY AND MOLECULAR COMPENSATION IN MICE LACKING THE OLIGODENDROCYTE-SPECIFIC GENE CNP1 ................................................................. 86
3.1 ABSTRACT .................................................................................. 87
3.2 INTRODUCTION ........................................................................... 88
3.3 MATERIALS AND METHODS ................................................... 90
3.4 RESULTS .................................................................................... 94
3.5 DISCUSSION ............................................................................... 107
4.0 GENERAL DISCUSSION ................................................................. 112
4.1 METHODOLOGICAL CONSIDERATIONS IN INVESTIGATING EMOTIONALITY ................................................................. 112
4.1.1 Z-score Normalization and Assessment of Emotionality ......... 113
4.1.2 Fear Conditioning and Assessment of Corticolimbic Function 114
4.1.3 Convergent Methodological Approaches Reveal Resilient Emotionality in CNP1KO Mice ........................................................................ 118
4.2 INFLUENCE OF EXAMINED RISK FACTORS ON BEHAVIORAL OUTPUTS

4.2.1 Effects of Risk Factors on Emotionality Outputs

4.2.1.1 Age

4.2.1.2 Sex

4.2.2 Effects of Risk Factors on Locomotor Outputs

4.3 INFLUENCE OF CNP1 ON THE NEUROBIOLOGY OF MOOD REGULATION

4.3.1 Molecular Effects of CNP1 Ablation

4.3.2 Cellular Effects of CNP1 Ablation

4.3.2.1 Neuronal Alterations

4.3.2.2 Oligodendrocyte Alterations

4.3.2.3 Paranodal Alterations

4.3.3 Circuit-Level Effects of CNP1 Ablation

4.3.4 Conclusions on the Neurobiological Consequences of CNP1 Ablation

4.4 DOES CNP1 PLAY A CAUSAL ROLE IN MDD?

4.5 OVERALL CONCLUSIONS AND FUTURE DIRECTIONS

APPENDIX A

APPENDIX B

APPENDIX C

5.0 REFERENCES
LIST OF TABLES

Table 2.1. P-values for 2-way ANOVA main effects corresponding to data shown in Fig. 3................................................................................................................................. 75
Table 3.1. Selection of significantly affected neuronal, oligodendrocyte and immune-related genes between CNP1\textsuperscript{KO} vs WT control mice................................. 105
Table 4.1. Comparison of human MDD symptoms to measurable mouse behaviors and corresponding tests ........................................................................................................... 116
Table B.1. Z-score values (Mean ± SEM) obtained by different combinations of 2 or 3 behavioral tests ................................................................................................. 150
Table B.2. P-values for 2-way ANOVA main effects calculated in different combinations of 2 or 3 behavioral tests.................................................................................. 152
Table C.1. Schedule of UCMS stressors and behavioral tests........................................ 173
Table C.2. List of 114 probesets found to be significantly changed in Cnp1\textsuperscript{KO} vs. WT mice ..................................................................................................................... 174
LIST OF FIGURES

Figure 1.1. Phylogenetic conservation of corticolimbic brain regions involved in mood regulation ................................................................. 25
Figure 1.2. Stress-induced changes in dendritic structure in rodents ........ 33
Figure 1.3. Circuits implicated in fear conditioning and extinction ........... 36
Figure 1.4. Structure of a myelinating oligodendrocyte ............................ 44
Figure 1.5. Decreased oligodendrocyte density in the amygdala of MDD patients................................................................. 49
Figure 1.6. Structure and localization of CNP1 ....................................... 51
Figure 2.1. Integrated emotionality z-scores in mice exposed to unpredictable chronic mild stress ........................................................................ 67
Figure 2.2. Chronic antidepressant treatment blocks stress-induced increase in emotionality z-scores (n=14-15/group) ........................................ 70
Figure 2.3. Integrated Emotionality z-score in corticosterone-treated male and female mice ................................................................. 73
Figure 2.4. Emotionality and locomotion scores in two animal models of anxiety/depression ................................................................. 77
Figure 2.5. Dissecting sex differences in baseline emotionality .................. 79
Figure 3.1. Cnp1KO baseline behavior at 3, 6, and 9 months of age ........... 97
Figure 3.2. Effects of two rodent models of depression in Cnp1KO mice ...... 100
Figure 3.3. Fear conditioning and c-Fos analysis ..................................... 103
Figure 3.4. BLA gene array analysis and qPCR validation ........................ 104
Figure 4.1. Summary diagram representing relative effects of individual risk factors on disease susceptibility in WT and CNP1KO mice 123
Figure 4.2. Model of potential circuitry changes in CNP1KO mice......................... 133
Figure 4.3. Proposed model of downstream consequences of altered paranode integrity...................................................................................................................... 134
Figure A.1. Diagrams of methods for producing genetically modified animals .. 144
Figure B.1. Z-scores values (Mean ± SEM) obtained by different combinations of 2 or 3 behavioral tests ........................................................................................................ 151
Figure C.1. Individual tests comprising 3 month emotionality and locomotion Z-scores.............................................................................................................................. 158
Figure C.2. Individual tests comprising 6 month locomotion Z-score .................. 159
Figure C.3. Individual tests comprising 9 month emotionality and locomotion Z-scores.............................................................................................................................. 160
Figure C.4. Baseline emotionality Z-scores excluding FST................................. 161
Figure C.5. Physiological measures in the baseline cohort................................. 162
Figure C.6. Individual tests comprising UCMS emotionality and locomotion Z-scores.............................................................................................................................. 163
Figure C.7. Individual tests comprising CORT emotionality and locomotion Z-scores.............................................................................................................................. 164
Figure C.8. Emotionality Z-scores excluding the forced swim test .................... 165
Figure C.9. Behavioral measures in a separate cohort exposed to an abbreviated (2 week) UCMS paradigm ................................................................. 166
Figure C.10. Physiological measures in the UCMS cohort................................. 167
Figure C.11. Physiological measures in the CORT cohort ................................. 168
Figure C.12. Hot plate test .................................................................................. 169
Figure C.13. Fear conditioning in a separate cohort of mice .............................. 170
Figure C.14. Extinction recall and fear renewal in the FC cohort....................... 171
Figure C.15. Correlation between WT-UCMS and KO-UCMS genes ............... 172
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LIST OF ABBREVIATIONS

ACC: anterior cingulated cortex
ATP: adenosine triphosphate
BA: basal nucleus of amygdala
BLA: basolateral amygdala
BDNF: brain derived neurotrophic factor
CASPR: contactin-associated protein
CE/CeA: central amygdala
CNP: 2'-3' cyclic nucleotide 3'-phosphodiesterase
CRH: corticotrophin releasing hormone
CORT: chronic corticosterone administration paradigm
CS: conditioned stimulus
DLPFC: dorsolateral prefrontal cortex
fMRI: functional magnetic resonance imaging
GABA: gamma-aminobutyric acid
GC: glucocorticoids
GFAP: glial fibrillary acid protein
GOI: gene of interest
GWAS: genome wide associated study
HPA: hypothalamic, pituitary adrenal axis
IL: infralimbic cortex (rodents)
KO: knockout
LA: lateral nucleus of amygdala
LPS: lipopolysaccharide
MAG: myelin associated protein
MAOI: monoamine oxidase inhibitor
MBP: myelin basic protein
MDD: major depressive disorder
MS: multiple sclerosis
OE: overexpressor
OPC: oligodendrocyte precursor cells
PFC: prefrontal cortex
PL: prelimbic cortex (rodents)
PLP: proteolipid protein
PTSD: post-traumatic stress disorder
QTL: quantitative trait locus
SERT: serotonin transporter
SNP: single nucleotide polymorphism
SSRI: selective serotonin reuptake inhibitor
UCMS: unpredictable chronic mild stress paradigm
US: unconditioned stimulus
vmPFC: ventromedial prefrontal cortex
WT: wild type mice
YLD: years of life lived with disability
1.0 INTRODUCTION

Major depressive disorder (MDD) is a genetically diverse and etiologically undefined disorder, likely involving complex molecular interactions in multiple brain regions. While there are ample clinical and postmortem human studies on MDD, conclusions regarding the mechanisms underlying MDD are difficult to draw because 1) external factors are difficult to control in human populations and 2) only minimally invasive techniques can be utilized. Thus, mechanistic studies on MDD rely heavily on studies in animal models where external factors can be more controlled and a single characteristic can be more thoroughly examined. However, interpreting results from animal studies and translating back to the human disorder can also prove challenging.

Both human and animal studies have implicated disturbances of oligodendrocytes in MDD and/or stress, but the functional consequences of oligodendrocyte disturbance on mood have not been investigated. The studies reported here use a knockout mouse to model a deficiency in oligodendrocyte function and also present a novel method for analyzing emotionality in mice. Below, I introduce current knowledge of clinical and neurobiological aspects of MDD focusing on two clinical factors relevant to these studies: age and sex. I then discuss corresponding neurobiological studies in animal models followed by a brief review of glial cell types and an overview of glial changes in MDD, focusing on oligodendrocytes. Finally, I discuss the structure and function of the oligodendrocyte-specific gene 2’-3’-cyclic nucleotide 3’-phosphodiesterase (CNP1) and evidence for its involvement in MDD and other psychiatric disorders.
1.1 MAJOR DEPRESSIVE DISORDER

Major depressive disorder (MDD), a devastating chronic illness of altered mood, is a syndrome defined by the core symptoms of excessive sadness (depressed mood) and anhedonia (inability to experience pleasure) for a minimum period of two weeks. A diagnosis of depression requires at least one of the core symptoms accompanied by five additional symptoms such as guilt, despair, weight gain or loss, impaired cognitive functions, and changes in sleep and eating patterns (DSM-IV-TR 2000). MDD has an unknown and potentially heterogeneous etiology but factors that are known to increase susceptibility to MDD include female sex, increasing age, genetic makeup, prior episode of MDD, and environmental factors (i.e. chronic stress). MDD is a significant contributor to the development and progression of systemic and organ diseases (Musselman et al. 1998; Ciechanowski et al. 2000; Schulz et al. 2000), and in MDD patients, lifetime mortality due to suicide reaches 15% (Mann 2003). The global impact of MDD is severe and major depression is considered the leading cause of disability worldwide (WHO 2008) in terms of years of life lived with the disability (YLD). In the United States, the annual cost of MDD was estimated to be well over $25 billion in direct medical costs alone in the year 2000 (Greenberg et al. 2003). However, equally significant contributors to the financial burden of depression are indirect medical costs such as absenteeism or lost productivity at work. While it is likely that effective treatment of MDD will offset medical costs by increasing worker productivity (Donohue and Pincus 2007), remission rates of individuals using a single pharmacological treatment are about 30% (Kennedy et al. 2001; Nemeroff et al. 2008). Even with combined treatment approaches a remission rate of only 50% is typically achieved (Blier et al. 2010). This clearly points toward a need to develop more effective antidepressant treatments, however, there is a paucity of novel molecular targets due to the lack of understanding regarding the basic molecular mechanisms of mood (Blier 2010).

While the exact mechanism of low mood is unclear, there are five leading hypotheses of the neurobiological causes underlying depression [Reviewed in
(Belmaker and Agam 2008; Krishnan and Nestler 2008)]. 1) The monoamine hypothesis: The advent and success of antidepressants such as selective serotonin reuptake inhibitors (SSRIs), which act to block the serotonin transporter (SERT), and monoamine oxidase inhibitors (MAOIs) initially generated focused research effort on the role of serotonin and other monoamines in MDD. However monoamine depletion studies and in depth molecular studies show that there are likely additional mechanisms underlying depression (Belmaker and Agam 2008). 2) The glucocorticoid hypothesis: MDD patients frequently have altered levels of circulating cortisol and alterations in corticotrophin releasing factor (CRH) levels, suggestive of dysfunction of the glucocorticoid system. While the exact mechanistic relationship between stress and depression has remained vague, stress is a predominant risk factor for MDD (Holsboer 2000). 3) The neurotrophic hypothesis: Indirect evidence in rodents indicates that antidepressants increase levels of brain-derived neurotrophic factor (BDNF) and infusion of BDNF into the hippocampus induces antidepressant effects (Shirayama et al. 2002; Delttheil et al. 2009). Direct evidence in humans is more limited and includes decreased circulating BDNF levels in MDD patients (Molendijk et al. 2010) and decreased BDNF levels in postmortem tissue from the hippocampus of MDD patients (Thompson Ray et al. 2011). However, it is likely that these effects are not causal, but rather have a specific modulatory role (Duman and Monteggia 2006; Castren et al. 2007). 4) The inflammatory hypothesis: People with infections, autoimmune diseases, or interferon treatments develop depression and some antidepressants have anti-inflammatory effects. Increases in certain cytokines, humoral mediators of immunity, have been implicated in depression, however, more research is necessary to elucidate the precise roles of the immune system in depression (Miller et al. 2009). 5) The GABAergic hypothesis: Reduced levels of gamma-aminobutyric acid (GABA) and altered GABA receptor subunit composition have been observed in MDD. Disruptions of GABA have also been tied to HPA axis regulation and monoaminergic transmission suggesting GABA may have downstream effects on other systems involved in mood (Luscher et al. 2011). While there is evidence for disruptions in all of these neurobiologic processes in MDD, each hypothesis on its own is likely too simplistic and
it is very probable that MDD involves a complex integration of these theories. Further research is also necessary to decipher whether these hypotheses represent causes, consequences, or confounds (neutral side effects) of the underlying pathophysiology of the disease.

1.1.1 Relevant Clinical Risk Factors for MDD

Similar to many psychiatric disorders, MDD is thought to arise from a combination of environmental, psychosocial, and neurobiological risk factors (Caspi and Moffitt 2006). The predominant environmental risk factors for MDD include adverse childhood experiences and stressful life events, such as hardships in job, health and relationship status. Predominant neurobiological risk factors include female sex, personality traits such as neuroticism, and genetic risk (Fava and Kendler 2000). However, MDD is known to have individual heterogeneity in clinical presentation, neurobiological factors, and treatment response (Rush 2007), which have led to the description of various subtypes (e.g. postpartum, atypical). Thus, environmental and underlying neurobiological predispositions combine to determine the relative risk of an individual. Below, I review two clinical factors that are relevant to the study: age and sex.

1.1.1.1 Sex Differences in MDD

One of the most replicated epidemiological findings in all of psychiatry is that women have higher rates of depression compared with men; a finding consistent across community-based epidemiological studies throughout the world (Weissman and Klerman 1985; Kessler et al. 1993). Approximately one in four women and one in ten
men will experience at least one debilitating episode of mood disorder in the course of a lifetime (Kendler 1998). Females are twice as likely to develop a single depressive episode compared to males (Kornstein et al. 2000), and are four times as likely to have recurrent episodes (Perugi et al. 1990). Females also tend to have more symptoms, different combinations of symptoms, and greater severity of the disorder (Angst and Dobler-Mikola 1984; Hirschfeld et al. 1984; Frank et al. 1988; Young et al. 1990; Grigoriadis and Robinson 2007). Even when controlling for factors such as willingness to seek help and gender bias of diagnosis, the risk for MDD is approximately two times higher in women than men (Kessler et al. 1994; Regier et al. 1998; Kornstein et al. 2000; Grigoriadis and Robinson 2007) and this sex difference persists into the elderly population (Krause 1986), suggesting an underlying biological predisposition for females to develop MDD.

While the underlying pathology of this sexual dimorphism in MDD is unknown, depressive episodes in women usually arise after the onset of puberty and often correspond with seasonal and biological rhythms (Kendler 1998; Becker et al. 2007). Women also experience higher rates of depression during times of low estrogen, such as premenstrual and postpartum periods (Fink et al. 1996), together suggesting potential hormonal origins of this sexual dimorphism rather than underlying genetic factors, a critical distinction [reviewed in (Grigoriadis and Robinson 2007)]. While few studies have examined sex differences in rodent models of depression, our lab has found that female mice lacking SERT, an MDD-associated gene, are more susceptible to developing a depressive-like syndrome following exposure to the unpredictable chronic mild stress paradigm (Joeyen-Waldorf et al. 2009). Ovarian hormones have also been shown to influence emotionality in female mice (Zimmerberg and Farley 1993; Mora et al. 1996), however, few studies have examined the effect of hormonal fluctuations on female vulnerability to a depressive-like syndrome. In an ongoing study, our lab recently found that vulnerability to depressive-like symptoms in female mice is likely due to organizational effects of hormones (i.e. permanent effects of developmental hormones on brain development) rather than activational effects (i.e. transient effects of gonadal hormones) [M. Seney, personal communication; reviewed in (Cooke et al. 2007)].
Thus, effects of sex hormones seem to be a critical factor involved in the precipitation of depressive symptoms and the underlying neurological and hormonal factors need to be more thoroughly examined.

1.1.1.2 MDD and Age

Greater than 50% of elderly MDD patients experience their first depressive episode after age 60 (Brodaty et al. 2001), a striking pattern representing a subtype of MDD termed late-onset depression. The predominant risk factors for late-onset depression include sleep disturbances, disability, and bereavement, which are mainly stress-related (Cole and Dendukuri 2003; Djernes 2006). However, characteristics of the natural aging process have also been implicated in the onset of late-onset MDD. For example, alterations in the serotonin system (decreased 5HT$_{2A}$ receptor binding), impaired endocrine function, cognitive deficits and dementia, and poor vascular health (hypertension and stroke) have all been implicated as potential biological factors involved in late-onset depression (Alexopoulos 2002; Schweitzer et al. 2002; Sheline et al. 2002; Blazer and Hybels 2005). While late-onset depression is a subclass of MDD and older adults appear to be at greater biological risk for developing MDD, the frequency of developing depression is actually lower in older adults compared with younger adults (Byers et al. 2010); [reviewed in (Blazer and Hybels 2005)]. Surprisingly, the co-morbidity of MDD with physical disorders also decreases with age, suggesting that the low MDD rates in elderly are not due to psychological confounds associated with physical disorders (Kessler et al. 2010). This lower rate of incidence could be due to factors such as age-related increases in psychological resilience (i.e. wisdom), religious involvement, and participation in valued activities (Hendrie et al. 2006). In accord, Fiske and colleagues recently proposed that a common pathway to depression onset in older adults may be a decrease of daily activities (Fiske et al. 2009).
Age-related illnesses have also been suggested to be causative for development of late-onset depression. There is substantial comorbidity between MDD and dementia and MDD is found in approximately 20% of all Alzheimer’s patients (Patterson et al. 1990). MDD, in combination with pre-morbid cognitive deficits, increases the risk for Alzheimer’s (Alexopoulos et al. 1993), suggesting that late-onset MDD may be a pre-morbid marker for other neurological disorders. Thus, late-onset MDD is a significant medical concern in the elderly and there is potential that late-onset MDD shares a common pathophysiology with certain age-related cognitive and neurodegenerative disorders (Olin et al. 2002).

1.1.2 Neurobiology of MDD

1.1.2.1 Neurocircuitry of MDD

Studies of anatomical regions underlying depression have revealed dysfunction of corticolimbic circuitry in MDD (Figure 1.1). Neuroimaging studies have shown failure of coordinated activity between cortical and limbic regions in MDD subjects (Mayberg 1997), reduced correlation between amygdala and prefrontal cortex (PFC) activity (Siegle et al. 2007), and reduced functional connectivity between the amygdala and perigenual cingulate cortex in individuals genetically susceptible to MDD (serotonin transporter s-allele carriers) (Pezawas et al. 2005). In addition, decreased functional coupling of the supragenual cingulate and amygdala was positively correlated with depressive symptom severity (Matthews et al. 2008). Using microarray data, robust coordinated shifts in gene transcript level (ie. gene synchrony) were reported between amygdala and cingulate cortex in MDD subjects (Gaiteri et al. 2010). A meta-analysis using both imaging data and mathematical modeling confirmed abnormal connectivity between corticolimbic regions and differentiated treatment responders from non-responders (Seminowicz et al. 2004).
Studies of anatomical regions suggest the amygdala plays a central role in mood-related disorders (Mayberg 1997; Krishnan and Nestler 2008). Functional magnetic resonance imaging (fMRI) studies revealed MDD patients had increased amygdala reactivity associated with exposure to increasingly sad faces (Surguladze et al. 2005), sustained amygdala activity in response to negative emotional stimulation (Siegle et al. 2002), and increased amygdala responsiveness to sad or fearful faces even when presented below the level of conscious awareness (Sheline et al. 2001; Suslow et al. 2010; Victor et al. 2010). In contrast, other studies in MDD subjects have revealed no alterations in amygdala reactivity perhaps highlighting the heterogeneity of the disorder or the diversity of the paradigms (Monk et al. 2008; Townsend et al. 2010). Neuroimaging studies have also revealed alterations in amygdala volume in MDD subjects (Sheline et al. 1998; van Eijndhoven et al. 2009; Lorenzetti et al. 2010), however, results are inconsistent and could be due to changes in the density of specific cell types (Campbell and MacQueen 2006; Rajkowska and Miguel-Hidalgo 2007). Other brain regions implicated in MDD include the anterior cingulate cortex (ACC), hippocampus, and nucleus accumbens, all regions that are functionally connected to the amygdala (Krishnan and Nestler 2008), supporting a central role for the amygdala in MDD. Rodent studies have further dissected the pathways involved in emotion and fear regulation and have revealed the basolateral nuclei in the amygdala (BLA) as a critically important structure in these processes (see 1.2.3).

The role of corticolimbic circuitry has also been well established in fear and anxiety in humans, primarily through fear conditioning and extinction paradigms. Individuals with post traumatic stress disorder (PTSD) show deficits in fear extinction and/or extinction retention, which are corticolimbic-specific tasks (Bremner et al. 2005; Wessa and Flor 2007; Milad et al. 2008); [reviewed in (Jovanovic and Ressler 2010)], and PTSD shares a strong comorbidity with depression (Kessler et al. 1995; Blanchard et al. 1998). A review of literature found that over 25 studies associated amygdala activation to fearful stimuli (Phan et al. 2002), and the ventromedial PFC (vmPFC) - amygdala circuit is thought to be disrupted in PTSD, similar to MDD (Rauch et al. 2006; Delgado et al. 2008). Also akin to MDD, PTSD patients frequently display disruptions of
cortisol levels (Yehuda et al. 2004) and cortisol administration reduces symptoms of PTSD (Aerni et al. 2004; Soravia et al. 2006). Together, this evidence suggests common mechanisms at play between MDD and PTSD. While investigations of these pathways in humans is limited to non-invasive techniques, fear conditioning and extinction circuits have been thoroughly dissected in rodents (see 1.2.3) and these pathways show robust phylogenetic conservation across species (Figure 1.1).
Figure 1.1. Phylogenetic conservation of corticolimbic brain regions involved in mood regulation. Human ventromedial prefrontal cortex (vmPFC) is reciprocally connected to amygdala (red arrows). Similarly, mouse prelimbic and infralimbic cortices are reciprocally connected to amygdala (red arrows). Human figure modified from (Tost and Meyer-Lindenberg 2010); mouse figure modified from (Heintz and Mamounas 2011). HYP=hypothalamus; ACG=anterior cingulated gyrus; PFC=prefrontal cortex; vmPFC=ventromedial PFC; AMY=amygdala; HPC=hippocampus.
1.1.2.2 Stress and Neuroendocrine Changes in MDD

The link between stress and the onset of MDD is well established (Hammen 2005). While stressful life events are a causal component in MDD (Kendler et al. 1999), depression, in turn, increases susceptibility to stressful life events which may account for chronicity and/or recurrence in the disorder (Hammen 1991); [reviewed in (Liu and Alloy 2010)]. In addition, numerous studies indicate that individuals exposed to early adverse experiences are more susceptible to developing mental disorders (Heim and Nemeroff 2001). In particular, childhood maltreatment and abuse are significant risk factors for developing MDD in women (McCauley et al. 1997; Kendler et al. 2004). Furthermore, stressful life events have been shown to precipitate MDD in genetically susceptible individuals (Caspi et al. 2003), and early life stress is suspected to lead to epigenetic susceptibility to MDD (McGowan et al. 2009), suggesting a need to consider both genetic and environmental factors when studying MDD.

Neurobiological stress mechanisms, such as the hypothalamic-pituitary-adrenal (HPA) axis, have also been implicated in MDD. Severe depressive episodes are frequently associated with high cortisol levels (hypercortisolaemia) (Brouwer et al. 2005) and patients with Cushing’s Syndrome (a disorder resulting in high circulating cortisol) are prone to depression (McEwen 2007). In contrast, atypical depression (characterized by hypersomnia and hyperphagia) is associated with lower cortisol levels (hypocortisolaemia) (Gold and Chrousos 2002; Brouwer et al. 2005). However, the level of cortisol in MDD also varies with sex, age, and genetic risk (Kaufman et al. 2004; Alexopoulos 2005; Kudielka and Kirschbaum 2005), indicating that a variety of external factors could contribute to the HPA disruptions. Patients with MDD consistently show blunted stress reactivity and impaired stress-recovery (Burke et al. 2005), and have alterations in both corticotropin releasing hormone (CRH) receptor and glucocorticoid receptor levels in limbic brain regions (Webster et al. 2002; Merali et al. 2004). Overall, it has been suggested that dysfunction of these critical stress mediators can disrupt the stability of the system leading to “allostatic load”, a concept whereby maladaptive stress
responses lead to wear and tear and eventually can evolve into disorders such as MDD (McEwen 2003).

1.1.2.3 Genetics of MDD

MDD is a polygenic, epistatic disorder with a heterogeneous etiology (Fava and Kendler 2000). Twin studies have revealed there is a strong genetic component to MDD (Kendler et al. 1995; Kendler and Prescott 1999) and a meta-analysis revealed that the heritability estimates of MDD range between 31-42% (Sullivan et al. 2000). Genetic risk factors that influence stressful life events are also correlated with genetic risk factors for MDD (Kendler and Karkowski-Shuman 1997), however, the complexity of MDD has made it difficult to find potential susceptibility genes and only a handful of genetic risk factors have been uncovered (Lopez-Leon et al. 2008). For instance, a polymorphism in the promoter region of the SERT gene combined with stressful life events was associated with risk for depression (Caspi et al. 2003) and specific epigenetic changes in the glucocorticoid receptor gene were associated with suicide victims who suffered childhood abuse (McGowan et al. 2009).

Genome wide association studies (GWAS) have been performed on MDD subjects with minimal success. Two recent GWAS studies failed to find any single nucleotide polymorphisms (SNPs) that contribute to the risk for MDD (Muglia et al. 2010; Shi et al. 2011). While a recent GWAS study found four MDD risk genes, the authors admit there is a likely possibility these are false discoveries (Bosker et al. 2011). Together, these results suggest that using GWAS to identify SNPs associated with MDD may be difficult due to heterogeneity of the disorder, uncontrolled environmental factors, and potentially small effect sizes of MDD-associated gene variants. Interestingly, a recent study assessing genetic variants in relation to cortisol secretion and depression found several SNPs in a single gene that were associated with both conditions (Velders et al. 2011). Thus, using GWAS for a more focused approach may help to identify variants related to subtypes of MDD or could play a role in individualization of treatment using pharmacogenetic approaches (Malhotra 2010).
1.2 USING RODENTS TO INVESTIGATE MDD

1.2.1 Difficulties in Using Rodents to Investigate MDD

Rodent models have been invaluable for studying emotion and fear-related behaviors as more invasive techniques can be used to explore the mechanism(s) underlying mood. However, modeling a complex human disorder such as MDD in a rodent poses many challenges. First, MDD is defined by symptomatology rather than neurobiology and the symptom heterogeneity between individuals can be vast (Hyman 2010), making it difficult for a single animal model or behavioral test to mimic all elements of the disorder. Second, MDD likely involves uniquely human neural circuitry, such as the much expanded prefrontal cortex, that has higher functioning than in a rodent (Crawley 2000). Another problem is that rodent models and behavioral tests are at risk for anthropomorphism (Crawley 2000). Emotions are highly species-specific and measuring abstract symptoms in rodents, such as depressed mood, presents a challenge as it is difficult to know the emotional capabilities of a rodent or what exactly a rodent is “feeling” (Cryan and Holmes 2005). Thus, when using or developing animal models for a psychiatric disorder such as MDD, it is critical to assess the level of validity it achieves. Animal models are typically assessed on construct, face, and predictive validity, which evaluate etiological, symptomatological, and pharmacological relevance respectively (Nestler and Hyman 2010). While it is unlikely we can ever fully reproduce MDD in a rodent, we can aspire to animal models (whether genetic, environmental, or
pharmacological) that recapitulate many aspects of the disorder in an attempt to model a depressive-like “syndrome”.

Overinterpretation of simple behavioral phenotypes is another concern in animal studies of MDD (Crawley 2000). A single phenotype or a single test are often wrongly interpreted as modeling the disease, rather than a single aspect or component of the disease. The focus on human endophenotypes, or a single heritable trait associated with the disorder, has provided more tangible measures to model in rodents (Cryan et al. 2005; Gould and Gottesman 2006). Currently, most labs use the approach that multiple tests are needed to assess convergent “rodent endophenotypes” of depressive-like behavior (Cryan and Mombereau 2004). For example, the forced swim test and tail suspension tests are not models of depression but they do isolate “simple, quantifiable endophenotypes or response elements that are sensitive to stress and antidepressant action and amenable to biological analysis” (Crowley and Lucki 2005). Thus, perhaps combining behavioral and physiological endpoints may provide a more tangible and comprehensive way to assess an MDD-like syndrome in rodents.

Another inherent problem in evaluating rodent behavior is variability in behavior due to environmental factors such as time of day, experimenter, animal colony-related events, etc. In a now classic study, several strains of inbred mice were simultaneously tested in three different laboratories. The authors went to great efforts to normalize protocols, equipment, and animal husbandry between the labs and, while large differences between inbred strains were replicated across labs, they found systematic differences between behavioral tests across labs, which they attributed to subtle environmental differences between labs (Crabbe et al. 1999; Wahlsten et al. 2003). The unpredictable chronic mild stress paradigm (see below), in particular, has suffered from inter-lab reliability issues (Cryan and Mombereau 2004) leading to a high degree of variability between successful protocols. In addition, there are multiple tests for assessing anxiety- and depressive-like behaviors in rodents and, adding to this issue, individual labs can even interpret results from a single test differently. For instance, the forced swim test, a classic test originally used to test antidepressant efficacy of compounds, is now frequently used to test for “depressive-like” behavior, and
interpretations of the behavior vary widely (Cryan and Mombereau 2004; Crowley and Lucki 2005; Cryan et al. 2005). Thus, multiple testing, along with a high degree of in-house validation, is necessary for assessment of complex phenotypes such as emotionality in rodents (Wahlsten et al. 2003).

1.2.2 UCMS and CORT Rodents Models of Depression

1.2.2.1 Behavioral Correlates of MDD

Two rodent models have been shown to recapitulate multiple symptoms of MDD and are thus highly utilized to study the neurobiological mechanisms underlying the disorder. The unpredictable chronic mild stress (UCMS) rodent model for depression mimics the role of “psycho-social” stress in precipitating depressive pathology in humans, while the chronic corticosterone model (CORT) recapitulates the physiological role of stress in MDD onset. The UCMS model consists of exposing mice to various randomized environmental and social stressors for at least four weeks (Ducottet et al. 2004; Willner 2005), while the CORT model consists of exposing mice to a low dose of exogenous corticosterone for at least four weeks (Ardayfio and Kim 2006; David et al. 2009), both attempts at construct validity. These models have been shown to induce a “depressive-like” syndrome (face validity), with symptoms reminiscent of the human disease [Reviewed in (Willner 2005; Sterner and Kalynchuk 2010)]. Following either paradigm, mice exhibit increased anxiety and depressive-like symptoms, increased anhedonia-like symptoms measured by decreased consumption of sucrose, and decreased fur coat state [CORT: (Ardayfio and Kim 2006; David et al. 2009; Gourley and Taylor 2009)], [UCMS: (Mineur et al. 2006; Ibarguen-Vargas et al. 2008; Surget et al. 2008)]. The UCMS paradigm has also been shown to mimic sex differences in human MDD patients (Dalla et al. 2005; Joeyen-Waldorf et al. 2009; Guilloux et al. 2011) and both paradigms
are sensitive to chronic antidepressant treatment (predictive validity), similar to human MDD patients (Surget et al. 2008; David et al. 2009). A reliable marker of the progression of both the UCMS and CORT syndromes is the degradation of fur coat quality, thought to be an impaired grooming response, which is a visual marker of the syndrome also reversed with chronic antidepressant treatment (Mineur et al. 2006; Surget et al. 2008; David et al. 2009). While both paradigms mimic multiple behavioral symptoms of MDD, a caveat is that they are exclusively dependent on environmental or physiological stressors, a precipitating factor for several other psychiatric disorders (e.g. anxiety disorders) as well as MDD. However, the cellular and physiological characteristics recapitulated by these models are more specific to those seen in MDD.

1.2.2.2 Cellular and Physiological Effects

Chronic stress has been shown to elicit enhanced dendritic arborization (hypertrophy) (Vyas et al. 2002) and enhanced synaptic connectivity (Vyas et al. 2006) in the BLA. Amygdala hypertrophy was found to persist for weeks after stress, unlike the reversible hypotrophy found in the hippocampus (Vyas et al. 2004). In addition, acute and uncontrollable stress, along with corticosterone administration, enhance BLA firing (Kavushansky and Richter-Levin 2006; Kavushansky et al. 2006), pointing toward an overall stress-induced increase in amygdala reactivity. In contrast to the amygdala, decreased glial cell proliferation and hypotrophy were observed in cortical regions following UCMS and chronic corticosterone administration (Wellman 2001; Banasr et al. 2007). Even a brief uncontrollable stress paradigm (3 days of forced swimming) elicited a significant retraction of dendrites in the infralimbic cortex (IL) (Izquierdo et al. 2006). Significant stress-related cellular changes have also been found in the hippocampus of rodents, including loss of neuronal plasticity in the CA3 region (Gould and Tanapat 1999; Sapolsky 2003) and decreased hippocampal neurogenesis (Gould et al. 1997; Gould et al. 1998). However, direct evidence supporting involvement of the hippocampus in MDD is limited to reports indicating that smaller hippocampal volumes
in MDD subjects are associated with loss of neuropil (Stockmeier et al. 2004). Interestingly, antidepressant-induced increases in hippocampal neurogenesis have recently been attributed to mediating effects of the BLA (Castro et al. 2010), thereby underscoring the pivotal role of this structure in mood regulation. Thus, chronic stress paradigms, such as UCMS and CORT, paradigms elicit cellular changes in regions impacted by MDD (Figure 1.2).

In addition to cellular alterations, UCMS exposure has been shown to induce alterations in the HPA-axis. UCMS exposure elicits increased glucocorticoid levels (Ayensu et al. 1995; Banasr et al. 2007) and mice exposed to the CORT paradigm have a blunted stress response exemplified by attenuated stress-induced corticosterone levels (David et al. 2009). UCMS also causes significant changes in both mRNA and protein levels of CRH receptors in the orbitofrontal cortex of mice (Anisman et al. 2007). Furthermore, chronic administration of CRF1 antagonists had antidepressant effects and reversed transcriptome changes in both cingulate cortex and amygdala following UCMS (Surget et al. 2008). Thus, similar to findings in MDD (see 1.1.2.2), UCMS and CORT elicit disruptions in the HPA-axis. In summary, the UCMS and CORT rodent models of depression achieve a crucial combination of construct, face, and predictive validity making them quite comprehensive models for MDD.
Figure 1.2. Stress-induced changes in dendritic structure in rodents. Chronic stress causes dendritic hypertrophy in the amygdala of rodents, but dendritic hypotrophy in the infralimbic prefrontal cortex of rodents, both regions implicated in MDD. These changes represent potential substrates for corticolimbic dysfunction in MDD, behaviors that are potentially detectable in fear conditioning paradigms. [Modified from (Rodrigues et al. 2009); originally from (Vyas et al. 2002; Izquierdo et al. 2006)].
1.2.3 Circuitry Underlying Fear and Emotion in Rodents

The amygdala has been implicated as a central region involved in MDD (section 1.1.2.1) and as previously discussed, there is significant conservation of corticolimbic circuitry between the mouse and human (Figure 1.1). This circuitry has been thoroughly examined in rodents using conditioned fear and the general pathway of fear assessment is widely acknowledged (Phelps and LeDoux 2005). Briefly, the BLA serves as a site of convergence for sensory information from the thalamus. This sensory input can then be modulated by regions such as hippocampus or prefrontal cortex and the information is then relayed to the central nucleus of the amygdala (CeA), the major output center of the amygdala. CeA outputs project to regions, such as the hypothalamus and periaqueductal gray, which control fear-associated responses [reviewed in (Phelps and LeDoux 2005)]. Below, I describe in depth how utilization of the fear conditioning paradigm in rodents has helped to shed light on the roles of these regions in mood regulation.

1.2.3.1 Fear Conditioning

A typical fear conditioning paradigm consists of learning of a fear association (fear learning), usually a conditioned stimulus (CS) (e.g. an audible tone) paired with an unconditioned fearful stimulus (US) (e.g. footshock), whereby the animal quickly learns to “fear” the CS, a response measured by freezing in rodents. This is often followed by fear extinction in which the animal is repeatedly exposed to the CS without the US, whereby the fear response to the CS is diminished (section 1.2.3.2). The BLA has been shown to play a central role in fear learning and is the site where sensory information about the CS and US converge during the learned fear association (Fanselow and LeDoux 1999). Lesioning the BLA prior to fear learning eliminates the learned association, but also eliminates freezing (Maren et al. 1996) and even natural variation
in BLA volume correlates with changes in fear response. For instance, a small BLA volume is associated with enhanced fear learning and an exaggerated glucocorticoid response (Yang et al. 2008). Fear learning also enhances firing in the BLA (Quirk et al. 1995) and disruption of BLA activity during learning prevents the learned association (Muller et al. 1997). On the other hand, the CeA is mainly involved in the output of the fear response, projecting to downstream targets in the brainstem and hypothalamus to coordinate motor and autonomic responses. However, recent evidence also implicates the CeA in the acquisition and storage of conditioned fear responses and supports differential roles for the lateral and medial portions of CeA (Wilensky et al. 2006; Zimmerman et al. 2007; Ciocchi et al. 2010).

1.2.3.2 Fear Extinction

There is evidence that extinction of the conditioned fear association is a separate learning process that actually forms a new memory which inhibits the conditioned response (Quirk 2002). The BLA is a critical component of fear extinction as well as fear conditioning, and it is necessary for acquisition of extinction (Herry et al. 2006; Sotres-Bayon et al. 2007). The basal (BA) and lateral (LA) nuclei have dissociable roles in these processes, and the BA is thought to be essential for accessing previously acquired fear memories (essential for extinction) (Anglada-Figueroa and Quirk 2005), while the LA may be involved in long-term storage of fear memories (Hobin et al. 2003; Rodrigues et al. 2004). While inhibiting the BLA with the GABA_A receptor agonist muscimol will reduce fear expression (i.e. freezing) during extinction, it does not impair extinction learning (i.e. the rate) (Muller et al. 1997; Akirav et al. 2006), suggesting the BLA is not the only component involved in extinction learning. Indeed, prefrontal regions including the prelimbic (PL) and infralimbic (IL) cortices (rodent analogues to the dorsal anterior cingulate cortex and vmPFC, respectively), have been implicated in extinction and fear expression (Peters et al. 2009). Reports indicate the PL modulates fear expression during extinction via projections to BLA, the “common final path” for fear.
expression, while the IL is necessary for extinction learning and retrieval (Sierra-Mercado et al. 2011). The resulting model is one where PL activation *drives* fear and IL activation *inhibits* fear through opposing connections with BLA (Figure 1.3). Intercalated neurons, an additional component of the circuit, mediate input from the IL to the CeA and are required for extinction learning (Likhtik et al. 2008).

**Figure 1.3. Circuits implicated in fear conditioning and extinction.** Cortical regions such as PL and IL modulate BLA and ITC which project directly to CE where fear outputs are determined. PL=prelimbic cortex, IL=infralimbic cortex, LA=lateral amygdala, BA=basal amygdala, ITC=intercalated cells, CE=central amygdala. Modified from (Peters et al. 2009).
1.2.3.3 Corticolimbic Coordination in Fear Conditioning

Similar to MDD, there is evidence for corticolimbic coordination in fear conditioning in rodents. The BLA and orbitofrontal cortex cooperate to encode learning associations (Schoenbaum et al. 1998), with the BLA encoding motivational significance and the orbitofrontal cortex controlling the appropriate behavioral outcome (Schoenbaum et al. 1999; Saddoris et al. 2005). The mPFC-BLA connection is critical during consolidation of the extinction memory, and the IL likely controls feed-forward inhibition to the BLA when the extinguished CS is re-experienced (Milad and Quirk 2002). Thus, the connection between BLA and PFC is considered crucial for encoding the motivational/emotional significance of a cue and determining the appropriate behavioral response (Davis and Whalen 2001; Rempel-Clower 2007).

1.2.3.4 Effects of Stress on Fear Conditioning

There is also well documented evidence that stress hormones influence the functioning of fear circuitry [reviewed in (Rodrigues et al. 2009)]. Exposure to stress and elevation of BLA glucocorticoid levels prior to conditioning enhances fear memory (Izquierdo et al. 2002; Rau et al. 2005), while blockade of glucocorticoid signaling in BLA after fear conditioning disrupts fear memory consolidation (Jin et al. 2007; Rodrigues and Sapolsky 2009). Glucocorticoid activation prior to extinction facilitates extinction learning and administration of glucocorticoid receptor antagonists or corticosterone inhibitors attenuates fear extinction (Cai et al. 2006; Yang et al. 2006). Therefore, all evidence suggests that activation of the HPA-axis is crucial for the formation and extinction of fear memories.

Rats selectively bred for increased susceptibility to the learned helplessness task (a paradigm which induces depression-like symptoms in rodents) showed enhanced fear learning and were resistant to fear extinction. Concomitant with these alterations, rats showed decreased sucrose preference, a rodent correlate to anhedonia (Shumake
et al. 2005), suggesting a connection between abnormal extinction mechanisms and a depressive phenotype. Several other studies have noted that chronic stress impairs recall of extinction (Miracle et al. 2006; Garcia et al. 2008; Farrell et al. 2010), but not fear expression or extinction learning. However, in two of these studies “extinction” was performed only 1 hour after initial fear conditioning, rather than the typical 24 hours, allowing no time for consolidation of the fear memory. Instead, “extinction recall”, performed on day 2, is more akin to typical extinction training. Taking this into consideration, the results obtained in these studies indicate chronic stress alters fear extinction and expression, an effect also shown to be sexually dimorphic (Baran et al. 2009). Notably, lesions of IL occlude this stress-induced impairment of extinction, an effect potentially mediated by stress-induced dendritic retraction in IL (Farrell et al. 2010). Furthermore, atrophy in IL neurons induced by brief stress is associated with resistance to fear extinction (Izquierdo et al. 2006). Animals exposed to prior stress also have facilitation of fear conditioning associated with decreased GABA control and neuronal hyperexcitability (Rodriguez Manzanares et al. 2005). Thus, responses to fear conditioning, particularly extinction learning, are altered by glucocorticoids and chronic stress paradigms indicating this task can be used to assess corticolimbic function associated with stress-related disorders such as MDD.

1.2.4 Using Knockout Mice

**Note on the following section:** Below is segment from a review article on the role of genetically modified animals in neuropharmacology that we wrote for the Encyclopedia of Neuropsychopharmacology (Authored by N. Edgar and E. Sibille). I have included an abbreviated version of the section entitled “Gene knockout technology” below to highlight the importance of using knockout animals to shed light on gene function, as I have used the CNP1KO mouse to elucidate the function of the gene in this dissertation. The entire article, along with definitions for words highlighted in blue can be found in Appendix A.
The creation of a traditional knockout (i.e. removal of a gene) mouse consists of disrupting all or part of the coding sequence of a gene of interest (GOI) for the purpose of exploring the phenotype in the absence of the gene. The GOI is removed by transfecting an embryonic stem cell with a construct designed for homologous recombination, which typically contains a selectable marker in place of the GOI. Mutated stem cells are then selected in vitro and are microinjected into a blastocyst and implanted into a pseudopregnant female mouse (Figure A.1b). Most of the offspring produced using this technique are chimeras (or mosaics) due to the presence of both mutated and non-mutated stem cells within the blastocyst. The next step consists of breeding chimeric mice to identify individuals with germ cells that have undergone homologous recombination. These founder mice will then be bred to produce homozygous mutant animals (Dale 2002).

While knockout organisms have been paramount in the goal to elucidate the function of specific genes, problems associated with knockout technology include that removal of a gene is often lethal or that the absence of the gene product during development leads to compensatory events that can obscure the analysis of the function of the missing gene. Specifically, these compensatory developments likely differ from the disease mechanisms that the knockout animal is intended to model, as disease processes rarely include full loss of gene function.

### 1.3 THE ROLE OF GLIA IN MDD

#### 1.3.1 Glial Structure and Function

There are two major classes of cells in the brain: neurons and glia. While neurons are often the primary focus of brain research, glial cells actually comprise 90% of brain cells. The two cell types differ in their electrical excitability. While neurons can generate an action potential, which then propagates through neural networks, glia are unable to generate an action potential, although they do express voltage-gated
channels. Glial cells are further delineated into macroglia (cells of neural [ectodermal] origin) and microglia (cells of non-neural [mesodermal] origin). Macroglia include astrocytes, oligodendrocytes, and ependymal cells, while microglia are in a class of their own and actually originate from macrophages that invade the brain early in life. Historically, glial cells were thought to be a “connective substance” which literally acted as the glue that held neurons together and which was devoid of cellular function (Verkhratsky and Butt 2007). While the functional significance of glial cells is often overlooked, increasing evidence is emphasizing the unique importance of glial cells and their role in mediating neuronal cell function.

1.3.1.1 Microglia

Microglia make up about 10% of all glial cells and are found throughout all regions of the brain. Microglia form the immune system of the brain, which can be activated during injury and/or disease and they contain both classical immunological receptors along with neuroreceptors. Each individual microglial cell covers an area of about 50,000μm², never coming into contact with another microglial cell. Within gray matter, microglial cells extend their processes in all directions; while in white matter, their processes are typically aligned perpendicular to white matter tracts. Microglial cells appear in one of three states: 1) Resting microglia are found under normal physiological conditions and their processes are constantly moving to scan their territory for noxious agents, 2) Resting microglia become activated when an insult is detected. The cells retract many of their processes and the remaining processes become thicker. The cell body enlarges, starts to produce immune response molecules, and becomes motile to remove any damaging agents (Rajkowska and Miguel-Hidalgo 2007). 3) If activated microglia are unable to clear the threat and cells begin to die, active cells become phagocytic, an irreversible state where the cells devour remnants of dead and dying neurons (Verkhratsky and Butt 2007).
1.3.1.2 Astrocytes

Astrocytes comprise about 80% of glial cells in the CNS and consist of about ten different subtypes (Verkhratsky and Butt 2007). The largest subgroup is the “true astrocytes”, including protoplasmic astrocytes, which are present in grey matter, and fibrous astrocytes, which are present in white matter. The other major subgroup is radial glia, which primarily assist in neuronal migration during development. The remaining groups are smaller, more specialized populations of astrocytes that are often region specific. With so many subtypes, astrocytes have diversified functional roles of which I will only touch on briefly here. One of the most critical functions of astrocytes is that of stem cells for both neurons and glia in the adult brain. Astrocytes are the stem elements found in the hippocampus and the subventricular zone that produce new neurons, and they are also the stem elements that produce all other types of glia throughout the brain. Astrocytes also play a crucial role in maintaining the blood brain barrier and regulating microcirculation within the brain. Astrocytes contain a variety of ion transporters for maintaining homeostasis of potassium and calcium in the extracellular space, along with glutamate transporters and aquaporins for regulating extracellular glutamate and water homeostasis, respectively (Rajkowska and Miguel-Hidalgo 2007). Astrocytes are also closely involved with regulating neuronal activity via tripartite synapses (an astrocyte synaptically associated with both pre- and post-synaptic neurons) (Araque et al. 1999) and glucocorticoid receptors that act to alter production of neurotrophic factors (Gubba et al. 2004). Finally, astrocytes modulate synaptic transmission by 1) reuptake and/or release of neurotransmitters at the synaptic cleft via specialized transporters or binding proteins; 2) morphological plasticity at the synapse [e.g. during lactation high levels of oxytocin trigger withdrawal of astrocyte processes thereby allowing more glutamate into the synaptic cleft; (Theodosis et al. 2006)]; and 3) complex connectivity between themselves, neurons, other glial cells, allowing signal propagation throughout the “network” (Verkhratsky and Butt 2007).
1.3.1.3 Oligodendrocytes

Oligodendrocytes, the main myelin forming cells of the central nervous system, comprise only about 5% of all glial cells, have fewer process than astrocytes, and are found in both grey and white matter. While they are morphologically distinguished into four phenotypes (Verkhratsky and Butt 2007), within the cortex and most subcortical regions, they are divided into perineuronal oligodendrocytes (found in grey matter; also termed Type I) and interfascicular oligodendrocytes (found in white matter; also termed Type II) (Rajkowska and Miguel-Hidalgo 2007). Both types of oligodendrocytes have a small round cell body and about four to six branching processes which can myelinate ten to thirty axons. Myelin consists of lipids (70%) and a variety of proteins (30%), including myelin basic protein (MBP), proteolipid protein (PLP) and 2',3'-cyclic nucleotide-3'-phosphodiesterase (CNP), which together comprise about 85% of all myelin proteins. When ensheathing axons, the cytoplasm is extruded from the cell processes and the opposing lipid bilayers are fused. The myelin then forms compacted concentric layers (lamellae) around the bare axon (typically 10-100 lamellae), with the cytoplasmic edges termed the inner and outer mesaxon. Oligodendrocytes play a role in regulating the development and periodicity of nodes of Ranvier, spaces of bare axon which contain ion channels critical for action potential propagation along the axon. At the ends of the internodal regions of myelin sheath, the myelin lamellae form a junction with the axon termed the paranode region (Figure 1.4). This region consists of septate-like junctions between the axon and paranodal myelin, with the axonal proteins Caspr (contactin-associated protein) and contactin interacting with the glial protein NF155 to form the junction. This region appears to have three primary roles in maintaining a stable saltatory conduction: 1) spatial separation of Na+ and K+ channels, 2) sealing the myelin sheath in a way that allows only selected nutrients to diffuse into the internodal periaxonal space (space between the myelin and axon), and 3) stabilization of the entire structure in the face of mechanical stressors (Lazzarini 2004; Rosenbluth 2009).

Functionally, mature oligodendrocytes provide critical insulation to facilitate axonal conduction. The myelin sheath increases the resistance and lowers the
capacitance of the axonal membrane which allows faster conductance speeds in myelinated axons compared to unmyelinated axons of the same diameter. While this function of myelin is well established, recent studies have indicated that myelin plays a more functional role than previously thought. Electrophysiological changes in oligodendrocytes can rapidly modulate axonal conduction velocity (Yamazaki et al. 2007). Activity in axons has been shown to promote astrocyte activity which in turn promotes myelination, suggesting an activity dependent myelination process (Ishibashi et al. 2006). Oligodendrocytes also monitor neural activity through a variety of receptors including glutamatergic (AMPA, NMDA, and kainate) (Patneau et al. 1994; Ziak et al. 1998) and GABAergic (GABA_A) (Berger et al. 1992) receptors, which both depolarize the cell, the latter due to high intracellular levels of Cl\(^-\). In addition, Nave suggests that trophic support from oligodendrocytes is required for mitochondrial energy metabolism in axons since the myelin sheath itself restricts axonal access to extracellular metabolic substrates (Nave 2010). Additional evidence for myelin trophic support comes from studies of mice lacking oligodendrocyte-specific proteins. While lack of MBP leads to loss of compact myelin (Brady et al. 1999), mice lacking PLP, myelin associated glycoprotein (MAG), and CNP all have relatively normal myelin assembly, but develop progressive neurodegeneration (Klugmann et al. 1997; Griffiths et al. 1998; Yin et al. 1998). In addition, PLP deficient mice also show disruption of fast axonal transport prior to axonal degeneration (Edgar et al. 2004). These studies indicate that a disruption in oligodendrocyte proteins can have a major impact on axon survival, pointing toward a pivotal role for myelin in axon trophic support. Together, these studies indicate that mature oligodendrocytes have a dynamic role in regulating neuronal signal transmission and support of axons.
Figure 1.4. Structure of a myelinating oligodendrocyte. Oligodendrocyte cells extend processes of their cell membrane to form the myelin sheath. Opposing sides of the cell membrane fuse together and this myelin sheath is wrapped in concentric circles around the axon. The segment of myelin sheath between the nodes of Ranvier (internode region) terminates at the paranode region, a site critical for axoglial interaction. From (Nave 2010).
1.3.1.4  NG2 Cells

Mature oligodendrocytes are derived from oligodendrocyte precursor cells (OPC) which develop in specific ventricular zones in the brain, migrate to their final site in the brain, and finally differentiate. During this time, various proteins are expressed that can mark the developmental stage. A specific subtype of OPCs, which expresses a novel chondroitin sulphate proteoglycan (NG2) (Belachew et al. 2001; Mallon et al. 2002), is worth mentioning. NG2 cells are a relatively newly discovered class of cells that share a common lineage with oligodendrocytes and express several markers specific to OPCs (e.g. platelet-derived growth factor) but do not express markers for mature oligodendrocytes. NG2 cells are thought to function as multipotent adult neural stem cells, as they can also differentiate into neurons and astrocytes. In addition, NG2 cells give rise to both mature oligodendrocytes and a separate population of NG2-glia called synantocytes (Belachew et al. 2003; Aguirre and Gallo 2004). While NG2 cells do not form contacts with each other, they do form contacts with neurons, oligodendrocytes, and astrocytes and express voltage-gated ion channels along with various receptors (Verkhratsky and Butt 2007). In addition, GABAergic and glutamatergic neurons establish contact with NG2 cells (Trotter et al. 2010), and they are able to fire action potentials (Karadottir et al. 2008), suggesting an active role in signal conduction. NG2 cells are also postulated to rapidly respond to changes in neuronal integrity to either stimulate formation of a glial scar, or to generate new neurons or glial cells if necessary (Butt et al. 2005). Thus, while the functional role of NG2 cells is still being investigated, they are clearly a critical element for maintaining interactions between neurons and glia. However, care must be taken in future studies to properly distinguish between NG2 cells and oligodendrocytes.
1.3.2 Glial Alterations in MDD

Glial disruptions are postulated to contribute to the pathophysiology of MDD and alterations have been seen in all the major glial subtypes in MDD. [Reviewed in (Rajkowska and Miguel-Hidalgo 2007; Hercher et al. 2009)]. Studies of brain regions implicated in MDD (particularly corticolimbic regions) have described reductions in glial density or glia/neuron ratio in the subgenual PFC (Ongur et al. 1998), dorsolateral prefrontal cortex (Rajkowska et al. 1999; Cotter et al. 2002), orbitofrontal cortex (Rajkowska et al. 1999) anterior cingulate cortex (Cotter et al. 2001), and amygdala (Bowley et al. 2002; Hamidi et al. 2004). In addition to glial cell density, increases in glial cell size have been reported in the dorsolateral PFC (Rajkowska et al. 1999) and anterior cingulate cortex (Chana et al. 2003) of MDD subjects.

The above studies used generalized nissl staining which does not allow distinction between different types of glial cells. However, studies with more specific immunostaining have revealed differences in subtypes of glia in MDD. While the neuroinflammatory hypothesis for MDD points toward potential alterations of microglia, the main cell type for active immune defense in the CNS (Muller and Schwarz 2007; Miller et al. 2009), only a single study has examined microglia pathology in MDD subjects. Steiner and colleagues showed increases in microglial density in a variety of brain regions in MDD subjects who committed suicide, but normal microglial densities in non-suicide MDD subjects (Steiner et al. 2008). They used a known microglial marker for neurodegeneration and neuroinflammation (HLA-DR), suggesting a role for these processes in suicidal behavior. In rats, proliferation of microglial cells in the hippocampus (a region implicated in stress and mechanism for antidepressant action) was reduced by chronic corticosterone administration and electroconvulsive seizure treatment reversed this effect (Wennstrom et al. 2006)

Alterations in astrocytes in MDD have primarily been found using immunostaining for GFAP (glial fibrillary acid protein) which stains for an intermediate filament protein found in astrocytes and ependymal cells. Decreases in GFAP have been found in the dorsolateral PFC and orbitofrontal cortex of MDD subjects, however, they have primarily
been found only in young (age 30-45) subjects with MDD (Johnston-Wilson et al. 2000; Miguel-Hidalgo et al. 2000; Si et al. 2004). Furthermore, there are reports of altered astrocytic excitatory amino acid transporter gene expression (EAAT1 and EAAT2) in cortical regions of MDD subjects (Miguel-Hidalgo et al. 2010). Rodent studies have revealed that rats with astrocytes specifically ablated in the frontal cortex showed depressive-like behaviors reversible with antidepressant treatment (Banasr and Duman 2008). Chronic stress in rodents decreased the number and somal volume of astrocytes in the hippocampus (Czeh et al. 2006) and impaired glutamatergic cycling in the PFC, a pattern reversible via pharmacological increase of glutamate uptake (Banasr et al. 2010). Together, this evidence suggests an astrocyte-mediated dysfunction/depletion of prefrontal cortex glutamatergic cycling and homeostasis in MDD [Reviewed in (Valentine and Sanacora 2009)].

While there is evidence for disruption in other glial subtypes, the role of oligodendrocyte alterations in MDD and other psychiatric disorders has become a primary focus of research (Fields 2008). In the amygdala and prefrontal cortex of MDD subjects, previously-reported decreases in glial cell number were attributed to reduced oligodendrocyte number, (Hamidi et al. 2004; Uranova et al. 2004) (Figure 1.5). In addition, patterns of downregulation of oligodendrocyte-related transcripts in amygdala (Sibille et al. 2009) and nearby temporal cortex (Aston et al. 2005) were seen in MDD subjects. Studies using diffusion tensor imaging have also revealed white matter abnormalities in cortical and temporal regions in MDD subjects (Alexopoulos et al. 2002; Taylor et al. 2004; Bae et al. 2006; Ma et al. 2007), and loss of white matter correlates with depressive symptom severity (Nobuhara et al. 2006).

Rodents exposed to chronic stress have a reduction in the proliferation of oligodendrocytes and NG2 cells in the PFC, a pattern that is reversible with antidepressant treatment (Banasr et al. 2007; Czeh et al. 2007). Similar to chronic stress, chronic corticosterone exposure in rodents resulted in decreased cortical and amygdala oligodendrocyte (Banasr et al. 2007) and NG2 proliferation (Alonso 2000; Wennstrom et al. 2006), suggesting oligodendrocytes may be particularly susceptible to corticosterone induced toxicity. Following electroconvulsive seizure therapy, an
antidepressant treatment in humans, non-stressed rats showed increased proliferation of NG2 cells in amygdala and hippocampus (Wennstrom et al. 2003; Wennstrom et al. 2004; Wennstrom et al. 2006) and increased proliferation of mature oligodendrocyte cells in the PFC (Madsen et al. 2005). However, in non-stressed rats treated chronically with the antidepressant fluoxetine, there were no changes observed in oligodendrocyte proliferation in prelimbic cortex (Kodama et al. 2004), suggesting oligodendrocyte changes are specific to chronic stress induced pathology and are not involved in the mechanism of pharmacological antidepressant treatment. Thus, it appears that oligodendrocytes are altered in corticolimbic regions affected in MDD and may be vulnerable to stress-related insults. However, it is unknown whether these changes represent a causal link to MDD or simply represent compensatory changes or neutral, confounding side effects.
Figure 1.5. Decreased oligodendrocyte density in the amygdala of MDD patients.
Oligodendrocyte cells were determined by morphology following Nissl staining and the density of oligodendrocytes was determined by dividing the total oligodendrocyte cell count by the volume of the amygdala. Densities were compared between control, MDD, and bipolar patients with only MDD exhibiting significantly decreased oligodendrocyte density compared with controls. Figure modified from (Hamidi et al. 2004).
1.4 CNP

1.4.1 Structure and Localization of CNP

The genetic loci for CNP are on mouse chromosome 11 and human chromosome 17 (Bernier et al. 1988; Douglas et al. 1992). The sequence homology of genomic DNA is highly conserved among species ranging from bullfrog to human, and the degree of homology between mouse and human is approximately 85% (Braun et al. 2004), suggesting a conserved function. The CNP gene contains four exons and gives rise to two RNA transcripts (CNP1 and CNP2) via alternative promoters, which are translated into protein isoforms [Reviewed in (Braun et al. 2004). However, the transcript for CNP2 contains alternative translation initiation sites for both CNP1 and CNP2, and is therefore able to produce both isoforms (O'Neill et al. 1997) (Figure 1.6A). CNP2 is the longer of the two isoforms as it contains a mitochondrial localization sequence on the N-terminus, which is cleaved upon entering the mitochondria leaving a truncated form similar to CNP1 (Lee et al. 2006). However, PKC-mediated phosphorylation can inhibit this translocation leaving an active CNP2 in the cytoplasm as well. CNP2 is also expressed at low levels in non-myelinating tissue, but is present only in mitochondria and is presumed to be involved in RNA and nucleotide processing (Lee et al. 2006).

While CNP1 is also expressed at much lower levels outside the nervous system, in the central nervous system it is found in both mature and immature oligodendrocytes where it comprises approximately 4% of myelin-associated protein (Braun et al. 2004), making it ideal as a marker for oligodendrocytes. Specifically, CNP1 is localized to the oligodendrocyte cell body, but is primarily found in the non-compact myelin of the inner mesaxon and paranodal loops (Trapp et al. 1988) (Figure 1.6B), the principle sites of communication between the axon and myelin sheath (see above). In this region, the myelin sheath of the internode is separated from the axon by 12-14nm (the periaxonal space) and CNP1 is localized to the cytoplasmic side of the periaxonal membrane. During development, OPCs first express CNP2 around embryonic day 15 in the rat, followed by expression of CNP1 beginning at postnatal day 1 (Scherer et al. 1994).
Expression of both CNP1 and CNP2 peaks just prior to the final differentiation stages preceding myelination (postnatal day 10 in the rat) (Amur-Umarjee et al. 1990), suggesting both forms are involved in myelin ensheathment, axon recognition, and process outgrowth (Verkhratsky and Butt 2007).

Figure 1.6. Structure and localization of CNP1. A). Diagram of the promoter regions of CNP DNA (top) and corresponding mRNA transcripts for CNP1 and CNP2 (bottom). The gene contains two promoter regions that alternately transcribe CNP1 or CNP2 [From (Braun et al. 2004)]. B) Electron micrograph of a cross-section of an axon showing immunoreactivity for CNP in the inner and outer mesaxon loops (arrows). Figures modified from (Braun et al. 1988).
1.4.2 Function of CNP

The high expression and paranodal localization of CNP would suggest an important functional role. As a phosphodiesterase, CNP is able to catalyze cleavage of 2',3'-cyclic nucleotides in vitro (Drummond et al. 1962; Sprinkle et al. 1978), however the physiological substrate in vivo remains unknown (or potentially non-existent), suggesting that the protein may serve a function other than enzymatic activity. Efforts to identify the function of CNP in vitro have found a role for the protein in cytoskeletal organization and cell signaling. OPCs express CNP in filopodia extensions that eventually form lamellae indicating a potential role in cytoskeleton organization and myelin construction (Braun et al. 1988). Furthermore, CNP associates with F-actin, serves as an anchor for tubulin (Dyer and Benjamins 1989; Bifulco et al. 2002), and also possesses microtubule polymerization activity (Bifulco et al. 2002). CNP overexpression in glial cells induces reorganization of the cytoskeletal network resulting in process outgrowth (Lee et al. 2005). Recent evidence has also shown that CNP is an RNA-binding protein which inhibits protein translation and mediates RNA binding to tubulin (Gravel et al. 2009). CNP has a binding site for ATP/GTP indicating it may be involved in signal transduction pathways (Stingo et al. 2007) and CNP has also been shown to associate with "lipid raft" membrane microdomains, which are presumed sites of organization for signaling complexes (Kim and Pfeiffer 1999). In healthy primates, CNP was found to accumulate with age due to failure of the proteosomal degradation system, potentially resulting in dysfunction of lipid rafts and eventual age-related disorders (Hinman et al. 2008). Thus, together this evidence suggests a role for CNP in cell signaling, perhaps mediating cytoskeletal assembly (Braun et al. 2004).

Similar to in vitro results, adult mice overexpressing CNP1 show redundant and aberrant myelination resulting in extension of myelin from internodal regions (Gravel et al. 1996). In addition, this myelin malformation was seen early in development prior to OPC differentiation, and resulted in relocation of CNP1 to compact myelin where it prevents fusion of the lamellae, likely by interfering with MBP binding (Yin et al. 1997). The CNP1 knockout mouse (CNP1KO) has normal myelin assembly, but has
progressive axonal degeneration and motor deficits with age (Lappe-Siefke et al. 2003). Furthermore, in the CNP1\textsuperscript{KO} mouse, both nodal sodium channels and paranodal adhesion proteins (e.g. Caspr) become progressively disorganized with age (Rasband et al. 2005) and examination of myelin ultrastructure found degeneration of small diameter axons as early as postnatal day 15 (Edgar et al. 2009). Together, the above suggests that removal of CNP compromises paranodal integrity leading to disrupted axoglial signaling and eventual axonal degradation.

Recent experiments from our lab indicated that CNP1 was downregulated in the amygdala of postmortem human MDD subjects and mice exposed to the UCMS model of depression (Sibille et al. 2009) and this downregulation was reversed by antidepressant treatment in rodents (Surget et al. 2008). The downregulation of this transcript was restricted to the amygdala, a critical brain region for mood regulation (Phelps and LeDoux 2005) and site of putative primary pathology in MDD (Drevets et al. 2002; Hamidi et al. 2004), and was not altered in anterior cingulate cortex or dentate gyrus, suggesting it is selectively mediated in a central brain region implicated in MDD. Downregulation of CNP1 transcripts were previously reported in whole brain homogenates and in the temporal cortex of MDD subjects (Aston et al. 2005; Sequeira et al. 2009). Furthermore, there is evidence of reduced levels of CNP in schizophrenia (Hakak et al. 2001; Davis et al. 2003; Flynn et al. 2003; Tkachev et al. 2003) and a single nucleotide polymorphism (SNP) linked to low CNP expression was significantly associated with schizophrenia (Peirce et al. 2006). Thus, there is evidence supporting the general hypothesis that altered CNP levels (and associated oligodendrocyte dysfunction) could be a factor in the development of psychiatric disorders. However, it remains to be determined whether oligodendrocyte-specific changes represent causative effects in MDD, or whether these changes are simply compensatory or neutral bystander effects.
1.5 SUMMARY OF INTRODUCTION AND SIGNIFICANCE OF RESEARCH

MDD is a devastating chronic disorder of altered mood and is the leading cause of disability worldwide in terms of years lived with the disability. While the specific mechanism(s) underlying MDD are not well understood, there is evidence for disrupted corticolimbic function, and the amygdala is thought to be a central brain region involved in mood regulation. There is also growing evidence for disrupted oligodendrocyte structure and function in the same corticolimbic regions in MDD subjects and in animal models of MDD. It is known that healthy oligodendrocytes are necessary to maintain optimal axon function (Nave 2010), however it remains to be determined whether oligodendrocyte-specific alterations are causal to MDD, or whether alterations are compensatory or simply neutral side effects in psychiatric disorders. CNP1 is a critical component of the paranode of oligodendrocytes, a region that is necessary to maintain the integrity of oligodendrocytes and proper axoglial interactions. Altered levels of CNP1 have also been found in postmortem tissue of MDD subjects, suggesting it may play a role in the pathophysiology of MDD.

Here, we investigated the effect of a lack of CNP1 on MDD-related features using the CNP1KO mouse. We developed and applied a novel Z-score normalization procedure to obtain a more long-term and comprehensive measure for emotionality (anxiety- and depressive-like behaviors) and also, to counteract inherent variability in behavioral measures (Chapter 2). We then examined whether the CNP1KO mouse 1) has altered baseline (trait) emotionality; 2) has altered vulnerability toward developing a depressive-like syndrome (state); and 3) has altered amygdala function and molecular characteristics (Chapter 3). These studies explored whether alterations in the integrity of oligodendrocytes contribute to the onset of depressive-like symptoms in mice and have implications for the pathophysiological mechanisms underlying MDD in humans.
2.0  PAPER 1: INTEGRATED BEHAVIORAL Z-SCORING INCREASES THE SENSITIVITY AND RELIABILITY OF BEHAVIORAL PHENOTYPING IN MICE: RELEVANCE TO EMOTIONALITY AND SEX

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\textbf{Note on my contributions to this paper:} I contributed a data set, participated in sections of the analysis, and helped with writing the manuscript.
2.1 ABSTRACT

Defining anxiety- and depressive-like states in mice (“emotionality”) is best characterized by the use of complementary tests, leading sometimes to puzzling discrepancies and lack of correlation between similar paradigms. To address this issue, we hypothesized that integrating measures along the same behavioral dimensions in different tests would reduce the intrinsic variability of single tests and provide a robust characterization of the underlying “emotionality” of individual mouse, similarly as mood and related syndromes are defined in humans through various related symptoms over time. We describe the use of simple mathematical and integrative tools to help phenotype animals across related behavioral tests (syndrome diagnosis) and experiments (meta-analysis). We applied z-normalization across complementary measures of emotionality in different behavioral tests after unpredictable chronic mild stress (UCMS) or prolonged corticosterone exposure - two approaches to induce anxious-/depressive-like states in mice. Combining z-normalized test values, lowered the variance of emotionality measurement, enhanced the reliability of behavioral phenotyping, and increased analytical opportunities. Comparing integrated emotionality scores across studies revealed a robust sexual dimorphism in the vulnerability to develop high emotionality, manifested as higher UCMS-induced emotionality z-scores, but lower corticosterone-induced scores in females compared to males. Interestingly, the distribution of individual z-scores revealed a pattern of increased baseline emotionality in female mice, reminiscent of what is observed in humans. Together, we show that the z-scoring method yields robust measures of emotionality across complementary tests for individual mice and experimental groups, hence facilitating the comparison across studies and refining the translational applicability of these models.
2.2 INTRODUCTION

Evaluation of behavioral and physiological parameters relating to emotion-like processes in animals is typically performed with several tests and without comprehensive analysis across paradigms. Mouse behavior is multimodal and full quantifiable assessment of emotionality (which covers anxiety-like and/or depressive-like behavior) is only possible when the same animal is exposed to multiple behavioral tests covering a wide range of behaviors over several days (Crawley et al. 1997; Crawley and Paylor 1997). However, closely-related behavioral parameters that are specific to each test and that relate to aspects of emotionality (for instance, entries into open field center or into open arms of the elevated plus maze) do not necessarily agree within animals and/or across time, leading to behavioral noise that is difficult to interpret. This behavioral variability can be caused by the time of day, the experimenter and recent activity in the colony, or may represent false positive/negative results in experiments with small numbers of test subjects (less than 10). More often, the cause of the variability is unknown, but it is thought to reflect natural fluctuations over the underlying mean value. Thus, as mice can be in different emotional states within short periods of time (Ramos 2008), correlation analyses of behavioral parameters obtained from different tests may result in lack of statistical power and affect principal component types of integrative analyses (Carola et al. 2002). Hence, to assess emotionality, we need simple and comprehensive tools that allow integration of behavioral parameters obtained in multiple (but complementary) behavioral tests.

It is important to note that convergent - rather than consistent - sets of symptoms are at the core of the clinical characterization of the human illness. Indeed, contrary to a putative “consistent” organ deficiency phenotype (i.e. muscle or liver function for instance), the manifestation of changes in emotionality can vary over time. This is one of the reasons why depression is diagnosed in humans by a set of variable symptoms (4-5 out of a list of 10) over time (2 weeks or more). It is not based on a single consistent behavior, but rather by a set of converging behavioral observations that together define a depressive syndrome. Here we are trying to provide a method to
operationalize this approach to rodent studies to increase the translational value of the models.

Here, we z-normalized results from rodent behavioral tests, experiments and cohorts, with the goal of assessing the emotionality dimension of mice. Z-normalization is a methodology that standardizes observations obtained at different times and from different cohorts, thus allowing their comparison and/or compilation. Its value is obtained by subtracting the average of observations in a population from an individual raw value and then dividing this difference by the population standard deviation. This type of normalization, compared to percentiles, allows data on different scales to be compared. Indeed, based on a translational application of the illness definition (i.e. a syndrome as a collection of variable symptoms), we actually may not expect systematically the same or "consistent" behavioral outputs, but we do expect converging results from emotionality measures over time. This may also be the reason why principal component analyses (PCA) have not been successful at summarizing emotionality behavioral data, as one of the assumptions under PCA is that "consistent" values should be systematically obtained (Carola et al. 2002; Milner and Crabbe 2008). Instead, the proposed z-score approach relies on testing whether a particular experimental group deviates from mean behaviors in converging directions across tests and time.

Furthermore, taking example from clinical study meta-analyses, where z-normalization is used to compile related measures performed in different studies but that assess the same illness dimension, we evaluated the possibility of comparing integrated measures of emotionality across different rodent experiments. We first validated the approach using two common methods to induce anxious-/depressive-like states in mice - UCMS and chronic corticosterone exposure - (Mineur et al. 2006; David et al. 2009) and then report its use in providing additional analytical opportunities, such as differentiating more subtle sex differences under baseline and induced high emotionality across studies.
2.3 METHODS

Animals

Male and female C57BL/6NTac mice (Taconic, Hudson, NY) were used. Mice were maintained under standard conditions (12/12 h light/dark cycle, 22 ± 1 °C, food and water ad libitum, 4-5 animals/cage), and the protocol was approved by the University of Pittsburgh Institutional Animal Care and Use Committee (protocol #0801794, Animal Assurance # A3187-01). Two different cohorts were used for each model (UCMS and corticosterone exposure) for a total of 4 cohorts. Baseline sex differences were established in 3 cohorts (Figs 2.1, 2.2, 2.4, 2.5).

Estrous cycle

Estrous state was monitored in female mice by vaginal smears in selected tests (Goldman et al. 2007). Briefly, 10 µl of saline was flushed into the vagina and then placed on a glass slide and coverslipped. Observation of stages of the estrous cycles was performed under light microscope with a 10X objective without staining. Vaginal smears were performed on the day of behavioral testing and on the day after to more accurately assess estrous stage.

Unpredictable Chronic mild stress (UCMS)

UCMS mimics the role of socio-environmental stressors in precipitating a depressive-like syndrome that shares characteristics with human depression, such as increased fearfulness/anxiety-like behavior, decreased consumption of palatable food and physiological changes (Santarelli et al. 2003; Pothion et al. 2004; Mineur et al. 2006). Importantly, the UCMS-induced syndrome is blocked and reversed by chronic antidepressant treatment (Surget et al. 2009). UCMS consisted of a 4-week regimen (or 6 weeks when fluoxetine was administered, see below) of pseudo-random unpredictable mild stressors: forced bath (~2 cm water in cage for 15 minutes), wet bedding, predator odor (1 hour exposure to fox urine), light cycle changes, social stress (rotate mice into previously occupied cage), tilted cage (45°), mild restraint (50mL
Falcon tube with air hold for 15 minutes) and bedding changes (Joeyen-Waldorf et al. 2009; Surget et al. 2009).

**Fluoxetine treatment**

Fluoxetine (Sigma, St. Louis, MO) was dissolved and administered in the drinking water (18 mg/kg/d) for 4 weeks, 15 days after the onset of UCMS, in order to reverse and block the development of the depressive-like phenotype (Santarelli et al. 2003; Surget et al. 2009).

**Corticosterone Treatment**

Corticosterone (Sigma, St. Louis, MO) was dissolved in vehicle (0.45% β-cyclodextrin) and delivered (35 µg/ml) in drinking water for 4 weeks, based on (David et al. 2009). Liquid consumption was monitored and bottles were changed every 3 days. This test models the elevated corticosteroid levels seen in some subjects with major depression (Brouwer et al. 2005; Antonijevic 2006). Chronic antidepressant treatment reverses the corticosterone-induced elevated emotionality (David et al. 2009; Gourley and Taylor 2009).

**Behavior**

Behavioral testing was performed using elevated plus-maze, open field and novelty suppressed feeding, three commonly used tests in the literature to measure components of emotionality. Tests were performed 3-5 days apart to minimize the impact of a previous test on the response for the same animals. Tests were performed in the order described below:

**Elevated Plus Maze Test (EPM)**

Behavior in the EPM was measured using a cross maze with two open and two closed arms (30 × 5 cm arms). Time spent in the open arms and ratio of entries into the open arms (entries into open arms divided by total entries into any arm x 100) during a
10 min test measured anxiety-related behaviors (Sibille et al. 2000). The total number of arm entries was used as an index of locomotor activity.

**Open Field Paradigm (OF)**

The time and distance ratio spent in the center of a 43x43 cm open chamber were recorded for 10 min to evaluate anxiety-related behaviors (center was defined as a 32x32 cm central arena). Here, we report time in the center of the open field and ratio of distance traveled in the center (distance traveled in the center divided by the total distance traveled x 100). The total distance traveled was used as an index of locomotor activity (David et al. 2009).

**The Novelty Suppressed Feeding test (NSF)**

As an index of emotionality, the latency to start eating a food pellet was monitored in food-deprived animals in a brightly illuminated chamber. Briefly, animals were food-deprived for 16 hours prior to the test. Testing was performed in a 50x50 cm box covered with bedding and illuminated by a 70-watt lamp. Mice were tested individually by placing them in the box for a period of 10 minutes. The latency to eat was timed. Immediately afterwards, the animal was transferred to its home cage and the amount of food consumed in the subsequent 5 minutes was measured, serving as a control for change in appetite as a possible confounding factor.

**Emotionality and Locomotion Z-score calculation**

Z-scores are dimensionless mathematical tools that allow for mean-normalization of results within studies and for subsequent comparison of related data across studies. Z-scores are standardized scores (by the group mean and group standard deviation) and no normal assumption is made. They indicate how many standard deviations ($\sigma$) an observation ($X$) is above or below the mean of a control group ($\mu$).

$$z = \frac{X - \mu}{\sigma}$$

$X$ represents the individual data for the observed parameter. $\mu$ and $\sigma$ represent
the mean and the standard deviation for the control group, respectively. Here as we investigated stress and sex effects, the male control group was defined as the control group (except for Figure 2.2 where effects of antidepressant in females were monitored and thus, the control group was the unstressed female group). Z-score values were calculated for test parameters measuring emotionality and locomotor activity. The directionality of scores was adjusted so that increased score values reflected increased dimensionality (emotionality or locomotion). Standard measures of anxiety-/depressive-like behaviors (Crupi et al. 2010; Post et al. 2010) were used here, but the approach can be customized to other tests, based on each lab's expertise.

For instance, decreased normalized OF center activity and increased NSF latency were converted into positive standard deviation changes compared to group means indicating increased emotionality. To avoid any weighted effect of locomotion on anxious behavior in the OF and EPM, distance ratios (center/total distance in the OF; or open arm entry ratio in the EPM) are typically used (Crupi et al. 2010; Post et al. 2010), thus integrated parameters were normalized for the locomotor component. In the NSF, the time necessary to initially approach the food pellet is orders of magnitude smaller than the time to overcome the conflict of the aversive environment, thus locomotor activity is typically not controlled for; rather appetite and food consumption are measured across groups. The selection of these specific dimensions was made based on the fact that these parameters are the most frequently used in the neuropsychopharmacology field so that readers could easily identify these components in their own studies. Furthermore, we selected for EPM and OF parameters that are associated in some principal component analyses studies (PCA) with "anxious behaviour" or "anxious locomotor activity" such as time in the open arms or time in the center (Carola et al. 2002); however other studies could not fully dissociate "unambiguously parameters fully reflecting ‘activity’ or ‘anxiety’" (Milner and Crabbe 2008). Finally, PCA analyses found that both these components had similar loads on anxious behaviour (Milner and Crabbe 2008).

As an example, z-score in the open-field (ZOF) was calculated for each animal using normalization of “time in the center” (TC) and “distance in periphery/total distance
ratio” (DR) values.

\[
Z_{OF} = \frac{\left(\frac{X - \mu}{\sigma}\right)_{TC} + \left(\frac{X - \mu}{\sigma}\right)_{DR}}{\text{Number of parameters}}
\]

Similarly, in the elevated plus maze for each animal, \(Z_{epm}\) calculation was performed using normalization of “time in the open arms” (TOA) and “Open/Closed arms entries ratio” (ER) values.

Finally, in the novelty suppressed feeding, \(Z_{nsf}\) was calculated for each animal using normalization of the latency time to eat the pellet.

Individual emotionality scores were then calculated by averaging z-score values across tests, thus leveraging potential biases induced by a single test. An emotionality z-score was calculated for each animal based on 3 different tests:

\[
\text{Emotionality Score} = \frac{Z_{OF} + Z_{EPM} + Z_{NSF}}{\text{Number of tests}}
\]

Finally group emotionality score means (and standard deviations) were obtained by averaging individual values within each group for each experiment (Figures 1-3) and by integrating similar groups across experiments (Figures 4-5).

**Statistical analysis**

Based on the experiment, the number of groups and treatments applied, Student t-tests, one-way or two-way ANOVA (sex, treatment, estrous state as co-factor), followed by post-hoc PLSD (when main effects were observed significant) and \(\chi^2\) analysis, were performed.
2.4 RESULTS

Z-score normalization confirmed elevated emotionality and identified robust sex differences in the UCMS model of depression

We employed emotionality z-scores to investigate the potential of combining results across different behavioral tests for anxiety- and depressive-like behaviors using the UCMS model, a validated paradigm to elicit anxious-/depressive-like behaviors. For this first analysis (Fig. 2.1), results from independent tests were as follows: in the OF, stress exposure did not affect time and relative distance traveled in the center of the OF (Fig 2.1A); in the EPM, UCMS-exposed animals spent significantly less time (p < 0.05) and entered proportionately less often (p < 0.05) into the open arms compared to controls (Fig 2.1A); in the NSF, there was a trend for UCMS-exposed animals to have increased latencies to eat the pellet (p = 0.09; Fig 2.1A).

Z-score normalization was then performed, first, within the respective behavioral parameters, hence transforming absolute values to numbers of standard deviations from the control means (see methods). As described in the methods, the control group used was the male non-stressed group. Male and female results are pooled in figures 2.1A-D, which underlie the slightly positive value for the "no stress" group that combine mean of z-normalized values of both sex (See further characterization in §3.4). This first step yielded, as expected, the same statistical p-values as before normalization (Fig 2.1B). We then averaged these normalized behavioral parameter z-scores to obtain a single value per mouse and per behavioral test (Fig 2.1C). Analyses of test-specific z-scores indicated a significant effect of stress exposure on EPM (p < 0.05), with UCMS-exposed animals displaying higher z-scores than controls. There was no effect of stress on OF z-score (p > 0.4), but a trend for an effect of stress on NSF z-score (p = 0.09). Finally, these values were averaged to obtain a single “Emotionality Score” for each mouse, describing the integrated output of that experiment (Fig. 2.1D). Note that all three tests are weighted similarly, as within-test parameters were averaged at the prior step (Fig. 2.1C). Here, the analysis of the combined normalized measures of emotionality resulted in augmented statistical significance of the stress main effect (p <
0.015 versus 0.03 < p < 0.09, depending on the test). We further compared males and females and show that the UCMS effect on emotionality was driven by a significant effect of stress exposure on emotionality score in females (p < 0.05), but not males (p > 0.1; Fig 2.1E). In summary, we showed that, in this particular experiment, z-scoring across complementary behavioral dimensions provided a more robust overall assessment of the effect of stress on emotionality (i.e. less sensitive to outlier values).
Figure 2.1. Integrated emotionality z-scores in mice exposed to unpredictable chronic mild stress. (A) Raw data obtained from three independent behavioral tests performed in the same animal in both males and females mice (OF, EPM and NSF; n=14-15/group/sex) (B) Normalization of data using z-score method was performed for each parameter as described in the methods using the control male group as the baseline. (C) Test z-values were then calculated by averaging individual z-scores, and (D) averaged to obtain Emotionality z-score. (E) Controls and stress groups were split by sex to investigate sex differences to stress exposure. Data represent mean ± SEM (n=14-15/group). A-E: * p<0.05, ** p<0.01, *** p<0.001 for effects of UCMS exposure compared to the no-stress group. # describe statistical trends (p<0.1).

Antidepressant reversal of elevated emotionality z-scores

As the observed effect of stress was greater in females (Fig 2.1E), we studied the reversal effects of chronic fluoxetine administration on female mice with altered behavior induced by stress (Fig 2.2). In this second cohort of UCMS-exposed animals, female mice were exposed to similar stressors and behavioral testing, and an independent group was exposed to chronic fluoxetine at the onset of the UCMS syndrome (Surget et al. 2009). Emotionality z-scores were calculated as described above. Results from individual tests were as follows: in the OF, no significant effects of UCMS or fluoxetine were observed on parameters measured (Fig 2.2A); in the EPM, UCMS-exposed females spent significantly less time (p < 0.001) and entered proportionately less often (p < 0.01) into the open arms compared to controls, while chronic fluoxetine treatment blocked the development of those effects for both parameters (p < 0.05); in the NSF, fluoxetine-treated animals displayed lower latency to start eating the food pellet compared to saline treated UCMS-exposed mice (p<0.05). These variable results are somewhat typical to behavioral studies, so to assess whether results reflected behavioral noise or fluctuations over a more stable underlying trend, we performed z-score normalization, first, within behavioral parameters (yielding the same statistical p-values as before normalization; Fig 2.2B), and, then, averaged results to
obtain a single value per mouse and per behavioral test (Fig 2.2C). Fluoxetine-treated and UCMS-exposed mice did not differ from controls on measures of emotionality in the OF and in the NSF. No significant effect was observed in the OF (Fig. 2.2C). The final z-score integration revealed a significant effect of UCMS, suggesting a stable underlying effect, although modest in this case. Control unstressed mice were compared to fluoxetine-treated stressed mice and no difference were observed for all experiments (Fig 2A-D). As expected, chronic SSRI treatment reversed the elevated stress-induced z-score measures of emotionality ($p < 0.01$, Fig 2D) (or blocked the development; see methods), bringing values back to baseline control levels. Together, this provides an additional example of using z-score normalization to extract a robust underlying trend out of more variable individual measures, and critically providing a pharmacological validation and a face validity of its application.
Figure 2.2. Chronic antidepressant treatment blocks stress-induced increase in emotionality z-scores (n=14-15/group). (A) Raw data obtained from three independent behavioral tests (OF, EPM and NSF; n=14-15/group) performed in the same animals. (B) Normalization of data using z-score method was performed for each parameter. (C) Test z-values were then calculated by averaging individual z-scores, and (D) averaged to obtain the Emotionality Score. Data represent mean ± SEM (n=14-15/group). **, p<0.01 ***, p<0.001 for effects of UCMS exposure compared to the no-stress group. $, p<0.05 $$$, p<0.01 for effects of 4-weeks fluoxetine treatment compared to the stressed group.

Elevated emotionality z-scores and increased statistical significance in the corticosterone-induced syndrome

To test the reliability of the z-normalization method across models, we then derived emotionality z-scores using behavioral results obtained in the chronic corticosterone model as an additional test case, since chronic exposure reliably increases emotionality in mice (David et al. 2009; Gourley and Taylor 2009). In light of sex differences in the UCMS model, we present data analyzed by sex (Fig 2.3). In the OF, corticosterone-exposed animals spent less time in the center than controls (main effect of corticosterone exposure, p < 0.05; Fig 2.3A). This result was driven by the fact that corticosterone-exposed males spent significantly less time in the open than control males (p < 0.01). There was also a significant effect of sex on time spent in the center (p < 0.05; Fig 2.3A), driven by a sex difference in corticosterone exposure, with treated males spending less time in the open than treated females (p < 0.05). For distance ratio in the OF, there was a main effect of treatment, with corticosterone-exposed animals having smaller distance ratios than controls (p < 0.01; Fig 2.3A). As for the time in the center of the OF, this result was driven by corticosterone-exposed males having smaller distance ratios than control males (p < 0.01). There was also a significant sex difference in distance ratio, driven by a sex difference in corticosterone exposure, with treated males having smaller ratios than treated females (p < 0.01; Fig 2.3A). In the EPM,
corticosterone-exposed animals spent significantly less time in the open arms than controls (main effect of corticosterone exposure, p < 0.05; Fig 2.3A). Corticosterone-treated animals also had a smaller open arm entry ratio than controls (overall, p < 0.01; males, p < 0.05; females, p < 0.05). In the NSF, there was a significant sex difference in latency (p < 0.01; Fig 2.3A), driven by the fact that corticosterone exposed males had longer latencies than treated females (p < 0.001). There was also a trend for an effect of treatment on latency (p = 0.09), with corticosterone exposed males having longer latencies than control males (p < 0.05).

Using these results, z-score transformation was performed, first, within behavioral parameters, yielding, as expected, exactly the same statistical p-values as before normalization (Fig 2.3B). Z-scores were then averaged to obtain a single value per behavioral test, and group differences were assessed (Fig 2.3C). There was a significant main effect of treatment on OF z-score (p < 0.01), driven by the fact that corticosterone-treated males had higher z-scores than control males (p < 0.01). There was also a significant main effect of sex on OF z-score (p < 0.05), driven by corticosterone-treated males having higher OF z-scores than treated females (p < 0.001). There was a significant main effect of treatment on EPM z-score, with corticosterone-treated animals displaying higher z-scores than controls (p < 0.01; males, p = 0.09; females, p < 0.05). There was a trend for a main effect of treatment on NSF z-score (p = 0.09), driven by corticosterone-exposed males having higher NSF z-scores than untreated males (p < 0.05). There was also a significant main effect of sex on NSF z-score (p < 0.01), driven by a sex difference in corticosterone exposure, with treated males having higher NSF z-scores than treated females (p < 0.001). Finally, these values were averaged to obtain a single “emotionality score” per mouse, then per experimental group (Fig. 2.3D). Note that all three tests are weighted similarly, as within-test parameters were averaged at the prior step (Fig. 2.3C). Here, z-scoring confirmed that the smaller and variable effect sizes in female mice reflected an overall less robust, although still significant, impact (p<0.05) of corticosterone exposure in female mice.
Combining normalized measures in group emotionality z-scores augmented the overall statistical significance of the corticosterone main effect (Table 2.1, $p < 0.0001$ versus $0.008 < p < 0.09$, depending on the test), thus emphasizing the low but measurable convergence of behavior between tests, and confirming that individual mice displayed similar directionality of effects across tests, suggesting that integrated z-scores provide a robust assessment (i.e. less sensitive to outlier values) of the effect of corticosterone on emotionality.
Figure 2.3. Integrated Emotionality z-score in corticosterone-treated male and female mice. (A) Raw data obtained from three independent behavioral tests (OF, EPM and NSF; n=14-22/group) performed in the same animals. (B) Normalization of data using the z-scoring method was performed for each parameter as described in the
methods. (C) Test z-values were obtained by averaging individual z-scores, and then combined to obtain emotionality z-scores. Data represent mean ± SEM (n=14-22/group). * p<0.05, ** p<0.01, *** p<0.001 for effects of corticosterone exposure compared to the sex-matched non-stress group. § p<0.05, §§§ p<0.001 indicate sex differences within groups.

Looking at the effect of sex, the emotionality z-score significance was lower in three out of five cases for the different parameters measured in the NSF, OF and EPM. The underlying cause of these more robust statistical parameters appears to rely on the fact that z-score normalization lowers the overall variance of the behavioral measurements (Table 2.1). This is consistent with the notion that the underlying changes in emotionality were similar in the three tests, but that test-specific variability in measures partly obscured its accurate measurement within individual tests. Hence, under these experimental conditions, emotionality z-scoring provided the best combination of low p-values, due to lower coefficient of variation of integrated values. Because the number of behavioral tests included in the analysis can also affect the overall statistical power and z-score values, we compared z-score values (Supplemental Figure B.1 or Supplemental Table B.1) and statistical results (Supplemental Table B.2) obtained by averaging data from either 2 or 3 behavioral tests. As expected the lowest variability in measures was observed when averaging z-scores from 3 tests, as demonstrated by a combination of a lower coefficient of variation and a higher statistical significance.
Combining emotionality z-scores across experiments and models provided additional analytical opportunities across independent studies: quantitative differences and sexual dimorphism in the UCMS and corticosterone models of altered mood states.

Results from the UCMS and corticosterone exposure studies suggested differences in opposite directions between males and females across the two models (Figs 2.1, 2.3). To further investigate this potential sexual dimorphism, we took advantage of the fact that, similar to clinical meta-analysis approaches, normalized z-scores can allow for comparison and pooling of results across experiments, hence increasing sample size and analytical power. Indeed, in meta-analysis, the same measure [e.g. a scaled measure of depressive state for example] is used in different studies, while here the same measure, emotionality Z-score, [e.g. an equivalent of a scaled diagnosis of animal behavioral state] was derived in different experiments and subjects and compared across studies. Here, combined experimental group sizes ranged from 22 to 51 animals per sex for each model. Integrated emotionality z-scores from two experiments using the UCMS paradigm confirmed that stress increases
emotionality in both sexes (male: p<0.01; female: p<0.001) and revealed a higher female response to UCMS (Fig. 2.4A, Female > Male, p<0.01) (Dalla et al. 2005; Joeyen-Waldorf et al. 2009). On the other hand, integrating results from two independent corticosterone experiments confirmed the robust effect in males (p<0.001), strengthened the conclusion of less robust female results (p<0.05), and revealed a significant sex difference in increased emotionality (Fig. 2.4A, Male > Female, p<0.001) while no group x sex interaction was observed (p=0.17, Fig. 2.4A). No baseline sex difference was observed (p=0.31), although more female mice displayed baseline emotionality scores greater than 0.5 (p<0.001; see next section).

In Fig 2.4B we present an alternate use of z-scoring, where locomotion z-scores were derived from related locomotor parameters across two tests (total ambulatory distance in the OF and total entries in EPM). Integrated locomotion z-scores from these same experiments using the UCMS and corticosterone exposure paradigms showed that (i) females had overall higher baseline locomotion activity compared to males (p<0.001), (ii) corticosterone induced a decrease in locomotor activity in males (p<0.001), but not in females (p=0.50), and that (iii) chronic stress induced no effect on locomotion parameters in either sex (males: p=0.06; females p=0.33). Estrous state did not correlate with altered behavior in individual tests.

Together, these results provide examples of the application of z-scoring across experiments initially performed separately. Here, for instance, integrated z-scores across behavioral tests and experiments revealed significant sex differences that were at best at trend level in individual experiments.
Figure 2.4. Emotionality and locomotion scores in two animal models of anxiety/depression. Use of z-score normalization allowed pooling of various experiments and multiple cohorts (n=22-51 mice/group), highlighting sex differences after chronic stress or corticosterone exposure. (A) Sex differences in emotionality responses to either stress or corticosterone exposure. Specifically, females (red) were significantly more sensitive to stress and less sensitive to corticosterone exposure compared to males (blue). (B) Applying similar normalization to locomotors parameters extracted from different behavioral tests (total crosses in OF and in EPM) revealed baseline sex differences, and in response to stress and corticosterone exposure. Data represent mean ± SEM (n=22-51/group). * p<0.05, ** p<0.01, *** p<0.001 for effects of corticosterone or stress exposure compared to the sex-matched control group. §§ p<0.01, §§§ p<0.001 indicate sex differences within groups.
Emotionality z-scores combined across cohorts revealed qualitative baseline sex differences

Elevated baseline emotionality was observed in female mice in some behavioral tests, but did not reach significance for individual experiments. Notably, highlighting consistent sex differences in mouse behavior can be difficult, as it requires a large group of animals, control for estrous state in females, and the direction of change can vary across different tests (Palanza 2001; Voikar et al. 2001). Here, we speculated that integrating results across these tests may reveal baseline differences, either in mean group differences or in the distribution of z-scores within groups. We thus integrated emotionality z-scores over three experiments and focused on control animals (n=42 males, 34 females; Fig. 2.5). Results revealed higher baseline emotionality in females (male, \( z = 0.00 \); female \( z = 0.574 \); \( p<0.001 \)). We next assessed the distributions of emotionality scores ("low", scores below -0.5; “normal”, scores between -0.5 and +0.5; “high”, scores greater than +0.5). This alternate use of z-scores revealed a highly significant shift to higher emotionality in females (\( \chi^2=16.8, \text{df}=2, \ p<0.001 \)), indicative of high baseline emotionality in 71% of female mice, but only in 24% of males. Notably, this difference did not correspond with estrous state in individual female mice, and in fact, represent integrated measures over a period of several days, hence encompassing most estrous states within individual mice.
**Figure 2.5. Dissecting sex differences in baseline emotionality.** Combining emotionality z-scores in control animals across several experiments shows that the distribution of baseline emotionality scores is significantly skewed towards higher values in females, as more females (red) show higher emotionality states compared to males (blue). Emotionality scores were separated in “low” (scores below -0.5), “normal”, (scores between -0.5 and +0.5) and “high” (scores greater than +0.5) (n=34-42 mice/sex extracted from 3 different cohorts). Relative proportions of animals in each group are indicated within bars. $\chi^2$ analysis on group distributions revealed sex differences (§§§§ $p<0.001$).
2.5 DISCUSSION

Principles of z-scoring methods adapted for behavioral measurements

To address inherent difficulties in behavioral phenotyping of mice over time and to obtain summarized results over tests and studies, we propose a method based on z-normalization principles for the quantification of behaviors in an integrative manner along coherent dimensions, such as shown here for emotionality. Indeed, it is often difficult to reconcile positive or intermediate findings across tests, especially for behavioral measures that are subject to known variability. We show that applying a z-normalization method across complementary behavioral measures related to aspects of emotionality can facilitate the "diagnosis" of an animal state. Emotionality in animal models is classically reflected by altered behavior monitored in different paradigms that can be restored after antidepressants (as performed here), by variations in physiological parameters (HPA axis, locomotor activity), and potentially through identification of brain region-specific genomic biomarkers of altered behavior (Krishnan et al. 2007; Sibille et al. 2009). Interestingly, since human mood is defined as an emotional state over time that is remote from proximal stimuli, we speculate that rodent emotionality z-scores may in fact represent the closest homolog of human mood. Indeed, they integrate behavioral states observed in various and multiple paradigms over several days of testing, including across various neuroendocrine states (i.e. sex hormones), hence capturing a more stable and enduring state of emotionality in mice. Similarly, the combined analysis of converging behavior can be assimilated to the clinical characterization of the human illness, which is diagnosed by a set of variable symptoms over time. So it is not based on a single consistent behavior, but rather by a set of converging behavioral observations that together define a depressive syndrome. A recent study aimed at the same goal by combining different behavioral tests into a single apparatus ("triple test"; composed of OF, EPM and Light/Dark test physically linked together) to phenotype animal's behavior using a similar comprehensive strategy based on multiple testing (Fraser et al. 2010). The future value of such a test will need to be assessed in multiple
studies. Notably, it is still based on a one-time assessment of animal's behavior, in contrast to our proposed analytical method for behavioral assessment over time.

Furthermore, emotionality z-scores – by allowing pooling of cohorts – can strengthen the reliability of effects and increase analytical opportunities. Specifically, we showed that emotionality z-scores reduced test-to-test variability for measures of dependent variables that are sensitive to multiple known (and unknown) environmental factors (time of day, animal facility-related events, experimenter, estrous phase, etc).

The rationale for using z-normalization, instead of, for instance, calculating percentage of control response for each parameter and averaging them across groups and cohorts, is that the standard deviations of z-normalization values are similar across parameters and tests. Thus, averaging z-values avoids weighted effects of one parameter or one test over another. Z-score methodology also differs from multivariate statistics, such as principal component analysis, which are performed to investigate whether behavioral measures assess a single and intangible entity. However, as discussed in the introduction, “emotionality” is by definition an underlying state that is vulnerable to timely fluctuations due to variable environmental and biological stimuli, and that may manifest as different behaviors, or “symptoms” over time. So we actually do not seek, and may not even expect, high correlation across tests, but rather we expect convergence of results obtained with integrated z-scores. Instead, we expect that a true underlying emotionality state will be revealed through similarities in effect size and directions over cohorts and tests over time. Similarly, other types of multivariate analysis, like MANOVA, assume linear relationships among dependent variables and covariates; therefore, when the relationship deviates from linearity – which might happen due to fluctuation in animal's behavior - the power of the analysis will be compromised. Similarly, multivariate analyses rely on similar assumptions of correlation rather than convergence, and therefore may not work as well. Finally, z-normalization within and across different behavioral tests results in a single score per mouse which may be seen as a quantitative “diagnosis” of their emotionality, a translational – and of course limited - equivalent to the way human depression is
quantified by structured interviews, such as the Global Assessment of Functioning scale or the Hamilton Depression Rating Scale.

Defining new tools for behavioral analysis in neuropsychopharmacology necessitates assessing their validity. As emotionality is an integrative behavioral entity that is composed of different parameters, such as anxiety, depression, and fear of novel environment, that are measured over time, our methodology has a strong face validity as it combines these multiple aspects. Predictive validity of the z-score method has been tested here by looking at antidepressant reversal of stress induced-emotionality.

Proof of concept: Application of z-scoring methods to two different models of altered mood disorders and to behavioral sex differences

Here we applied behavioral z-scoring methods to the quantification of emotionality in two rodent models that are frequently used to induce higher anxiety- and depressive-like behavior in mice. UCMS is based on chronic psychosocial stress, while chronic corticosterone exposure relies on neuroendocrine dysfunction. In our studies, main effects of either UCMS or corticosterone exposure were observed in most, but not all, of the single tests performed within individual cohorts (Fig 2.1A and 2.3A). Z-score normalization appeared to increase the robustness of the analyses by decreasing the variability of integrated measures (Fig 2.1D and 2.3D; Table 2.1). Combining emotionality z-scores across experiments revealed significant sex differences in response to stress or corticosterone exposures (Fig. 2.4), hence demonstrating the value of the approach at detecting effects that were either not significant or at trend levels in experiments performed separately. Notably, the goal of these integrated analyses is not to “increase statistical significance”, but rather to extract underlying trends out of apparently variable results. For instance, we showed that, compared to males, females were more sensitive to chronic stress, but less sensitive to chronic corticosterone administration. Greater female behavioral and physiological stress sensitivity has previously been reported (Dalla et al. 2005; Joeyen-Waldorf et al. 2009), associated with higher corticosterone levels after various stressors (Handa et al. 1994). Although corticosterone administration can induce high emotionality in males (Gourley
et al. 2008; Murray et al. 2008; Zhao et al. 2008; David et al. 2009) and females (Ardayfio and Kim 2006), sex differences had not yet been directly studied. Using emotionality z-scores, we were able to combine individual experiments and showed that females were overall less sensitive than males to corticosterone exposure, thus consolidating a large literature on sex-related differences in response to glucocorticoids and in HPA-axis dysregulation in rodents (Galea et al. 1997; Liu et al. 2006) and humans (Young and Ribeiro 2006; Bremmer et al. 2007; Young et al. 2007; Binder et al. 2009). Of course, since both the rodent and human literature are mostly male-biased, an alternative interpretation is that males are more sensitive to corticosterone exposure and less to the effects of chronic stress. In summary, these results support our hypothesis that z-scoring normalization of related behavior can reveal consistent and stable changes in underlying emotionality in mice, despite apparent and often unexplained variability. Using this approach augmented the translational validity of the models by suggesting similar directions for sex differences that are observed in human subjects.

Application of behavioral z-scores

By definition, z-scores normalize results across tests, experiments and cohorts, as they take into consideration differences from mean group values in terms of numbers of standard deviations from the control mean (See methods). The approach is not new by itself, as it is commonly applied in clinical and epidemiological studies. An important feature of its application to behavioral data is to ensure conformity with the direction of effects. For instance, increased emotionality in mice is revealed by decreased values of dependent variables in some tests (OF and EPM) and by increased values in other tests (NSF), and thus all measures indicative of increased emotionality should be reflected by positive numbers of standard deviations from the control group mean. While our z-score calculation here was based on data extracted from three behavioral tests commonly used in neuropsychopharmacology, it could be extended to other behavioral tasks that measure other parameters related to emotionality, such as number of fecal boluses in a new environment, elevated O maze, marble burying, light/dark transition, etc. Here we
use the term of emotionality to cover both anxiety-like and depressive-like behaviors, as they are difficult to fully dissociate in rodents, but the approach can be expanded to include more specific tests. However, while multiple testing in the same animal is necessary to robustly assess emotionality, experimenters should verify that response to one behavioral test was not altered by prior testing. Notably, the integrated approach does not detract from the analysis of distinct components of individual tests, which may reveal nuances in behavioral responses and changes. The potential application of behavioral z-scoring is quite extensive, from dissociating emotionality-related behavior in stressed/control animals, knockout or transgenics/wild-type (using combined group scores), to identify consistent outliers or segregate resilient from responder animals to environmental exposure or pharmacological treatment (e.g. through score histograms), or to measure antidepressant-predictive behaviors or antidepressant reversal of induced behavioral syndromes.

Behavioral z-scores can also be applied to other behavioral dimensions (memory tests, addiction tests, etc.). Here we briefly showed a similar approach applied to locomotion. Indeed, while emotionality z-scores already include locomotor-controlled parameters extracted from each test, normalization of locomotion-specific parameters can further evaluate overall locomotor activity under baseline conditions, between males and females and after experimental manipulations for instance (i.e. UCMS or corticosterone exposure).

Some of the critical aspects and potential limitations that need to be further characterized include, amongst others: (i) reliable behavioral protocols across experiments (Wahlsten et al. 2006), (ii) combining results across strains (Milner and Crabbe 2008; Yalcin et al. 2008), especially in the context of sex differences (Voikar et al. 2001), and (iii) careful consideration of behavioral dimensions to be integrated.

Conclusion

In summary, we suggest that using an easy-to-apply and "generalizable" z-score methodology can increase the reliability and comprehensiveness of behavioral testing from a variety of non-exclusive tasks, but along cohesive behavioral dimensions, for
complex behaviors such as emotionality of animals. Here, the application of this method to quantify emotionality in mice demonstrated that mice display subtle baseline emotionality sex differences that are similar to those observed in humans (Brebner 2003), support the use of chronic mild stress as a comprehensive model to induce an anxiety-like/depressive-like syndrome, and points to corticosterone exposure as a model for male neuroendocrine vulnerability to mood disorders.

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3.0 PAPER 2: RESILIENT EMOTIONALITY AND MOLECULAR COMPENSATION IN MICE LACKING THE OLIGODENDROCYTE-SPECIFIC GENE CNP1

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Running Title: The role of CNP1 in mood regulation
3.1 ABSTRACT

Altered oligodendrocyte structure and function is implicated in major psychiatric illnesses, including low cell number and reduced oligodendrocyte-specific gene expression in major depressive disorder (MDD). These features are also observed in the unpredictable chronic mild stress (UCMS) rodent model of the illness, suggesting that they are consequential to environmental precipitants; however, whether oligodendrocyte changes contribute causally to low emotionality is unknown. Focusing on 2’-3’-cyclic nucleotide 3’-phosphodiesterase (CNP1), a crucial component of axoglial communication deregulated in the amygdala of MDD subjects and UCMS-exposed mice, we show that altered oligodendrocyte integrity can play an unexpected functional role in affect regulation. Mice lacking CNP1 (knockout, KO) displayed a decreased anxiety- and depressive-like phenotype (i.e. low emotionality) at baseline, which developed progressively between 3 and 9 months of age. This phenotype was accompanied by increased motor activity, but was evident prior to neurodegenerative-associated motor coordination deficits (≤9-12 months). Notably, CNP1\(^{\text{KO}}\) mice were less vulnerable to developing a depressive-like syndrome after either UCMS or chronic corticosterone exposure. CNP1\(^{\text{KO}}\) mice also displayed reduced fear expression during extinction despite normal amygdala c-Fos induction after acute stress, together implicating dysfunction of an amygdala-related neural network, and consistent with proposed mechanisms for stress resiliency. However, the CNP1\(^{\text{KO}}\) behavioral phenotype was also accompanied by massive upregulation of oligodendrocyte- and immune-related genes in the basolateral amygdala, suggesting an attempt at functional compensation. Together, we demonstrate that the lack of oligodendrocyte-specific CNP1 leads to resilient emotionality. Yet, combined with substantial molecular changes and late-onset neurodegeneration, these results suggest the low CNP1 seen in MDD may cause unsustainable and maladaptive molecular compensations contributing to the disease pathophysiology.

Keywords: oligodendrocyte, CNP, depression, UCMS, emotionality, myelin, stress
3.2 INTRODUCTION

Disruptions in neuronal signaling have long been the focus of research on major depressive disorder (MDD). More recently, however, glial disruptions have been postulated to contribute to the pathophysiology of MDD (Fields 2008; Hercher et al. 2009; Fields 2010), and the roles of specialized glial subtypes are now being investigated. While alterations in microglia (Muller and Schwarz 2007; Steiner et al. 2008; Miller et al. 2009) and astrocyte-related components (Choudary et al. 2005; Rajkowska and Miguel-Hidalgo 2007; Valentine and Sanacora 2009; Banasr et al. 2010) are observed in MDD, oligodendrocyte alterations in MDD are becoming a primary focus of research (Fields 2008). Oligodendrocytes, the main myelin-forming cells of the central nervous system (CNS), provide both structural and trophic support for neurons, and facilitate axonal conduction. In the amygdala and prefrontal cortex of MDD subjects, previously-reported decreases in glial cell number were attributed to reduced oligodendrocyte number (Hamidi et al. 2004; Uranova et al. 2004), consistent with patterns of downregulation of oligodendrocyte-related transcripts in amygdala (Sibille et al. 2009) and nearby temporal cortex (Aston et al. 2005). Changes in NG2 cells, a cell type sharing a common lineage with oligodendrocytes (Belachew et al. 2001; Mallon et al. 2002), have also been associated with depression-related characteristics in rodents. For instance, both chronic corticosterone exposure and chronic stress in rodents decreased cortical and limbic oligodendrocyte (Banasr et al. 2007) and NG2 cell proliferation (Alonso 2000; Wennstrom et al. 2006). Rats given electroconvulsive seizure therapy, an antidepressant treatment for otherwise non-responsive MDD subjects, showed increased proliferation of NG2 cells in the amygdala and hippocampus (Wennstrom et al. 2003; Wennstrom et al. 2004; Wennstrom et al. 2006). Thus, evidence suggests that oligodendrocytes, a crucial element for maintaining optimal neuronal function, may be vulnerable to stress-related insults and may contribute to the pathophysiology of MDD.

We previously identified a set of dysregulated gene transcripts in the amygdala of male subjects with familial MDD, including robust downregulations of multiple
oligodendrocyte-related genes (Sibille et al. 2009). This pattern was also observed in mice following unpredictable chronic mild stress (UCMS) (Surget et al. 2008) and appeared restricted to the amygdala (not anterior cingulate cortex or dentate gyrus). 2'-3' cyclic nucleotide 3'-phosphodiesterase (CNP1) was one of several oligodendrocyte-specific genes significantly downregulated across species. Additionally, CNP1 was previously implicated in MDD (Aston et al. 2005; Sequeira et al. 2009) and schizophrenia (Hakak et al. 2001; Peirce et al. 2006; Che et al. 2009), supporting the hypothesis that altered CNP1 function may participate in the pathophysiology of psychiatric disorders. While CNP1 is expressed at low levels in the periphery, it is expressed at higher levels in NG2 cells and it comprises ~4% of myelin-associated proteins in mature oligodendrocytes (Braun et al. 2004). CNP1 is localized to non-compact myelin in the inner mesaxon and paranodal loops (Trapp et al. 1988), the principle sites of contact with the axon. CNP1 binds microtubules and regulates mRNA expression and transport at the paranode (Gravel et al. 2009), although its exact role in axoglial communication is not known. The lack of CNP1 protein in mice results in disorganization of nodal sodium channels and paranodal adhesion proteins (e.g. Caspr and Nav) (Lappe-Siefke et al. 2003; Edgar et al. 2009). Adult CNP1KO mice do not initially show an overt behavioral phenotype, but develop progressive axonal degeneration and motor deficits after 6 months of age, leading to premature death (Lappe-Siefke et al. 2003; Edgar et al. 2009).

Healthy oligodendrocytes are necessary to maintain optimal axon function (Nave 2010), however it remains to be determined whether oligodendrocyte-specific alterations are causal to MDD, or are simply compensatory or neutral side effects in psychiatric disorders. Here, we investigated the effect of a lack of CNP1 on emotionality (i.e. anxiety and depressive-like behaviors) in mice, under baseline conditions (trait) and after chronic stress or corticosterone exposure, two validated paradigms for inducing high emotionality states. As the amygdala is a central region in affect regulation and since our initial findings showed low CNP1 in the amygdala of MDD patients (Siegle et al. 2002), we also assessed amygdala function in CNP1KO mice using fear conditioning, c-Fos induction and gene expression profiling. The experiments show that lack of CNP1
results in low emotionality under baseline and induced states, along with reduced corticolimbic fear expression, a suggested mechanism for stress resilience.

3.3 MATERIALS AND METHODS

Animals. Adult male and female CNP1\textsuperscript{KO} and wild-type (WT) littermate mice (C57BL/6 background) (Lappe-Siefke et al. 2003) were obtained from heterozygous crossings and microchipped for identification. 5 cohorts were used: Baseline Cohort (N=11-17/group; 6 and 9 months baseline), UCMS Cohort (N=8-12/group, 6 months of age), CORT Cohort (N=10-14/group; 3-month baseline and CORT exposure), Fear conditioning (FC) Cohort (N=16-19/group, 3 and 6 months of age, FC) and cFos Cohort (N=6/group, 6 months of age). Mice were maintained under standard conditions (group housed, 12/12-hour light/dark cycle, 22±1°C, food and water \textit{ad libitum}). All testing was conducted in compliance with the NIH laboratory animal care guidelines and with protocols approved by the Institutional Animal Care and Use Committee.

Behavioral measures. Major depression is defined as a syndrome (i.e. collection of symptoms) including low mood or anhedonia, accompanied by cognitive (e.g. attention, concentration) and physiological symptoms (e.g. weight, locomotor and sleep pattern changes), and frequently co-morbid with significant anxiety symptoms. Thus, its emotionality component is best characterized in mice by a comprehensive panel of behavioral tests for anxiety-like and depressive-like emotionality, and for antidepressant-like behavior. Hence, baseline, UCMS, and CORT cohorts were tested in the elevated plus maze (EPM), open field (OF), novelty suppressed feeding (NSF), forced swim test (FST), and rotarod (RR), as described (Joeyen-Waldorf et al. 2009) and in the following order: EPM, OF, NSF, FST, RR, separated by a minimum of 1-2 days. Detailed methods are in the Supplemental Material.
**Behavioral z-scoring.** To address behavioral variability and obtain comprehensive and integrated measures in each group, emotionality- and locomotion-related data were normalized using a Z-score methodology previously described (Guilloux et al. 2011). Briefly, for each behavioral measure, Z-scores for individual animals were calculated using the formula below, which indicates how many standard deviations (σ) an observation (X) is above or below the mean of a control group (μ).

\[
z = \frac{X - \mu}{\sigma}
\]

Z-scores for behavioral measures were first averaged within test, and then across test to insure equal weighting of the four tests comprising the final Z-score (Figure 3.1B-C). Separate Z-scores were calculated for the Baseline, UCMS, and CORT cohorts using the means and standard deviations of the respective control groups (i.e. “WT” for Baseline or “WT control” for UCMS and CORT). Locomotion Z-scores were similarly obtained from EPM (total crosses) and OF (total distance traveled) data.

**Estrous phase.** Female mice in the UCMS and 3-month baseline cohorts were assessed for estrous phase on the day of behavioral testing after the EPM, OF or NSF test to control for potential effects of hormonal fluctuations on behavior. Estrus, metestrus, diestrus, and proestrus were determined via vaginal cytology (Goldman et al. 2007) as previously described (Guilloux et al. 2011).

**Unpredictable chronic mild stress (UCMS).** Single housed mice were subjected to four weeks of a randomized schedule of 1-2 mild stressors per day, seven days per week. Detailed methods are in the supplements and full table of stressors in Supplementary Table C.1.

**Chronic corticosterone treatment (CORT).** As described previously (Guilloux et al. 2011), mice were given corticosterone (35µg/ml; Sigma-C2505) dissolved in 20%
cyclodextrin (Sigma H107) as their only water supply for four weeks. Due to reduced cohort size, behavior of all mice was tested both prior to (3-month baseline) and following CORT exposure.

**Fear conditioning.** The protocol was performed over three days using a computer-controlled system (Coulbourn Instruments, Allentown, PA) (Figure 3.3A). On day 1, mice were trained to associate a conditioned stimulus ([CS]; 80db, 2KHz, 15s tone) to an unconditioned stimulus ([US]; 0.5mA, 0.5s footshock) in context A (shock floor, silver/aluminum walls, 70% ethanol cleanser). On day 2, mice received 30 trials of the CS only in context B (non-shock grid floor, black walls, Windex cleanser). On day 3, mice were placed back in context B (extinction recall) followed by context A (fear renewal) 1hr later; and freezing was recorded during 5 CS exposures in each context. On each day, a 2-minute acclimation period preceded and followed testing. Trials were presented with a variable inter-trial interval (25-35s) and percent freezing was measured during the 15s CS.

**Immunohistochemistry.** Stress-induced c-Fos immunoreactivity was performed as described (Sibille et al. 1997). Brains were collected by perfusion 120 minutes following a 15-minute restraint stress. 40um sections (6/mouse) were incubated with c-Fos antibody (polyclonal rabbit anti-c-Fos; 1:5000 dilution; Calbiochem, San Diego, CA), followed by secondary biotinylated goat-anti-rabbit IgG (1:500 dilution; Vector Laboratories, Burlingame, CA), and visualized with diaminobenzidine solution (DAB Peroxidase Substrate Kit; Vector Laboratories, Burlingame, CA). Total numbers of c-Fos positive cells were counted in the basolateral amygdala (BLA).

**Gene array.** Brains from the UCMS cohort (n=11-13 mice per group) were selected based on emotionality Z-scores that were closest to the means of their group. Following dissection, brains were immediately flash frozen on dry ice. As the other hemisphere was allotted for other experiments, the left BLA was dissected directly on the cryostat using a 0.5mm micropunch, tissue samples were frozen at -80°C until extraction, and
total RNA was extracted using an RNeasy Mini Kit (Qiagen, Germantown, MD). Concentration and purity were tested using a Bioanalyzer and Nanodrop Spectrophotometer (260/280≥1.8). One half of each RNA sample (~150ng) was processed on Illumina Mouse WG-6v2 Expression BeadChips at the Keck Microarray facility (Yale University) and expression levels were determined using the Illumina BeadArray Reader. The other half was used for independent verification by qPCR. Two internal controls verified the validity of the arrays (see results): 1) CNP1 is downregulated/absent in CNP1KO and 2) Cnp1 is downregulated in WT mice following UCMS (Surget et al. 2008).

Real time quantitative polymerase chain reaction (qPCR). Total RNA was converted into cDNA using the qScript cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD). As previously described, qPCR reactions were assessed by SYBR green fluorescence signal (Invitrogen, Carlsbad, CA) using the Opticon Monitor DNA Engine (Bio-Rad, Berkeley, CA) (Sibille et al. 2009). Briefly, samples were run in quadruplicate and ∆Ct values were determined by comparison to the geometric mean of three reference genes (housekeeping genes: actin, GAPDH, cyclophilin). Signal intensities (SI=100*2^-∆Ct) were used for comparison to microarray expression values.

Statistical Analysis. There were no significant effects of sex or estrous phase in all examined cohorts, so male and female groups were combined, where appropriate. Genotype differences in Baseline and CORT cohorts were assessed using repeated measures ANOVA for age and treatment, respectively. Genotype comparisons with the 3-month baseline group were made using one-way ANOVA. UCMS Z-scores were assessed using a two-way ANOVA for genotype and treatment. Weekly measures in UCMS (fur rating, body weight, and corticosterone levels) were assessed using two-way repeated measures ANOVA for week with genotype and treatment as co-factors. Genotype differences in the FC cohort were assessed using repeated measures ANCOVA with age as a covariate for days 1 (conditioning) and 2 (extinction) and one-way ANCOVA for day 3 (recall and renewal). Genotype differences in the c-Fos cohort
were determined using two-way ANOVA for genotype and stress. Gene expression changes across genotype were assessed in four subgroups: WT-Control (WC), WT-UCMS (WU), KO-Control (KC) and KO-UCMS (KU), using ANOVA followed by two-group student t-tests for gene selection. LSD and Tukey's tests were also used for post-hoc analysis (Supplementary Table 2). Selection criteria were set at p<0.01 and effect size greater than 30%. Genotype differences in qPCR were assessed with one-way ANOVA.

3.4 RESULTS

CNP1KO mice display low baseline/trait emotionality and high locomotor activity

Consistent with prior reports, CNP1KO mice showed normal motor coordination in the rotarod test at 3 and 6 months of age and deficits at 9 months of age (Figure 3.1A). Mice were grossly impaired at 12 months of age and could not perform the rotarod test (data not shown). Therefore, we tested the impact of reduced CNP1 on emotionality in male and female mice at 3, 6 and 9 months of age (i.e. prior to and during mild neurodegenerative-related stages), using four behavioral paradigms (EPM, OF, NSF and FST). To extract stable patterns underlying behavioral variability, emotionality-related measures were normalized to the 3-month WT group, and averaged per mouse across tests to obtain integrated “emotionality Z-scores” (Guilloux et al. 2011) (See methods, and example of 6-month time-point in Figure 3.1B). CNP1KO were comparable to WT mice at 3 months of age and displayed progressively decreasing emotionality z-scores at 6 and 9 months of age (Figure 3.1C and Supplementary Figures 3.1-3.3). It is unlikely that changes were due to repeated testing (at 6 and 9 months) since a pattern of decreased emotionality in WT mice would be expected, although CNP1KO-specific memory-related events cannot be ruled out (See fear-conditioning tests). While EPM and OF emotionality measures were controlled for locomotor activity, FST results could reflect differences in activity. Removing the FST from the emotionality Z-score
decreased power, but the same progressive pattern of low emotionality was observed in CNP1KO mice (6mo p=0.07; 9mo p<0.0004; Supplementary Figure C.4).

Integrated Z-score measures of locomotor activity revealed a concomitant progressive increase from 3 to 9 months of age in CNP1KO mice (Figure 3.1D and Supplementary Figures C.1-C.3). CNP1KO mice also displayed lower body weight at 6 and 9 months compared to WT, but had normal levels of stress hormones (Supplementary Figures C.5A-B). Together, these results are supportive of a time-dependent decrease in emotionality in CNP1KO mice, which is paralleled by a progressive elevated locomotor phenotype.
Figure 3.1. **CNP1KO baseline behavior at 3, 6, and 9 months of age.** A) Latency to fall on the rotarod. CNP1KO mice show significant motor coordination deficits at 9 months. B) Example of individual behavioral measures combined in the emotionality Z-scores (6-month time-point). CNP1KO mice showed significant changes in EPM and FST, and non-significant changes in NSF and OF. In NSF, no differences in weight loss or post-test food consumption were noted (data not shown). EPM, OF, NSF, and FST measures were normalized using WT means and standard deviations, and averaged per group (see Methods and (Guilloux et al. 2011)). Breakdown of z-scores for other age groups are presented in supplementary figures C.1-C.3. C) High emotionality Z-scores indicate elevated anxiety-related and depressive-like behaviors. D) Similarly-derived locomotion Z-scores indicate elevated locomotor activity. Z-scores are normalized to the 3 month WT group for both emotionality and locomotion. Red asterisks represent within-genotype age comparisons. Black asterisks represent across genotype comparisons. Data represent mean ± SEM (n=9-18/group). *p<0.05, **p<0.01, ***p<0.001, # represent statistical trends (p<0.1).

**CNP1KO mice are resistant to developing high emotionality states in two distinct rodent models of depression**

To test whether low baseline emotionality also conferred reduced vulnerability to develop high emotionality states, we exposed two independent cohorts of CNP1KO mice to environmental (UCMS) or neuroendocrine (CORT) stressors for a period of four weeks. In both experiments, WT groups responded with robust and characteristic increases in emotionality (Figures 3.2A-B). CNP1KO mice responded with increased emotionality after UCMS compared to non-stressed CNP1KO mice (Figure 3.2A; Supplementary Figure C.6), but displayed a trend toward lower emotionality following CORT exposure (Figure 3.2B; Supplementary Figure C.7). Notably, following both paradigms, CNP1KO mice remained at emotionality levels that were lower than WT non-stressed groups (Figure 3.2A-B). These differences remained when FST was excluded.
from Z-score analyses (Supplementary Figure C.8A-B) and were confirmed in an independent cohort (Supplementary Figure C.9). Note that the UCMS cohort was 6-months of age at testing, so the low emotionality and high locomotion of CNP1\(^{\text{KO}}\) mice match prior results (Figure 3.1), hence independently confirming the baseline phenotype.

UCMS increased locomotion in WT (trend level) but not in CNP1\(^{\text{KO}}\) mice (Figure 3.2C; Supplementary Figure C.6). CORT exposure reduced locomotion in WT and increased locomotion in CNP1\(^{\text{KO}}\) mice (Figure 3.2D; Supplementary Figure C.7). No notable genotype differences were seen in weight gain, fur rating, or corticosterone levels in either paradigm (Supplementary Figures C.10A-C and C.11A-C). In summary, CNP1\(^{\text{KO}}\) mice displayed reduced vulnerability to develop high emotionality behaviors after chronic environmental and neuroendocrine challenges, as emotionality z-scores never reached WT levels. Notably, dissociations between changes in emotionality and locomotor activity were observed between genotype groups (Figure 3.2E-F).
Figure 3.2. Effects of two rodent models of depression in CNP1KO mice. Emotionality Z-scores in the unpredictable chronic mild stress (UCMS) (A) and chronic corticosterone treatment (CORT) (B) cohorts. Locomotion Z-scores in the UCMS (C) and CORT (D) cohorts. Z-scores were normalized to WT control groups within each experimental cohort. Differences in CNP1KO control groups between cohorts reflect age differences (UCMS, 6 months; CORT, 3 months). Schematic diagrams of emotionality and locomotion interactions in WT and CNP1KO mice across age (E) and after UCMS or CORT exposure (F). WT mice showed a dissociation in emotionality/locomotion effects between the two depression models, while changes in CNP1KO remained along the same axis. Red asterisks represent within genotype age comparisons. Black asterisks represent across genotype comparisons. Data represent mean ± SEM (n=10-14/group). *p<0.05, **p<0.01, ***p<0.001, # represent statistical trends (p<0.1).

Normal fear conditioning and cellular reactivity to stress, but reduced fear expression, suggest low encoding of emotional salience in CNP1KO mice

Our motivating study indicated reduced CNP1 levels in the amygdala of MDD subjects and UCMS-exposed mice. To test whether the observed behavioral phenotype included disrupted amygdala function, we assessed CNP1KO mice in the fear conditioning paradigm (Figure 3.3A), a test relying on amygdala processing. Similar to WT, CNP1KO mice learned to associate the conditioned stimulus (CS) with the unconditioned stimulus (US) on day 1 (Figure 3.3B-left panel). On day 2, both groups showed intact extinction learning, but CNP1KO mice displayed lower CS-induced freezing from the onset, resulting in significantly lower freezing throughout the extinction paradigm (Figure 3.3B-right panel), an indication of low fear expression (Sierra-Mercado et al. 2011). No significant differences in pain sensitivity were observed in the hot plate test (Supplementary Figure C.12). These results were confirmed in a separate cohort (n=8-12/genotype; p<0.01 for extinction; Supplementary Figure C.13). This robust genotype difference was consistently observed during the following extinction recall and
fear renewal, and was not due to lack of consolidation of fear memory (Supplementary Figure C.14).

To assess whether the observed differences could reflect baseline changes in stress-induced activation of the amygdala, we measured the expression of the immediate early gene c-Fos in the BLA of a separate cohort of animals following a 15-minute restraint stress. WT and CNP1<sup>KO</sup> mice displayed similar baseline (although at trend level for low reactivity in CNP1<sup>KO</sup> mice) and stress-induced number of c-Fos positive cells (Figure 3.3C-D), indicating intact BLA response to acute stress in CNP1<sup>KO</sup> mice.
A.

Day 1: Training

Context A

Day 2: Extinction

Context B

Day 3: Extinction
Recall and Fear Renewal

Context B

CS: 80db, 2 kHz, 15 sec Tone
US: 0.5 mA, 0.5 sec footshock

B.

% Freezing

Baseline CS1 CS2 CS3 CS4 CS5 First 15 Last 15

Fear Conditioning Context A

Extinction Context B

WT KO

C.

Non Stressed

WT KO

Stressed

D.

Total cFos+ Cells

WT KO

Non-Stress Stress

*** ***
**Figure 3.3. Fear conditioning and c-Fos analysis.** A) Fear conditioning protocol. Percent freezing during (B) fear conditioning and extinction. C-D) Analysis of stress-induced cFos expression in BLA of WT and Cnp1KO mice. C) c-Fos expression in the amygdala under 10x magnification, Bar=0.1mm. D) Total c-Fos positive cell counts. While there was a trend (p<0.1) toward a genotype difference in the non-stressed groups, no significant differences were found between WT and CNP1KO. In all panels, red asterisks represent within-genotype comparisons. Black asterisks represent across-genotype comparisons. Data represent mean±SEM (n=16-19/group), (n=9-18/group). *p<0.05, **p<0.01, ***p<0.001, # represent statistical trends (p<0.1).

**Upregulated oligodendrocyte- and immune-related gene transcripts in the BLA of CNP1KO mice**

To investigate putative underlying amygdala-related mechanisms, large-scale gene expression was assessed by microarray in the BLA of WT and CNP1KO mice from the UCMS cohort (Figure 3.4A). Internal verification of the array data confirmed that CNP1 levels were undetectable in CNP1KO mice (Figure 3.4B) and downregulated in UCMS-exposed WT (WU) mice for two out of three probes (Figure 3.4C). (Surget et al. 2008; Sibille et al. 2009) UCMS-exposed CNP1KO (KU) mice showed a similar pattern of transcript changes as UCMS-exposed WT (WU) mice (R=0.72; Supplementary Figure C.15), indicating that the broad biological response to stress is intact. However, due to the phenotypic differences, these UCMS-related genes are unlikely to be related to the behavioral phenotype in CNP1KO mice. Instead, based on consistent low emotionality in control and UCMS-exposed CNP1KO mice, we focused on genes displaying similar changes under control and UCMS conditions, as potential “mediators” of the behavioral phenotype. 114 “CNP1KO-associated probesets” were identified by the following criteria: 1) significant difference in CNP1KO under control (KC vs WC) and UCMS-exposed (KU vs WU) conditions (p<0.01; effect size>30%), 2) consistent directionality of effect in both groups, and 3) no change in WT after UCMS (p>0.1).
Figure 3.4. BLA gene array analysis and qPCR validation. A) BLA tissue micropunch. B) Absence of expression of the three CNP1 probes in CNP1\textsuperscript{KO} mice. Expression levels were at the threshold of detection (\(~100-120\) units; gray shading). C) Downregulation of two out of three CNP1 probes in WT mice exposed to UCMS. D) RT-qPCR confirms significant upregulation of 8 out of 9 genes. Data represent mean±SEM (Microarray \(n=11-13\)/group; qPCR \(n=6\)/group). *\(p<0.05\), **\(p<0.01\), ***\(p<0.001\), # represent statistical trends (\(p<0.1\)).
Based on prior identification of relative glial/neuronal enrichment of transcript origin (Sibille et al. 2008), 97% of the 114 identified transcripts were enriched in glial or mixed glial/neuronal origin. Specifically, a systematic upregulation of oligodendrocyte-related transcripts was observed (24% of CNP1\(^{\text{KO}}\)-associated genes), including genes involved in structure, function and production of the myelin sheath (Table 3.1). In parallel, we observed a significant upregulation of immune-related transcripts (32% of KO-associated genes) including genes associated with the immune complement system and major histocompatibility complex (Table 3.1; see also Supplementary Table C.2). Ingenuity pathway analysis of CNP1\(^{\text{KO}}\)-associated genes identified a gene network that closely links oligodendrocyte with immune genes, suggesting that the upregulation of immune-related genes may be synchronized with the dysregulation of oligodendrocyte-related genes in CNP1\(^{\text{KO}}\) mice. Only two neuronal-enriched genes were identified (Cortistatin and Serpina3n) suggesting minimal structural and/or functional adjustment in neurons.

Table 3.1. Selection of significantly affected neuronal, oligodendrocyte and immune-related genes between CNP1\(^{\text{KO}}\) vs WT control mice. Number of redundant probes are shown following the gene symbol. Red=neuronal-enriched genes; Blue=oligodendrocyte-enriched genes; Underline=Immune-related genes. Alr=average log ratio (CNP1\(^{\text{KO}}\)/WT). The listed p-values represent the main genotype effect from the ANOVA analysis (See Supplementary Table C.2).
<table>
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<td>7.82E-17</td>
</tr>
<tr>
<td>Cd68</td>
<td>CD68 antigen</td>
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<td>7.72E-16</td>
</tr>
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<td>kelch-like 6 (Drosophila)</td>
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<td>1.49E-15</td>
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<tr>
<td>Fcer1g</td>
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<td>adenosine synthase synthase like 1</td>
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<tr>
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<tr>
<td>C1qc</td>
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<tr>
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<tr>
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<td>2.77E-12</td>
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<tr>
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<td>3.69E-12</td>
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<td>N-myc downstream regulated gene 1</td>
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<td>lysozyme 2</td>
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<td>Lgals3</td>
<td>lectin galactose binding soluble 3</td>
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<td>5.27E-10</td>
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<td>CD74 antigen (major histocompatibility complex class II antigen-associated) transcript variant 2</td>
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<tr>
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3.5 DISCUSSION

Focusing on an oligodendrocyte gene (CNP1) that is critical for neuronal support and that is dysregulated in MDD, we tested the potential mechanistic link between altered oligodendrocyte function and emotionality in mice. We show that removal of CNP1 in mice leads to unexpected reduced baseline emotionality, reduced fear expression (during extinction) and lower vulnerability to develop high emotionality states using two rodent paradigms to induce depressive-like states. This behavioral profile suggests the presence of a dysfucntional amygdala-related network that is consistent with proposed mechanisms for stress resiliency. Gene array analysis in the BLA of CNP1\(^{\text{KO}}\) mice revealed a robust upregulation of oligodendrocyte- and immune-related transcripts, potentially representing functional compensations.

CNP1\(^{\text{KO}}\) mice exhibit a low baseline emotionality phenotype (trait) that appears progressively over time, but that is observed prior to the onset of motor coordination deficits (9-12 months), indicating that it is not due to the late-onset widespread axonal degeneration observed in CNP1\(^{\text{KO}}\) mice (Lappe-Siefke et al. 2003). Rather, more discrete molecular changes in myelin structure have been observed at earlier ages, which may contribute to the observed phenotype. For instance, the direct functional consequence of CNP1 ablation is not known, but disorganization of critical proteins (e.g. Caspr, Nav) at the paranode region at 3 months and degeneration in some small diameter axons as early as post-natal day 15 were reported in CNP1\(^{\text{KO}}\) mice (Rasband et al. 2005; Edgar et al. 2009). So deregulated paranode function may translate into suboptimal support for neuronal axons, hence priming the system for deregulated physiological responses to stress/fear.

CNP1\(^{\text{KO}}\) mice also display a concomitant increase in locomotor activity. Tests of emotionality control for activity, but locomotor and emotionality phenotypes are often difficult to dissociate in rodent models. In fact, quantitative trait loci mapping studies have identified regions on chromosomes 2, 7, and 8 that encode for both anxiety-related behavior and locomotion in rats (Conti et al. 2004), and the well-characterized high emotionality and low locomotion phenotypes of mice (Holmes et al. 2003) or rats
lacking the serotonin transporter associate with common genetic loci (Homberg et al. 2010). So, instead of a confounding factor, altered locomotor activity may represent an epistatic outcome of genetic disruptions of emotionality regulators, which are nevertheless dissociable under certain genetic and/or stress-induced conditions (Figure 3.2E-F).

CNP1KO mice have normal amygdala cellular reactivity (c-Fos measures) and fear acquisition, but display low fear expression during extinction learning (Figure 3.3), which was not likely due to poor consolidation of the fear memory (Supplementary Figure C.14). Instead, we speculate that reduced fear expression in CNP1KO mice may result from low encoding of emotionality salient stimuli (e.g. the original fear association). Accordingly, CNP1KO mice respond to chronic behavioral (UCMS) or physiological (CORT) stress (Figure 3.2A), but their induced emotionality states remained significantly below the levels associated with depressive-like states in WT mice (Figure 3.2A-B). Together, this behavioral pattern is consistent with stress resilient phenotype. Indeed, in humans, resilience is associated with the ability to adapt to chronic stress (Feder et al. 2009) and to perceive stressful events in a less threatening way (Kobasa 1979; Tugade and Fredrickson 2004; Southwick et al. 2005), both characteristics observed here in CNP1KO mice. Fear expression is dependent on BLA function (Herry et al. 2006; Sotres-Bayon et al. 2007), but is also modulated by regions of the prefrontal cortex (Jovanovic and Ressler 2010; Sierra-Mercado et al. 2011). Similarly, in humans, the ventromedial prefrontal cortex (vmPFC) modulates amygdala function during fear extinction (Delgado et al. 2008), and potent vmPFC inhibition of the amygdala is postulated to occur in resilient individuals (Liberzon and Sripada 2008; Feder et al. 2009). Together, this suggests the presence of a dysfunctional amygdala-related neural network in CNP1KO mice, consistent with proposed mechanisms for stress resilience. As disruption in related networks including prefrontal and/or hippocampal regions cannot be excluded, site-specific and time-dependent alterations could further refine the role of CNP1.

Microarray evaluation of transcript levels within the BLA of CNP1KO mice indicated a robust pattern of upregulated oligodendrocyte-related transcripts. This
suggests an attempted compensation for the lack of structure/function due to missing CNP1, and potentially reflecting prior reports of enlargement of the myelin inner tongue in small diameter axons and of whirls of excess redundant myelin sheaths seen early in CNP1KO mice (Edgar et al. 2009). Microarray assessment also revealed a pattern of upregulated immune-related transcripts (MHC class II, complement system, inflammatory mediators: Table 3.1), consistent with reports of reactive gliosis and microglial activation in CNP1KO mice (Lappe-Siefke et al. 2003). Interestingly, components of the complement system can be either deleterious or beneficial (Shen and Meri 2003). Recent evidence of upregulated immune-related genes (e.g. c1qc, cd74, serpina3, tyrobp) was associated with protected cognition in old subjects (Katsel et al. 2009), suggesting neuroprotective potential. Here, upregulated immune-related genes in CNP1KO mice show ~40% overlap with the gene set implicated in that study, suggesting potential mixed deleterious/neuroprotective effects of immune-related gene changes in CNP1KO mice. Surprisingly, only two neuronal-associated transcripts were found changed (upregulated) in CNP1KO mice, and both are associated with immune function. Cortistatin, a neuropeptide, has anti-inflammatory and neuroprotective properties in mice (Gonzalez-Rey et al. 2006) and humans (Carrasco et al. 2008; van Hagen et al. 2008), while Serpina3, a serine peptidase inhibitor, is upregulated in response to inflammation in the rodent brain (Tsuda et al. 1996; Takamiya et al. 2002) suggesting a protective role for these genes in CNP1KO mice.

Whether increased immune- and oligodendrocyte-related transcripts may be an attempt of the system to ”repair” damage and/or protect against further damage due to the lack of CNP1-related function, is not known. However, Ingenuity-based functional analyses suggest that the two processes are inter-related, a finding consistent with reports in most mental illnesses (Ryan et al. 2006; Rao et al. 2010; Shelton et al. 2010) and highlighted by the comorbidity of emotion regulation disorders (i.e. anxiety, depression) in patients with multiple sclerosis, an inflammatory demyelinating CNS disease (Hogancamp et al. 1997; Chwastiak and Ehde 2007). Consequently, CNP1 could be a critical component of the mechanistic link between oligodendrocytes and
immune function underlying psychiatric and other CNS disorders (Nave 2010; Konradi et al. 2011).

While the molecular phenotype of the CNP1\(^{KO}\) appears confined to non-neuronal compartments, one of the primary functions of myelinating oligodendrocytes is to support electrical signal conduction along the axon (Poliak and Peles 2003). The paranode region, where CNP1 is localized, is critical for maintaining axonal integrity (Garcia-Fresco et al. 2006; Rosenbluth 2009). CNP1 and other gene products in this region are thought to maintain optimal functioning of axonal mitochondria and it is suggested that perturbations at the paranode could disrupt this fragile metabolic coupling (Nave 2010). Electrophysiological changes in oligodendrocytes can modulate axonal conduction velocity (Yamazaki et al. 2007) and the firing of action potentials in NG2 cells is dependent on axonal synapses (Karadottir et al. 2008), highlighting the functional connectivity between neurons and oligodendrocytes (Fields 2008). In addition, structural changes in oligodendrocytes specifically at the paranode region, are suggested to be a potential mechanism for subtle alterations in axonal conduction and associated loss of signal integrity (Edgar and Nave 2009; Yamazaki et al. 2010). Accordingly, improper signal conduction in amygdala-related circuitry in CNP1\(^{KO}\) mice (due to paranode disruption) could account for the low encoding of emotionally-salient information, and related phenotypic abnormalities.

In summary, initial studies reported low CNP1 in MDD and in response to chronic stress (Surget et al. 2008; Sibille et al. 2009) (Figure 3.4). We now demonstrate that disruption of oligodendrocyte function (via CNP1 ablation) can impact circuits mediating emotionality in mice, leading to a resilient emotionality phenotype. Thus, although these studies indicate that CNP1 disruption is not likely a causal factor in MDD, however, the current results suggest that low CNP1 and/or disruption of a critical axoglial junction may contribute to clinical symptoms of mood disorders in an unexpected and potentially maladaptive way (due to massive molecular changes), and should therefore be further examined in relation to psychiatric illnesses and potential treatments.
Acknowledgements

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MDD is a devastating disorder with severe personal and societal consequences, but the fundamental neurobiology underlying MDD is complex and not well understood. It is crucial to gain insight into these mechanisms in order to stimulate the development of new interventions and therapies. Oligodendrocyte disturbances are consistently found in both MDD subjects and animals exposed to chronic stressors, however, it is unclear if these disturbances play a functional role in the precipitation of the disorder. The studies in this dissertation investigated the consequences of oligodendrocyte disruption (ablation of CNP1) on behavioral and molecular aspects of emotionality in mice and emphasize new approaches for examining emotionality in mice. We demonstrate that CNP1KO mice display resilient emotionality, altered corticolimbic function, and molecular disruptions in the BLA. Below, I discuss the broad implications of these findings and propose a role for CNP1 in maintaining the integrity of axoglial interactions and ensuing downstream processes.

4.1 METHODOLOGICAL CONSIDERATIONS IN INVESTIGATING EMOTIONALITY

The complex symptomatology and neurobiology of MDD make it difficult to use animals to model the precise nuances of the disorder (Cryan and Holmes 2005). Thus, there is a need for new approaches to investigate depressive-like symptoms in rodents. In this portion of the discussion, I will describe how we have initiated use of Z-score normalization to both counteract variability and gain a more comprehensive view of
depressive-like symptoms in rodents. In addition, I describe the application of the fear conditioning paradigm as a way to assess corticolimbic function related to emotionality in mice. Finally, I discuss how application of these approaches revealed a pattern of resilient emotionality in CNP1KO mice.

4.1.1 Z-score Normalization and Assessment of Emotionality

While the use of animals is crucial for investigating neurobiological facets of MDD, a wide range of difficulties exist when attempting to model aspects of the disorder (see section 1.2.1). In particular, the current strategy in the field is to use multiple behavioral tests to assess convergent “endophenotypes” of depressive-like behavior (Cryan and Mombereau 2004). However, in rodents, behavioral tests often suffer from both internal and external variability sometimes making it difficult to assign conclusions. In Chapter 2, we extend the notion of a behavioral test battery to model a single aspect of MDD: emotionality in mice. We show that Z-score normalization and multiple test integration allows for a more comprehensive assessment of a depressive-like phenotype in mice across several dimensions. First, Z-scoring stabilized variability between tests due to external factors (e.g. time of day, experimenter, etc.). Second, combining multiple common features of tests by application of the Z-score methodology revealed trends in the data that may not have otherwise been revealed (see below). Third, Z-scoring can be used to pool multiple data sets, thereby increasing analytical power and potential for different analyses. Finally, the diagnosis of human MDD has a temporal component, where the symptomatology must be present for a minimum of 2 weeks. Z-score normalization can take this temporal component into account by combining test results from multiple days allowing an assessment of the emotionality “state” of the animal over time.

Specific applications of Z-score normalization in Chapters 2 and 3 revealed trends in emotionality that may have otherwise gone undetected. In Chapter 2, combining several experimental cohorts of WT mice using Z-score methodology revealed intriguing sex differences in baseline emotionality and vulnerability to develop
a depressive-like syndrome. In Chapter 3, application of Z-score normalization revealed robust and unexpected patterns of altered emotionality in CNP1\textsuperscript{KO} mice. CNP1\textsuperscript{KO} mice had decreased emotionality at baseline and decreased vulnerability to a depressive-like syndrome in two rodent models (UCMS and CORT) compared with WT mice. While the amount and variety of data from these experiments could promote over-interpretation of single parameters, Z-score analysis instead allowed us to gain a broad assessment of the emotionality phenotype in CNP1\textsuperscript{KO} mice. The aforementioned applications of Z-score methodology display the power of this technique to reveal both underlying trends and global patterns of emotionality in mice.

Using the Z-score to combine emotionality parameters or “endophenotypes” into one comprehensive score may be the closest researchers can get to assessing an actual “syndrome” in mice. Indeed, the future of behavioral phenotyping may be in determining behavioral patterns in animal models which could parallel subtypes of complex human disorders or even patterns of pharmacological responsiveness. For instance, a pattern of high emotionality in EPM and OF, but not NSF, may be indicative of an anxiety-like phenotype, whereas high emotionality in NSF and FST might be indicative of a depressive-like phenotype. Going one step further, Z-scoring could be used to assess other parameters, such as cognition, locomotion (also shown here), or even physiological measures. Potentially, an algorithm could be developed where multiple Z-scores, each containing a different combination of test parameters, are assessed (and perhaps weighted) to determine the disorder most closely related with the observed “pattern”. In conclusion, the Z-score method has the potential to enhance the translational validity of animal testing in MDD and potentially other complex disorders.

4.1.2 Fear Conditioning and Assessment of Corticolimbic Function

PTSD and MDD share a strong comorbidity (Blanchard et al. 1998) and there is considerable overlap between pathways implicated in both disorders, suggesting common mechanistic underpinnings. Indeed, corticolimbic function has been shown to
be dysregulated in both disorders. Yet, while the fear conditioning paradigm has been used to assess corticolimbic dysfunction in PTSD (Jovanovic and Ressler 2010), only a single study has investigated fear conditioning in MDD subjects. This study found MDD patients display enhanced fear learning, however, fear extinction or extinction recall was not investigated in these subjects (Nissen et al. 2010). In animals, mice exposed to the UCMS model of depression have been shown to have abnormal extinction learning (Baran et al. 2009) and one report found impaired extinction recall in SERT knockout mice (Wellman et al. 2007), which display classic depressive-like symptoms. In addition, results in Chapter 3 show decreased depressive-like symptoms concurrent with low fear expression during extinction (i.e. less freezing during entire extinction period), together suggesting a connection between depressive-like symptoms and extinction mechanisms in rodents (see also 4.1.3). Accordingly, the fear conditioning paradigm may be useful in assessing an “endophenotype” of MDD-related corticolimbic dysfunction, a parameter that could also be utilized in the Z-score method.

What type of fear conditioning pattern might we expect to see in an MDD patient? Neurobiologically, MDD patients display heightened amygdala reactivity and decreased corticolimbic association (Surguladze et al. 2005; Matthews et al. 2008). During the fear conditioning paradigm, the amygdala plays a central role in fear learning, extinction, and recall; and prefrontal regions conventionally are thought to inhibit amygdala output (i.e. fear) (Sierra-Mercado et al. 2011). Thus, in MDD subjects exposed to a fear conditioning paradigm we could expect to see one or more of the following: 1) increased fear learning [shown in (Nissen et al. 2010)], 2) heightened fear expression during extinction, 3) decreased/impaired fear extinction, 4) increased fear renewal, and/or 5) decreased extinction recall. While these predicted characteristics may share some overlap with those seen in PTSD (Jovanovic and Ressler 2010), it would be worthwhile to tease apart disorder-specific patterns, as subtle differences between PTSD and MDD subjects have been observed in fear inhibition during a startle paradigm (Jovanovic et al. 2010).

Another challenge in using animal research to investigate MDD is that many symptoms of depression (e.g. sadness, guilt, rumination, negative thinking) are difficult
to accurately recapitulate in animals (Nestler and Hyman 2010). There is evidence that the fear conditioning paradigm has the potential to portray some of these “human-associated” traits in mice (Table 4.1). First, individuals with MDD tend to assess interactions and events at a more negative value and anticipate more negative future events (Lavender and Watkins 2004), an action akin to encoding a stimulus at a high emotional valence, potentially reflected by enhanced fear learning, increased fear expression, or impaired fear extinction. Second, MDD subjects frequently exhibit stress vulnerability (or lack of resilience), a characteristic that appears to be reflected during extinction portions of the paradigm (see section 4.1.3). Finally, rumination, an unintentional and recurrent thought process, either self-directed or directed at a perceived object of despair (Nolen-Hoeksema 1991), has been shown to involve regions of the prefrontal cortex (Hamilton et al. 2011). A rodent equivalent of rumination could be reflected by an inability to extinguish fear, ineffective extinction recall, or enhanced fear renewal. Consequently, these apparently abstract features of MDD may simply be manifestations of a more tangible corticolimbic dysregulation. While it could be argued that the circuitry affected in MDD may involve uniquely human pathways (Crawley 2000), the corticolimbic pathway described in rodent fear conditioning is most certainly phylogenetically conserved (Quirk et al. 2010) making it an excellent paradigm for examining the conserved or “primitive” emotional pathway.

Table 4.1. Comparison of human MDD symptoms to measurable mouse behaviors and corresponding tests. The ten listed human MDD symptoms correspond to the diagnostic and statistical manual of mental disorders (DSM-IV TR) criteria for MDD, with more measurable characteristics listed below each symptoms category. Tests highlighted in red are components of a fear conditioning paradigm which can be used to test corticolimbic function. OF=open field, EPM=elevated plus maze, NSF=novelty suppressed feeding.
<table>
<thead>
<tr>
<th><strong>HUMAN</strong></th>
<th><strong>MOUSE</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Symptom</strong></td>
<td><strong>Behavior</strong></td>
</tr>
<tr>
<td>Low or depressed mood / comorbid anxiety (Kessler 1996)</td>
<td>Anxiety-/Depressive-like behavior fearfulness</td>
</tr>
<tr>
<td>• Increased acute stress reactivity (i.e. Lack of stress resilience)</td>
<td>Increased stress reactivity</td>
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<tr>
<td>• Increased chronic stress vulnerability</td>
<td>Increased fear / emotionality</td>
</tr>
<tr>
<td>• Abnormal risk/fear assessment</td>
<td>Assignment of high emotional valence to fear or risk</td>
</tr>
<tr>
<td>Sadness / hopelessness</td>
<td>No correlate</td>
</tr>
<tr>
<td>Anhedonia</td>
<td>Reduced consumption of palatable food</td>
</tr>
<tr>
<td>Feelings of worthlessness/guilt</td>
<td>Decreased fear extinction or extinction recall</td>
</tr>
<tr>
<td>• Ruminaton</td>
<td>Social avoidance or attack</td>
</tr>
<tr>
<td>Social withdrawal</td>
<td>Abnormal sleep patterns</td>
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<tr>
<td>Sleep disturbances</td>
<td>Weight loss change in food consumption</td>
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<tr>
<td>Appetite and weight changes</td>
<td>Activity</td>
</tr>
<tr>
<td>Fatigue / low energy</td>
<td>Locomotor activity following acute or chronic stress</td>
</tr>
<tr>
<td>Psychomotor agitation</td>
<td>Poor working memory</td>
</tr>
<tr>
<td>Difficulty concentrating</td>
<td>Poor coat condition</td>
</tr>
<tr>
<td>Poor hygiene</td>
<td>Impulsivity</td>
</tr>
<tr>
<td>Suicidality</td>
<td>No correlate</td>
</tr>
<tr>
<td>• Suicidal attempts/completion</td>
<td></td>
</tr>
<tr>
<td>• Suicide thoughts</td>
<td></td>
</tr>
</tbody>
</table>
4.1.3 Convergent Methodological Approaches Reveal Resilient Emotionality in CNP1KO Mice

Downregulation of CNP1 has been implicated in MDD (Aston et al. 2005; Sequeira et al. 2009) and a recent study in our lab found that CNP1 was specifically downregulated in the amygdala of human MDD patients and mice exposed to the UCMS model of depression (Surget et al. 2008; Sibille et al. 2009). Utilization of the Z-score normalization method and fear conditioning paradigm in CNP1KO mice revealed a surprising pattern. The results presented in Chapter 3 show that CNP1KO mice have decreased vulnerability to develop a depressive-like syndrome following the UCMS and CORT rodent models of depression (Figure 4.1). In addition, CNP1KO mice displayed low fear expression during the extinction learning phase of our fear conditioning paradigm, suggestive of low encoding of emotionality salient stimuli. In humans, emotional resilience is defined as the ability to successfully adapt to chronic stress (Charney 2004; Feder et al. 2009) and the ability to perceive stressful events in a less threatening way (Southwick et al. 2005), both characteristics displayed by the CNP1KO mouse. In addition, Feder (2009) suggests that resilient individuals have optimal functioning of fear extinction mechanisms (Feder et al. 2009) and Charney (2004) states that resilience is "an ability to quickly attenuate learned fear through a powerful extinction process" (Charney 2004). Together, this evidence indicates that the pattern exhibited by CNP1KO mice is characteristic of stress resilience.

Reports indicate that resilience is not only the absence of vulnerability to stress but, is itself, an active neurobiological process (Krishnan et al. 2007). This is supported by investigations showing that stress vulnerable and stress resilient mice actually have quite different transcriptional profiles, rather than opposite patterns (Bergstrom et al. 2007). There is evidence that resilience is mediated by the same corticolimbic regions involved in MDD and fear (Charney 2004). Similar to our findings in Chapter 3, mice lacking the intracellular signaling molecule ERK (extracellular signal-regulated kinase) displayed both enhanced fear extinction and reduced depressive–like behaviors (Tronson et al. 2008). In accord with these findings, a polymorphism (G196A) in the
BDNF (brain-derived neurotrophic factor) gene has been associated with both stress resilience and altered fear extinction in mice (Krishnan et al. 2007; Soliman et al. 2010). These examples demonstrate that, similar to depressive symptoms (section 4.1.2), stress resilience is closely associated with fear extinction mechanisms, a notion that should be more thoroughly explored in the context of MDD.

### 4.2 INFLUENCE OF EXAMINED RISK FACTORS ON BEHAVIORAL OUTPUTS

Here, I describe the effects of putative MDD risk factors (age, sex, and stress) on behavioral outputs in both WT and CNP1^KO^ mice. Notably, a distinct dissociation was found between WT and CNP1^KO^ mice, with WT mice responding with increased emotionality to all risk factors while CNP1^KO^ mice responded variably to individual risk factors, suggesting the CNP1 gene interacts with various risk factors differentially (Figure 4.1). In the following sections, I first discuss the effects of age and sex on emotionality in WT and CNP1^KO^ mice followed by observed locomotor effects.

#### 4.2.1 Effects of Risk Factors on Emotionality Outputs

##### 4.2.1.1 Age

While older individuals often have reduced rates of depression due to greater psychological resilience (i.e. wisdom), late-onset depression is a subclass of MDD that develops in older individuals. Similar to this sub-class, previous studies in our lab showed increased emotionality in older C57BL/6 WT mice compared to younger animals (Joeyen-Waldorf et al. 2009). In Chapter 2, we also show that older WT mice exhibit a slight increase in emotionality suggesting: 1) age needs to be carefully controlled when designing studies for emotionality in mice and 2) certain strains of mice,
such as C57BL/6, could be used to examine subclasses of MDD, such as late-onset depression.

In contrast to WT animals, CNP1 KO mice show a pronounced and progressive decrease in emotionality with age (Chapter 3) (Figure 4.1). Similar to previous studies (Lappe-Siefke et al. 2003), we found motor coordination deficits occur in these mice around 9 months of age, and are likely due to large scale axonal degeneration (Lappe-Siefke et al. 2003). However, we saw decreased emotionality as early as 6 months of age. Previous studies have shown that CNP1 KO mice have disorganization of paranodal proteins (e.g. Caspr) at 3 months of age (Rasband et al. 2005) and degenerative changes in small diameter axons as early as 15 days (Edgar et al. 2009). Consequently, emotionality circuits may be sensitive to these more discrete alterations in myelin structure (e.g. at the paranode), rather than overt axonal degeneration.

In healthy rhesus monkeys, age-related increases in dysfunctional CNP1 cause disruption of lipid raft signaling systems (Hinman et al. 2008), implying that dysfunction of CNP1 (whether ablation or overexpression of the aberrant protein) may be related to age-associated or progressive disorders. Similar to this situation, both the PLP KO and the PLP overexpressor mouse exhibit progressive axonal degeneration (Griffiths et al. 1998; Griffiths et al. 1998), providing support that overexpression of CNP1 in vivo may result in a similar progressive neurodegeneration as seen in the knockout (see also 4.3.2.3).

Although CNP1 KO mice display emotional resilience rather than susceptibility, concurrent dysregulation of both of these traits is reminiscent of some forms of dementia that are preceded by MDD (Alexopoulos et al. 1993; Cannon-Spoor et al. 2005). In fact three recent longitudinal studies indicate that depression and number of depressive episodes are risk factors for development of cognitive disorders, particularly Alzheimer’s disease (Dotson et al. 2010; Saczynski et al. 2010; Wilson et al. 2010). This evidence implies that MDD may share a common neuropathology with Alzheimer’s disease. Accordingly, a recent review implicates the MDD-induced dysfunction of the stress pathway as a precipitating factor in the onset of Alzheimer’s disease (Aznar and Knudsen 2011). Our results show that the CNP1 KO has pre-motor affective
dysregulation and significant changes in several Alzheimer's-related genes (Serpina3n, Cort, c1qB; see Table 3.1), suggesting these genes, along with CNP1, may share roles in both affect and neurodegenerative pathways. Therefore, it may be relevant to examine cognitive function in CNP1\(^{\text{KO}}\) mice.

### 4.2.1.2 Sex

Female subjects are twice as likely to develop MDD than male counterparts and fluctuations in hormones correlate with depressive episodes (Kornstein et al. 2000). While few studies have purposely examined sexual dimorphism in animal studies, there is evidence that female rodents are more susceptible to UCMS than males (Dalla et al. 2005; Joeyen-Waldorf et al. 2009). In Chapter 2, we combine data from WT animals across several experimental cohorts to reveal sexual dimorphism in two models of depression. Similar to previous studies, we found female mice had higher emotionality at baseline and were more susceptible to UCMS. However, males were more susceptible to chronic CORT treatment (Figure 4.1). These results reveal interesting sex differences between stress and neuroendocrine responses, and add to an increasing literature of stress-related sex differences in both rodents (Galea et al. 1997; Liu et al. 2006) and human MDD patients (Young and Ribeiro 2006; Binder et al. 2009). These models could therefore provide future avenues for investigating MDD-related sex differences.

In Chapter 3, we examined sex differences in several cohorts of CNP1\(^{\text{KO}}\) mice, and consistently observed no main effect of sex in all examined tests. On the surface, this would suggest CNP1 does not interact with sex hormones to alter vulnerability. An alternative interpretation could be that CNP1 does interact with sex because the typical (WT) sex difference is not seen in the CNP1\(^{\text{KO}}\) mouse, indicating that absence of CNP1 somehow counteracts female vulnerability to UCMS.

Some subtypes of MDD are associated with hormonal fluctuations (Becker et al. 2007; Grigoriadis and Robinson 2007). An ongoing study in our lab revealed that behavioral sexual dimorphism following UCMS is primarily due to organizational effects.
of hormones (permanent effects of developmental hormones) rather than activational effects (transient effects of gonadal hormones) (M. Seney, personal communication). In Chapters 2 and 3, we found no differential effects of estrous phase on depressive-like behaviors in female WT or CNP1\(^{K0}\) mice exposed to UCMS or CORT, further supporting that there are not robust activational effects of hormones on depressive-like susceptibility in mice. Thus, the UCMS and CORT models appear to be associated with organizational rather than activational effects of hormones, suggesting they would not be robust models for subtypes of MDD associated with circulating sex hormones (e.g. postpartum depression). However, these effects may be obscured by a dysregulation of the estrous cycle in females exposed to UCMS (Dalla et al. 2005), and a more directed analysis may reveal subtle influences of circulating hormones.
Figure 4.1. Summary diagram representing relative effects of individual risk factors on disease susceptibility in WT and CNP1\textsuperscript{KO} mice. In humans, both clinical and neurobiological factors determine the relative risk of an individual. Here, we tested putative risk factors in WT and CNP1\textsuperscript{KO} mice. WT mice respond with increased emotionality to all risk factors. CNP1\textsuperscript{KO} mice respond with decreased emotionality to age and CORT, increased emotionality to UCMS, and neutrally to female sex. Hypothetically, a certain level of emotionality may push an individual across the “disease threshold”, defined here as the level of emotionality significantly different from the WT no risk group (red dashed line). Critically, CNP1\textsuperscript{KO} mice never reached the “disease threshold” and remained well below all WT groups, highlighting their resilience to a depressive-like syndrome.

4.2.2 Effects of Risk Factors on Locomotor Outputs

Psychomotor changes often occur in MDD and changes in locomotion can be a confounding factor when examining emotionality in mice (Conti et al. 2004; Strekalova et al. 2005; Leventhal et al. 2008). While we weren’t specifically examining locomotor changes in our studies, we wanted to both carefully control for locomotion, and explore whether locomotor changes parallel emotionality changes in these studies. In Chapter 2, we found WT females tend to have increased locomotor activity at baseline. Following CORT exposure, females again had increased locomotor activity while males had decreased locomotor activity, indicating sexually dimorphic factors may play a role in locomotor activity. In Chapter 3, we show CNP1\textsuperscript{KO} mice have increased locomotor activity with age. UCMS exposure unexpectedly decreased locomotion and CORT exposure increased locomotion in CNP1\textsuperscript{KO} mice, the opposite of the pattern seen in WT mice. Overall, it appears that female sex positively regulates locomotion, while absence of CNP1 completely deregulates locomotor behaviors, highlighting the need to consider locomotion parameters in study design.

Quantitative trait locus (QTL) mapping studies have pinpointed certain chromosomal regions that encode both anxiety and locomotor behavior, suggesting an
epistatic interaction between these traits (Conti et al. 2004). In addition, genes associated with hyperactivity in mice (e.g. in KO mice) are expressed at much higher levels in “mood-associated” regions such as the amygdala, cerebral cortex and hippocampus, when compared with non-locomotion-associated genes (Mignogna and Viggiano 2010), providing further support for a connection between emotionality and locomotion. Classic examples of this association in mutant mice include the serotonin transporter KO mouse (Holmes et al. 2003) and the 5HT1AR KO (Heisler et al. 1998; Parks et al. 1998; Ramboz et al. 1998), which both show increased anxiety and decreased locomotion. In addition, stress paradigms commonly elicit hyperactivity in WT animals, along with heightened emotionality (Strekalova et al. 2005). In Chapter 3, we show that WT animals (males and females grouped together) exposed to UCMS display increased emotionality and increased locomotion as expected, but CORT elicits increased emotionality and an unexpected decrease in locomotion. Thus, the CORT and UCMS paradigms elicit different locomotor changes, again suggesting these models may work through different mechanisms. In summary, our data provide further supporting evidence that there are connections between locomotion and emotionality, although, it appears that these phenotypes are dissociable under stress conditions (i.e. CORT).

4.3 INFLUENCE OF CNP1 ON THE NEUROBIOLOGY OF MOOD REGULATION

In this portion of the discussion, I discuss how the ablation of CNP1 has influenced the neurobiology of mood regulation, on a molecular, cellular, and circuit level, expanding on topics touched on briefly in the discussion of Chapter 3. I also discuss predictions regarding how this ablation might impact neurobiological components not directly tested here. Lastly, I present a potential model incorporating potential neurobiological consequences of CNP1 alteration or ablation.
4.3.1 Molecular Effects of CNP1 Ablation

While the exact function of CNP1 remains unknown, it appears to play a role in diverse processes including axoglial communication, outgrowth of the myelin sheath, RNA binding, and microtubule and actin binding (section 1.4). In Chapter 3, CNP1 ablation led to a robust upregulation of other oligodendrocyte-related transcripts in the BLA, indicating an attempted compensation for the absence of structural and functional roles of CNP1. Alternatively, this upregulation could be a non-specific attempt for the cell to regain functional and structural homeostasis. While a seemingly minor difference, we could examine the molecular profile of CNP1 overexpressor (CNP1\textsuperscript{OE}) mice to determine if we see an opposite transcriptional profile (CNP1-specific changes) or a similar pattern of upregulation (non-specific homeostasis-related changes).

Patients with MDD have been shown to have increases in immune-mediators, and injections of lipopolysaccharide (LPS; a cytokine inducer) can induce depressive-like symptoms in humans and mice (Reichenberg et al. 2001; Dantzer et al. 2008). In contrast to these reports, we saw an unexpected upregulation of immune-related transcripts in parallel with decreased emotionality and resilience in CNP1\textsuperscript{KO} mice (Chapter 3). Interestingly, rats that were resilient to a chronic mild stress (CMS) paradigm displayed an upregulation of immune response genes in the hippocampus relative to control animals, and this response reflected neuroprotective immune regulator genes (Bergstrom et al. 2007). In addition, a report revealed that many of the same immune-associated genes found in our study were involved in protected cognition (Katsel et al. 2009), together suggesting this subset of immune regulator genes may be involved in resilience and/or neuroprotective mechanisms.

Adding to the notion that CNP1 ablation may induce neuroprotective inflammatory responses, the only two neuronal-associated transcripts found to be significantly upregulated in the CNP1\textsuperscript{KO} were cortistatin, a neuroprotective anti-inflammatory neuropeptide, and SPI-3n, a serine peptidase inhibitor. SPI-3 has been shown to be upregulated in the brain after LPS treatment (Tsuda et al. 1996; Takamiya et al. 2002), and is transcriptionally regulated by interleukin-6 and glucocorticoids.
Similarly, cortistatin has been shown to downregulate inflammatory mediators in mice (Gonzalez-Rey et al. 2006). It also plays a role in mediating immune function in humans (van Hagen et al. 2008) and low cortistatin expression is associated with increases in GFAP and apoptosis (Carrasco et al. 2008). Additionally, recent findings in our lab have revealed downregulation of cortistatin in the amygdala and anterior cingulate cortex of MDD subjects (E. Sibille, personal communication), supporting the notion of cortistatin as a positive mediator of resilience. Overall, these results indicate that the observed increases in certain immune- and oligodendrocyte-related genes may be an attempt of the system to “repair” damage and/or protect against future damage due to the removal of CNP1.

Our results in Chapter 3 also suggest a connection between altered oligodendrocyte components and immune-related components in CNP1KO mice. Prior reports indicate oligodendrocytes interact with microglial cells, the main immune response cells in the CNS. For instance, microglial processes have been shown to interact directly with NG2 cells (Nishiyama et al. 1997). In vitro, oligodendrocyte stress signals recruit nonreactive microglia (Nicholas et al. 2003), which play a role in survival and maturation of OPCs (Nicholas et al. 2001). However, active microglia can also induce contact-dependent death of oligodendrocytes (Nicholas et al. 2003). Genetic perturbation of oligodendrocytes is thought to trigger an influx of certain immune-mediators, leading to a detrimental cycle of inflammation and degeneration (Nave 2010). Supporting this notion, mice overexpressing oligodendrocyte-specific, PLP, exhibit secondary inflammation including microgliosis and T-cell infiltration (Anderson et al. 1998). Blockade of T-cell function in this mouse ameliorates the phenotype, pointing toward an active role for immune-related factors during oligodendrocyte distress (Ip et al. 2006; Kroner et al. 2010). The relationship between oligodendrocytes and immune function is highlighted in multiple sclerosis (MS), an inflammatory demyelinating CNS disease. Patients with MS also share a strong comorbidity with MDD (Chwastiak and Ehde 2007). Interestingly, a recent report found approximately 44% of multiple sclerosis patients had autoantibodies against CNP1 (Lovato et al. 2008). Combined with our data from chapter 3, we can infer that CNP1 could serve as a molecular link.
between altered oligodendrocyte function and immune/microglial activation, a notion that could be further explored in CNP1\(^{\text{KO}}\) mice or mice exposed to LPS.

### 4.3.2 Cellular Effects of CNP1 Ablation

#### 4.3.2.1 Neuronal Alterations

Similar to stress-induced oligodendrocyte alterations, stress and glucocorticoid treatment have been shown to affect neuronal plasticity in emotionality-related brain regions in rodents [reviewed in (Roozendaal et al. 2009)], suggesting a potential link between neuronal and oligodendrocyte cellular changes. Specifically, acute glucocorticoid treatment and chronic stress result in hypertrophy of BLA neurons (Vyas et al. 2002; Vyas et al. 2004; Mitra and Sapolsky 2008), and chronic stress produces atrophy in IL [(Izquierdo et al. 2006); see also Figure 1.2]. Moreover, dendritic hypotrophy in the BLA was found to be a marker for resilience to anxiety-like behaviors (Mitra et al. 2009) and stress-induced atrophy in IL neurons was correlated with resistance to fear extinction (Izquierdo et al. 2006). Based on these studies and the fear extinction phenotype of CNP1\(^{\text{KO}}\) mice, we might expect to see neuronal changes such as amygdala hypotrophy and increased IL branching in CNP1\(^{\text{KO}}\) mice. In fact, a recent study found that CNP interacts with the neurite outgrowth inhibitor, NOGO-A, indicating that alterations (or ablation) of the oligodendrocyte-specific gene CNP may influence neuronal outgrowth (Sumiyoshi et al. 2010) and pointing toward CNP1 as a potential link between stress-induced neuronal and oligodendrocyte alterations. These plausible neuronal characteristics could be measured in the CNP1\(^{\text{KO}}\) using Golgi staining and subsequent analysis with Sholl or a similar morphological procedure.
4.3.2.2 Oligodendrocyte Alterations

Decreased oligodendrocyte density and downregulation of oligodendrocyte-related transcripts have been reported in MDD and rodents exposed to chronic stress or glucocorticoids (Hamidi et al. 2004; Aston et al. 2005; Banasr et al. 2007; Sibille et al. 2009). However, whether these alterations are causal or simply side effects of the disorder is unknown. In Chapter 3, we compromised oligodendrocyte function via CNP1 ablation and found resilient emotionality in mice, along with a robust upregulation of oligodendrocyte-related genes in the BLA of CNP1^KO^ mice. The latter finding is consistent with a reported excess and swelling of myelin in the CNP1^KO^ (Edgar and Nave 2009), and may reflect compensatory changes attributed to lack of oligodendrocyte structure and function. It is also plausible that this upregulation reflects increased oligodendrocyte number in the CNP1^KO^, a hypothesis that could be tested via cell counts or density analysis, and would be particularly interesting in light of the reported decreased oligodendrocyte density in MDD (Hamidi et al. 2004). While it is clear that stress leads to decreased oligodendrocyte number, we have shown that disruption of oligodendrocyte function (but not number/density) results in resilient emotionality. Thus, it is still unclear whether decreased oligodendrocyte number/density leads to depressive-like symptoms. To address this, it would be beneficial to examine effects of global or region-specific decreases in oligodendrocyte number/density to see if a depressive-like phenotype emerges. This could be accomplished globally by systemic administration of the copper chelator, cuprizone, which induces apoptosis in oligodendrocytes [method reviewed in (Matsushima and Morell 2001)]; or in a region-specific manner with other demyelinating toxins such as lysolecithin (Woodruff and Franklin 1999).

Our results in Chapter 3 indicate that CNP1^KO^ mice overall have resilient emotionality compared with WT mice. Considering the vulnerability of oligodendrocytes to stress and glucocorticoid toxicity, this could suggest that absence of CNP1 alters the stress-responsiveness of oligodendrocytes. Interestingly, it has been proposed that mechanisms of allostasis (adaptation to challenge) in corticolimbic regions may be
crucial to understanding stress resilience (Charney 2004; McEwen 2007; Krishnan and Nestler 2008). The evidence provided here implicates CNP1 as a potential genetic factor involved in oligodendrocyte-mediated resilience. Considering that CNP1 is localized to the paranode region of oligodendrocytes, our data support that the paranode region could be a critical site for regulating stress responsiveness, potentially through allostatic mechanisms (see below).

### 4.3.2.3 Paranodal Alterations

One of the primary functions of oligodendrocytes is to promote axonal conduction and actively regulate neuronal properties (Poliak and Peles 2003) and depolarization of oligodendrocytes has been shown to disrupt axonal conduction (Yamazaki et al. 2007; Yamazaki et al. 2010). CNP1 is localized to the paranodal junction of oligodendrocytes, the critical site of interaction between the myelin sheath and axon (Trapp et al. 1988; Salzer 2003; Rosenbluth 2009) (see section 1.3.1.3). In mice lacking the paranodal protein, Caspr, the paranode region fails to form and these mice exhibit decreased nerve conduction velocity (Bhat et al. 2001), suggesting the paranode region is critical for proper axonal signal transmission. While a single report found no difference in compound action potential between CNP1KO and WT mice (Edgar et al. 2009), reports suggest it may be the more subtle leak currents that are impacted by paranode disruptions resulting in more (or less) rapid depolarizations (Yamazaki et al. 2010).

Evidence suggests that disruption of CNP1 may lead to aberrant signal conduction and downstream dysregulation of circuitry and behavior (Figure 4.3). Therefore, it would be highly relevant to thoroughly examine cellular and electrophysiological properties in CNP1KO mice.

While alterations in oligodendrocytes impact neuronal firing (see above), repetitive action potential propagation, in turn, leads to paranodal swelling (Wurtz and Ellisman 1986), indicating a fine-tuned bidirectional balance between axonal activity and the paranode. The concept of a homeostatic relationship between oligodendrocytes and neurons is also supported by Nave, who hypothesized that oligodendrocyte
mutations cause reduced metabolic coupling between the myelin and the axon (Nave 2010). Ablation of proteins, such as CNP1 and Caspr, cause disorganization at the paranode (Rasband et al. 2005; Sun et al. 2009), potentially generating a disruption in axoglial homeostasis. It is difficult to determine from these studies if the observed phenotype of CNP1KO mice in Chapter 3 is due to disrupted paranodal integrity (i.e. homeostasis) or a dose-dependent effect of CNP1. Examination of CNP1 heterozygous or overexpressor mice would reveal if there is a dose-dependent effect of CNP1 on the emotionality phenotype. While expectations may be that CNP1 is a positive regulator of axoglial function, and a CNP1 overexpressor mouse would have an opposite phenotype (i.e. depressive-like symptoms), this may not be the case. An excess of CNP1 at the axoglial junction, may instead, disrupt paranodal homeostasis resulting in a similar phenotype as CNP1KO mice (i.e. resilience). In support of this notion, and as previously mentioned, PLPKO and PLP overexpressor mice both show axonal degeneration implying that an imbalance (rather than linear dose) of PLP elicits disruptions of axoglial communication (Griffiths et al. 1998; Griffiths et al. 1998). Examination of emotionality in other oligodendrocyte mutant mice (e.g. Caspr) may also shed light on the role of specific myelin compartments.

4.3.3 Circuit-Level Effects of CNP1 Ablation

A large body of research implicates dysregulation of corticolimbic networks in MDD and points toward the amygdala as the central region involved in mood regulation (Mayberg 1997; Seminowicz et al. 2004). The fear conditioning paradigm is well-established in both humans and mice and has been shown to be dependent upon corticolimbic circuitry, specifically the BLA (Sah and Westbrook 2008; Jovanovic and Ressler 2010). In Chapter 3, we used a fear conditioning paradigm to examine amygdala-based corticolimbic function in CNP1KO mice and found that they have normal fear conditioning, but decreased fear expression during extinction. Our results in this paradigm are similar to studies that pharmacologically inactivate either BLA or PL, a manipulation which produces low freezing (i.e. fear expression) throughout the
extinction phase (Sierra-Mercado et al. 2011). Therefore, on a circuit level, it appears that CNP1KO mice have abnormal corticolimbic function which may be a result of hypofunction of the amygdala or decreased “top-down” control from the PL (Figure 4.2). Supporting this notion, Charney (2004) states that resilience “may be due to inhibition of amygdala activity mediated by the medial prefrontal cortex”. This hypothesis could be tested in CNP1KO mice by: 1) electrophysiological recordings (e.g. compound action potential) in BLA and PL to examine firing rate during the extinction phase, 2) examining BLA and PL C-Fos expression following extinction learning, or 3) pharmacologically inactivating (GABA_A agonist muscimol) or activating (GABA_A antagonist bicuculline) BLA or PL and examining resulting fear expression behavior. Alternatively, a PL or BLA specific CNP1 knockdown or overexpressor (using means outlined in Appendix A) could be used to test the region-specific effects of CNP1 alteration on behavioral or electrophysiological outputs.

The fear conditioning paradigm can be used to examine both fear memory (amygdala based) and contextual memory (hippocampus based) (LeDoux 2000); processes we attempt to dissociate in Chapter 3. Extinction recall and fear renewal results, indicated contextual memory, and therefore hippocampal function, was intact in CNP1KO mice (Figure 3.3). However, large differences in baseline freezing between WT and CNP1KO mice on days 2 and 3 make this difficult to accurately confirm. Thus, it might be beneficial to perform memory based cognitive tasks on CNP1KO mice such as the Morris water maze or Y maze. Performance during extinction (day 2) indicates CNP1KO mice display low freezing during the initial extinction trials, a behavior that could be the result of either a fear memory deficit or a low threat assessment of the CS. In support of a low threat assessment, we saw a significant change in freezing behavior during early extinction trials compared to late extinction trials (i.e. rate of extinction), implying that fear memory (memory of the CS-US pairing) is intact. In addition, results from a pilot study where mice are re-exposed to five trials of the CS-US pairing indicate CNP1KO mice initially display a fear response similar to WT mice, but quickly display a diminished fear response (Appendix D). This suggests: 1) they remember the CS and 2) they temper their response after re-exposure to the pairing. These results support
that both contextual and fear memory are intact in CNP1\textsuperscript{KO} mice, and instead, provide evidence that CNP1\textsuperscript{KO} mice encode the CS at a low threat level.
Figure 4.2. Model of potential circuitry changes in CNP1KO mice. WT mice receive input from PFC to the BLA which together determine input to CeA and subsequent fear output. Results from the fear conditioning paradigm suggest that CNP1KO animals likely have either reduced PFC input to BLA (green) or normal PFC function and hypofunction of BLA (red). Either scenario would result in decreased input to CeA and low fear output. The proposed changes are testable hypotheses (see text) and would result in the pattern of low expression observed during the extinction phase of the paradigm. Modified from (Vidal-Gonzalez et al. 2006).

4.3.4 Conclusions on the Neurobiological Consequences of CNP1 Ablation

In previous studies from our lab, CNP1 was found to be downregulated in the amygdala of MDD subjects and WT mice exposed to UCMS. Here, our results indicate that CNP1KO mice have: 1) low emotionality, 2) decreased vulnerability to develop a depressive-like syndrome, 3) deregulation of amygdala-mediated corticolimbic circuitry, and 4) robust upregulation of oligodendrocyte and immune-related transcripts. As CNP1 is localized to the paranode region, we suspect paranode function may be a critical factor involved in homeostasis and/or allostasis of MDD-associated brain regions. Disruption of oligodendrocyte function at the paranode can impact circuits mediating emotionality in mice, resulting in resilient emotionality (Figure 4.3). In support of this notion, the white matter abnormalities found in human MDD subjects using diffusion tensor imaging have been suggested to disrupt neural circuits involved in MDD (Ma et al. 2007). In conclusion, dysfunction of this region could translate into suboptimal support for neuronal axons, priming the system for deregulated physiological responses to stress and inducing compensatory/neuroprotective changes.
There is evidence that disruption of paranode integrity (e.g. CNP1 ablation) [1] causes altered structure and function of oligodendrocytes [2]. This, in turn, could lead to aberrant signal transmission along the axon [3] resulting in altered integration of emotionality salient stimuli within the amygdala (e.g.) [4]. Abnormal amygdala function may cause downstream deregulation of corticolimbic circuitry involved in mood regulation [5], ultimately resulting in abnormal emotionality behaviors [6]. Molecular changes such as increased oligodendrocyte and immune-related components could be side effects of specific points of disruption. See text for supporting information.

4.4 DOES CNP1 PLAY A CAUSAL ROLE IN MDD?

The goal of the studies outlined in this dissertation was to determine whether downregulation of CNP1 could be a causative factor in the onset of MDD. We examined the emotionality phenotype, amygdala-specific behavior, and the molecular profile of CNP1KO mice to determine whether CNP1 might play a causal role in MDD onset. Criteria for a causal MDD-associated gene may be 1) altered levels (i.e.
upregulated or downregulated) in MDD subjects, 2) comparable alteration of the gene in an animal model of MDD, 3) an induced change of the gene in an animal elicits depressive-like symptoms, and 4) an induced change of the gene in an animal elicits molecular and cellular changes similar to those seen in MDD. CNP1 met criteria 1 and 2. However, outcomes for criteria 3 and 4 were opposite of what was anticipated, indicating CNP1 is not a causal MDD-associated gene. Instead, they indicate downregulation of CNP1 in MDD may elicit a phenotypic compensation in MDD. However, it is difficult to conclusively make this determination in a global CNP1 KO mouse since diseases rarely include full loss of gene function and the phenotype we see could be due to compensatory effects (e.g. absence of CNP1 during development) unrelated to CNP1. In addition, CNP1 is found throughout the brain making it difficult to pinpoint regional effects and warranting further exploration using heterozygous and site- and temporal-specific CNP1 mutants. While there are many unknowns about the function of CNP1 in MDD, we provide clear evidence that disruption of CNP1 does play a role in mood regulation. Thus, while not a causative gene in MDD, it is a potentially relevant gene in secondary aspects of mood regulation, axoglial interactions, or in therapeutic aspects of MDD (see below) and other psychiatric disorders.

Our results indicate that CNP1 KO mice display resilience, raising the possibility that CNP1 could be a potential therapeutic target. However, our results combined with prior evidence would suggest that CNP1 is not likely a good target because a reduction in CNP1 has maladaptive effects that eventually lead to widespread axonal degeneration and death. Since CNP1 is highly expressed throughout the brain, it might be difficult to achieve site-specific effects with pharmacological agents, increasing the likelihood of numerous side effects. Several organophosphorus compounds have been reported to decrease levels of CNP1 (Olajos 1987). Notably, aniline, an organic compound used to make dyes, plastics, and even drugs, first increases then drastically decreases levels of CNP1 when given to rats orally. The eventual decrease in CNP1 correlates with increased swelling of oligodendrocyte cytoplasm and ultrastructural myelin splitting (Kanno et al. 2010), which both raises concern about the harmful
neurological properties of organophosphorus compounds and implies pharmaceutical targeting of CNP1 may have detrimental side effects.

While our studies indicate CNP1 is not causative to MDD and may not be a suitable target for pharmacological intervention, investigations of CNP1 function can help to clarify the roles of oligodendrocytes and paranodal junctions in MDD. Since CNP1 is widely used as a marker for oligodendrocytes, some tools have been developed that may be utilized for this purpose. For instance, mice with markers attached to the CNP1 promoter have been developed (e.g. CNP-β-galactosidase and CNP-GFP), which could be useful in isolating cells that selectively express CNP1 (Chandross et al. 1999; Roy et al. 1999; Belachew et al. 2001). In addition, most of the traditional viral vectors used in viral-mediated gene delivery (e.g. adeno-associated virus or lentivirus) infect mainly neurons and not oligodendrocytes, which might make it difficult to perform site-specific manipulations of CNP1. An alternative approach may be to use an organophosphorus compound such as aniline which directly targets CNP1.

4.5 OVERALL CONCLUSIONS AND FUTURE DIRECTIONS

Investigations into the role of oligodendrocytes in MDD and other psychiatric disorders are gaining momentum as more evidence points toward oligodendrocytes as active and integral components of neural functioning. Here, we explored CNP1 as a potential MDD-associated gene and component of proper oligodendrocyte functioning. We applied a novel Z-score normalization procedure and traditional fear conditioning paradigm to assess emotionality and associated corticolimbic function in a more comprehensive and translational manner. Z-score analysis in WT animals revealed that age, sex, and locomotion are factors that can influence emotionality and should be taken into consideration when designing, analyzing and interpreting behavioral measures in mice. Z-score analysis also allowed for a broad assessment of the behavioral state of CNP1KO mice, revealing an unexpected behavioral pattern indicative of resilience. CNP1 was implicated as a component involved in mediating the function
of corticolimbic regions responsible for mood regulation. Our data suggest that downregulation of CNP1 may lead to a phenotypic compensation in MDD, but long-term downregulation of CNP1 has an unsustainable and maladaptive trajectory. Together, our results suggest a model where disrupted axoglial interaction (via the paranode) has a direct impact on the function of the amygdala and associated corticolimbic circuits resulting in subsequent altered emotionality (Figure 4.3). Furthermore, it suggests the paranode region of oligodendrocytes may be involved in maintaining homeostasis of the axoglial interface. This region may also be a critical site for regulating stress responsiveness (allostasis).

Future studies should be aimed at deciphering the role of CNP1 using temporal and site-specific mutants, characterizing the role of the paranode region in MDD, exploring more causal roles of oligodendrocyte alterations in MDD, and examining polymorphisms in the CNP1 gene and their relation to MDD. Future research into oligodendrocyte function may change the current notion that neurons and neuron-networks are the solitary substrate for higher level functions such as complex behavior, emotion, and cognition.
APPENDIX A

PAPER 3 – GENETICALLY MODIFIED ANIMALS

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Definition of a genetically modified animal
An animal whose genetic material has been altered by the use of genetic engineering or recombinant DNA technology. In biomedical sciences, genetically modified animals are typically generated for the purpose of studying the function of a particular gene.

Principles and Role in Psychopharmacology
One of the main goals of the field of genetics is to classify and functionally characterize individual genes. The investigative approach to studying genes in living organisms has principally been divided into three strategies: 1) analysis of natural variation, 2) random mutagenesis and, 3) targeted mutagenesis and transgenesis (Rudolph and Mohler 1999). Analysis of natural variation (e.g. spontaneous mutations) and random mutagenesis (e.g. chemical or irradiation) are the primary approaches of ‘forward genetics’ where the genetic cause (genotype) of an altered or abnormal phenotype is investigated. However, with random mutagenesis, many chromosomal loci are often targeted and it is difficult to trace any phenotype back to a specific genetic origin. The development of ‘reverse genetic’ approaches, where a particular gene is
altered and the phenotype is investigated, provided tools to investigate specific gene function in a more targeted manner (Brusa 1999). Since the development of these tools in the 1980s and 1990s, their use in the field of biomedical research and pharmacology has been substantial due to the ability to develop suitable animal models of specific diseases, the ability to genetically dissect the underlying mechanisms of disease, and the ability to identify and verify molecular targets of pharmacological agents.

In the past two decades, a variety of techniques have been developed to introduce genetic modifications in various species for specific research purposes. Among the most targeted species are Drosophila melanogaster, Caenorhabditis elegans and mice, which have each played integral roles in identifying genes involved in development, aging, cell differentiation, and other major biological functions. Other genetically modified animals that have been developed include xenopus, zebrafish, rabbits, pigs, and cows. More recently, transgenic and knockout rats have been developed which will allow more extensive research in the neurosciences due to their extensive use in behavioral paradigms (Abbott 2004). In addition, the first transgenic primate disease model (for Huntington’s disease) was recently created (Yang et al. 2008). While a wide variety of genetically modified organisms have been created to date for numerous research purposes, techniques for genetically modifying mice are the most advanced and the most applicable to the field of psychopharmacology, which represent the main focus of the remainder of this article.

**Transgenic Technology**

While the term “transgenic” has grown to include any type of genetically modified animal, the traditional definition of a transgenic organism is one containing foreign DNA, whether from the same species or a different one. The expression of foreign DNA in a mouse is a valuable technique since it allows for the investigation of the functional role of this gene in a living organism. For instance, transgenic mice overexpressing a particular gene are often generated to analyze exaggerated phenotypes. The expression of a human gene or a mutated gene in mice is also often used to explore gene function, particularly in the context of a specific disease.
There are several ways to create a transgenic mouse, however, all methods consist of first designing a DNA fragment, or “genetic construct”, which contains the gene of interest (GOI) and other features necessary for the expression of this gene in a mammalian system (e.g. gene promoter, enhancer, polyA signal, etc). The traditional transgenic method consists of physically injecting the transgenic construct into the nucleus of a fertilized egg (pronuclear microinjection), allowing it to develop in vitro to the blastocyst stage, and then implanting the egg into a pseudopregnant female (Fig A.1a). The embryos must then be screened for the presence of the transgene and unlike the production of knockouts, the transgene typically occurs in an all or none fashion, with the embryo either containing the transgene in every cell or in no cells at all. Alternatively, transgenic animals can be produced by viral infection of the fertilized embryo (see below) or transfection of embryonic stem cells (ES) with the gene of interest (Dale 2002). While the transgenic approach is fast and efficient, limitations of the technology include: 1) The GOI may randomly integrate into the genome which can result in expression in ecotopic sites, interference with the endogenous gene, or severe disruption of the homeostasis of the cells and organism; 2) The level of gene expression is unable to be controlled and is dependent upon where the gene inserts into the host genome and on the number of copies inserted. Overexpression of the gene could have unexpected detrimental effects, including lethality; 3) Mosaic or chimeric animals are sometimes produced, particularly when the transgenic animal is generated with the viral method, due to infection of only a subset of cells within the blastocyst; 4) The genetic background of mice can considerably influence the ability of the manipulated egg to survive microinjection, implant in the uterus, and develop to term (Brusa 1999; Dale 2002)

**Gene Knockout Technology**

The creation of a traditional knockout (i.e. removal of a gene) mouse consists of disrupting all or part of the coding sequence of the GOI, with the purpose of exploring the phenotype in the absence of the gene. The exact locus of the GOI can be targeted by creating a genetic construct that is homologous to the region of the GOI on a
particular chromosome. The genetic construct is injected into embryonic stem cells where rare homologous recombination events can occur with the endogeneous GOI at the intended chromosomal locus. In the knockout approach, the GOI is often replaced with neomycin (Neo) or another selectable marker which allows for in vitro selection and identification of the stem cell colonies that have undergone appropriate recombination (Fig. A.1b). Selection against random integration into the genome is often attained by using a second selectable marker outside the inserted region. Once the mutated stem cells are identified and verified, they are microinjected into a blastocyst and implanted into a pseudopregnant female as in a transgenic mouse. Most of the offspring produced using this technique are chimeras (or mosaics) due to the presence of both mutated and non-mutated stem cells within the blastocyst. Embryonic stem cells from a different color strain of mice than the fertilized egg are often used because the chimeric mice are then easily identifiable due to bi-colored fur. The next step consists of breeding chimeric mice to identify individuals with germ cells that have undergone homologous recombination. These founder mice will then be bred to produce homozygous mutant animals (Dale 2002). Similar to knockout mice, knockin technology, where one or more exons of a certain gene are replaced with an altered version (Fig. A.1b), is often used to study specific polymorphisms, or the human equivalent of the GOI.

While knockout organisms have been paramount in the goal to elucidate the function of specific genes, problems associated with knockout technology include that removal of a gene is often lethal or that the absence of the gene product during development leads to compensatory events that can obscure the analysis of the function of the missing gene. Specifically, these compensatory developments likely differ from the disease mechanisms that the knockout animal is intended to model, as disease processes rarely include full loss of gene function.

*Conditional Knockout Technology*

The desire for greater specificity and the need to bypass potential developmental compensations and occasional lethality has lead to the development of conditional or inducible knockouts, where the experimenter has either temporal or regional control
over expression of the GOI. Several systems have been developed for this purpose including the Cre-LoxP system, Flp-FRT system, and the Tetracycline-inducible system. The Cre-LoxP system of bacterial origin, employs Cre recombinase, which mediates site-specific recombination by targeting a DNA sequence called LoxP (Houdebine 2007). To implement this system, one strain of mice is engineered to express Cre under a tissue- or developmental stage-specific promoter and these mice are bred to a second strain of mice in which the GOI has been flanked with LoxP sites. The resulting offspring then have the GOI removed only in the tissues (or at the developmental timepoint) where/when Cre is expressed (Fig. A.1c). The Flp-FRT system originates in yeast and is used in the same manner as the Cre-LoxP system with Flp recombinase excising the GOI flanked by FRT DNA sequences (Houdebine 2007). Importantly, these two systems can be used together to allow for a range of additional approaches.

The tetracycline-inducible system is based on the tetracycline resistant gene from bacteria and has been modified for use in mammals to essentially act as a switch to initiate or terminate gene expression. In bacteria, the tetracycline resistance gene is typically kept in the off position by a repressor bound to a specialized DNA sequence in the promoter of the gene, the tetracycline operator (tetO) sequence. For use in mammalian transgenics, the repressor protein was modified into a transactivator (tTA) which allows constitutive expression of genes bound to tetO. Thus, in the presence of tetracycline, gene expression is terminated when the drug binds to tTA, removing it or preventing its binding to tetO. A modified version of tTA (rtTA) requires the presence of tetracycline to bind to tetO thereby allowing activation of a gene in the presence of tetracycline. For these systems to work, two transgenes in a single animal are needed; one expressing tTA under the control of a site- or temporal-specific promoter and the GOI under control of the tetO (Fig. A.1d) (Brusa 1999).

RNAi and Gene Transfer in vivo

The recent discovery and exploitation of the endogenous RNA interference (RNAi) mechanism has aided in the development of loss of function models. The endogenous mechanism consists of short sequences of double stranded RNA which
bind and cleave complementary mRNA sequences thereby silencing or inducing downregulation of the specific gene (Dunn et al. 2005). Short sequences of double stranded RNA (~22 nucleotides), termed small interfering RNA (siRNA), are easily synthesized and can be delivered in vivo using a variety of gene transfer techniques (see below and Fig. A.1e). Similar to RNAi, antisense technology is the expression of the reverse complement of mRNA which interferes with normal translation thereby reducing protein synthesis (Dale 2002).

While conditional and inducible knockouts provide certain level of site and temporal specificity, they are dependent upon the availability of promoters that provide the desired specificity. The development of viral-mediated gene transfer has allowed more flexibility in producing the desired manipulations. In addition to an alternative method of producing transgenic animals (discussed above), viral vectors can be used to transfer genetic material in a temporal and site specific manner in both neonatal and adult mice. Modified virons such as herpes, lenti-, adeno-, and adeno-associated viruses can be engineered to carry a transgene, siRNA, or other genetic material. These viral vectors are then able to infect cells and transmit the desired genetic material. Viral-mediated gene transfer has the additional advantage over conditional mutants that the genetic manipulation occurs only at the site of infection and cell-type specific promoters can be used for additional specificity. Depending upon the type of virus used, the genetic material may be integrated into the genome or may remain epichromosomal. The type of virus also influences the infection rate, type of cells infected, and the size of DNA insert (Dunn et al. 2005). While viral-mediated gene transfer technology is widespread, non-viral gene transfer to the central nervous system has also been achieved using in vivo electroporation and both intracerebroventricular and intrathecal infusion (Gilmore et al. 2006).
Figure A.1. Diagrams of methods for producing genetically modified animals.
The creation of the first knockout mouse in 1989 let to a noble prize for Sir Martin Evans (Cardiff University in Wales), Oliver Smithies (University of North Carolina at Chapel Hill), and Mario Capecchi (University of Utah in Salt Lake City) in 2007. Since the development of the technology, using knockout mice along with other types of mutants to investigate critical questions in psychopharmacology has become standard practice. In particular, genetically modified mice have been essential in psychopharmacology for 1) Elucidating both the function of a gene and the molecular elements associated with a gene;  2) Creating animal models of human disease; 3) Identifying and validating drug targets and drug specificity and; 4) Examining temporal aspects of gene function.

One of the first major breakthroughs in the field of neuroscience using targeted mutagenesis came from Eric Kandel’s group at Columbia University. In a series of experiments using the tetracycline inducible system, they were able to express a calcium-independent form of the forebrain specific calcium dependent kinase, calcium-calmodulin kinase II (CaMKII) and found deficits in spatial memory and hippocampal long-term potentiation (LTP) (Mayford et al. 1996). These groundbreaking experiments provide a classic example of the power of how spatial and temporal control over molecular elements can aid in elucidating the function of specific genes and their role in higher brain function. The CaMKII promoter still remains one of the most popular promoters to express numerous genetic constructs in the mouse forebrain.

Another critical role for transgenic animals is the development of animal models for human disease, by either the introduction of a mutated gene or the elimination of a gene putatively involved in the illness. In the field of neuroscience this has been particularly useful in modeling a wide variety of disorders, including Alzheimer’s disease, Huntington’s disease, neuropsychiatric disorders, and cerebral ischemia. In particular, Alzheimer’s disease (AD) is characterized by the formation of neurofibrillary tangles of hyper-phosphorylated tau protein and by amyloid β-peptide (Aβ) plaques. Mutations in the amyloid precursor protein (APP), presenilin 1 and 2 (PS1, PS2) and apolipoprotein E (APOE) are all implicated in the disease. Studies now show that mice overexpressing
APP and PS1 form Aβ plaques and display memory deficits, both characteristic symptoms of Alzheimer’s disease (Brusa 1999), consequently highlighting the important role of genetically modified animals in testing potentially causal mechanisms involved in human disease. In some cases it is necessary to replace the murine gene with the human gene due to distinct structural differences between the human gene and mouse homologue at the molecular level (Rudolph and Mohler 1999).

The development of genetic animal models for human diseases has provided a solid foundation for drug discovery and for the identification of drug targets. The specificity of the genetic manipulation (i.e. removing a gene coding for a particular receptor subtype) ensures virtually absolute selectivity thereby offering a great advantage over classical pharmacological approaches. For instance, the function of receptor subtypes can be examined using both knock-in and knockout approaches. A particularly relevant example is that of the utilization of transgenic mice in determining the role of specific GABA receptor subunits in distinct actions of the benzodiazepine, diazepam. Diazepam is known to act on GABA A receptors containing the α1-, α2-, α3-, or α5- subunits. By examining mice carrying point mutations in the benzodiazepine binding sites of each subunit, investigators were able to genetically dissect the different functions of diazepam (e.g. sedative vs. anxiolytic) acting at otherwise similar GABA A receptors (Rudolph and Mohler 1999).

Other critical uses of genetically modified animals are the dissection of second messenger signaling pathways and the determination of critical developmental time-windows for gene function. The latter was elegantly demonstrated by the use of a tetracycline inducible knockout of the serotonin 1A receptor. Gross et al. 2002, showed that when the 1A receptor was knocked out during development, it resulted in behavior similar to the knockout mouse (increased anxiety). However, when the receptor was knocked out in adults, the phenotype was absent (anxiety levels were normal) thereby implicating the 1A receptor as a critical developmental factor for normal emotional behavior (Gross et al. 2002).

In summary, the primary goal of reverse genetics is to create a targeted mutation and then investigate the resulting phenotype. We briefly discussed several methods of
targeted mutagenesis including the development of transgenic animals and the techniques for developing global, conditional and inducible knockouts. The values of this technology have been far-reaching and have played a considerable role in psychopharmacology. We have highlighted some of the most common uses for genetically modified animals in this field including the dissection of molecular mechanisms, modeling human disease, drug discovery and validation, and the investigation of critical time windows in gene function. While several other approaches for creating genetically modified animals exist (e.g. the use of modified male gametes) along with other applications for these animals (e.g. pharming), we focused on the role of genetically modified mice in psychopharmacology, as their impact on this field has been substantial. New approaches to developing and using genetically modified organisms are quickly evolving, including modifications and combinations of the discussed systems, which will likely further impact psychopharmacology.

**Short Definitions from Paper 3: Genetically Modified Animals**

**Transgenic Organism**

**Synonyms**

Mutant

**Definition**

Traditionally, an organism with the addition of foreign DNA, whether from the same species or a different one. More recently the term transgenic has been used to refer to any genetically modified organism.

**Knockout / Knockin**

**Synonyms**

Global knockout; Constitutive knockout

**Definition**
Knockout - The removal or complete disruption of a specific gene in an animal from the blastocyst stage through adulthood. Knockin – The introduction of a mutated version of a specific gene in place of the wild type version.

**Conditional knockout**

**Synonyms**
Site-specific knockout, region-specific knockout, cell type-specific knockout

**Definition**
The removal or complete disruption of a specific gene in a manner that controls the cell types and brain region or site where the disruption occurs. The Cre/loxP system is frequently used to produce conditional knockouts and in this system, the promoter expressing Cre recombinase will give rise to the specificity of the excised gene.

**Inducible knockout**

**Synonyms**
Time-specific knockout, temporal knockout

**Definition**
The use of a system where the experimenter controls the timing of gene removal. The tetracycline inducible system is frequently used to produce animals where a simple injection of tetracycline will either terminate or initiate gene transcription.

**RNAi**

**Synonyms**
siRNA

**Definition**
An endogenous system where short sequences of double stranded RNA molecules induce the cleavage of matching mRNA resulting in downregulation of a particular gene. This system can be manipulated for experimental by using exogenous siRNA to downregulate a GOI.
Chimera
Synonyms
Mosaic
Definition
An animal in which individual cells contain genetic material from only one of two potential lineages. These animals are often produced in the creation of knockout mice where a mutated ES cell is introduced into the blastocyst containing wild type ES cells.

Forward Genetics / Reverse Genetics
Synonyms
Forward genetics – Random mutagenesis; Reverse genetics – Targeted mutagenesis
Definition
Forward genetics is the examination of the genetic cause of an altered or abnormal phenotype introduced by a chemical mutagenesis or mutation by irradiation (e.g. phenotype → genotype). In reverse genetics, a particular gene is altered and the phenotype is investigated (e.g. genotype → phenotype).

Phenotype/Genotype
Synonyms
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Definition
Genotype is the specific genetic constitution of an organism including the gene allelic make-up. Phenotype is the physical trait or characteristic arising from the genotype.

Pseudopregnant
Synonyms
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Definition
A hormonal state similar to pregnancy that is induced in mice by mating a female with a vasectomized male. In this state, the uterus is receptive to an implanted embryo.
Table B.1. Z-score values (Mean ± SEM) obtained by different combinations of 2 or 3 behavioral tests.

<table>
<thead>
<tr>
<th>Tests</th>
<th>Male Vehicle</th>
<th>Male Corticosterone</th>
<th>Female Vehicle</th>
<th>Female Corticosterone</th>
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</thead>
<tbody>
<tr>
<td>EPM/OF</td>
<td>-0.02±0.20</td>
<td>0.70±0.12</td>
<td>-0.12±0.18</td>
<td>0.33±0.21</td>
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<td>EPM/NSF</td>
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<td>-0.07±0.19</td>
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<td>0.67±0.10</td>
<td>-0.27±0.15</td>
<td>0.25±0.17</td>
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</tbody>
</table>
Figure B.1. Z-scores values (Mean ± SEM) obtained by different combinations of 2 or 3 behavioral tests
Table B.2. P-values for 2-way ANOVA main effects calculated in different combinations of 2 or 3 behavioral tests.

<table>
<thead>
<tr>
<th>Tests</th>
<th>Main effect of sex</th>
<th>Main effect of cort</th>
<th>Sex x cort interaction</th>
<th>Coefficient of variation</th>
</tr>
</thead>
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<td>&lt; 0.001</td>
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<td>5.08</td>
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<tr>
<td>EPM/OF/NSF</td>
<td>0.018</td>
<td>&lt; 0.001</td>
<td>0.511</td>
<td>3.04</td>
</tr>
</tbody>
</table>
APPENDIX C

SUPPLEMENTAL INFORMATION PAPER 2

Resilient emotionality and genetic compensation in mice lacking the oligodendrocyte-specific gene Cnp1

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C.1 SUPPLEMENTARY METHODS

C.1.1 Behavioral Measures

_Elevated plus maze (EPM):_ The EPM test consisted of four elevated runways (81 cm height): two open arms (without walls) and two closed arms (with walls) (36 x 6 cm arms). Mice were placed in the EPM for 10 minutes and anxiety-like behaviors were assessed by measuring the amount of time spent on the open arms (relative to the 10 minute total time in the maze) and the ratio of crosses into the open arms. Total number of crosses into either open or closed arms was used as a measure for overall locomotor activity.

_Open field (OF):_ Behavior in the OF was measured using ActiVMeter Open Field Cages (dimensions: 45 x 45 cm; BioSeb Inc., Vitrolles Cedex, France). This system measures the movement of the animal using a weight and vibration-sensitive platform, accurately recording the spatial position of the animal. Mice were placed in the OF for 10 minutes and anxiety-like behavior was measured by time spent in the center (relative to the 10 minute total time in the maze) and the ratio of distance traveled in the center. Locomotor activity was measured by total distance traveled.

_Novelty suppressed feeding (NSF):_ The NSF test consisted of food-depriving mice overnight (~16 hours), then providing them with a single food pellet placed in the middle of a novel, aversive environment (a brightly lit 51 x 51 cm enclosure). The latency to start feeding (during 12 min. assay) was used as a measure for depressive-like behavior. Food consumption (8 minutes post-test) and weight loss were measured as controls for potential feeding differences.

_Forced Swim Test (FST):_ Mice were tested for acute stress response using the forced swim test. Mice were placed in a one liter beaker filled with room temperature water (~22°C) for 6 minutes. The time spent swimming during the last four minutes of testing was used as a measure of stress-responsiveness.
Rotarod (RR): Motor deficits have been previously reported in Cnp1KO mice after 6 months of age (Lappe-Siefke et al. 2003). Here, mice were placed on a rotating, accelerating rod (0-40 rpm over 60 seconds) (Rotamex-5; Columbus Instruments, Columbus, OH) and the latency to fall was recorded over 10 consecutive trials. The average latency of trials 8-10 was used as a measure for motor coordination.

C.1.2 Physiological Measures

Stress hormone levels were assessed in Baseline, UCMS and CORT groups prior to the start of the emotionality phenotyping test battery. To monitor glucocorticoid levels we used a non-invasive technique assessing corticosterone metabolites in fecal samples of mice, which has been extensively validated for this species (Touma et al. 2003; Touma et al. 2004). Since sampling of feces can be performed completely non-invasively and allows even frequent sampling of the same individual or group, the monitoring of fecal hormone metabolites avoids all stress effects related to common blood sampling procedures in mice. Additionally, in the feces circulating hormone levels are integrated over a certain period of time and probably the production rate is reflected rather than actual steroid concentrations (i.e. hormone metabolite concentrations in feces reflect the cumulative secretion and elimination of hormones over a number of hours (Touma and Palme 2005). Fecal samples, therefore, do not only represent a single sample point like blood samples but also are less affected by episodic fluctuations of hormone secretion. Consequently, steroid metabolite concentrations measured in the feces might represent the animal's hormonal status more accurately. For sample collection, the bedding of the mouse cages was changed the night prior to feces collection, insuring that older feces were not accidentally collected, and feces were grouped according to genotype and sex (n=3-5 samples/group consisting of 3-4 animals per sample). Following collection, fecal samples were stored at -20°C until 0.05g of the dried sample were extracted using 80% methanol and corticosterone metabolite levels were determined in a 5alpha-pregnane-
3beta,11beta,21-triol-20-one enzyme immunoassay (for details see (Touma et al. 2003; Touma et al. 2004)).

**Body weight:** Body weight (g) was recorded at each age in animals in the baseline cohort and weekly in UCMS and CORT cohorts to monitor weight gain in all experimental groups.

**Fur rating** has previously been shown to be an accurate marker of the progression of the depressive-like state (Mineur et al. 2003). Here, individual animals in the UCMS and CORT cohorts were assessed weekly using a rating scale of 1-4, with 1 indicating a well-groomed coat and 4 indicating a tousled coat with bald patches.

### C.1.3 Unpredictable chronic mild stress (UCMS)

UCMS-exposed mice were maintained under standard laboratory conditions, however, UCMS-exposed animals were single housed throughout the paradigm, as an additional stressor. Control mice were group housed to avoid the stress of single housing. The UCMS group was subjected to 4 weeks of 1-2 mild stressors per day. Stressors include forced bath (~2 cm of water in a rat cage for 15 min), wet bedding, aversive smell (1 hr exposure to fox urine), light cycle reversal or disruption (variable times), social stress (rotate mice into previously occupied “dirty” cages), tilted cage (45º tilt), restraint (50 ml tube with airhole for 15 min), no bedding, and bedding change (replace soiled bedding with clean bedding). Two or three stressors were sometimes used simultaneously to contribute to the random nature of the paradigm. Full schedule of stressors can be viewed in Supplementary Table C.1.
C.1.4 Hotplate test for pain sensitivity

In rodents, pain sensitivity is modeled by acute pain tests that measure the threshold to high-intensity stimuli. To determine whether $Cnp1$ KO mice have the same pain sensitivity as WT mice, the same cohort of mice used in the fear conditioning (FC) experiment was tested in the hotplate test. Mice were placed on a hotplate (Columbus Instruments, Columbus, OH) set to 55°C and surrounded by clear plexiglass walls and a lid. The latency for the mouse to withdraw and lick a hindpaw is measured. Differences in the latencies between genotypes were assessed using one way ANOVA.
Figure C.1. Individual tests comprising 3 month emotionality and locomotion Z-scores. In NSF, there was no difference in weight loss but reduced food consumption in Cnp1KO, which may have confounded the negative results in that test (data not shown). Z-scores are normalized to the WT group. Data represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, # represent statistical trends (p<0.1).
Figure C.2. Individual tests comprising 6 month locomotion Z-score. See text (and Figure 1b) for breakdown of 6 month emotionality Z-score. Z-score is normalized to WT group. Data represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, # represent statistical trends (p<0.1).
Figure C.3. Individual tests comprising 9 month emotionality and locomotion Z-scores. In NSF, similar to the 3-month time-point (but not 6-month), there was no difference in weight loss but reduced food consumption in the Cnp1\(^{KO}\), which may have confounded the negative results in that test (data not shown). Z-scores are normalized to the WT group. Data represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, # represent statistical trends (p<0.1).
Figure C.4. Baseline emotionality Z-scores excluding FST. While emotionality measures in the EPM and OF are controlled for locomotion, hyperactivity could affect results in the FST. Here, with FST removed from the emotionality Z-score calculation, we still see a pronounced and progressive low emotionality phenotype in the Cnp1KO. Data represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, # represent statistical trends (p<0.1).
Figure C.5. Physiological measures in the baseline cohort. A) Fecal corticosterone metabolite levels. At each age, there were no genotype differences. Differences between ages were not examined as they likely reflect unknown factors in the animal colony on the day of sample collection. B) Body weight. Cnp1KO had lower body weight at 6 and 9 months. Data represent mean ± SEM (n=9-18/group). *p<0.05, **p<0.01, ***p<0.001, # represent statistical trends (p<0.1).
Figure C.6. Individual tests comprising UCMS emotionality and locomotion Z-scores. Cnp1KO mice exposed to UCMS lost more weight during the NSF food deprivation (P<0.001), but consumed less food following the test (p<0.05), suggesting the decreased latency in the NSF is not due to increased appetite (data not shown). Z-scores are normalized to the WT control group. Red asterisks represent within genotype age comparisons, while black asterisks represent across genotype comparisons. Data represent mean±SEM (n=9-18/group). *p<0.05, **p<0.01, ***p<0.001, # represent statistical trends (p<0.1).
**Figure C.7. Individual tests comprising CORT emotionality and locomotion Z-scores.** In the NSF, $Cnp1^{KO}$ mice consumed less food post-test, but showed no difference in %weight loss at baseline. Following CORT exposure, $Cnp1^{KO}$ mice lost more weight during NSF food deprivation (p<0.05), but had no difference in post-test food consumption (data not shown). Z-scores are normalized to the WT baseline group. (n=21-26/group). Red asterisks represent within genotype age comparisons, while black asterisks represent across genotype comparisons. Data represent mean±SEM. *p<0.05, **p<0.01, ***p<0.001, # represent statistical trends (p<0.1).
Figure C.8. Emotionality Z-scores excluding the forced swim test. A) UCMS cohort. B) CORT cohort. Red asterisks represent within genotype age comparisons, while black asterisks represent across genotype comparisons. Data represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.
Figure C.9. Behavioral measures in a separate cohort exposed to an abbreviated (2 week) UCMS paradigm. Data represent mean±SEM (n=20WT, 12KO; age 6 mo.). *p<0.05, **p<0.01, ***p<0.001, # represent statistical trends (p<0.1).
Figure C.10. Physiological measures in the UCMS cohort. Since there were physiological sex differences in these measures, reports were split by sex. Week 0 measures were taken prior to the start of the UCMS treatment, followed by weekly measures during treatment. A) Body Weight. Male and female Cnp1KO mice had significantly lower body weights during week 0. B) Fur Rating. In females, mice exposed to UCMS had higher fur rating ratings at week 4 (WT; black asterisks; Cnp1KO blue asterisk). Male Cnp1KO had higher fur ratings at week 4 (trend level). C) Fecal corticosterone metabolite levels showed no significant differences between the groups. Data represent mean± SEM (n=9-18/group). *)p<0.05, **p<0.01, ***p<0.001, # represent statistical trends (p<0.1).
Figure C.11. Physiological measures in the CORT cohort. Since there were physiological sex differences in these measures, reports were split by sex. Week 0 measures were taken prior to the start of the CORT treatment, followed by weekly measures during treatment. A) Body Weight across week. B) Fur Rating across week C.) Fecal corticosterone metabolite levels before and during CORT treatment. Red asterisks represent within genotype comparisons. Data represent mean±SEM (n=9-18/group). *p<0.05, **p<0.01, ***p<0.001, # represent statistical trends (p<0.1).
Figure C.12. Hot plate test. Test for pain sensitivity in the fear conditioning cohort. Data represent mean ± SEM (n=16-18/group). No significant differences were found (p>0.1).
Figure C.13. Fear conditioning in a separate cohort of mice. Fear acquisition (left panel) was normal in both WT and Cnp1KO mice, while fear extinction revealed low fear expression in Cnp1KO mice (right panel). Red asterisks represent within genotype comparisons, while black asterisks represent across genotype comparisons. Data represent mean±SEM (n=8-12/group). *p<0.05, **p<0.01.
Figure C.14. Extinction recall and fear renewal in the FC cohort. Lower fear expression during extinction on day 2 (i.e. decreased freezing time) could suggest an improper consolidation of fear memory (see Figure 3b, right panel). So on day 3, we assessed extinction recall and fear renewal. Both groups displayed recall freezing times similar to their pre-extinction levels, suggesting sub-optimal extinction recall. However, in both conditions, CNP1\textsuperscript{KO} mice display significantly lower freezing compared with WT animals, a pattern similar to results during the extinction phase. Data are displayed as the mean percent freezing in context B or A during exposure to the conditioned tone (5 total exposures). Asterisks represent between genotype comparisons. Data represent mean±SEM (n=8-12/group). **p<0.01, ***p<0.001.
Figure C.15. Correlation between WT-UCMS and KO-UCMS genes. Genes significantly changed in WT mice exposed to UCMS correlate with genes changed in Cnp1KO mice exposed to UCMS (p<0.01 and 30% effect size), indicating an overall conservation of stress-associated gene changes. R=0.72, p=4.67x10^-18.
Supplementary Table 1. Schedule of UCMS stressors and behavioral tests. For descriptions of stressors, see supplementary methods (above). EPM=elevated plus maze; OF=open field; NSF=novelty suppressed feeding; FST=forced swim test. Individual stressors are color coded. Multiple simultaneous stressors are coded green.

<table>
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Table C.2. List of 114 probesets found to be significantly changed in Cnp1<sup>KO</sup> vs. WT mice. Genes are ordered by p-value. Alr=average log ratio.

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<th>Gene Symbol</th>
<th>Gene Title</th>
<th>Genotype Effect (KO vs. WT)</th>
<th>Post-Hoc Comparison (KO-control vs. WT-control)</th>
<th>Post-Hoc Comparison (KO-UCMS vs. WT-UCMS)</th>
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178
| Tmem1 0 | transmembrane protein 10 | 1.103 | 6.76E-16 | 1.016 | 1.24E-07 | 0.00E+00 | 2.26E-09 | 1.185 | 6.22E-07 | 0.00E+00 | 1.92E-11 |
| Tmem1 25 | transmembrane protein 125 | 0.805 | 9.05E-14 | 0.783 | 4.13E-08 | 0.00E+00 | 3.96E-08 | 0.822 | 1.41E-06 | 0.00E+00 | 7.34E-09 |
| Trem2 | triggering receptor expressed on myeloid cells 2 | 0.698 | 8.75E-15 | 0.616 | 1.31E-06 | 0.00E+00 | 6.88E-08 | 0.773 | 6.81E-09 | 0.00E+00 | 1.19E-10 |
| Tspan2 | tetraspanin 2 | 0.953 | 1.51E-16 | 0.943 | 2.45E-09 | 0.00E+00 | 1.80E-10 | 0.959 | 5.53E-09 | 0.00E+00 | 6.66E-11 |
| Tyrobp | TYRO protein tyrosine kinase binding protein | 0.732 | 5.52E-16 | 0.686 | 7.76E-08 | 0.00E+00 | 3.12E-09 | 0.772 | 1.11E-07 | 0.00E+00 | 7.57E-11 |
5.0 REFERENCES


downregulation of the ubiquitin cycle and alterations in synaptic genes." Mol Psychiatry 11(10): 965-78.


