THE ROLE OF DENDRITIC BDNF TRANSCRIPTS ON ALTERED INHIBITORY CIRCUITRY IN AGING AND DEPRESSION

by

HYUNJUNG OH

B.S. in Biology, Yonsei University, 2001

M.S. in Biology, Yonsei University, 2003

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SCHOOL OF MEDICINE

This dissertation was presented

by

Hyunjung Oh

It was defended on

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and approved by

Dr. Charles W. Bradberry, Professor, Psychiatry

Dr. Linda Rinaman, Professor, Neuroscience

Dr. Teresa G. Hastings, Associate Professor, Neurology, Neuroscience

Dr. Kirk I. Erickson, Associate Professor, Psychology

Dr. Francis S. Y. Lee, Professor, Psychiatry, Weill Cornell Medical College

Dissertation Advisor: Dr. Etienne Sibille, Associate Professor, Psychiatry

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Hyunjung Oh, M.S., Ph.D.

University of Pittsburgh, 2015

Low neurotrophic support and a GABA deficit have been suggested as mechanisms underlying structural and functional abnormalities of the brain in depressed subjects. A parallel downregulation of brain-derived neurotrophic factor (BDNF) and GABA function-related genes including somatostatin (SST), a marker of GABA interneurons targeting the dendritic compartment of pyramidal cells, has been consistently observed during normal aging and in major depression. Here, translational research combining cell culture, animal and human postmortem studies has been conducted in search of a possible link between BDNF and GABA interneurons.

I found that dendritic-targeting interneuron markers displayed a higher BDNF dependency compared to other GABAergic genes in BDNF-knockdown mice. To explore the nature and extent of the biological components linking BDNF and SST, we analyzed the top 200 genes positively correlated with BDNF expression in the human brain and found that age-related BDNF reduction may induce synaptic alterations which are likely responsible for age-associated cognitive decline.

Interestingly, SST and the α5 subunit of GABAA receptor (GABRA5), a subunit considered to be enriched in the post-synaptic compartment of SST (+) interneurons, are included in the top 200 genes, with GABRA5 displaying the highest correlation with BDNF expression among ~ 300,000 probes examined with the arrays. These data suggest that

the synaptic target of SST (+) interneurons, the distal dendrite, may act as a bridge between BDNF and SST. Therefore, we hypothesized that MDD is associated with reduced dendritic BDNF which results in low BDNF supply to SST (+) interneurons. Indeed, 3' untranslated region (UTR)-containing-BDNF mRNA, which is known to migrate to the distal dendrites of pyramidal cells, showed downregulation in the dorsolateral prefrontal cortex (dlPFC) of depressed subjects and medial prefrontal cortex of stressed mice. Furthermore, such changes were closely linked to changes in dendritic-targeting interneuron markers. Knockdown of BDNF long 3' UTR was sufficient to induce dendritic shrinkage, depressive-/anxiety-like behavior, and SST downregulation. Finally, pharmacological potentiation of TrkB prevented the development of depression-like behaviors following chronic stress in rodents.

Together, I provide a mechanistic link between the GABA and neurotrophic hypotheses of major depression, which data indicate may be through dysfunctional dendritic-targeting interneuron populations.

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PREFACE

First and foremost, I would like to express my gratitude to my dissertation advisor, Dr. Etienne Sibille. As a great scientist, he continuously stimulated me to look at the data from a different point of view and to see the big picture. As a great mentor, he patiently guided me through the process of learning to write and present scientifically. Also, he was always there to provide necessary help whenever I encountered a problem that left me confused and frustrated. I feel very fortunate to have been under his tutelage during my graduate training.

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Finally, I would like to thank my family and friends. Without their unconditional support and love, I would not be able to go through hard times during these years and I will always be thankful for having them in my life.

LIST OF ABBREVIATIONS

sgACC: subgenual anterior cingulate cortex

AD: antidepressant

AAV: adeno-associated virus

BDNF: brain-derived neurotrohpic factor

CCK: cholecystokinin

CORT: cortistatin

CALB2: calretinin

CT: cookie test

DBS: deep brain stimulation

DLG: disks large homolog

dlPFC: dorsolateral prefrontal cortex

DSM: diagnostic and statistical manual of mental disorders

EPM: elevated plus maze

FACS: fluorescence activated cell sorting

GABA: gamma-aminobutyric acid

GAD: glutamic acid decarboxylase

KD: knockdown

KO: knockout

MDD: major depressive disorder

mPFC: medial prefrontal cortex

NPY: neuropeptide Y

NSF: novelty suppressed feeding

OF: open field

PMI: post-mortem interval

PV/PVALB: Parvalbumin

qPCR: quantitative polymerase chain reaction

RIN: RNA integrity number

SNRI: selective noradrenaline reuptake inhibitor

SSRI: selective serotonin reuptake inhibitor

SST: somatostatin

TMS: transcranial magnetic stimulation

TrkB: tropomyosin receptor kinase B

UCMS: unpredictable chronic mild stress

UTR: untranslated region

vGAT: vesicular GABA transporter

vGLUT: vesicular glutamate transporter

1.0 INTRODUCTION

1.1 MAJOR DEPRESSIVE DISORDER

1.1.1 Overview: epidemiology, economic burden, diagnosis and treatment

Major depressive disorder (MDD) is the most common psychiatric disease affecting 350 million people worldwide (WHO, 2012). Lifetime prevalence of the disease is 17 % (Kessler et al., 2005), 7 % of adults aged 18 or older reported that they had at least one major depressive episode in the past year (Substance Abuse and Mental Health Services Administration, 2013). MDD is one of the leading cause of disability (WHO, 2012), and costs the US more than 200 billion dollars annually (Greenberg et al., 2015).

To date, no reliable biomarkers have been discovered for the diagnosis and prognosis of MDD. MDD is diagnosed when a person experiences depressed mood or anhedonia, and five or more other symptoms including diminished ability to think or concentrate, feelings of worthlessness, recurrent thoughts of death, weight changes, sleep disturbance, or fatigue for at least 2 weeks (Diagnostic and Statistical Manual of Mental Disorders, 5th Edition, American Psychiatric Association, 2013). The majority of MDD patients also suffer from additional co-morbid mental disorders such as anxiety (57%), substance abuse (30%) and personality disorder (44%) (Davis et al., 2008; Melartin et al., 2002).

Pharmacotherapy is usually offered as an initial treatment for mild-to-moderate MDD. Pharmaceutical action of common antidepressants, such as selective serotonin reuptake inhibitors (SSRIs) and selective serotonin-norepinephrine reuptake inhibitors (SNRIs), works through blocking membrane transporters, and therefore increasing monoamine level in the synaptic cleft. Although a large number of antidepressants are available in the market, significant problems remain to be solved. For example, these treatments may cause a wide range of side effects such as nausea, weight gain, sexual disability, fatigue, insomnia, dry mouth and blurred vision. Moreover, it takes several weeks before a significant improvement in symptoms is reached, and remission rates following 14 weeks of SSRI treatment are only 30 % (Trivedi et al., 2006).

For patients with severe symptoms and/or resistance to antidepressant treatment, electroconvulsive therapy (ECT) and transcranial magnetic stimulation (TMS) are recommended. Nonetheless, ECT causes memory loss in some patients, cannot be used for patients with unstable heart problems due to its effect raising heart rate and blood pressure (American Psychiatric Association, 2008). TMS is reported to produce fewer side effects; however, its effectiveness appears to be lower than that of ECT (Eranti et al., 2007; Padberg and George, 2009). MDD is highly recurrent; at least 50% of patients who achieve remission from the first episode relapse in their lifetime and experience more severe symptoms and a longer duration of illness in recurrent episodes (Burcusa and Iacono, 2007; Ramana et al., 1995).

The heterogeneous symptoms, high comorbidity with other diseases, low treatment efficacy and high relapse rate (Ferguson, 2001; Huynh and McIntyre, 2008; Trivedi et al., 2006) emphasize the need for an increased understanding of the pathophysiology of MDD and the development of better treatments.

1.1.2 Biological mechanisms of MDD

Despite decades of intensive research, the biological mechanisms of MDD are far from clear. Failure of genome-wide association studies (GWAS) to identify genetic risk factors for major depression suggests that the disease results from the sum of multiple small factors rather than one single major deficit. However, there are several hypotheses that have been supported by repeated observations.

1.1.2.1 Monoamine hypothesis

The monoamine theory of MDD, which proposes that depression is caused by a monoamine deficit in the brain, was first introduced following the observation that drugs which deplete extracellular monoamine levels lead to depressive symptoms (Schildkraut, 1965) and further developed during investigations of the mechanism of antidepressant action.

The early antidepressants were discovered by serendipity; isoniazid, a monoamine oxidase inhibitor (MAOI), and imipramine, a tricyclic antidepressant (TCA), were initially developed to treat tuberculosis and psychosis, but clinicians observed their anti-depressant effect in the treated patients. Both compounds increase brain concentrations of monoamines by inhibiting the enzyme breaking down monoamines (MAOI) and inhibiting reuptake of serotonin and noradrenaline as well as blocking diverse neurotransmitter receptors (TCA). Following the discovery that monoamines are taken up presynaptically (Axelrod et al., 1961; Thase et al., 1992), selective monoamine reuptake inhibitors were developed and still commonly used to treat MDD (reviewed in (Prins et al., 2011).

The important role of optimal monoamine transmission on mood regulation is undeniable; however, the fact that current antidepressants need several weeks to months to show

therapeutic effects, despite immediate enhancement of monoamine neurotransmission after drug administration (Nestler et al., 2002), implies that additional mechanisms are involved and long-term adaptations in the brain are essential.

1.1.2.2 Neurotrophic hypothesis

Brain-derived neurotrophic factor (BDNF) is a neurotrophic factor widely expressed throughout the central nervous system including the hippocampal formation, cerebral cortex, thalamus, hypothalamus, amygdala, cerebellar granule cell layer and spinal cord (reviewed in (Edelmann et al., 2014)). It plays an important role in neurogenesis, differentiation, plasticity and survival. The BDNF gene has a complex structure, composed of at least 9 exons including 1 protein-coding and 8-10 non-coding exons (Figure 1) (Aid et al., 2007), as well as two polyadenylation sites (see Chapter 4 for more detailed information of splice variants). Each exon has its own promoter which regulates BDNF expression in response to various environmental factors such as stress (Rasmusson et al., 2002; Smith et al., 1995; Ueyama et al., 1997), enriched environments (Chourbaji et al., 2008; Rossi et al., 2006), diet (Molteni et al., 2002) and exercise (Cotman and Berchtold, 2002; Russo-Neustadt et al., 1999; Russo-Neustadt et al., 2001; Vaynman et al., 2004). These factors are also known to be associated with MDD.

Decreased BDNF expression has been proposed as a mechanism underpinning structural changes of the brains of MDD subjects and stressed animals including cerebral atrophy, dendritic shrinkage, synaptic loss and impaired brain connectivity (Figure 2) (Drevets, 2000; Drevets et al., 1998; Drevets et al., 1997; Kang et al., 2012; Rajkowska, 2000a; Rajkowska et al., 1999; Rajkowska et al., 2007; Stockmeier et al., 2004a; Videbech and Ravnkilde, 2004). This hypothesis is supported by limited, but direct evidence of low BDNF level in blood (Bocchio-Chiavetto et al., 2010), and in postmortem tissue from the hippocampus (Dunham et al., 2009;

Thompson Ray et al., 2011) and amygdala (Guilloux et al., 2012) of MDD patients. Rodent studies provide further evidence that antidepressant treatments restore BDNF levels (Duman and Monteggia, 2006; Duman and Vaidya, 1998; Nibuya et al., 1996; Russo-Neustadt et al., 1999; Russo-Neustadt et al., 2001), that hippocampal infusion of BDNF produces antidepressant effects (Deltheil et al., 2008; Deltheil et al., 2009; Shirayama et al., 2002), and that blockade of BDNF signaling diminishes action of antidepressants (Shirayama et al., 2002).

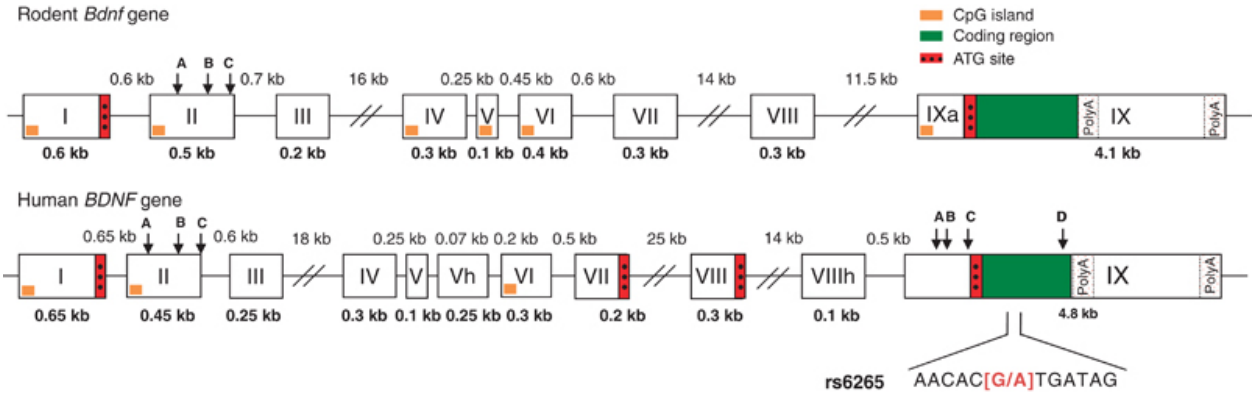

Figure 1. Structure of the human and rodent BDNF genes

Exons are shown as boxes and the introns as lines. Numbers of the exons are indicated in roman numerals and the size of exons and introns are indicated in Arabic numerals. The 3′ coding exon (exon IX) contains two polyadenylation sites (poly A). The red boxes represent the translation start codon (ATG) and the green box shows protein coding region, including the rs6265 genetic variant implicated in the Val66Met polymorphism. Arrows indicate within-exon alternative splice sites (A, B, C, D). CpG islands were predicted with Methprimer software and determined as sequences of at least 200 pairs of bases with a GC percentage greater than 50%. From (Boulle et al., 2012).

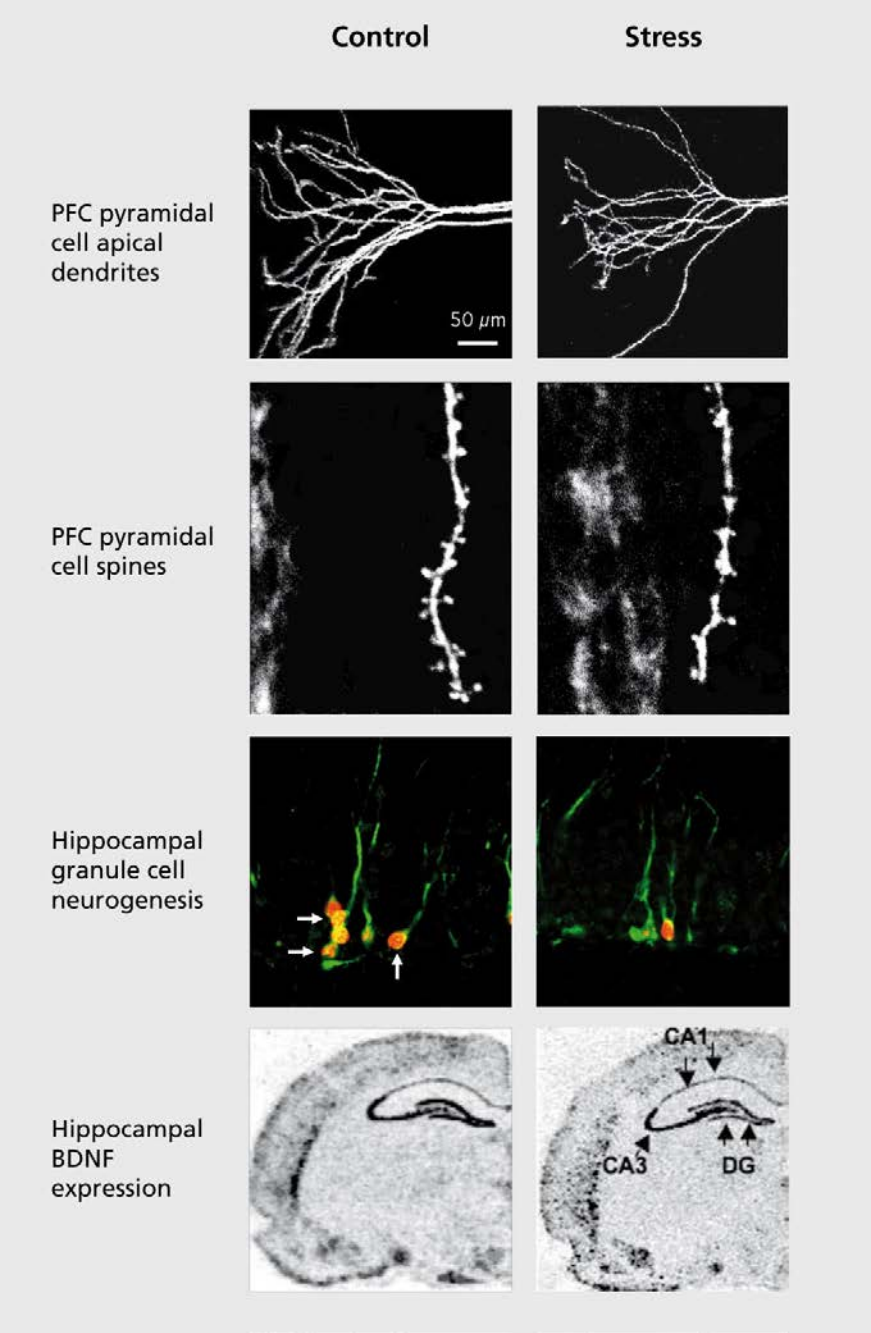

Figure 2. Influence of stress on the morphology and proliferation of neurons and neurotrophic factor expression

Immobilization stress decreases the complexity of pyramidal cell apical dendrites, and the number of spines in the prefrontal cortex (PFC). Stress also decreases neurogenesis and BDNF expression in the adult hippocampus (HP). From (Duman, 2009).

Recent discovery of a single-nucleotide polymorphism (SNP) in BDNF, Val66Met, in which Valine is substituted to Methionine at codon 66, has introduced interesting aspects to this hypothesis. Val66Met is a common polymorphism; allele frequency related to Met varies 18 to 41 % of populations, depending on ethnicity (Shimizu et al., 2004). It has been found that the Met polymorphism is associated with disrupted subcellular translocation, activity-dependent secretion, neurogenesis and reduced dendritic complexity (Bath et al., 2012; Chen et al., 2006; Egan et al., 2003). Consistent with these findings, human studies found cognitive impairment (Bath and Lee, 2006), defective fear extinction (Soliman et al., 2010), poor episodic memory, abnormal activity (Egan et al., 2003; Hariri et al., 2003) and reduced volume of the hippocampus (Bueller et al., 2006; Hajek et al., 2012; Molendijk et al., 2012), prefrontal cortex (Kim et al., 2013; Pezawas et al., 2004), amygdala (Montag et al., 2009) in 66Met carriers.

There are discrepancies between studies investigating the association between Val66Met polymorphism and depression. Nevertheless, given that the combination of genetic and environmental risk factors implicated in the etiology of MDD and the 66Met allele is related to the dysregulation of hypothalamic-pituitary-adrenal axis activity, disrupted BDNF function may increase stress susceptibility rather than directly contribute to the disease (Alexander et al., 2010; Dougherty et al., 2010; Shalev et al., 2009; Yu et al., 2012).

Although there is controversy regarding whether BDNF deficiency is a cause or consequence of MDD, growing evidences indicates that BDNF deficiency and/or disrupted function is associated with many aspects of the disease, as well as stress susceptibility and the therapeutic effect of antidepressants.

1.1.2.3 GABA hypothesis

Lastly, research has suggested that major depression is associated with an impaired excitation and inhibition balance, likely caused by GABA dysfunction. The GABA hypothesis of affective disorders was originally proposed by Emrich et al. based on the mood-stabilizing effect of sodium valproate, a GABAergic anticonvulsant (Emrich et al., 1980). Additionally, human studies reported decreased GABA concentrations in MDD patients in the occipital and frontal cortices, which were reversed after SSRI treatment or ECT (Gabbay et al., 2012; Hasler and Northoff, 2011; Hasler et al., 2007; Levinson et al., 2010; Sanacora et al., 2003; Sanacora et al., 2002). Animal studies revealed a close linkage between diminished GABA transmission and behavioral deficits (Earnheart et al., 2007; Guidotti et al., 1985; Lloyd et al., 1983; Martin et al., 1989; Petty and Sherman, 1980, 1981; Poncelet et al., 1987).

GABA interneurons projecting to pyramidal neurons can be classified into two major groups based on their synaptic targets: (1) perisomatic- or (2) dendritic-targeting (Figure 3). Each interneuron population can be further subdivided by their molecular markers. Fast-spiking, perisomatic-targeting interneurons express parvalbumin (PV) and control spike timing of the output neurons (Ali and Thomson, 2008; Kawaguchi and Kubota, 1997; Muller et al., 2007). SST-expressing interneurons, including CORT- and NPY- positive populations, target the dendritic compartments of pyramidal cells and provide fine-tuning (Lin and Sibille, 2013). Consistent with reduced density of GABA interneurons in depressed brains (Guilloux et al., 2012; Rajkowska et al., 2007), we observed depression-related downregulation of dendritic-targeting interneuron markers in diverse corticolimbic areas including the dorsolateral prefrontal cortex, amygdala, and subgenual anterior cingulate cortex (sgACC) (Guilloux et al., 2012; Sibille et al., 2011; Tripp et al., 2011; Tripp et al., 2012). The markers of perisomatic-targeting

interneurons (CCK and PV) were relatively unaffected. Using a mouse model of depression, we showed that chronic stress produced a reduction in SST expression and most importantly, knockout of SST was sufficient to induce depressive-/anxiety-like phenotype in mice (Lin and Sibille, 2015).

Studies have reported alterations in SST-positive dendritic-targeting GABA neurons across neurological disorders and normal aging, implying that these neurons might be highly susceptible to various biological challenges. While we found some clues that the selective changes in SST are related to low BDNF signaling (Guilloux et al., 2012; Tripp et al., 2012), detailed mechanisms have not yet been elucidated (see Chapter 4 for in-depth discussion).

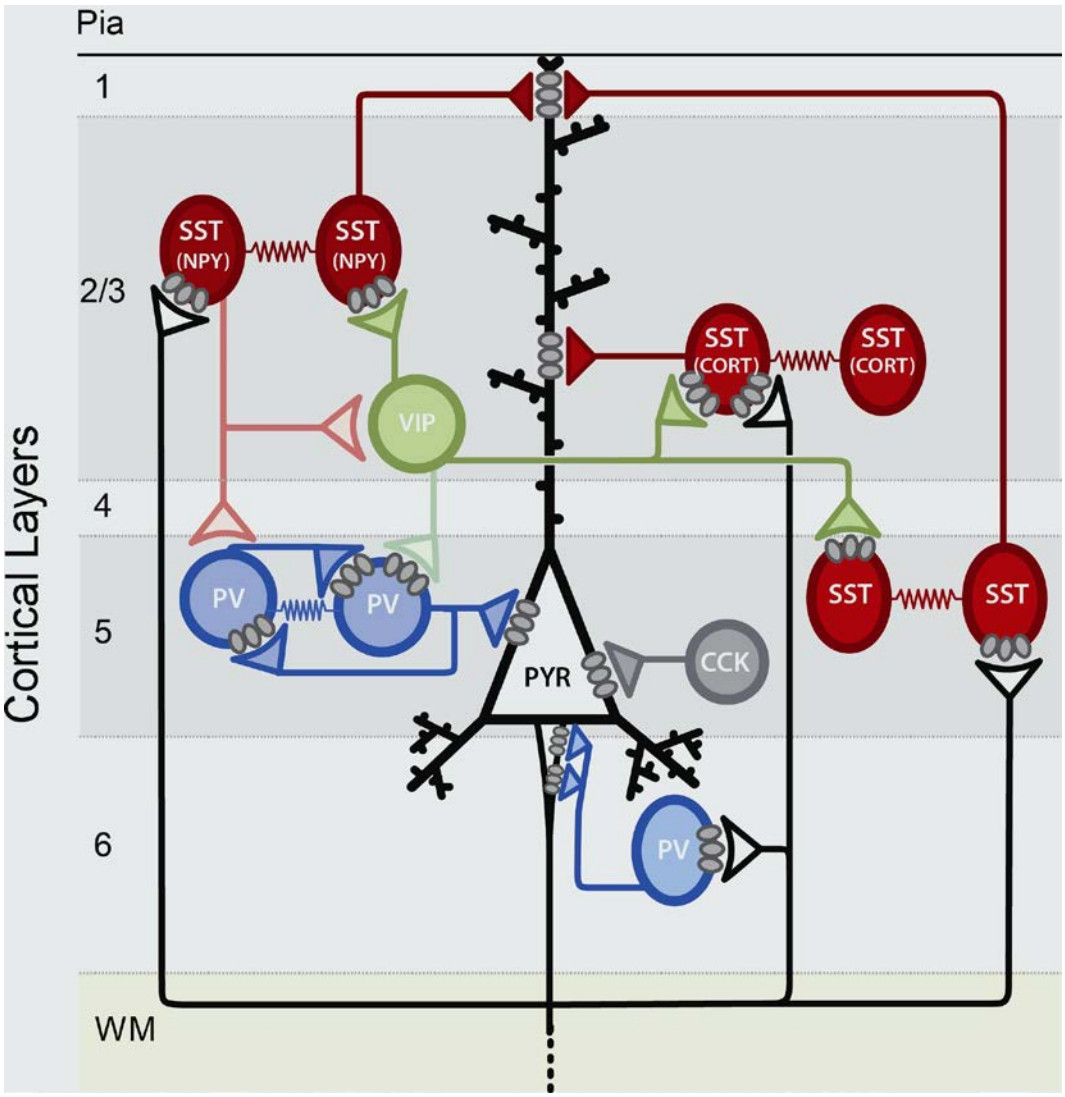

Figure 3. GABA microcircuitry

GABA neurons expressing somatostatin (SST), neuropeptide Y (NPY), and cortistatin (CORT) innervate and inhibit the distal dendrites of pyramidal neurons (PYR). Parvalbumin (PV) and cholecystokinin (CCK)-expressing interneurons target the cell body and axon initial segment, and vasoactive intestinal polypeptide (VIP)-expressing neurons target other GABA neurons. From (French et al., 2014).

1.1.3 UCMS: an animal model of MDD

Although there are concerns about modeling a complex disease such as major depression in a rodent, it is undeniable that rodent models can provide great insights into the pathology of the disease. Models allow us to investigate molecular changes in the brain at a crucial time point and to examine the causality relationship between depression and various genetic and environmental factors. Furthermore, proper animal models are essential for the development and testing of novel therapeutic strategies for MDD.

Unpredictable chronic mild stress (UCMS) is an animal model of depression commonly used in the field. The UCMS procedure consists of exposing rodents to a variety of randomized environmental and social stressors (e.g. light/dark cycle changes, predator scent, tilted cages, wet/no bedding, and cage mate changes) for several weeks. Publications report that animals exposed to this protocol show MDD-like physiological and behavioral changes such as fur degradation and weight loss, decreased intake of palatable food (interpreted as anhedonia-like behavior), decreased sexual behaviors, and changes in sleep patterns (reviewed in (Willner, 2005)). Importantly, most of these behavioral changes can be reversed by chronic antidepressant treatment (Surget et al., 2008; Surget et al., 2009; Willner et al., 1992; Willner et al., 1987).

1.1.4 Interaction of MDD and brain aging

Age is one of the most potent risk factors for neurological disorders. Many neurodegenerative and psychiatric disorders have a typical range of age-of-onset: schizophrenia and bipolar disorder typically begin in early adulthood, autism is often first identified in toddlers, and Alzheimer's and Parkinson's disease are late-onset.

The effect of aging on clinical depression is controversial (Blazer, 1994; Glaesmer et al., 2011; Kessler et al., 2010). The incidence of major depression in the community of individuals over the age of 65 is reported between 1% and 5% (Fiske et al., 2009), which is lower than that of young adults; however, studies using different rating scales demonstrate different results. The anhedonia-focused depression rating scale, Hospital Anxiety and Depression Scale (HADS-D), demonstrated a significant increase of incidence of depression in the community-dwelling elderly (Solhaug et al., 2012), and 15-30% of elderly individuals experience various depressive symptoms (Glaesmer et al., 2011; Koenig and Blazer, 1992; McKinney and Sibille, 2012). Overall, it is apparent that sub-threshold depression and depressive symptoms are more common in later life (Beekman et al., 1995). This may not be surprising given that the elderly population is more likely to experience negative life events such as age-related illnesses (Alzheimer's disease, Parkinson's disease, arthritis), social isolation because of death of a spouse and retirement, and cognitive or physical disability. Although 65- to 75-percent of late-life depression is treatable, elderly people sometimes accept depression as a normal part of aging.

On the other hand, major depression is associated with an increased risk of developing cardiovascular diseases, dementia, diabetes and other diseases commonly observed in later life. This notion led to a hypothesis that MDD might accelerate aging (Verhoeven et al., 2014; Wolkowitz et al., 2010; Wolkowitz et al., 2011c). Interestingly, telomere length, considered a cellular marker of biological age, has been found shorter in MDD patients (Hartmann et al., 2010; Hoen et al., 2011; Lung et al., 2007; Simon et al., 2006; Verhoeven et al., 2014; Wikgren et al., 2012; Wolkowitz et al., 2011a).

Accumulating evidence suggests the potential interactive effects of stress and aging on the vulnerable neuronal population (Figure 4). Mood symptoms often precede the onset of

neurodegenerative disorders (Jost and Grossberg, 1996; Tolosa et al., 2007). Similar to MDD, decreased brain volume, synaptic loss, and functional impairment of corticolimbic areas, including the dlPFC, have been observed in older subjects who do not show obvious neurological disease symptoms (Arnsten, 2009b; Bloss et al., 2011; Cook and Wellman, 2004; Holmes and Wellman, 2009; McEwen and Gianaros, 2011; Morrison and Baxter, 2012; Radley et al., 2004; Wang et al., 2011). Human postmortem studies from our group found a profound overlap between normal aging- and depression-related gene expression changes (Douillard-Guilloux et al., 2013; Erraji-Benchekroun et al., 2005; Glorioso et al., 2011; Glorioso and Sibille, 2011) including downregulation of SST and BDNF. Animal studies reported that both stress and aging impair the same class of spines, i.e., thin, highly plastic spines (Bloss et al., 2010; Bloss et al., 2011; Dumitriu et al., 2010). Interestingly, it seems that the effect of stress on molecular, anatomical and functional changes is augmented in older animals (Juster et al., 2010; Lupien et al., 2009; McEwen and Morrison, 2013). Aged animals display higher basal and post-stress corticosterone levels compared to young animals (Issa et al., 1990), and fail to restore dendritic complexity in the absence of stress (Bloss et al., 2010), thus indicating a loss of cellular resilience. Together, these results suggest that the emotion regulatory system may be selectively affected by early homeostatic changes during normal aging (further review is available in Appendix A). Understanding the underlying mechanisms in addition to the possible moderators and effectors of normal brain aging will provide important insights into pathological declines in brain function with age.

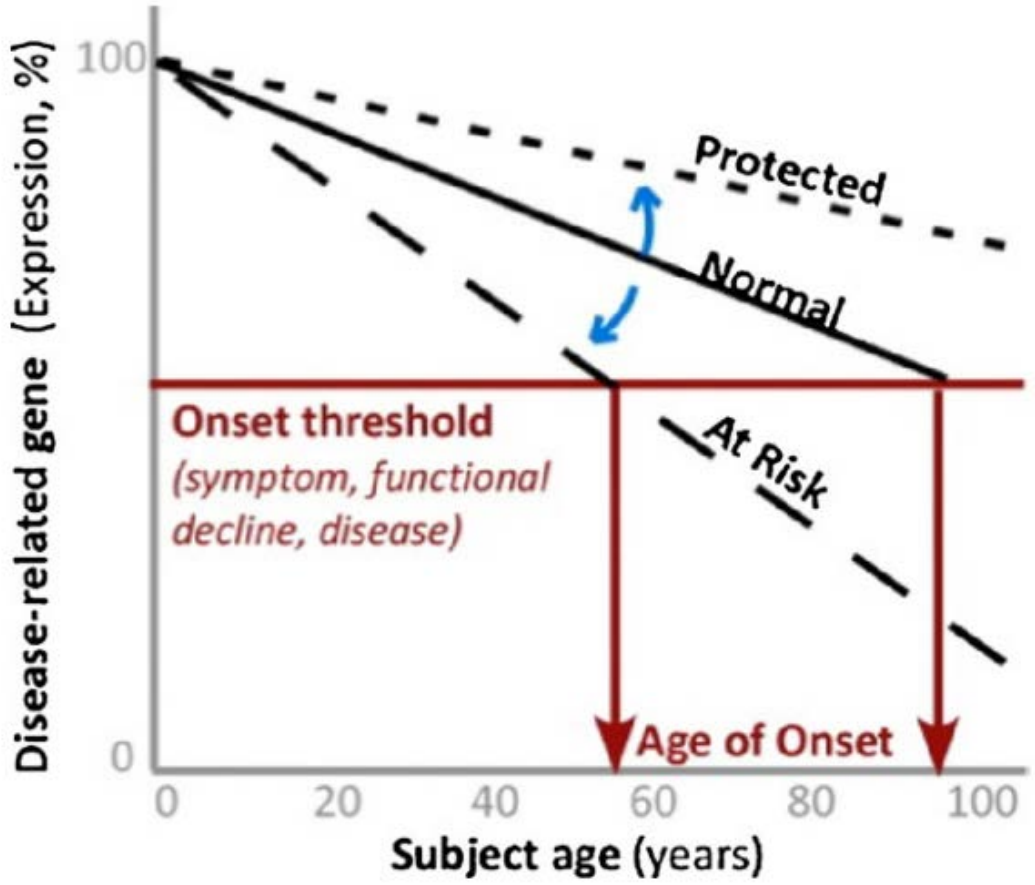

Figure 4. A proposed model of age-by-disease interaction

Reduction of disease-related genes below threshold (horizontal red line) marks the onset of symptoms. Changes in the trajectory of age-related changes of disease-related genes (Y-axis) determine at what age (X-axis), or if, an individual may develop symptoms (vertical red arrows). Modulators (blue arrows) may thus place individuals on ‘‘at risk’’ or ‘‘protected’’ trajectories. From (Glorioso and Sibille, 2011), originally from (Glorioso et al., 2011).

1.2 AIMS OF THIS DISSERTATION

Impaired structural and functional connectivity, GABA deficits, and selective downregulation of dendritic-targeting interneuron markers have been repeatedly observed in major depression. The

aim of this dissertation is to identify a mechanistic link between these different phenomena by focusing on the well-known neurotrophic factor BDNF. As a starting point, I determined the degree of BDNF dependency of GABA-related genes in the cingulate cortex of transgenic mice with low BDNF expression, and compared the findings with human postmortem MDD brains (Chapter 2). In order to (1) find a possible link between BDNF and SST and (2) assess how normal age-related BDNF changes contribute to transcriptome remodeling - which may provide an important insight into the early stage of pathological declines in brain function - I identified genes potentially regulated by BDNF in the prefrontal cortex of control subjects and directly addressed the causality question via temporal inhibition of BDNF/TrkB signaling (Chapter 3). Finally, I investigated a mechanism underlying high BDNF dependency of SST (+) dendritic-targeting interneurons and tested the antidepressant effect of a TrkB agonist, 7,8-dihydroxyflavone (Chapter 4).

All of my research efforts across correlative (human) and causative (cell and mouse) studies have provided (i) evidence for the interactive influence of aging and stress on depression, (ii) the biological mechanism underlying the high vulnerability of dendritic-targeting interneurons that is widely observed in stress- and aging-related neurological disorders, and (iii) targets for the development of preventive interventions.

2.0 PAPER 1: BRAIN-DERIVED NEUROTROPHIC FACTOR SIGNALING AND SUBGENUAL ANTERIOR CINGULATE CORTEX DYSFUNCTION IN MAJOR DEPRESSION

Adam Tripp[1, 2], Hyunjung Oh[1,2,3], Jean-Philippe Guilloux[4], Keri Martinowich[5,6], David A. Lewis[2,3], Etienne Sibille[2,3§]

[1]denotes co-first authorship, both contributed equally to this manuscript

[2]Department of Psychiatry, University of Pittsburgh, PA 15312, USA

[3]Center For Neuroscience, University of Pittsburgh, PA 15312, USA

[4]Université Paris-Sud EA 3544, Faculté de Pharmacie, Châtenay-Malabry cedex F-92296, France

[5]Genes, Cognition and Psychosis Program, National Institute of Mental Health, Bethesda, MD 20892, USA

[6]Lieber Institute for Brain Development, Johns Hopkins Medical Campus, Baltimore, MD 21205, USA

[§]To whom correspondence should be addressed. E-mail:sibilleel@upmc.edu

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2.1 INTRODUCTION

Major depressive disorder is a debilitating disorder of low affect and altered mood regulation that affects approximately 17% of the population at some point in life, resulting in serious personal, social, and economic burdens (Kessler et al., 2003). The prevalence of major depressive disorder is two times higher in women than in men. Female patients with the disorder tend to have higher symptom numbers, a more severe type of depression, and greater risk of recurring episodes compared with male patients, but the underlying biological vulnerabilities have not been characterized (Perugi et al., 1990). Changes in the structure, function, and coordinated activity of several brain regions may underlie impaired mood regulation in depression (Seminowicz et al., 2004). Increased metabolic activity in one of these regions, the subgenual anterior cingulate cortex, has been consistently reported in the induction of the depressive state, and subgenual anterior cingulate cortex metabolism is reversed by pharmacological treatment (Mayberg et al., 2000) and deep brain stimulation (Mayberg et al., 2005).

Low neurotrophic support in limbic brain regions has been proposed as a unifying hypothesis for the reduced density or cell numbers in the frontal cortex (Rajkowska et al., 2001) and amygdala (Bowley et al., 2002) and the reduced hippocampal volume observed in individuals with major depression (Videbech and Ravnkilde, 2004). Rodent studies have demonstrated that various antidepressant treatments increase brain-derived neurotrophic factor (BDNF) expression (Schmidt and Duman, 2007), and BDNF infusion into the hippocampus is sufficient to produce an antidepressant-like effect (Shirayama et al., 2002). Despite abundant animal studies supporting the close relationship between BDNF and depression, direct evidence in humans is limited to reports of low circulating peripheral BDNF levels, which are normalized by antidepressant treatment (Molendijk et al., 2011), and studies demonstrating reduced pro-

BDNF and BDNF levels in the postmortem amygdala of depressed female subjects (Guilloux et al., 2012) and in the hippocampal tissue in depressed patients (Dunham et al., 2009; Thompson Ray et al., 2011). Additionally, studies have reported that individuals who die by suicide exhibit low hippocampal and midbrain BDNF levels (Dwivedi et al., 2003), reduced activity-dependent BDNF expression by hypermethylation of promoter/exon IV of the BDNF gene (Keller et al., 2010), and, in carriers of the BDNF Met allele, increased risk for violent suicide (Perroud et al., 2008; Pregelj et al., 2011), together providing additional evidence that BDNF has a role in the psychopathology of major depression.

In parallel, human imaging and basic science studies have suggested excitation/inhibition impairment in individuals with major depression that is potentially mediated by decreased GABA content (Luscher et al., 2011). We recently reported down-regulation of several GABA-related genes in the dorsolateral prefrontal cortex (Sibille et al., 2011), subgenual anterior cingulate cortex (Tripp et al., 2011), and amygdala (Guilloux et al., 2012) in patients with major depression, potentially affecting somatostatin-positive dendritic targeting interneurons. We further demonstrated that a set of amygdala-related gene changes (affecting the TAC1, CORT, NPY, SST, RGS4, and SNAP25 genes [Table 1]) correlate with reduced BDNF expression in depressed patients and in mice with reduced BDNF function, hence identifying a pattern of reduced BDNF-dependent gene expression in major depression (Guilloux et al., 2012) and providing supporting evidence for a link between the neurotrophic (Schmidt and Duman, 2007) and GABA (Luscher et al., 2011) hypotheses implicated in depression.

In the present study, we investigated molecular evidence for a low BDNF and reduced GABA function pathway in the subgenual anterior cingulate cortex in individuals with major depressive disorder. We first tested the degree of BDNF dependency on gene expression of a set

of GABA- and BDNF-related genes in the cingulate cortex of mice with reduced BDNF function. Our choice of genes was determined by our previous study of the amygdala in depressed postmortem subjects (Guilloux et al., 2012) to enable comparative analyses across the two studies. Based on this information, we assessed changes in three sets of genes, with high, intermediate, or no BDNF dependency, in human subgenual anterior cingulate cortex using postmortem brain samples from a large cohort of subjects with major depressive disorder and matched comparison subjects. Exploratory analyses were performed on putative sex differences in expression patterns, given the greater female vulnerability and the previous findings of more robust somatostatin down-regulation in women with major depression (Tripp et al., 2011).

2.2 MATERIALS AND METHODS

2.2.1 Human postmortem subjects

After consent from the next of kin, brain samples were obtained during autopsies performed at the Allegheny County Medical Examiner's Office (Pittsburgh) using procedures approved by the University of Pittsburgh Institutional Review Board and the Committee for Oversight of Research Involving the Dead. Consensus DSM-IV diagnoses were made by an independent committee of experienced clinical research scientists using information from clinical records, toxicology results, and standardized psychological autopsies (Glantz et al., 2000). Fifty-one pairs of subjects were analyzed. Each pair consisted of one subject with major depressive disorder and a comparison subject matched for sex; group means for age, postmortem interval, and brain pH were nearly identical (see Table 5 in the Appendix B). Subgenual anterior cingulate cortex

samples containing all six cortical layers were harvested from coronal sections as described elsewhere (Sibille et al., 2009b).

2.2.2 Mice

BDNF-heterozygous ($Bdnf^{+/-}$) mice (3–4 months old) were bred on a mixed S129/Sv×C57BL/6 genetic background (Korte et al., 1995). BDNF exon-IV knockout ($Bdnf^{KIV}$) mice were crossed on C57BL/6 as described elsewhere (Sakata et al., 2009). All mice were maintained under standard conditions (i.e., in a 12/12-hour light-dark cycle, in 22°C [SD=1], and with food and water ad libitum). Brains were rapidly removed and flash frozen on dry ice. The left and right cingulate cortices were micropunctured using 0.5-mm diameter punches (Sibille et al., 2009b) and stored in TRIzol (Invitrogen, Carlsbad, Calif.) at –80°C. All animal care and treatment was in accordance with the Guide for Care and Use of Laboratory Animals established by the National Institutes of Health.

2.2.3 Real-time quantitative polymerase chain reaction (PCR)

Total RNA was isolated from TRIzol homogenates of the subgenual anterior cingulate cortex in all 51 pairs of postmortem subjects (major depression and comparison subjects) and of the cingulate cortex in rodents. The samples were purified using RNeasy spin columns (Valencia, Calif.), and RNA integrity was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Walbronn, Germany). To generate cDNA, 1 μg total RNA was mixed with oligo-dT primers and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, Calif.) per the manufacturer's protocol. PCR products were amplified in quadruplets on a Mastercycler real-

time PCR machine (Eppendorf, Hamburg, Germany) using universal PCR conditions as described elsewhere (Tripp et al., 2011). Results were calculated as the geometric mean of threshold cycles normalized to three validated internal controls (actin, glyceraldehyde-3-phosphate dehydrogenase, and cyclophilin G).

2.2.4 Gene selection

We assessed mRNA expression of several genes that we previously demonstrated to be affected in the amygdala in individuals with major depression and that display various levels of BDNF dependency, based on expression levels in the amygdala in the same strains of mice with reduced BDNF functions ($Bdnf^{+/-}$ and $Bdnf^{KIV}$) used in the present study (Korte et al., 1995; Sakata et al., 2009). Our set includes genes related to BDNF signaling (BDNF and TRKB), BDNF-dependent genes (CORT, NPY, SST, VGF, TAC1, SNAP25, and RGS4), and GABA-associated genes (GAD1, GAD2, GABRA1, SLC6A1, CALB2, PVALB, SST, NPY, and CORT) (Table 1).

2.2.5 Protein isolation and BDNF quantification

Following RNA extraction, acetone precipitation of proteins was carried out from the TRIzol samples, and Western blot analysis was performed as described elsewhere (Sibille et al., 2009b). Dual signals were detected using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, Neb.), and BDNF signal ratios to actin were calculated. Samples were processed in matched pairs on the same gel, and results were replicated for a total of three different Western blots. Test assays were run with 5–50 μg of total protein using the following antibodies: mouse antihuman BDNF (R&D Systems, Minneapolis), antihuman TrkB (sc-8316; Santa Cruz

Biotechnology, Santa Cruz, Calif.), and antiactin (A2228; Sigma-Aldrich, St. Louis). We also measured BDNF using an enzyme-linked immunosorbent assay (ELISA) kit (Promega, Madison, Wisc.).

2.2.6 Statistical analysis

Differences in diagnosis-dependent gene expression were determined by analysis of covariance (ANCOVA) using SPSS (SPSS, Chicago). To determine which covariates to include in the gene-specific models, each nominal factor was tested as the main factor using analysis of variance (ANOVA), scale covariates were tested using Pearson's correlation, and repeated measures were corrected using the modified Holm-Bonferroni method (see Table 6 in the Appendix B). Samples were analyzed for the presence of antidepressant medication at the time of death. Given the small number of observations (N=9), benzodiazepines were not formally analyzed. No other psychotropic medications were present at the time of death. ANCOVA models, including significant cofactors, were then applied to the combined and sex-specific analyses. For the rodent analyses, ANOVA models with sex as a cofactor were applied to the combined analysis, and unpaired two-tailed t tests were performed in the separate male and female analyses. Values were then adjusted across genes for multiple testing using a second modified Holm-Bonferroni test (i.e., false discovery rate-corrected). Uncorrected p values for diagnosis effects are listed in Table 1.

2.3 RESULTS

2.3.1 BDNF-dependent gene expression in the cingulate cortex in mice with altered BDNF function

Since BDNF expression and function vary significantly depending on brain regions, sex, and cellular activity, we first tested the role of BDNF in regulating the expression of the genes of interest in the cingulate cortex of mice that were heterozygous for a constitutive deletion of the Bdnf gene ($Bdnf^{+/-}$) or that had a targeted disruption of exon IV ($Bdnf^{KIV}$), with the latter mutation resulting in blockade of activity-dependent BDNF protein expression (Sakata et al., 2009). Bdnf mRNA levels were reduced in $Bdnf^{+/-}$ mice (Table 1). When the groups were separated by sex, this decrease reached statistical significance only in male mice, potentially reflecting reduced analytical power in smaller groups. Bdnf mRNA levels were also reduced in $Bdnf^{KIV}$ mice, in both the combined and sex-specific groups (Table 1). These latter results reveal that constitutive and activity-dependent functions of BDNF are not only reduced in the cingulate cortex in $Bdnf^{KIV}$ mice, which is consistent with an independent study of the same $Bdnf^{KIV}$ mouse strain reporting activity-dependent $Bdnf^{KIV}$ promoter-driven transcription abolishment in the cortex (Martinowich et al., 2011), but they are also significantly reduced in basal transcription by promoters I, II, III, VI, and IXa. The levels of Trkb, the main receptor of Bdnf, were not affected in the two mouse models.

Using the $Bdnf^{+/-}$ and $Bdnf^{KIV}$ mouse models, we next investigated the degree of BDNF regulation on target genes (defined as BDNF dependency) within the cingulate cortex. The quantitative PCR (qPCR) analysis revealed robust and significant decreases in gene transcript levels for Cort, Vgf, Sst, Tac1, and Npy expression (false discovery rate-corrected significance

in the combined male-female group in at least one strain of mice) but less robust effects on Snap25 and Gad2 expression (uncorrected significance in the combined male-female group in at least one strain of mice) and little or no effect of decreased BDNF function on Gad1, Pvalb, Rgs4, Gat1, and Gabra1 expression (no difference in the combined male-female group and uncorrected significance in the separate sex groups in one strain of mice or no change observed at all). Combined, these findings provided us with three sets of genes with a gradient of BDNF dependency in the cingulate cortex. Results of this analysis are summarized in Table 1.

Table 1. Assessment of Brain-derived neurotrophic factor (BDNF) dependency on target gene expression in Bdnf $^{+/-}$ and Bdnf^{KIV} mice[a]

		Bdnf$^{+/-}$ Mice					
		Combined Male-Female		Male		Female	
Gene Code	Gene Name	Average Log Ratio[b]	p	Average Log Ratio[b]	p	Average Log Ratio[b]	p
Bdnf-IX	Brain-derived growth factor	−0.56†	0.02†	−0.74†	0.01†	−0.34	0.17
Trkb (*Ntrk2*)	Tyrosine kinase receptor	0.03	0.43	−0.01	0.48	0.07	0.34
High BDNF dependency							
Cort	Cortistatin	−0.68‡	0.001‡	−0.64‡	0.002‡	−0.72†	0.04†
Vgf	Neurotrophic growth factor inducible	−0.64‡	0.001‡	−0.56†	0.04†	−0.74‡	0.002‡
Sst	Somatostatin	−0.59‡	0.003‡	−0.41†	0.01†	−0.80†	0.004†
Tac1	Protachykinin-1	−0.73‡	5.1E-05‡	−0.66‡	0.002‡	−0.81‡	0.001‡
Npy	Neuropeptide Y	−0.55†	0.01†	−0.45†	0.05†	−0.68†	0.05†
Intermediate BDNF dependency							
Snap25	Synaptosomal-associated protein 25	−0.37†	0.04†	−0.44†	0.04†	−0.29	0.20
Gad2 (*Gad65*)	Glutamate decarboxylase 2	−0.33†	0.04†	−0.54†	0.02†	−0.08	0.32
Low or no BDNF dependency							
Gad1 (*Gad67*)	Glutamate decarboxylase 1	−0.09	0.30	−0.33†	0.03†	0.19	0.25
Pvalb	Parvalbumin	0.03	0.44	−0.35	0.1	0.47	0.07
Rgs4	Regulator of G protein signaling 4	−0.13	0.36	−0.33†	0.04†	0.11	0.45
Slc6a1	GABA transporter 1	−0.31	0.10	−0.42	0.03	−0.17	0.35
Calb2	Calretinin	0.32	0.32	−0.48	0.10	1.25	0.15
Gabra1	GABA-A receptor, alpha 1	0.13	0.16	0.05	0.40	0.23	0.1

[a] Mice heterozygous for a constitutive deletion of the Bdnf gene are identified as *Bdnf*$^{+/-}$ (N=13 [seven were male, and six were female]; comparison mice: N=13 [seven were male, and six were female]), and mice with a targeted disruption of exon IV are identified as Bdnf^{KIV} (N=12 [seven were male, and five were female]; comparison mice: N=12 [seven were male, and five were female]).
[b] Data indicate average log ratio of the experimental group relative to the comparison group.
† Significant at $p<0.05$.
‡ Significant after correction for multiple testing.

Bdnf^{KIV} Mice					
Combined Male-Female		Male		Female	
Average Log Ratio[b]	p	Average Log Ratio[b]	p	Average Log Ratio[b]	p
−0.70†	0.02†	−0.62†	0.03†	−0.80‡	0.001‡
−0.08	0.24	−0.02	0.45	−0.16	0.07
−1.30‡	1.9E-04‡	−0.92†	0.05†	−1.82‡	4.4E-06‡
−0.24	0.10	−0.53†	0.02†	0.15	0.26
−0.60‡	1.4E-04‡	−0.64‡	0.003‡	−0.55†	0.01†
−0.82‡	<0.001‡	−0.70†	0.03†	−1.00‡	4.7E-05‡
−0.42‡	0.001‡	−0.47†	0.01†	−0.34†	0.03†
0.02	0.44	−0.27	0.11	0.43‡	0.004‡
−0.02	0.46	−0.10	0.33	0.10	0.12
0.03	0.39	−0.16	0.12	0.29†	0.05†
−0.09	0.22	−0.19	0.16	0.04	0.39
−0.05	0.32	0.04	0.41	−0.18	0.14
0.10	0.28	−0.10	0.35	0.39†	0.02†
0.20	0.18	0.29	0.20	0.09	0.32
0.00	0.5	0.09	0.23	−0.12	0.22

2.3.2 BDNF-dependent gene expression changes in the subgenual anterior cingulate cortex in postmortem subjects with depression

To evaluate subgenual anterior cingulate cortex BDNF function in the postmortem brain of subjects with major depression and comparison subjects, we used qPCR to measure the mRNA expression levels of BDNF, of the BDNF receptor TRKB, and of the three sets of genes with variable BDNF dependency described above. The qPCR measures were first evaluated for the effect of relevant cofactors to include in the main ANCOVA models for each gene, as described earlier. None of the investigated cofactors displayed consistent effect. The cofactor analyses and ANCOVA inclusions are summarized in Table 6 in the Appendix B.

Results from the ANCOVA analyses for the effects of major depression are summarized in Table 2. Although BDNF mRNA levels were not changed, mRNA levels of the BDNF receptor TRKB were significantly reduced by approximately 30% after correction for multiple

testing. In view of the negative BDNF mRNA finding and the complex regulation of BDNF mRNA at the protein level, we attempted to measure extracts using quantitative Western blot tests and ELISA. However, the pro- and mature forms of BDNF were below detection levels in the subgenual anterior cingulate cortex samples using both approaches. Similarly, the signal-to-background ratio for TrkB protein levels was low in the samples and precluded robust quantification.

Reduced expression was identified for CORT (–33%), SNAP25 (–38%), VGF (–35%), SST (–34%), NPY (–37%), GAD1 (–25%), GAD2 (–28%), and PVALB (–33%). Reduced expression was also observed for TAC1 (–25%) but only at nominal uncorrected significance. The RGS4, GABA transporter 1/SLC6A1, calretinin/CALB2, and GABRA1 genes were unchanged.

When the groups were segregated by sex, BDNF levels remained unchanged, and TRKB was similarly reduced in both male (–29%) and female (–32%) depressed subjects compared with the respective comparison subjects, although results were less robust and only showed nominal uncorrected significance. Overall, male depressed subjects exhibited more robust decreased expression across the gene panels than female depressed subjects compared with the respective comparison subjects. Out of the eight genes displaying false discovery rate-corrected significance in the combined male-female group, six remained significant at false discovery rate-corrected p values and two at uncorrected values in the male cohort, compared with two and three genes, respectively, in the same categories in the female cohort. Out of the four genes showing no effect in the combined group, calretinin/CALB2 was decreased in the male cohort at false discovery rate-corrected significance.

When summarized by BDNF-dependent categories (Table 2), these results suggest a robust decrease in BDNF signaling in the subgenual anterior cingulate cortex in depressed subjects, with changes related to GABA functions also including genes with moderate or low BDNF dependency.

Table 2. Alterations in proximal Brain-derived neurotrophic factor (BDNF) signaling machinery and distal BDNF-dependent genes in the subgenual anterior cingulate cortex of postmortem subjects with major depressive disorder

	Combined Male-Female		Male		Female	
Gene	Average Log Ratio[a]	p	Average Log Ratio[a]	p	Average Log Ratio[a]	p
BDNF	0.04	0.75	0.01	0.95	0.08	0.68
TRKB	−0.52‡	0.003‡	−0.49†	0.03†	−0.55†	0.04†
High BDNF dependency						
CORT	−0.58‡	1.69E-05‡	−0.69‡	0.002‡	−0.48‡	3.00E-03‡
VGF	−0.63‡	0.001‡	−0.81‡	3.76E-04‡	−0.49	0.09
SST[b]	−0.59‡	0.001‡	−0.40†	0.04†	−0.83†	0.009†
TAC1	−0.42†	0.02†	−0.27	0.22	−0.57†	0.02†
NPY	−0.66‡	1.01E-04‡	−0.75‡	0.004‡	−0.58‡	0.002‡
Intermediate BDNF dependency						
SNAP25	−0.69‡	4.53E-0.4‡	−0.98‡	0.001‡	−0.39	0.12
GAD2 (GAD65)	−0.48‡	2.00E-0.3‡	−0.60‡	0.005‡	−0.36	0.11
Low or no BDNF dependency						
GAD1 (GAD67)	−0.41‡	1.00E-03‡	−0.76‡	4.39E-04‡	−0.06	0.66
PVALB (PV)	−0.58‡	0.005‡	−0.76†	0.03†	−0.41†	0.05†
RGS4	−0.42	0.10	−0.22	0.49	−0.63	0.13
SLC6A1 (GAT1)	−0.06	0.82	−0.11	0.74	−0.01	0.98
*CALB2 (*calretinin*)*	−0.13	0.23	−0.41‡	0.001‡	0.16	0.38
GABRA1	0.00	0.97	−0.11	0.21	0.11	0.25

[a] Data indicate average log ratio of the experimental group relative to the comparison group.
[b] Data for *SST* quantitative polymerase chain reaction were reanalyzed from a previous study (21).
† Significant at $p<0.05$.
‡ Significant after correction for multiple testing.

2.3.3 BDNF-dependent mRNA sex differences in comparison postmortem subjects

To investigate potential sources of the discrepancy in altered major depression-related gene expression between male and female subjects, we compared expression differences between male and female comparison subjects (i.e., individuals without psychiatric diagnoses). We found

no sex differences in BDNF or TRKB mRNA expression, but several genes within the investigated panel, including CORT, NPY, CALB2, and TAC1, showed reduced baseline expression in women (Figure 5). This suggests that levels of expression in the female comparison subjects may already be closer to the low expression levels observed in depressed subjects.

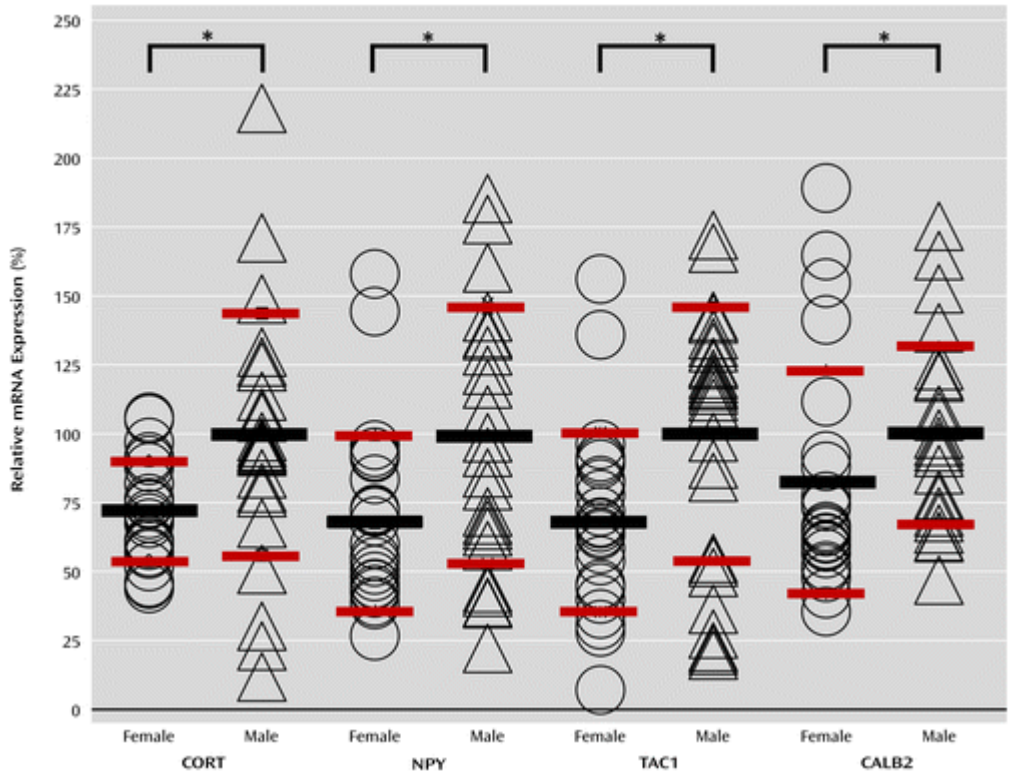

Figure 5. Significant sex differences in gene expression in postmortem comparison subjects[a]

[a] Comparisons are between male and female comparison subjects without psychiatric diagnoses. SST mRNA expression was lower in female subjects at a level that fell short of significance ($p<0.1$). Black bars indicate mean values, and red bars indicate the coefficients of variation. The asterisk indicates statistical significance ($p<0.05$).

2.4 DISCUSSION

Seeking molecular evidence in support of a low BDNF and reduced GABA function pathway in the subgenual anterior cingulate cortex in individuals with major depression, we first relied on two strains of mice with reduced BDNF function to determine the extent to which our genes of interest depend on BDNF for expression in the cingulate cortex (Table 1). Translating this information to human subjects with major depression and measuring expression levels in their subgenual anterior cingulate cortex, we observed no changes in BDNF itself, reduced expression of TRKB, the main receptor through which BDNF signals, and reduced mRNA levels of several genes for which expression depends on BDNF (Table 2). Among these BDNF-dependent and depression-affected genes, the reduced expression of several markers of GABA-ergic interneurons that specifically target the dendritic compartment of pyramidal neurons (SST, NPY, and CORT) suggests the presence of a reduced dendritic inhibition phenotype in individuals with major depression, downstream from low BDNF signaling. GABA-related changes also extended to genes with modest or no evidence for BDNF dependency (based on mouse studies [also see Table 1 and Table 2]), suggesting that additional factors lead to reduced GABA function in depression. Overall, results were more robust in men, which is contrary to our previous observations in the amygdala, in which depressed women exhibited reduced BDNF levels (but not TRKB levels) and greater BDNF-dependent gene changes compared with men. Together, these results suggest a core BDNF-/GABA-related pathology in major depression that affects markers of interneurons targeting pyramidal cell dendrites and that displays sex- and brain region-specific features.

With the exception of sex and, to a lesser extent, age, none of the clinical, demographic, and technical parameters had any consistent detectable effects on gene expression in our

relatively large cohort of human subjects. Death by suicide has been associated with reduced BDNF expression, but differences observed in the present study appeared to be more robust in subjects who did not die by suicide, compared with the respective comparison subjects (see Table 7 in Appendix B).

When the groups were segregated by sex, we observed similar changes in TRKB expression and overall lower statistical significance of changes in depression-related gene down-regulation in female subjects, although not systematically (Table 2). Notably, the expression levels of three BDNF-dependent genes (CORT, NPY, and TAC1) were already lower in female comparison subjects relative to male comparison subjects (Figure 5). Despite lower baseline levels, expression changes for CORT, TAC1, and NPY still displayed greater or equal statistical significance and effect size in depressed female subjects compared with depressed male subjects. Thus, despite a less robust profile of molecular changes, the low female baseline expression for some genes may result in a greater propensity to reach the threshold of low pathophysiological function (Table 2). Finally, the overall male-female similarities in gene changes downstream from low BDNF in the two mouse strains (Table 1) and the reduction or absence of changes in human female subjects for genes with intermediate and low BDNF dependency (SNAP25, GAD1/GAD2 [Table 2]) suggest the presence of additional sex-specific moderating factors in human subjects. This is consistent with our previous study of the amygdala in depressed male (Sibille et al., 2009b) and female (Guilloux et al., 2012) subjects, in which BDNF and BDNF-dependent genes were robustly affected in women but not in men. Together, these results support the concept that sex differences in the vulnerability to and the expression of major depression may not result from different pathophysiological mechanisms but rather from moderating

biological factors acting on a core phenotype implicating a putative reduction in GABA function and dendritic targeting interneuron vulnerability.

SST, NPY, and CORT are three neuropeptide coding genes with overlapping patterns of expression in mice that are found in approximately 20% of interneurons and that have the functional characteristic of providing GABA-mediated inhibition to distal dendrites of pyramidal neurons. Moreover, TAC1, the fourth neuropeptide coding gene that is similarly down-regulated in the subgenual anterior cingulate cortex and amygdala in individuals with major depression (Figure 6), encodes for substance P, a gene product with putative antidepressant activity (Ratti et al., 2011), which in the cortex mainly activates SST-positive cells through NK1R receptor binding (Vruwink et al., 2001). Since all four genes are dependent on BDNF for their expression (Table 1), low BDNF signaling may orchestrate a synergy between decreased TAC1, SST, NPY, and CORT expression, leading to reduced inhibition onto the dendritic trees of targeted pyramidal neurons. VGF and SNAP25, two genes involved in synaptic function and previously implicated in major mental illnesses, were also found to be BDNF dependent and down-regulated in the subgenual anterior cingulate cortex in subjects with major depression, suggesting that a broader BDNF-dependent module may be affected. Finally, expression of GAD1, a gene encoding an enzyme that produces GABA, and expression of PV, a gene encoding a marker for fast spiking GABA interneurons targeting the cell body and axon initial segment, were also down-regulated. For PV and GAD1, the mechanism appears to be independent of reduced BDNF function (Table 1, Table 2). Notably, PV levels were not affected in other brain regions (the dorsolateral prefrontal cortex [e.g., Sibille et al. 2011] and the amygdala [e.g., Guilloux et al. 2012]), and calretinin (CALB2), a marker for a third interneuron subset, displayed reduced baseline expression and no depression-related changes in the subgenual anterior cingulate cortex

but was decreased in the amygdala in depressed female subjects (Figure 6). Together, these findings put forward critical observations of the pathology of major depression, which may relate to three consecutive biological scales: 1) molecular function, manifested by altered BDNF-/TrkB- and GABA-associated gene function; 2) cellular microcircuitry, in which findings appear to be clustered by function (i.e., dendritic inhibition); and 3) circuit moderators, in which sex-related factors and brain regions are relevant modulators to gene expression in major depression. Hence, reduced GABA-mediated inhibition of incoming information in pyramidal dendrites may represent a putative microcircuitry-level phenotype underlying the increased activation of the subgenual anterior cingulate cortex and amygdala that is frequently reported in studies of patients with major depression (Mayberg et al., 1999; Siegle et al., 2007). In turn, restoring dendritic inhibitory function may reduce pyramidal cell activation and excitatory tone and contribute to the reduction in activation of the subgenual anterior cingulate cortex with positive treatment response to therapeutic intervention (e.g., deep brain stimulation, antidepressants) (Mayberg et al., 2000; Mayberg et al., 2005).

Some of the limitations of these results are inherent to investigation of heterogeneous cohorts and of postmortem brain samples. Large numbers of clinical, demographic, and technical parameters have to be taken into consideration, and results are mostly correlative and cannot provide insight into developmental processes in major depression. Our relatively large cohort size allowed us to rule out major effects of putative confounds (details are summarized in Table 6 in Appendix B), but the results will need to be confirmed in independent cohorts.

The causal link between reduced BDNF signaling and altered gene expression was inferred from analyses of rodents with genetically induced reduction in BDNF function, but species differences may exist, and thus the different labels of gene-specific BDNF dependency

may vary. The fact that regional variations in BDNF dependency were also observed in rodents suggests that aspects of the human gene regulation patterns are conserved across species. This latter observation supports the need for further studies of rodent models with more refined genetic manipulations affecting specific interneuron populations, for instance, to assess the effect on reduced dendritic inhibition on the local microcircuitry in the amygdala and cingulate cortex and downstream behavioral phenotypes. Indeed, it is evident that the complexity of the putative BDNF-mediated cellular and signaling phenotype observed in human major depression is not fully replicated in currently available genetic rodent models, and thus caution should be applied when interpreting rodent behavioral outputs downstream from broad genetic changes; for instance, $Bdnf^{+/-}$ and $Bdnf^{KIV}$ mice exhibit normal and increased emotionality, respectively (Ibarguen-Vargas et al., 2009; Sakata et al., 2010). Disruption of forebrain-specific BDNF leads to higher emotionality when combined with exposure to chronic stress in female mice (Autry et al., 2009; Monteggia et al., 2007). Conversely, low ventral striatum BDNF can have antidepressant-like effects (Berton et al., 2006). Our observation in humans of reduced expression of BDNF in the amygdala and of reduced TRKB in the subgenual anterior cingulate cortex suggests that altered BDNF signaling may represent a complex integration of currently unidentified upstream events (e.g., stress factors, developmental trajectories, and genetic variation), which result in similar core downstream changes (i.e., reduced markers of dendritic inhibition) and that are further moderated by numerous factors (e.g., sex, brain region, and brain activity).

Finally, it is also becoming evident that the observed findings are not specific to major depression, since similar reductions in BDNF and SST expression have been reported in studies of other neuropsychiatric (e.g., schizophrenia (Hashimoto et al., 2005b; Mellios et al., 2009) and

bipolar disorder (Fernandes et al., 2011)) and neurological (e.g., Alzheimer's disease and Huntington's disease) disorders. Thus, our findings may more accurately reflect a molecular and cellular endophenotype that implicates BDNF signaling and GABA microcircuitry and that has its own etiological factors. However, the restricted scope on markers affecting dendritic inhibition that we observed differs from observations in studies of other diseases in which changes occur in the context of other core pathologies, such as robust PV-related GABA dysfunction in schizophrenia (Hashimoto et al., 2008) or neurodegenerative processes in Alzheimer's disease (Podtelezhnikov et al., 2011). Investigating the etiological factors and phenotypic outputs of these respective molecular and cellular endophenotypes outside the restriction of the categorical definitions of psychiatric and neurological illnesses may provide dimensional insight into relevant proximal pathophysiological mechanisms to be targeted for therapeutic purposes, while their patterns of co-occurrences may be informative of mechanisms underlying clusters of symptoms that are enriched in clinically defined disorders.

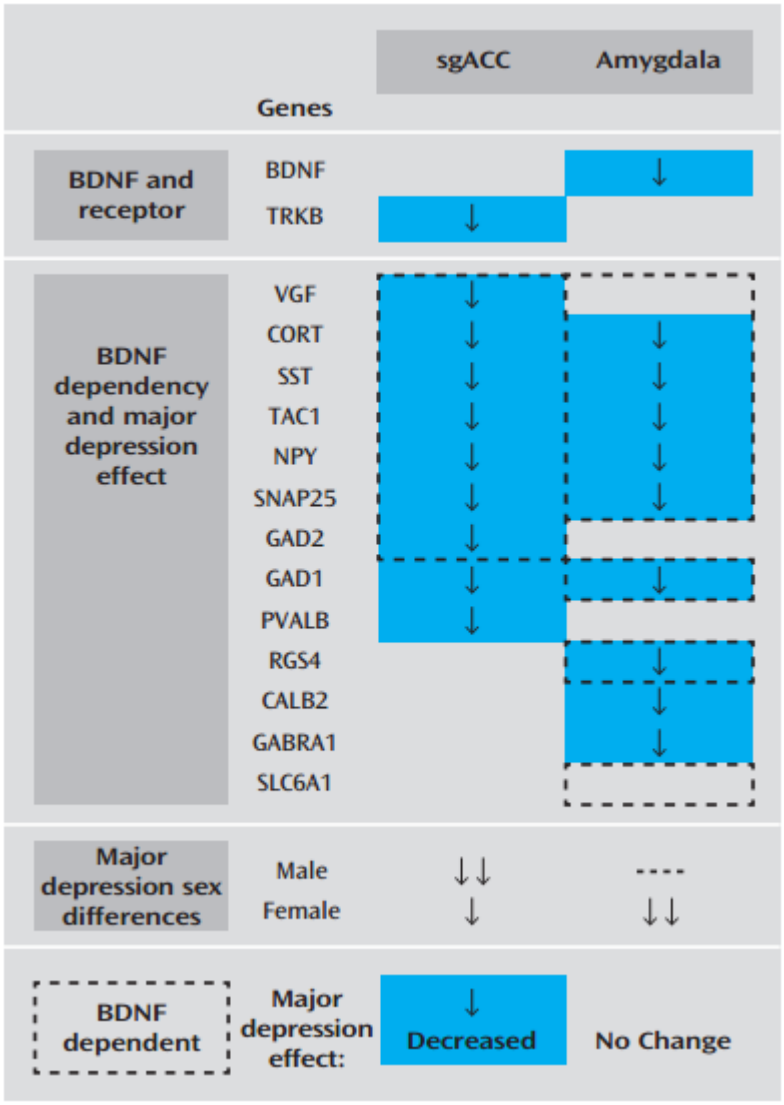

Figure 6. BDNF/TRKB and associated GABA marker dysregulation in the subgenual anterior cingulate cortex and amygdala in postmortem subjects with major depressive disorder[a]

[a] Reduced BDNF signaling in major depressive disorder is suggested by the findings of low BDNF in the amygdala and low TrkB in the subgenual anterior cingulate cortex. Analyses of mice with reduced BDNF functions suggest that co-occurring GABA-related gene changes are partly downstream from low BDNF signaling, but the exact nature and extent of downstream gene changes are moderated by brain region- and sex-specific factors. Comparison findings in the amygdala are taken from a previous study (12). SgACC=Subgenual anterior cingulate cortex.

3.0 PAPER 2: THE ROLE OF BDNF IN AGE-DEPENDENT CHANGES OF SYNAPTIC MARKERS IN HUMAN PREFRONTAL CORTEX

Hyunjung Oh[1], David A. Lewis[1], Etienne Sibille[1,2§]

[1]Department of Psychiatry, Center for Neuroscience, University of Pittsburgh, PA, USA

[2]Campbell Family Mental Health Research Institute of CAMH; Departments of Psychiatry, and Pharmacology and Toxicology, University of Toronto, Toronto, Canada

[§]To whom correspondence should be addressed. E-mail: Etienne.Sibille@camh.ca

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3.1 INTRODUCTION

With dramatic growth of the older population, the need to understand the mechanism and consequence of aging on the brain has become critically important. The effect of aging on the incidence of psychiatric disease remains controversial (Blazer, 1994; Glaesmer et al., 2011; Kessler et al., 2010). While sub-threshold depression and depressive symptoms are more common in later life (Beekman et al., 1995), they are often misattributed to aging *per se*, leading to under-diagnosis of depression. Animal studies show that the effects of stress on cognitive, neuroendocrine, biochemical and anatomical changes are augmented in older subjects (Juster et al., 2010; Lupien et al., 2009).

Recent advances in understanding the molecular and cellular bases of altered mood regulation in adult depressed subjects, and the observation of a considerable overlap between normal aging and depression-related brain changes (McKinney et al., 2012; McKinney and Sibille, 2013), suggest that aspects of mood regulatory mechanisms may be selectively vulnerable to early homeostatic changes during normal aging. Indeed, older age is a potent risk factor for the functional decline of the prefrontal cortex (PFC), which is important for cognitive function, working memory, and emotion regulation in all species, ranging from rodents to humans. Imaging studies report smaller PFC volume in elderly subjects without obvious neurological disease (Tisserand et al., 2002). Consistent with functional impairment and brain atrophy, a decrease in neuronal body size, dendritic length, and loss of synapses without loss of neurons have been observed in postmortem studies (Morrison and Baxter, 2012; Rajkowska, 2000b; Stockmeier et al., 2004b).

BDNF, a small secreted protein, and its receptor, TrkB, play important roles in neuronal development, differentiation, maintenance and plasticity throughout life. In the brain, two

isoforms of TrkB are abundantly expressed: full-length (TrkB-FL) and truncated form (TrkB-T1). In contrast to TrkB-FL which binds to BDNF and activates downstream kinase cascades, TrkB-T1 lacks intracellular catalytic domain and therefore act as an endogenous inhibitor of TrkB-FL by competing for the available BDNF pool (Eide et al., 1996; Gupta et al., 2013). Low BDNF and/or TrkB expression has been reported in multiple brain disorders and normal brain aging which are often accompanied by mild brain atrophy, reduced neuronal function, and synaptic loss (Durany et al., 2001; Guilloux et al., 2012; Hattiangady et al., 2005; Howells et al., 2000; Phillips et al., 1991; Romanczyk et al., 2002; Thompson Ray et al., 2011). Brain-specific deletion of BDNF or TrkB in mouse induces neuronal shrinkage, dendritic retraction in the cortex, and cognitive and learning deficit (Gorski et al., 2003a; Gorski et al., 2003b; Xu et al., 2000). These data imply that normal aging-associated BDNF and TrkB changes in the PFC might compromise synaptic integrity and function, although this link has not been tested in humans.

The biological role of BDNF has been thoroughly studied, however, investigating BDNF-driven changes in vivo and/or in human subjects is challenging due to its low endogenous level, and complex regulation by multiple promoters. Most studies were conducted with in vitro models, sometimes with the aid of overexpression or exogenous application of BDNF. Moreover, whereas changes in BDNF levels seem an obvious candidate biological event for age-related structural and functional changes, numerous other age-related complex processes occur, including among others, increased inflammation, reduced blood flow, accumulated free radical damage to macromolecules; all potentially contributing to the molecular, structural and functional alterations of the brain.

In the present study, we investigated age-related BDNF change in the human prefrontal cortex and specifically focused on its putative contribution to altered transcriptome profile occurring during normal aging. We then used a conditional genetic mouse model to directly test the putative causal role of BDNF/TrkB signaling in altered aging-associated expression patterns. We predicted that reduced BDNF directly contribute to reduced synaptic function through altered expression of markers for inhibitory and excitatory neurons and synaptic genes.

3.2 MATERIALS AND METHODS

3.2.1 Human postmortem subjects

Postmortem brains were collected from the Allegheny County Coroner's Office (Pittsburgh, PA) after consent from next of kin. The neuropsychiatric-related resources of this brain collection have been characterized in microarray and anatomical studies (Bloss et al., 2011; Hashimoto et al., 2005a; Lewis et al., 2001; Mirnics et al., 2000; Mirnics et al., 2001; Sibille et al., 2009a; Sweet et al., 2003). In short, for all cases, a committee of experienced clinical scientists examined clinical records, toxicology and standardized psychological autopsy data (Glantz et al., 2000). Individuals were also screened for the absence of neurodegenerative disorders by neuropathological examination (Glantz and Lewis, 1997; Sibille et al., 2004; Sweet et al., 2004). All procedures were approved by the University of Pittsburgh's Committee for the Oversight of Research Involving the Dead and Institutional Review Board for Biomedical Research. After careful examination of clinical and technical parameters, 209 control subjects without DSM-IV diagnosis were selected. All six cortical layers of orbitoventral frontal cortex (Brodmann area 47)

were harvested and stored in TRIzol (Invitrogen Life Technologies, Carlsbad, CA) at -80˚C until processed for RNA isolation. Due to the large cohort size, a sub-cohort was generated for qPCR validation based on younger (≤42 years; n=40) and older (≥60 years; n=40) age. These two groups did not differ in other measures (Table 3).

Table 3. Characteristics of human postmortem samples used in array and qPCR

No significant group differences in clinical variables were observed, except for age.

	Array	qPCR	
		Younger (≤42 yrs)	Older (≥60 yrs)
n	209	40	40
Age (years)	50.7 ± 15.0 (range 16-96)	29.7 ± 8.6	67.8 ± 8.1
Sex	M 165, F 44	M 33, F 7	M 33, F 7
Race	W 178, B 31	W 37, B 3	W 37, B 3
Postmortem interval (hours)	17.1 ± 5.9	16.8 ± 6.0	17.1 ± 6.3
pH	6.7 ± 0.3	6.7 ± 0.3	6.7 ± 0.3
RNA integrity number	8.0 ± 0.7	8.1 ± 0.7	8.0 ± 0.7

3.2.2 RNA extraction

Human RNA was isolated from brain homogenate in TRIzol and further purified with RNeasy spin columns (QIAGEN, Valencia, CA). RNeasy micro plus kit (QIAGEN) was directly used to isolate total RNA from mouse cortices.

3.2.3 Gene arrays and expression analysis

RNA samples were processed for microarray analysis using Affymetrix GeneChip Human Gene 1.1ST, according to manufacturer's protocol (Affymetrix, Santa Clara, CA). Gene expression data was extracted using Expression Console build 1.2.1.20 and normalized with quantile normalization method to eliminate batch effects. To remove age effect on gene expression, each expression value was fitted to a regression model using potential confounding covariates. The residuals of each gene were further adjusted by a power function regression model for age effect only.

3.2.4 BDNF coexpression network analysis

Pearson's correlation values were calculated between BDNF and each gene examined by array. The top 200 genes positively correlated with BDNF ($R>0.575$) were analyzed and visualized using Cytoscape (Shannon et al., 2003) (version 3.1.1) with ClueGO plugin (Bindea et al., 2009) (version 2.1.7) and further examined with DAVID Functional Annotation Clustering Tool (Huang da et al., 2009a, b). The group p-values that are reported in the article are adjusted with Bonferroni step down method.

3.2.5 Animals and drug treatment

$TrkB^{F616A}$ heterozygote mice (Chen et al., 2005) were obtained from Jackson laboratories (Bar Harbor, ME) and intercrossed to generate homozygote mice. Male homozygote mice (9-10 weeks old) were fed with 25 μM 1NMPP1 or vehicle (0.0003% DMSO) via drinking water for 3

weeks and sacrificed. Left and right medial prefrontal cortices were collected and stored at -80˚C until RNA isolation.

3.2.6 Real-time Quantitative Polymerase Chain Reaction (qPCR)

cDNA was synthesized with total RNA using qScript cDNA supermix (Quanta BioSciences, Gaithersburg, MD). PCR products were amplified in triplicate on a Mastercycler real-time PCR machine (Eppendorf, Hamburg, Germany) using universal PCR conditions. Results were calculated as the geometric mean of threshold cycles normalized to three validated internal controls (actin, glyceraldehyde-3-phosphate dehydrogenase, and cyclophilin G). Based on the BDNF coexpression network analysis, candidate genes for qPCR verification were selected: (i) pan-BDNF; (ii) 2 TrkB isoforms (TrkB-FL, TrkB-T1); (iii) 5 excitatory synapse-related genes: vesicular glutamate transporter 1 (vGLUT1), glutamate receptor, AMPA1 (GRIA1), glutamate receptor, NMDA 2A & 2B (GRIN2A & 2B), discs large homolog 4 (DLG4, also known as PSD95); (iv) 8 inhibitory neuronal genes (presynaptic markers): SLC32A1 (vGAT1), 7 interneuron markers (SST, NPY, CORT, PVALB, CCK, GAD 1 & 2) (v) 3 postsynaptic GABA receptors (GABRA 4/5, GABRB3).

3.2.7 Statistical analysis

Gene expression differences were determined by analysis of covariance (ANCOVA) using SPSS (SPSS, Inc., Chicago, IL). To determine covariates included in the gene-specific models, each nominal factor was tested as the main factor by ANOVA, scale covariates were tested by Pearson correlation. Repeated measures were corrected by modified Holm-Bonferroni test and ANCOVA

models including significant co-factors were then applied. For the rodent studies, animals were separated into two cohorts to minimize age difference. Unpaired t-tests were performed for the separate cohorts and then p-values were combined using Stouffer's z-trend method (Whitlock, 2005) and reported in the article.

3.3 RESULTS

3.3.1 BDNF and BDNF-associated transcriptome changes in aging human brain

Large-scale gene expression was obtained by microarray analysis in the orbitofrontal cortex of 209 postmortem samples from subjects without psychiatric or neurologic illness, ranging from 16 to 96 years of age. BDNF expression gradually decreased with age (Pearson's correlation value to Age ($R=-0.36$, $p<0.0000001$; Figure 7.A). To identify BDNF-related transcriptome changes, we calculated Pearson's correlation values between BDNF and each gene probeset. Overall, genes with higher correlation to BDNF showed larger age-related changes (Figure 7.B).

To investigate biological pathways potentially affected by BDNF, the top 200 genes positively correlated to BDNF ($R>0.575$, whole list is available in Table 8 in Appendix C) were analyzed with Cytoscape with ClueGO (Figure 7.C, full annotations are available in Figure 24 in Appendix C). Among 75 GO terms with $p<0.01$, 9 functional groups were identified: synaptic transmission (66 genes, p=2.4E-22), neuron projection development (47 genes, p=3.2E-15), neuron part (49 genes, p=1.3E-17), neuron development (46 genes, p=5.7E-16), signal release (20 genes, p=2.9E-07), dendrite (24 genes, p=1.6E-10), cell-cell signaling (54 genes, p=3.8E-18), synaptic vesicle (12 genes, p=3.7E-08), growth cone (9 genes, p=1.9E-05). DAVID Functional

Annotation Clustering Tool confirmed that the most enriched gene cluster in the BDNF coexpression network included synapse-related genes (enrichment score=6.93). The second and third clusters identified by DAVID analysis were also associated with synapse (Figure 7.D and Table 9 in Appendix C). Genes in top 3 clusters are related to voltage gated channels (CACNA2D1, KCND2, SCN3B, KCNAB1, SCN2A, CACNB1, CACNA1E, CACNG3, SCN8A, KCNK1, KCNV1), neurotransmitter receptors (GABRB3, GRIN2B, GABRA4, GRIA1, GLRA3, GABRA5, GRIN2A, GABBR2, HTR2A), and synaptic vesicle-related molecules (SLC17A7, SVOP, RAB3C, GRIN2B, SYT4, SYT5, SV2B, ATP6V0D1), synaptic cell adhesion molecules (NLGN1, NRXN3) (Figure 7.D).

An important control analysis is to rule out a general effect of age underlying the correlation between BDNF and the expression of other genes. Accordingly, we performed the same analysis using data in which the effect of age was analytically subtracted (i.e., using age-residual values). Results indicated very similar patterns of correlation values at the level of individual genes, and for the functional clustering analysis: 145 of top 200 were overlapping and synapse-related genes enriched in BDNF coexpressed genes (Figure 25 in Appendix C).

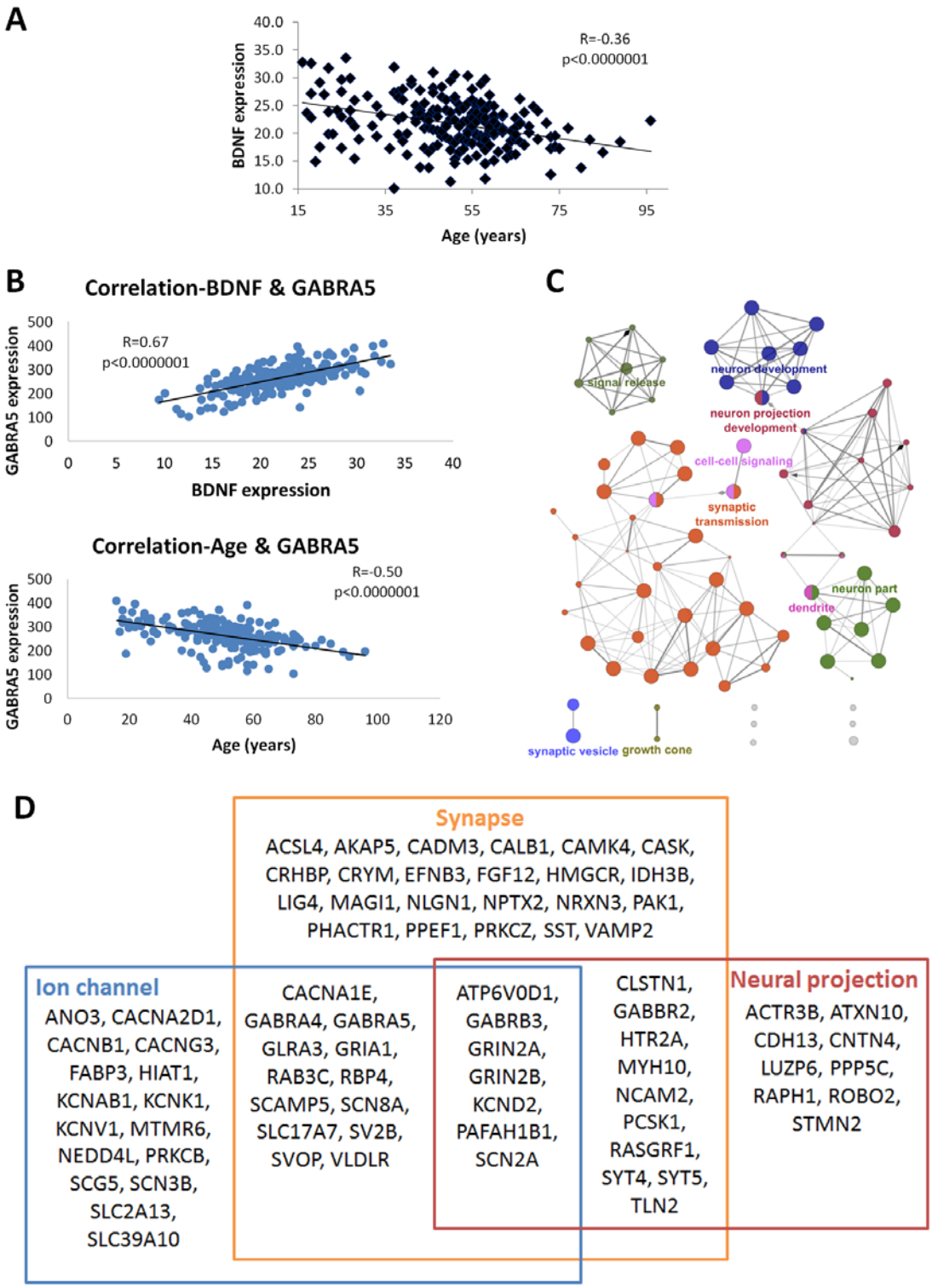

Figure 7. Age-dependent changes in BDNF expression and identification of BDNF-coexpressed genes

(A) Age-related reduction of BDNF in human frontal cortex revealed by array (R=-0.36, p<0.0000001) (B) Example of BDNF and age opposite correlations for genes of interest: GABRA5 expression is positively correlated with BDNF and negatively with age. (C) Sets of GO terms enriched in top 200 genes correlated with BDNF in human frontal cortex as identified by Cytoscape with ClueGO. (D) Synapse related genes enriched in BDNF coexpression network as identified by David functional analysis.

3.3.2 Excitatory and inhibitory synaptic genes demonstrate age-related changes that parallel changes in BDNF

DAVID functional annotation tool revealed that GABA as well as glutamate receptor activity-related genes were significantly enriched in the top 200 BDNF coexpression network; GABA receptor activity-related genes (GO:16917): GABBR2, GABRA4/5, GABRB3 (fold enrichment=13.7, p=0.003), glutamate receptor activity-related genes (GO:0008066): GABBR2, GRIA1, GRIN2A/2B (fold enrichment=10.6, p=0.006). Therefore, we went back to the expression dataset and investigated whether additional markers of excitatory and inhibitory synapses undergo aging-related changes in a BDNF-dependent manner. Overall, age-related downregulation was observed across excitatory and inhibitory synaptic markers (percentage of genes with significant negative correlation to age: 68.4% of GABA synaptic genes, 63.0% of glutamate synaptic genes, Table 4), suggesting a general suppression of neurotransmission. Although the degrees of correlation to BDNF expression were slightly reduced using age-residual values, 79 out of 84 synaptic genes showed the same directionality and significance of correlation using residual or uncorrected expression values (94% matching; Table 2). Compared to excitatory synapse-related genes, inhibitory synaptic markers showed a trend towards higher correlation to BDNF (gene expression: p=0.088, age residuals: p=0.072).

Table 4. Correlation between age-related BDNF expression and expression level or age-adjusted residual expression values of synapse-related genes

Close correlation to BDNF is maintained after aging effect on gene expression is removed.

		Excitatory synapse related					
		Correlation and p-values					
Gene Symbol	mRNA Accession	genes to Age	p-value	genes to BDNF	p-value	residuals to BDNF	p-value
DLG1	NM_001098424	0.12	0.043	0.11	0.056	0.16	0.012
DLG2	NM_001364	-0.40	<1.E-07	0.52	<1.E-07	0.39	<1.E-07
DLG3	NM_021120	-0.41	<1.E-07	0.64	<1.E-07	0.54	<1.E-07
DLG4	NM_001365	-0.47	<1.E-07	0.48	<1.E-07	0.31	3.E-06
GLS	NM_014905	-0.09	0.087	0.50	<1.E-07	0.44	<1.E-07
GLUL	NM_002065	0.18	0.005	-0.46	<1.E-07	-0.35	<1.E-07
GLUL	NM_002065	0.23	3.E-04	-0.46	<1.E-07	-0.33	5.E-07
GRIA1	NM_000827	-0.33	1.E-06	0.63	<1.E-07	0.52	<1.E-07
GRIA2	NM_001083619	-0.29	1.E-05	0.51	<1.E-07	0.42	<1.E-07
GRIA3	NM_007325	-0.09	0.087	0.48	<1.E-07	0.41	<1.E-07
GRIA4	NM_000829	-0.41	<1.E-07	0.54	<1.E-07	0.42	<1.E-07
GRID1	NM_017551	0.06	0.213	-0.19	0.003	-0.14	0.019
GRID2	NM_001510	-0.34	<1.E-07	0.48	<1.E-07	0.37	<1.E-07
GRIK1	NM_175611	-0.60	<1.E-07	0.52	<1.E-07	0.32	1.E-06
GRIK2	NM_175768	0.11	0.050	-0.09	0.109	-0.03	0.321
GRIK3	NM_000831	-0.50	<1.E-07	0.55	<1.E-07	0.40	<1.E-07
GRIK4	NM_014619	-0.18	0.004	0.12	0.042	0.07	0.162
GRIK5	NM_002088	-0.32	2.E-06	0.23	0.001	0.14	0.026
GRIN1	NM_007327	-0.29	9.E-06	0.39	<1.E-07	0.29	1.E-05
GRIN2A	NM_001134407	-0.51	<1.E-07	0.61	<1.E-07	0.47	<1.E-07
GRIN2B	NM_000834	-0.52	<1.E-07	0.62	<1.E-07	0.49	<1.E-07
GRIN2C	NM_000835	0.10	0.082	-0.38	<1.E-07	-0.33	5.E-07
GRIN2D	NM_000836	-0.12	0.041	-0.06	0.197	-0.08	0.127
GRIN3A	NM_133445	-0.57	<1.E-07	0.57	<1.E-07	0.44	<1.E-07
GRIN3B	NM_138690	0.00	0.497	-0.27	5.E-05	-0.27	5.E-05
GRINA	NM_000837	-0.16	0.011	0.29	1.E-05	0.21	0.001
GRIP1	NM_021150	0.05	0.241	0.21	0.001	0.20	0.002
GRIP2	NM_001080423	-0.13	0.027	-0.12	0.037	-0.19	0.003
GRM1	NM_001114329	-0.22	0.001	0.55	<1.E-07	0.46	<1.E-07
GRM2	NM_000839	-0.43	<1.E-07	0.41	<1.E-07	0.29	1.E-05
GRM3	NM_000840	-0.57	<1.E-07	0.32	2.E-06	0.15	0.015
GRM4	NM_000841	-0.06	0.185	-0.06	0.185	-0.09	0.108
GRM5	NM_001143831	-0.27	5.E-05	0.44	<1.E-07	0.34	<1.E-07
GRM6	NM_000843	0.06	0.179	-0.12	0.037	-0.11	0.062
GRM7	NM_181874	-0.32	2.E-06	0.28	2.E-05	0.19	0.003
GRM8	NM_001127323	-0.18	0.006	0.43	<1.E-07	0.40	<1.E-07
HOMER1	NM_004272	-0.67	<1.E-07	0.55	<1.E-07	0.44	<1.E-07
HOMER2	NM_199330	-0.19	0.003	0.07	0.167	0.01	0.429
HOMER3	NM_004838	0.04	0.266	-0.40	<1.E-07	-0.39	<1.E-07
SLC17A1	NM_005074	0.15	0.013	-0.33	5.E-07	-0.23	4.E-04
SLC17A2	NM_005835	-0.01	0.450	-0.07	0.172	-0.07	0.153
SLC17A6	NM_020346	-0.19	0.004	0.45	<1.E-07	0.34	<1.E-07
SLC17A7	NM_020309	-0.40	<1.E-07	0.60	<1.E-07	0.50	<1.E-07
SLC17A8	NM_139319	-0.08	0.114	0.01	0.450	-0.02	0.368
SLC1A1	NM_004170	-0.26	8.E-05	0.34	<1.E-07	0.25	1.E-04
SLC1A4	NM_003038	0.08	0.129	-0.21	0.001	-0.17	0.008

		Inhibitory synapse related					
		Correlation and p-values					
Gene Symbol	mRNA Accession	genes to Age	p-value	genes to BDNF	p-value	residuals to BDNF	p-value
APITD1-CORT	NM_198544	-0.45	<1.E-07	0.55	<1.E-07	0.40	<1.E-07
CALB1	NM_004929	-0.68	<1.E-07	0.59	<1.E-07	0.47	<1.E-07
CALB2	NM_001740	-0.40	<1.E-07	0.39	<1.E-07	0.25	1.E-04
CCK	NM_000729	-0.18	0.005	0.42	<1.E-07	0.35	<1.E-07
GABARAP	NM_007278	-0.05	0.216	0.28	2.E-05	0.23	5.E-04
GABARAPL1	NM_031412	-0.20	0.002	0.40	<1.E-07	0.32	2.E-06
GABARAPL2	NM_007285	-0.01	0.418	0.26	6.E-05	0.21	0.001
GABBR1	NM_001470	-0.31	2.E-06	0.36	<1.E-07	0.21	0.001
GABBR1	NM_001470	-0.30	4.E-06	0.36	<1.E-07	0.23	4.E-04
GABBR1	NM_001470	-0.29	9.E-06	0.34	5.E-07	0.23	4.E-04
GABBR2	NM_005458	-0.50	<1.E-07	0.59	<1.E-07	0.48	<1.E-07
GABRA1	NM_000806	-0.21	0.001	0.54	<1.E-07	0.46	<1.E-07
GABRA2	NM_000807	-0.18	0.004	0.11	0.054	0.08	0.123
GABRA3	NM_000808	-0.26	8.E-05	0.57	<1.E-07	0.47	<1.E-07
GABRA4	NM_000809	-0.35	<1.E-07	0.63	<1.E-07	0.53	<1.E-07
GABRA5	NM_000810	-0.50	<1.E-07	0.67	<1.E-07	0.58	<1.E-07
GABRA6	NM_000811	0.07	0.157	-0.12	0.044	-0.09	0.088
GABRB1	NM_000812	0.06	0.192	0.14	0.019	0.23	4.E-04
GABRB2	NM_021911	-0.22	0.001	0.53	<1.E-07	0.44	<1.E-07
GABRB3	NM_021912	-0.39	<1.E-07	0.63	<1.E-07	0.51	<1.E-07
GABRD	NM_000815	-0.22	0.001	0.48	<1.E-07	0.35	<1.E-07
GABRE	NM_004961	0.11	0.053	-0.19	0.004	-0.09	0.100
GABRG1	NM_173536	0.05	0.259	-0.37	<1.E-07	-0.30	6.E-06
GABRG2	NM_198904	0.05	0.250	0.44	<1.E-07	0.43	<1.E-07
GABRG3	NM_033223	-0.24	3.E-04	0.33	5.E-07	0.26	7.E-05
GABRP	NM_014211	-0.04	0.262	-0.06	0.195	-0.08	0.139
GABRQ	NM_018558	-0.15	0.015	0.42	<1.E-07	0.38	<1.E-07
GABRR1	NM_002042	0.04	0.285	-0.23	5.E-04	-0.22	0.001
GABRR2	NM_002043	0.10	0.075	-0.23	5.E-04	-0.17	0.007
GABRR3	NM_001105580	0.10	0.072	-0.09	0.098	-0.09	0.108
GAD1	NM_000817	-0.20	0.002	0.50	<1.E-07	0.39	<1.E-07
GAD2	NM_000818	-0.27	3.E-05	0.51	<1.E-07	0.39	<1.E-07
GPHN	NM_020806	-0.15	0.016	0.16	0.010	0.13	0.031
NPY	NM_000905	-0.23	4.E-04	0.36	<1.E-07	0.21	0.001
PVALB	NM_002854	-0.09	0.088	0.30	5.E-06	0.19	0.003
SLC32A1	NM_080552	-0.30	6.E-06	0.44	<1.E-07	0.28	2.E-05
SST	NM_001048	-0.50	<1.E-07	0.59	<1.E-07	0.45	<1.E-07
VIP	NM_003381	-0.46	<1.E-07	0.42	<1.E-07	0.29	1.E-05

3.3.3 Validation and extension of age-dependent changes in BDNF, GABA- and glutamate-related genes

To extend and validate microarray results, we performed qPCR with primers for BDNF and TrkB genes in a selected cohort of 40 younger and 40 older subjects. The array data did not differentiate between the isoforms of TrkB so we included primers for the TrkB-FL and the TrkB-T1. Sixteen glutamatergic and GABAergic synapse-related genes were also assessed by qPCR. BDNF showed downregulation (-10.7±3.6% compared to young, F=4.44, p=0.038) with older age. Whereas expression of the full-length TrkB decreased (-19.1±2.7%, F=27.44, p=1.4E-06), expression of the truncated form increased in older brain (+21.9±6.8%, F=8.92, p=0.004). Most examined synaptic markers displayed lower expression in older subjects (Figure 8.A-D): vGLUT1 (-13.8±2.4%, F=12.58, p=0.001), GRIN2A (-22.4±3.8%, F=19.07, p=3.8E-05), GRIN2B (-25.9±5.2%, F=9.38, p=0.003), DLG4/PSD95 (-18.9±2.6%, F=19.04, p=3.9E-05), SST (-46.2±3.8%, F=49.04, p=8.2E-10), NPY (-21.7±5.0%, F=9.49, p=0.003), CORT (-37.5±2.7%, F=74.02, p=6.4E-13), CCK (-16.6±3.6%, F=14.23, p=3.2E-04), vGAT1 (-31.9±4.3%, F=35.33, p=7.9E-08), GAD1 (-12.9±3.6%, F=5.68, p=0.020), GABRA4 (-14.1±3.6%, F=8.53, p=0.005), GABRA5 (-23.7±2.1%, F=43.36, p=4.8E-09), GABRB3 (-29.3±2.8%, F=52.73, p=2.7E-10). Overall, the qPCR data replicated the array findings with high positive correlation (R=0.74, p=4.8E-04; Figure 26 in Appendix C).

Notably, the expression of synaptic genes showed the highest correlation to TrkB-FL expression among 3 BDNF signaling genes (average R=0.63, p<0.0000001; Figure 8.E), suggesting that low TrkB-FL expression in the older brain may drive age-related changes of synaptic genes.

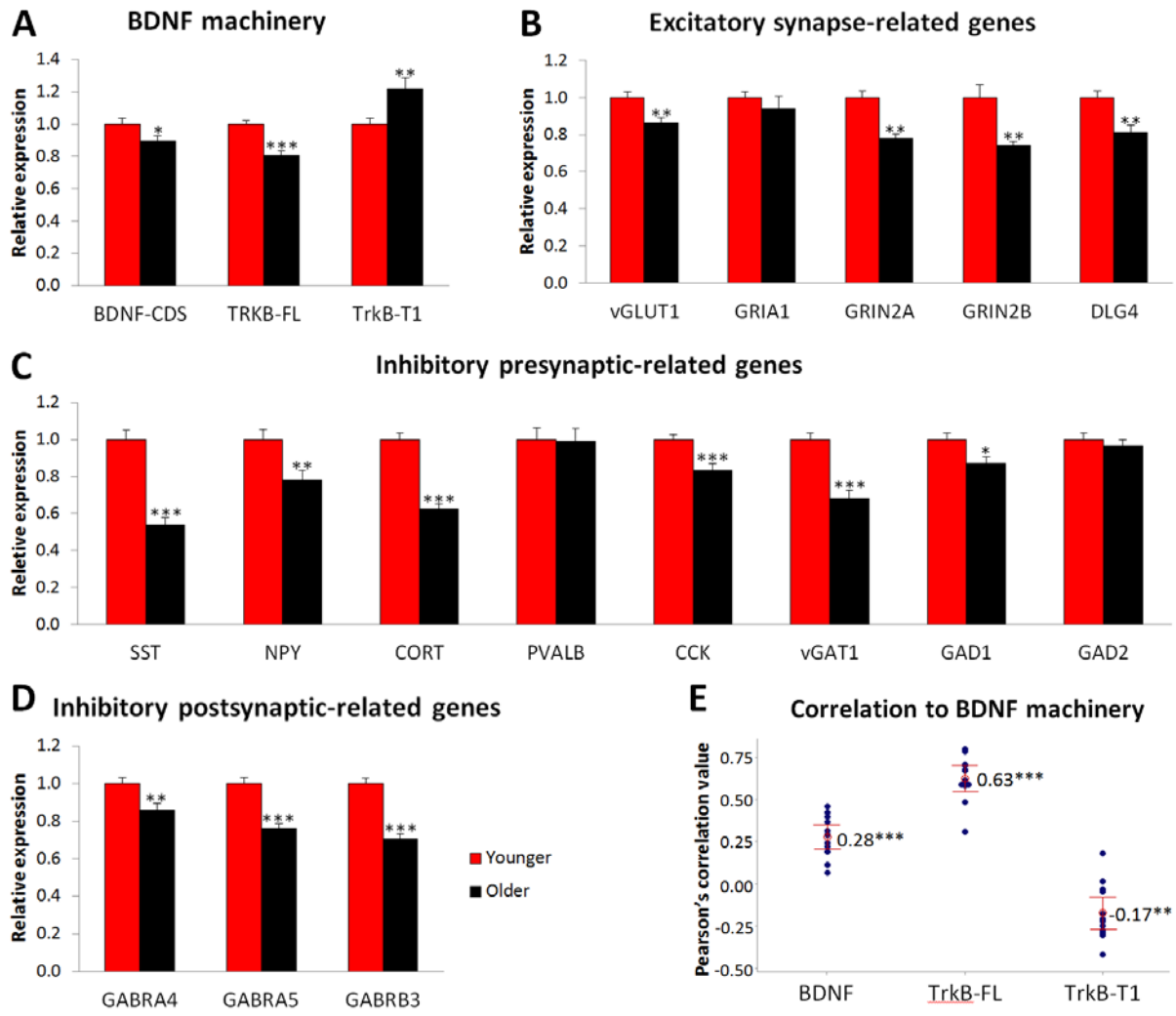

Figure 8. qPCR validation of age-related gene expression changes in two different age groups

(A) Relative expression level (old/young) of BDNF signaling (BDNF, TrkB-FL, TrkB-T1) and (B~D) selected sets of synapse-related genes in aged subjects compared to the young (Young: ≤42 years, Old: ≥60 years, n=40/each group). (E) Pearson's correlation between BDNF signaling and synaptic markers in aging cohort (one sample t-test to 0, $^{*}p<0.05$ $^{**}p<0.01$ $^{***}p<0.001$).

3.3.4 Temporal blockade of BDNF signaling is sufficient to induce age-like changes in inhibitory synapse-related genes

To investigate if low BDNF signaling is sufficient to induce age-related synaptic gene changes, we utilized transgenic mice, $TrkB^{F616A}$, which harbor a point mutation in the ATP binding pocket of TrkB that is selectively blocked by ATP competitive kinase inhibitor 1NMPP1, resulting in a blockade of TrkB-mediated signaling (Chen et al., 2005). Three weeks of 1NMPP1 treatment did not result in changes in BDNF expression, but increased expression of TrkB-FL (+48.5±11.9%, p=0.001) and TrkB-T1 (+30.6±9.5%, p=0.014) in the frontal cortex of $TrkB^{F616A}$ homozygote mice (Figure 9.A). Among tested synaptic markers, seven genes showed significant aging-like changes: DLG4 (-12.0±3.4%, p=0.016), SST (-35.6±6.0%, p=0.001), NPY (-28.8±4.8%, p=2.4E-05), CORT (-31.7±4.7%, p=0.003), CCK (-36.6±4.4%, p=0.001), GAD1 (-38.2±4.5%, p=0.016), GABRA5 (-17.0±3.8%, p=0.041) (Figure 3.B-D). Glutamate receptors and GABRB3 were upregulated: GRIA (+38.6±8.7%, p=0.008), GRIN2A (+55.3±14.4%, p=0.006), GRIN2B (+81.6%±22.7%, p=0.001), GABRB3 (+37.8±7.1%, p=0.003) (Figure 9.B and D).

These results indicate that the temporal blockade of TrkB activity in the frontal cortex of adult mice is sufficient to induce an aging-associated pattern in expression of markers for inhibitory presynaptic but not excitatory synaptic genes.

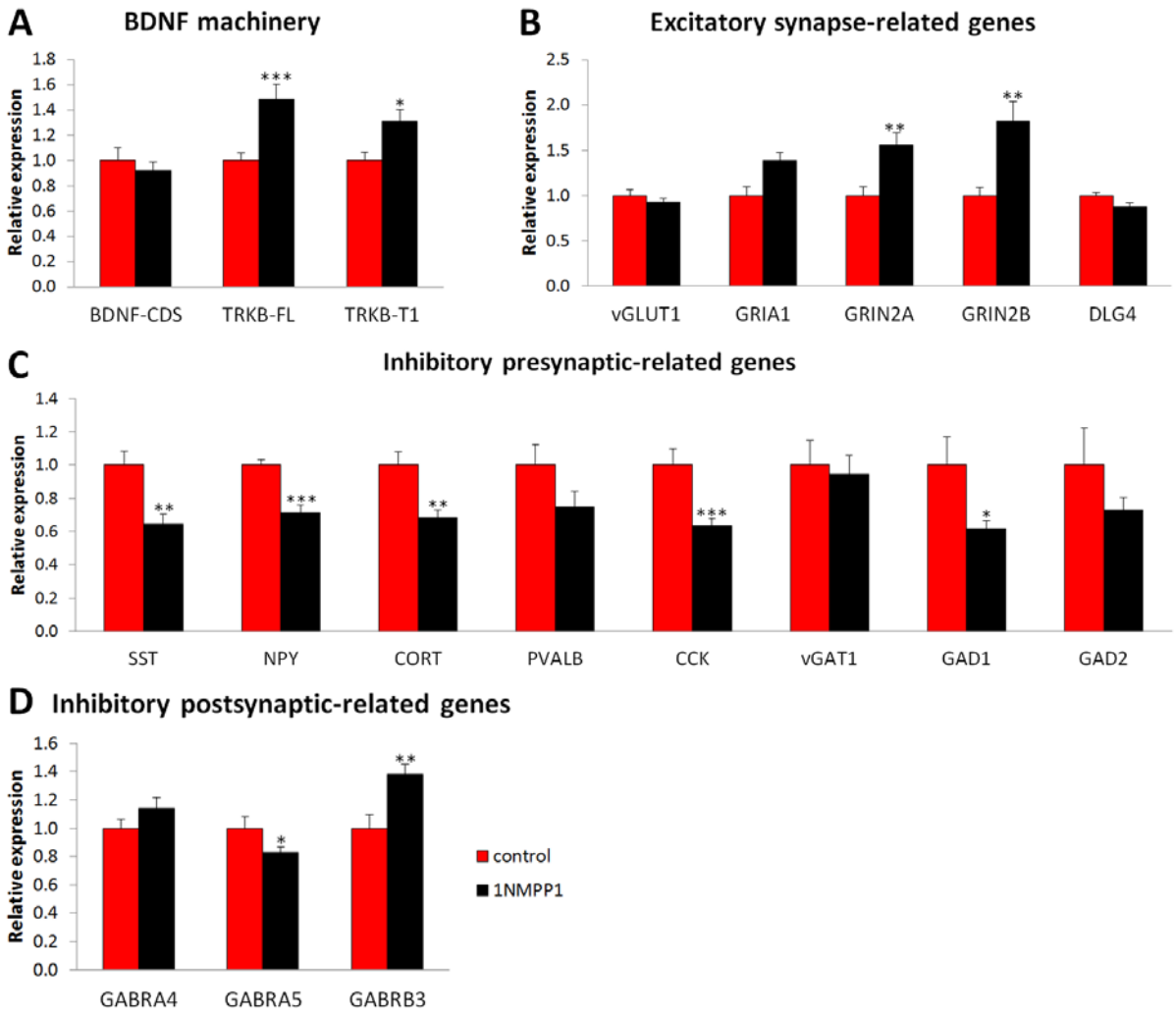

Figure 9. Gene expression changes by temporal blockade of TrkB

(A) Relative expression level (1NMPP1/vehicle) of BDNF signaling (BDNF, TrkB-FL, TrkB-T1) and (B~D) selected sets of synapse-related genes in 1NMPP1-treated adult mice compared to the control (n=12/each group).

3.4 DISCUSSION

Normal brain aging is associated with progressive cellular and structural changes, cognitive decline, and increased vulnerability to neurobiological diseases. Reduced BDNF function has been suggested as a contributor to the molecular and cellular causes of age-related deficits. In the present study, we observed changes in BDNF and TrkB transcripts consistent with reduced BDNF function in the older human brain (Figure 7 and 8). The analysis of the BDNF gene coexpression network shows that the expression of multiple synapse-related genes is closely associated with BDNF function (as measured by BDNF/TrkB-FL/T1 expression; Figure 7) and also downregulated with aging (Table 4). Our isoform-specific qPCR investigation revealed that all BDNF signaling genes, BDNF, TrkB-FL and TrkB-T1, changed in the direction of lower BDNF function in the older human brain (Figure 8). Finally using transgenic mice we demonstrated that a transient downregulation of BDNF signaling, through TrkB blockade in the adult brain, is sufficient to cause an aging-like gene expression profile, specifically affecting GABA-related presynaptic markers (Figure 9). These results extend previous correlative findings on age-associated decrement of BDNF and TrkB in elderly humans and animal models (Hassel et al., 2008; Hattiangady et al., 2005; Hayashi et al., 2001; O'Callaghan et al., 2009; Romanczyk et al., 2002; Silhol et al., 2007; Silhol et al., 2005) in two ways: first, by delineating in the human brain the extend of the putative impact of reduced BDNF signaling on the age-related transcriptome, and second, by demonstrating in rodents a causal link between reduced neurotrophic support and synaptic-related genes. Finally, the preferential impact or reduced BDNF signaling on GABA-related markers (Figure 9) provides insights into the dynamics of gene changes with aging and has implications for mechanisms of brain disorders.

It is widely accepted that synaptic impairments, rather than neuronal loss, underlie brain aging. Human studies have reported breakdown in network connectivity as well as impaired white matter integrity with aging (Bishop et al., 2010). Non-human primate studies have provided more direct evidence that cognitive impairment might result from the loss of cortico-cortical synapses in PFC: First, age-associated synaptic loss is most prominent for axospinous synapses in layer 3 where cortico-cortical synapses predominate. Second, the extent of synaptic loss correlates with the degree of cognitive impairments (Dumitriu et al., 2010). And lastly, poor working memory is associated with a higher incidence of presynaptic boutons harboring malformed, donut-shaped mitochondria that form abnormally small synaptic contacts (Hara et al., 2014). In agreement with these reports, we observed in older brain decrements of expression for markers of presynaptic boutons, such as vGLUT1, which is enriched in cortico-cortical axon terminals. In vitro studies have shown that BDNF plays a key role in synaptogenesis and synaptic maturation, not only for excitatory synapses but also for inhibitory synapses (Kohara et al., 2007). The present transcriptome and coexpression network analysis confirmed the close link between BDNF and the downregulated expression of many synapse-related genes. These correlations remained significant even after gene expression was normalized by aging effect (i.e., using age-residual expression values), implying that age-dependent changes in BDNF expression mediate, rather than moderate, specific age-related transcriptome changes in the human brain, potentially contributing to the observed structural, connectivity and functional changes during aging.

Notably, we show that GABA receptors (GABBR2, GABRA4/5, and GABRB3), glutamate receptors (GRIA1, GRIN2A & 2B) and multiple genes associated with both excitatory and inhibitory functions are closely linked to BDNF expression and downregulated with age.

This overall age-related downregulation of synaptic markers may contribute to maintain brain function, namely through adjusting the excitation-inhibition balance with increasing age. BDNF regulates many cellular functions related to differentiation, maintenance and plasticity, and is thus considered a valid biological candidate to orchestrate such complex changes in the human brain. Our postmortem human study provides supporting evidence for low BDNF leading to decreased synaptic markers during brain aging. However, these findings do not demonstrate causation. In previous studies, high BDNF dependency was observed for interneuron markers in constitutive (Guilloux et al., 2012; Tripp et al., 2012) and adult (Glorioso et al., 2006) BDNF knockout mice. Yet, constitutive or chronic reduction of BDNF can lead to biological compensations and the observed gene expression changes could themselves be the result of those compensatory mechanisms. Further, age-related gene expression changes in human seem to be more specifically affected by TrkB changes. Utilization of $TrkB^{F616A}$ mice allowed us to address causality while circumventing those limitations. Contrary to our expectation that both excitatory and inhibitory synaptic genes would be decreased by a temporal TrkB blockade, we observed aging-like changes mainly in markers of GABA neurons inhibitory presynaptic functions: SST, NPY, CORT, CCK and GAD1. Potential reasons for these findings and implications for aging and brain disorders are discussed next.

Several reasons might account for the differential effect of aging and temporal blockade of BDNF signaling. First, species differences might account for the discrepancy; indeed, age-related alterations in gene expression patterns differ between mice and humans (Loerch et al., 2008). Second, 1NMPP1 treatment to $TrkB^{F616A}$ mice is known to exhibit a TrkB-null phenotype (Chen et al., 2005), so contrary to an aging-related gradual decrease of BDNF function, this accelerated dramatic change in BDNF/TrkB activity may be too short for evoking the more

gradual and long-term compensatory changes that occur during aging. We believe that this temporal de-coupling allowed us here to differentiate the effects of BDNF on markers of distinct cell populations. Third and related, interneurons might be more dependent on BDNF than pyramidal cells and consequently are more likely to exhibit a response to low BDNF signaling. This is consistent with prior findings using constitutive KO lines (Glorioso et al., 2006; Guilloux et al., 2012; Tripp et al., 2012). GABAergic cells cannot produce BDNF and rely on BDNF supply from other cell population such as excitatory neurons (Cellerino et al., 1996; Gorba and Wahle, 1999; Jin et al., 2003; Kohara et al., 2007; Marty et al., 2000; Rocamora et al., 1996). Together with the findings from human post-mortem study, the animal data suggests that age-repressed BDNF signaling may result in lower GABA function, which with time may lead to glutamate signaling downregulation to preserve excitatory-inhibitory balance (Figure 10).

One can assume that this mechanism of low BDNF function - driving synaptic alterations without breaking the excitatory-inhibitory balance - is crucial for healthy aging. On the other hand, deregulation in this homeostatic mechanism may lead to pathological changes and brain disorders. Interestingly, MDD has been associated with reduced central BDNF function (Guilloux et al., 2012; Tripp et al., 2012) and with an accelerated pattern of age-related transcriptome changes (Douillard-Guilloux et al., 2013). However, these MDD- and BDNF-related changes have been preferentially associated with altered/reduced pre-synaptic GABA function rather than excitatory glutamatergic-related functions (Ding et al., 2015; Guilloux et al., 2012; Tripp et al., 2012). This suggests that MDD may result from a failure for excitation to compensate for BDNF and GABA-related gene changes. This is consistent with an age by disease interaction model, where the normal trajectory of age-dependent changes provides substrates for pathological changes. This model suggest that whenever those changes occur out

of their biological context (e.g., too early) or do not lead to appropriate homeostatic adaptations, this may result in biological imbalance and pathophysiological entities (McKinney et al., 2012; Sibille, 2013). Here, the mechanism underpinning reduced BDNF during aging was not investigated, however, hypermethylation in BDNF gene was observed in older subjects of the same cohort (McKinney et al., 2015). It would be interesting to investigate how those (and other) age-related regulatory mechanisms are affected in MDD.

In summary, the findings from these human and mouse studies show that age-related reduction in BDNF signaling causes synaptic alterations in the prefrontal cortex, which is potentially related to cognitive changes in older subjects. The early event may involve alterations in GABA pre-synaptic markers, whereas excitatory synaptic changes may follow to maintain the excitation/inhibition balance.

Limitations

First, no gene or cellular functional analysis was performed in this study. Although animal and imaging studies imply that synaptic disturbance is the underpinning mechanism of age-related cognitive decline, we cannot directly determine if synaptic gene expression changes are responsible for functional deficit. Second, gene expression profile was analyzed in tissue homogenate containing all 6 layers of gray matter. Considering that age-associated cognitive impairment has been suggested to correlate with layer 3 pyramidal neurons, the molecular changes might be diluted and information such as cell specific-gene alteration pattern would be lost. Finally, functional activity was inferred by RNA analyses and not assessed at the protein level, since expression and integrity of small neuropeptides can be affected by many factors associated with under human postmortem conditions.

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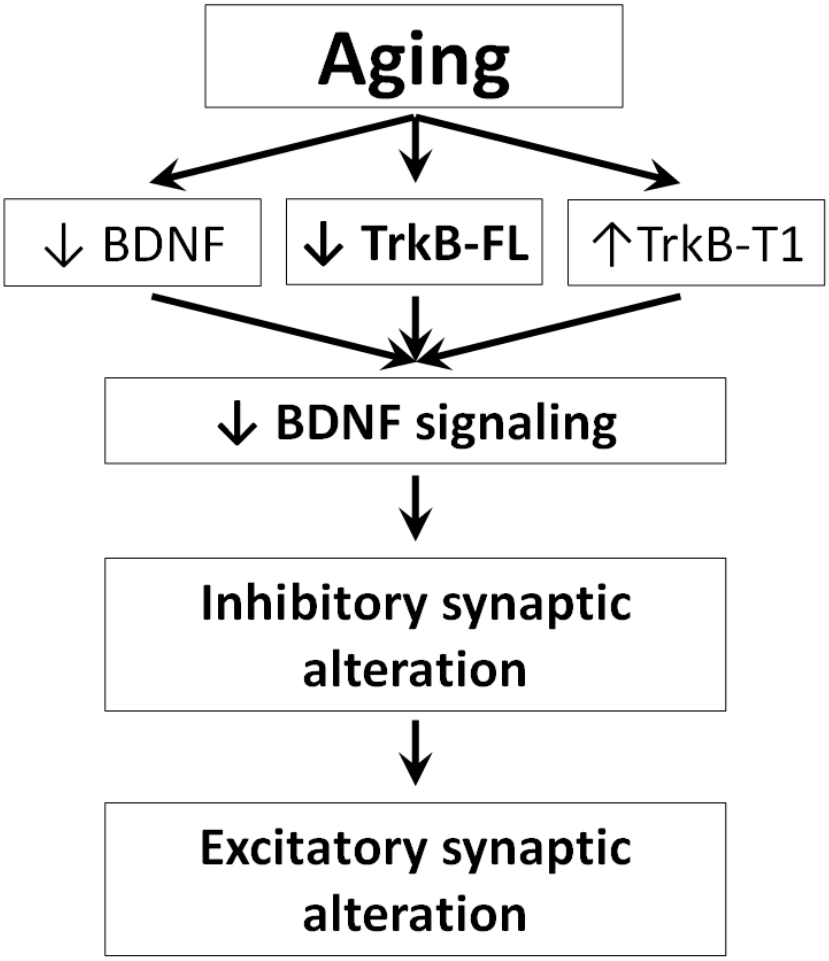


Figure 10. Proposed sequence of BDNF-induced brain changes during normal aging

Results from this study show that brain aging suppresses BDNF and TrkB-FL expression, and promotes TrkB-T1 expression, all together in the direction of repressing BDNF activity. Studies in rodents with temporal blockade in BDNF/TrkB signaling suggest that reduced function in the GABA presynaptic compartment may first occur, leading in time to reduced function in excitatory synaptic function, together maintaining homeostasis and excitation/inhibition balance.

4.0 PAPER 3: THE ROLE OF DENDRITIC BDNF TRANSCRIPTS ON ALTERED INHIBITORY CIRCUITRY IN MAJOR DEPRESSIVE DISORDER

Hyunjung Oh[1], David A. Lewis[1], Simon Watkins[2] and Etienne Sibille[1,3§]

[1]Department of Psychiatry, Center for Neuroscience and [2]Center for Biologic Imaging, Department of Cell Biology and Physiology, University of Pittsburgh, PA, USA

[3]Campbell Family Mental Health Research Institute of CAMH; Departments of Psychiatry, and Pharmacology and Toxicology, University of Toronto, Toronto, Canada

[§]To whom correspondence should be addressed. E-mail: Etienne.Sibille@camh.ca

4.1 INTRODUCTION

Major depressive disorder is the most common psychiatric disease affecting 350 million people worldwide (WHO, 2012). Depressive disorders were the second leading cause of disability in 2010 (Ferrari et al., 2013) and with low remission and high relapse rates, MDD imposes billions of dollars of economic burden on US society (Greenberg et al., 2015; Greenberg et al., 2003; Kessler, 2012). MDD is defined as a syndrome of mood dysregulation, physiological symptoms and cognitive impairment (DSM-5; American Psychiatric Association, 2013). These multifactorial features imply that various brain regions are involved in the pathophysiology of disease (Seminowicz et al., 2004).

Structural and functional abnormalities of the prefrontal cortex (PFC) have been consistently reported in MDD. The PFC plays a critical role in cognitive function, working memory, emotion regulation and is highly affected by stress (Arnsten, 2009b; Cerqueira et al., 2007; Cerqueira et al., 2005; Cook and Wellman, 2004; Holmes and Wellman, 2009; McEwen and Gianaros, 2011; Radley et al., 2008; Radley et al., 2004; Wellman, 2001). Consistent with functional impairment and atrophy (Arnsten, 2009a; Drevets, 2000, 2007; Drevets et al., 1998; Drevets et al., 1997; Liston et al., 2006; Tan et al., 2007), human postmortem studies reported decreased neuronal body size, spine loss, and decreased cellular density in the PFC of subjects with MDD (Kang et al., 2012; Rajkowska, 2000a; Stockmeier et al., 2004a).

Decreased GABA neurotransmission has been implicated in MDD, suggesting imbalance of excitation and inhibition (E/I) in the illness (Levinson et al., 2010; Sanacora et al., 2004; Sanacora et al., 1999; Sequeira et al., 2009). Studies from our lab have shown decreased GABA-related genes in diverse corticolimbic areas including the dorsolateral PFC (dlPFC), amygdala, and subgenual anterior cingulate cortex (sgACC) of MDD patients (Guilloux et al., 2012; Sibille

et al., 2011; Tripp et al., 2011; Tripp et al., 2012). Downregulation of SST, one of the molecular markers of dendritic targeting interneurons, in addition to reduced SST (+) cell number, was consistently found across different brain regions, whereas parvalbumin (PVALB), the marker of perisomatic-targeting interneurons, remained relatively unaffected.

One of the mechanisms underpinning this selective vulnerability of SST-expressing interneurons could be the loss of local neurotrophic support. BDNF is a neurotrophic factor that plays a crucial role in neuronal development, plasticity and maintenance throughout life. MDD, prolonged stress and chronic administration of corticosterone reduce BDNF expression (Duman and Monteggia, 2006; Dunham et al., 2009; Durany et al., 2001; Dwivedi et al., 2003; Guilloux et al., 2012; Hashimoto et al., 2005a; Howells et al., 2000; Mao et al., 2014; Phillips et al., 1991; Thompson Ray et al., 2011), which might compromise structural integrity (Radley et al., 2008; Radley et al., 2004). GABAergic cells cannot produce BDNF and rely on BDNF supply from other cell populations such as excitatory neurons (Cellerino et al., 1996; Gorba and Wahle, 1999; Jin et al., 2003; Kohara et al., 2007; Marty et al., 2000; Rocamora et al., 1996). In human subjects with MDD, SST reduction is accompanied by BDNF and/or TrkB downregulation (Guilloux et al., 2012; Tripp et al., 2012), and potentially accelerated by normal age-downregulation of BDNF expression (Douillard-Guilloux et al., 2013). Animal studies using mice with reduced BDNF expression revealed high BDNF dependency of SST (Glorioso et al., 2006; Guilloux et al., 2012; Tripp et al., 2012), but the underlying mechanism has not been identified.

Local action of BDNF is closely associated with neural plasticity. In addition to its autocrine action regulating dendritic and synaptic plasticity of pyramidal cells (Tanaka et al., 2008; Wang et al., 2015), BDNF can alter the presynaptic GABA system in a paracrine manner

(Bolton et al., 2000; Hyman et al., 1994; Kohara et al., 2007; Ohba et al., 2005; Wuchter et al., 2012; Yamada et al., 2002). Of the ~300,000 probes tested by microarray, we found that the expression of GABRA5 (gamma-aminobutyric acid A receptor, alpha 5), which is specifically enriched nearby dendritic GABA synapses (i.e. perisynaptic), showed the highest correlation to BDNF level in the human prefrontal cortex (Oh et al., submitted). This suggests that dendritic BDNF function may be crucial to maintain normal function of SST cells. Interestingly, BDNF transcripts are present in dendrites (An et al., 2008; Baj et al., 2011) and cellular localization of BDNF transcripts is highly linked to phospho-TrkB immunostaining pattern (Baj et al., 2011), suggesting that BDNF transcripts can be locally translated and act in dendrites. Accumulating evidence implies that dendritic BDNF mRNA is closely associated with stress: dendritic BDNF transcripts are decreased by stress (Berry et al., 2015; Luoni et al., 2014; Luoni et al., 2015), and increased by antidepressant treatment (Baj et al., 2012). Knockdown of dendritic BDNF impairs structural integrity of hippocampal neurons (Baj et al., 2011; Orefice et al., 2013).

In the current study, we first determined whether dendritic BDNF transcripts were changed in the PFC of MDD subjects and in the mPFC of mouse exposed to chronic stress, and tested whether such changes were linked to dendritic-targeting inhibitory interneuron markers. Using small hairpin RNA (shRNA), we knocked down dendritic BDNF mRNA to test whether low dendritic BDNF expression is associated with MDD- and stress-related phenotype. Finally, we investigated whether enhanced TrkB signaling could prevent and reverse chronic stress-induced behavioral and molecular changes. We predicted that decreased local neurotrophic support at dendrites contributes to selective disturbance in dendritic-targeting GABA interneurons and finally results in mood dysregulation.

4.2 MATERIALS AND METHODS

4.2.1 Human postmortem subjects

Postmortem brains were collected from the Allegheny County Coroner's Office (Pittsburgh, PA) after consent from next of kin. The neuropsychiatric-related resources of this brain collection have been amply characterized in microarray and micro-anatomical studies (Bloss et al., 2011; Hashimoto et al., 2005a; Lewis et al., 2001; Mirnics et al., 2000; Mirnics et al., 2001; Sibille et al., 2009a; Sweet et al., 2003). In short, for all cases, a committee of experienced clinical scientists examines clinical records, toxicology exam and standardized psychological autopsy data (Glantz et al., 2000). Consensus DSM-IV diagnosis is established for psychopathology, medical and social histories, and history of substance abuse. This latter characterization incorporates a structured interview, conducted by a licensed clinical psychologist with family members of the index subject, to assess diagnosis, psychopathology, medical, social and family histories, as well as history of substance abuse. Individuals were also screened for the absence of neurodegenerative disorders by neuro-pathological examination (Glantz and Lewis, 1997; Sibille et al., 2004; Sweet et al., 2004). All procedures were approved by the University of Pittsburgh's Committee for the Oversight of Research Involving the Dead and Institutional Review Board for Biomedical Research. Upon brain collection, coronal blocks are cut in ~2 cm blocks through the rostro-caudal extent of the brain and stored at –80°C. The RNA integrity of each brain is assessed by chromatography using the Agilent Bioanalyzer instrument (Agilent Bioanalyzer; Santa Clara, CA, USA). After careful examination of clinical and technical parameters, 19 pairs of MDD and control subjects were selected (detailed characteristics of the cohort is available in Table 10 in Appendix D). Each subject was matched for sex and as closely as possible for age

with control subjects. Subject groups did not differ in mean age, PMI, RNA integrity number (RIN), RNA ratio, comorbid substance abuse, or brain pH as determined by one way ANOVA ($p>0.05$).

4.2.2 Animals and unpredictable chronic mild stress

Young adult C57BL/6J male mice were divided into two groups and submitted to control housing condition or UCMS consisting of an 7-week regimen 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 or bob cat urine), light cycle changes, social stress (rotate mice into previously occupied cage), tilted cage (45°), mild restraint (50 mL Falcon tube with air hole for 15 minutes) and bedding changes. The progression of the UCMS syndrome was monitored weekly by assessing the degree of coat state degradation and weight changes for each mouse.

4.2.3 Drug treatment

To test whether enhanced BDNF signaling can prevent chronic stress-induced behavioral/molecular changes, male C57/BL6 mice received intraperitoneal injections of TrkB agonist, 7,8-dihydroxyflavone (7,8-DHF, 5mg/kg) or vehicle after 2 weeks of chronic mild stress for 5 weeks (n=12/group). Anxiety- and depression-related behaviors were determined with cookie test, elevated plus maze, open field and novelty suppressed feeding test. At the end of study, the brains were flash frozen on dry ice.

4.2.4 Behavioral testing

After UCMS, common tests for depression and anxiety-like phenotype were conducted. Behavior evaluated using the Elevated Plus Maze (EPM) used a cross maze with 2 open and 2 closed 30×5 cm arms. Time spent in the open arms and percent entries (entries into open arms divided by entries into open or closed arm×100) in the open arms was recorded for 10 min to measure anxiety-like behavior. The total number of entries into any arm was used as an index of locomotor behavior. The Open Field test (OF) was performed with aid of the ANY-maze video tracking system for the measurement of anxiety-like behavior. The center of the OF was defined as the centermost 51×51 cm arena. The time spent and percent distance traveled (distance in center divided by total distance×100) in the center of the arena was recorded for 10 min. The total distance travelled was recorded as an index of locomotor activity. The latency for food-deprived mice to feed in an aversive, novel environment was be used as an index of emotionality in the Novelty Suppressed Feeding test (NSF). Mice were food-deprived for 16 h prior to exposure to NSF. Testing was performed in a brightly lit 51×51 cm arena covered in bedding. Latency to eat a food pellet placed in the aversive center of the arena was recorded during a 12-minute session. Food consumption in the home cage (food eaten divided by body weight) during 5 min following NSF testing and percent weight lost during food deprivation was measured as controls for appetite differences. The Cookie Test (CT) is based on the conflict between the drive for the stimulus and the neophobic behavior of the mouse and utilizes a device containing three aligned compartments with the same dimension (20 × 20 × 20 cm). Mice were first familiarized with a chocolate cookie (Keebler® Fudge Stripes, US) 5 days before the first testing. At the time of testing, a small amount (2±1 g) of chocolate cookie was placed at the corner of the black compartment. The mouse was initially placed in the white compartment of the apparatus. The

time of first cookie consumption (bites) was recorded within the 12 min test period. Next day, another session of testing was performed. An elongated time prior to cookie consumption was interpreted as anhedonia, a habituation deficit or a combination of both effects. After behavioral tests, all mice were sacrificed by Avertin overdose. After quick perfusion, brains were extracted quickly and flash frozen on the dry ice.

4.2.5 Emotionality z-score

To reduce behavioral variabilities which are thought to be caused by natural fluctuations over time, we integrated emotionality-related measures across tests as described earlier (Guilloux et al., 2011). Z-scoring is a mathematical tool used to normalize results within studies. These values "indicate how many indicate how many standard deviations (σ) an observation (X) is above or below the mean of the control group (μ)".The equation is as follows: $z=\frac{X-\mu}{\sigma}$

After adjusting directionality of the scores so that increased score values reflected increased depressive-/anxiety-like behavior, z-scores of individual tests were averaged for group emotionality z-score.

4.2.6 RNA extraction and Real-time quantitative polymerase chain reaction

For human studies, all six layers of dlPFC were collected in TRIzol and further purified with Qiagen RNeasy spin columns (QIAGEN, Valencia, CA). For mice, mPFC punches were taken using 19G corer and processed for RNA extraction with Qiagen RNeasy micro kit. To generate cDNA, 100 μg of total RNA was mixed with SuperScript III supermix (Invitrogen, Carlsbad,

CA) according to manufacturer's protocol. PCR products were amplified in quadruplets on a Mastercycler real-time PCR machine (Eppendorf, Hamburg, Germany) using universal PCR conditions. Results were calculated as the geometric mean of threshold cycles normalized to three validated internal controls (actin, glyceraldehyde-3-phosphate dehydrogenase, and cyclophilin G). Candidate genes for qPCR verification were (i) 6 BDNF transcript variants: BDNF-exon 1, 2, 4, 6, CDS, long 3' UTR; (ii) 2 TrkB isoforms (FL, T1); (iii) 7 Excitatory neuronal gene: vGLUT 1, GRIA1, GRIN 2A & 2B, DLG4; (iv) 12 Inhibitory synapse-related genes: 9 presynaptic genes, vGAT1, 8 interneuron markers (SST, NPY, CORT, PVALB, CCK, GAD 1 & 2), 3 Postsynaptic (GABRA 4/5, GABRB3).

4.2.7 Protein isolation, immunoprecipitation, and western blotting

After micropunch, the remaining frontal cortices were collected in lysis buffer (50mM Tris-Cl, 150mM NaCl, 1% Triton-X100, 0.1% SDS, 1X phosphatase inhibitor cocktails (P0044; Sigma-Aldrich, St. Louis, MO,), 1X protease inhibitors (539131; EMD Millipore, Billerica, MA)). Protein concentration was measured using a Pierce BCA protein assay kit.

For p-Trk immunoprecipitation, 500 µg of protein was diluted with PBS to 1 ml and incubated overnight at +4ºC with 2.5 µl of phospho-TrkA(Y674/675)/TrkB(Y706/707) antibody (4621; Cell signaling technology, Danvers, MA). Immunocomplexes were precipitated with 20 µl of Protein A/G agarose bead (20412; Life technologies, Grand Island, NY) for 2 hours at room temperature and separated on 4-15% gradient gel (4561083; Bio-Rad Laboratories, Hercules, CA) under reducing conditions and blotted to a PVDF membrane. After blocking with Licor blocking buffer (927-40000; LI-COR Biosciences, Lincoln, NE), membranes were incubated with rabbit anti-total TrkB antibody (sc-8316; 1:500, Santa Cruz Biotechnology Inc.

Santa Cruz, CA) and mouse anti-actin antibody (A2228; 1:10,000, Sigma-Aldrich, St. Louis, MO) at 4 ºC overnight. After rinse with PBST, fluorescent dye-conjugated secondary antibodies (LI-COR Biosciences) were treated and visualized using LI-COR Odyssey Infrared imaging system.

4.2.8 Small hairpin RNA, DNA constructs and Adeno-associated virus (AAV)

Small hairpin RNA (shRNA) sequence targeting ggaaggctcggaagcaccct of long 3' UTR of mouse BDNF was adapted from previous work (Orefice et al., 2013). Lentiviral vectors containing shRNA against BDNF long 3' UTR or scrambled shRNA, and eGFP as a reporter gene were purchased from GeneCopoeia (Rockville, MD). AAV9-BDNF long 3' UTR shRNA-GFP and control virus with scrambled shRNA were commercially prepared with the U6 promoter (Virovek, Hayward, CA). Briefly, Bac-to-bac system (Invitrogen) was used to generate recombinant baculoviruses. First, shRNA was sub-cloned into pFastBac shuttle vector and transformed into DH10Bac competent bacteria. After antibiotic selection, recombinant Bacmid containing E. coli were cultured and miniprep DNAs were prepared. Recombinant Baculovirus particles were generated by transfecting Sf9 cells with miniprep DNAs and amplified once. For AAV vector production, Sf9 cells were double-infected with recombinant baculovirus containing the shRNA and a helper recombinant baculovirus containing the AAV Rep and Cap genes. Three days post-infection, the Sf9 cells were harvested and lysed in SF9 lysis buffer (50mM Tris-HCl, pH7.8, 50mM NaCl, 2mM $MgCl_2$, 1% Sarkosyl, 1% Triton X-100, and 140 units/ml Benzonase). After genomic DNA digestion, cell debris was removed by centrifugation at 8,000rpm for 30 min. To purify AAV vectors, the cleared lysates were loaded onto CsCl step-gradient and subjected to ultracentrifugation at 55,000 rpm for 20 hours. The viral band was drawn through a syringe with

an 18-gauge needle and loaded onto a second CsCl and subjected to linear-ultracentrifugation at 65,000rpm for 20 hours. Then the viral band was drawn and passed through two PD-10 desalting columns (GE HealthCare) to remove the CsCl and detergents and at the same time exchanged to PBS buffer containing 0.001% pluronic F-68. Quantitative real-time PCR (qPCR) was performed to determine the virus titer with primers corresponding to the target gene.

4.2.9 Primary culture and transfection of mouse cortical neurons

Primary cortex neurons obtained from E18 embryos of C57BL/6J mouse were purchased from Life Technologies (Cat. No. A15585). After rapid-thaw, 3 X105 viable neurons were cultured on poly-D-lysine coated 12 mm coverslips in Neurobasal media (Invitrogen) supplemented with 2% B27, 0.5 mM GlutaMAX™-I Supplement, and 1% penicillin-streptomycin. Half of the medium was replaced with fresh medium every third day to feed cells. At 7 days in vitro (DIV7), transfection was performed using 1 µl of Lipofectamine 3000 (Invitrogen) and plasmid DNA (0.5 µg) in 50 µl of OPTI-MEM. Half of the culture media was replaced with fresh media after 3 days.

4.2.10 Fluorescent-activated cell sorting (FACS) and RNA knockdown yield test

After 5 days of transfection, media was removed and rinsed with 500 µL of DPBS twice. 300 µl of 0.5% trypsin-EDTA was added to detach cells from the plate. After 10 min at 37C, trypsin reaction was stopped by adding 300 µl of saline with 1% BSA, 4 mM EDTA, 20 U/ml of DNase1. Cells were collected by centrifugation at 300G, 5 min at 4C) and resuspended in 0.3 ml of FACS buffer (PBS, 0.5 % BSA, 2 mM EDTA bisodium salt, 10 U/ml of DNAse1). GFP-

expressing cells were directly collected to lysis buffer containing microtube by FACSAriaII in collaboration with McGowan Institute for Regenerative Medicine Flow Cytometry Facility. Total RNA was extracted using Qiagen RNeasy micro kit (QIAGEN, Valencia, CA) following the manufacturer's protocol. Total RNA sample was mixed with qScript cDNA supermix (Quanta BioSciences) to generate cDNA. PCR products were amplified in triplets on a Mastercycler real-time PCR machine (Eppendorf, Hamburg, Germany) using universal PCR conditions. Results were calculated as threshold cycles normalized to three housekeeping gene: actin, cyclophilin, GAPDH.

4.2.11 Immunocytochemistry and image analysis

After 5 days of transfection, cells were rinsed twice with PBS and fixed with 4% PFA for 25 minutes. After subsequent rinses, cells were stained with GFP (A21311; rabbit anti-GFP (2 µg/ml), Life Technologies) and MAP2 (M9942; mouse anti-MAP2 (2 µg/ml), Sigma-Aldrich) to visualize dendrites of transfected cells. Images were obtained using Nikon 90i microscope and the morphology of dendrites were automatically traced and reconstructed by Imaris software (Bitplane AG, Concord, MA). Quantification of the total length of dendrites, number of dendritic segments, Sholl analysis with concentric circles drawn every 10 µm from the soma were performed by the program.

4.2.12 Animals and stereotaxic surgery

Young adult C57BL/6J male mice were bilaterally injected with 400 nl of AAV9-BDNF long 3’ UTR shRNA-GFP virus or with AAV9-scrambled shRNA-GFP (control) with a titer of ~ 2 X

10^{12} genome copies/ml into the medial PFC (A/P +2.0 mm, M/L ±0.4 mm, D/V -2.0 mm). After 2-3 weeks of recovery, mice were exposed to UCMS. For gene expression analysis, mice received BDNF long 3' UTR shRNA in one hemisphere and scrambled shRNA in the other for intra-subject comparison.

4.2.13 Immunohistochemistry and image analysis

Mice were anesthetized and perfused through the heart with PBS, followed by fixative solution containing 4% paraformaldehyde in PBS. Fixed brains were removed from the skull, postfixed overnight, and then cryoprotected for 24-48 hours in 30% sucrose in PBS. Coronal sections with a thickness of 35 µm were collected in six serially adjacent sets and stored at 4°C in PBS with 0.1% sodium azide.

After blocking with 10% normal goat serum, 0.3M Glycine in PBS with 0.3% Triton X-100, sections were incubated with antibodies diluted in PBS containing 0.3% Triton X-100 and 1% normal goat serum. Antibodies used for immunohistochemistry were as follows: Alexa Fluor® 488 conjugated rabbit anti-GFP (1:1000, Life Technologies, A21311), chicken anti-GFP (1:1000, abcam, ab13970), rabbit anti-SST (1:200, Santa Cruz Biotechnology, sc-13099), mouse anti-PVALB (1:1000, Sigma-Aldrich, P3088), Cy3 conjugated goat anti-rabbit (1:250), Alexa Fluor® 568 conjugated goat anti-mouse (1:250), Alexa Fluor® 488 conjugated goat anti-chicken (1:250). Immunoreacted sections were mounted onto charged glass microscope slides (SuperFrost Plus; Fisher Scientific), air-dried and coverslipped with Vectashield hardset mounting media (Vector, H-1400).

4.2.14 Statistical analysis

Gene expression differences were determined by analysis of covariance (ANCOVA) using SPSS (SPSS, Inc., Chicago, IL, USA). To determine covariates to include in the gene-specific models, nominal factors were each tested as the main factor by ANOVA, scale covariates were tested by Pearson correlation, and repeated measures were corrected by modified Holm-Bonferroni test. ANCOVA models including significant co-factors were then applied.

4.3 RESULTS

4.3.1 Parallel downregulation of BDNF-long 3’ UTR and dendritic targeting interneuron markers in the frontal cortex of human subjects with MDD

The BDNF gene is composed of at least nine exons and makes diverse transcript variants by combination of alternative splicing and two polyadenylation sites (Figure 11.A). The spatial segregation of BDNF mRNA is encoded by different untranslated regions (UTRs); transcript variants with exon 2, 6 and the long 3' UTR can migrate to distal dendrites whereas mRNAs containing exon 1, 4 and the short 3' UTR are restricted to the soma (Baj et al., 2011; Waterhouse and Xu, 2009) (Figure 11.B; modified from Baj et al., 2011). Therefore, we designed primers targeting different UTRs related to cellular localization of BDNF transcripts to investigate if depression is associated with defects in specific transcript variants. A primer set targeting protein coding sequence (cds) of BDNF was also included to measure total BDNF level. Among tested BDNF UTRs, only the long 3’ UTR was significantly decreased in the dlPFC of

MDD subjects (-20.8±5.1%, p=0.012, Figure 11.C), suggesting significant decrease of dendritic BDNF transcripts.

A broader panel of tested genes indicated no expression changes for TrkB isoforms and excitatory synaptic-related genes. However, inhibitory synaptic genes displayed significant disease-related downregulation: SST (-33.9±7.2%, p=4.6E-04), NPY (-32.4±6.0%, p=2.5E-03), CORT (-30.8±4.8%, p=8.5E-05), vGAT1 (-28.6±5.1%, p=6.6E-03), GAD1 (-21.8±3.1%, p=8.3E-03) GAD2 (-20.8±2.1%, p=8.4E-03), GABRA5 (-20.0±2.7%, p=3.0E-03) (Figure 11.D-G, p-values are corrected for multiple comparisons). Interestingly, all three dendritic-targeting GABA interneuron markers (SST, NPY, and CORT) and GABRA5 were significantly decreased in MDD, whereas PVALB and cholecystokinin (CCK), the molecular markers of perisomatic-targeting interneurons, did not show significant changes, together suggesting selective and specific effects of MDD on dendritic-targeting interneurons.

We previously reported that SST displayed an age-related decline in sgACC of control subjects, but not in MDD because of low SST expression in MDD across ages (Tripp et al., 2011). Similarly, age-related reduction was observed in BDNF-long 3' UTR, SST and CORT in control subjects; however, such gene expression alterations were diminished or decreased in MDD (Table 11 in Appendix D). Sex of the patient, antidepressant treatment at time of death, and mode of death (i.e. suicide or not) did not explain group differences in our genes of interest.

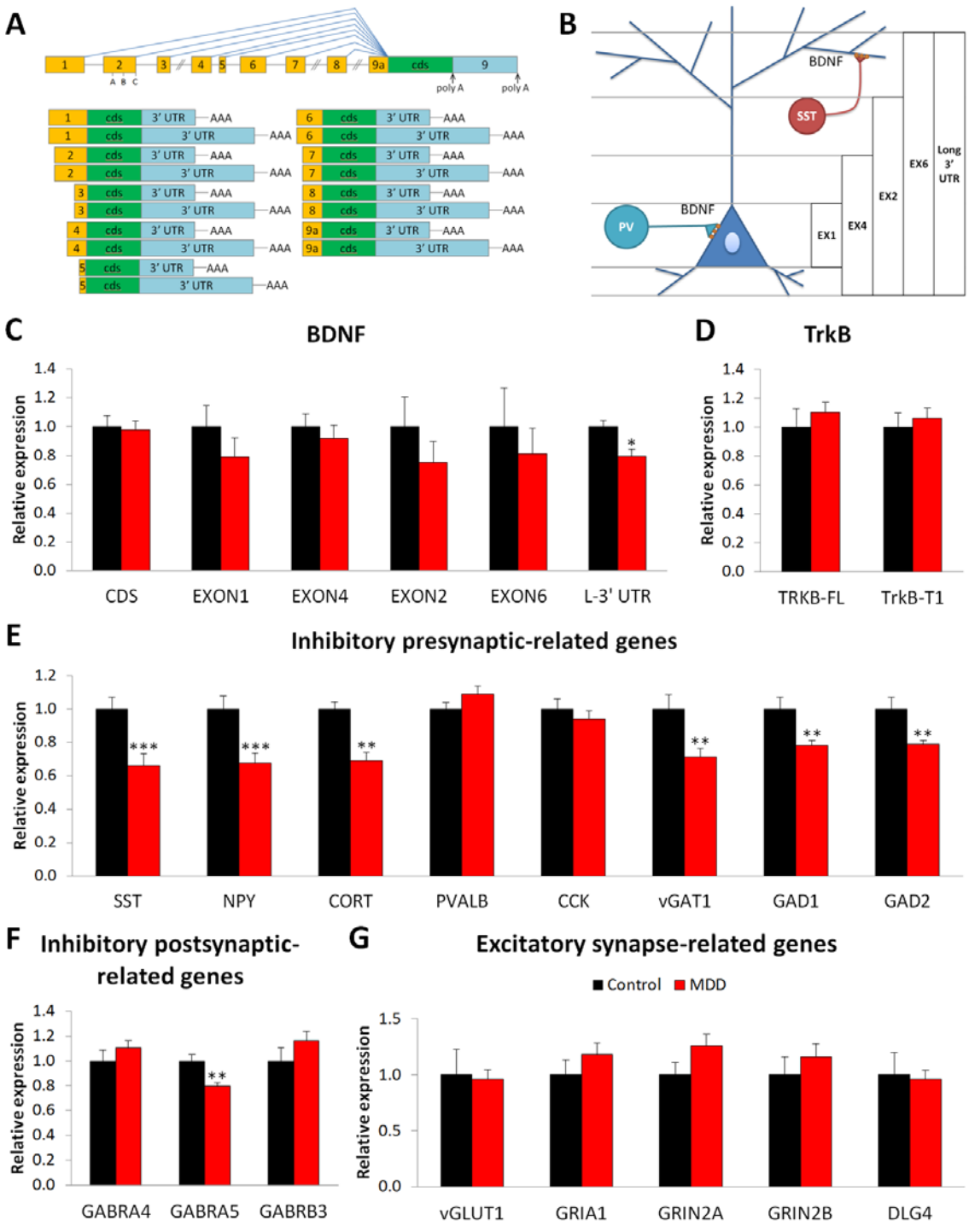


Figure 11. MDD-related changes in BDNF transcript variants and synaptic markers in human dlPFC

(A) Rodent BDNF gene structure and splice variants. (B) Schematic representation of microcircuitry and relative cellular distribution of BDNF transcripts. (C) Relative expression level (MDD/control) of BDNF transcripts, (D) TrkB isoforms, (E-G) selected sets of synapse related genes (n=19/group; *p<0.05, **p<0.01, ***p<0.001).

To investigate if low dendritic BDNF mRNA is associated with inhibitory synaptic gene changes, we calculated Pearson's correlation values between BDNF long 3' UTR and genes changed or unchanged in the brain of MDD subjects. While genes without disease-related changes did not show significant correlation, most MDD-related synaptic genes were positively correlated to the level of BDNF long 3' UTR (Figure 12): Pearson's correlation to BDNF-long 3' UTR: SST (R=0.53, p=6.8E-04), NPY (R=0.49, p=1.9E-03), CORT (R=0.58, p=1.5E-04), vGAT1 (R=0.35, p=0.033), GAD1 (R=0.37, p=0.021), GAD2 (R=0.250, p=0.129), GABRA5 (R=0.47, p=2.7E-03). Average correlation value between BDNF to MDD-related genes was 0.42±0.11 (p=6.1E-06), mean correlation value to MDD-unrelated genes was 0.10±0.16 (p=0.092; Figure 12.E).

In summary, selective downregulation in dendritic BDNF and dendritic-targeting interneuron markers were observed in the dlPFC of MDD subjects. Close correlation between BDNF long 3' UTR and GABA synaptic gene expression suggests that that low dendritic BDNF may drive such changes.

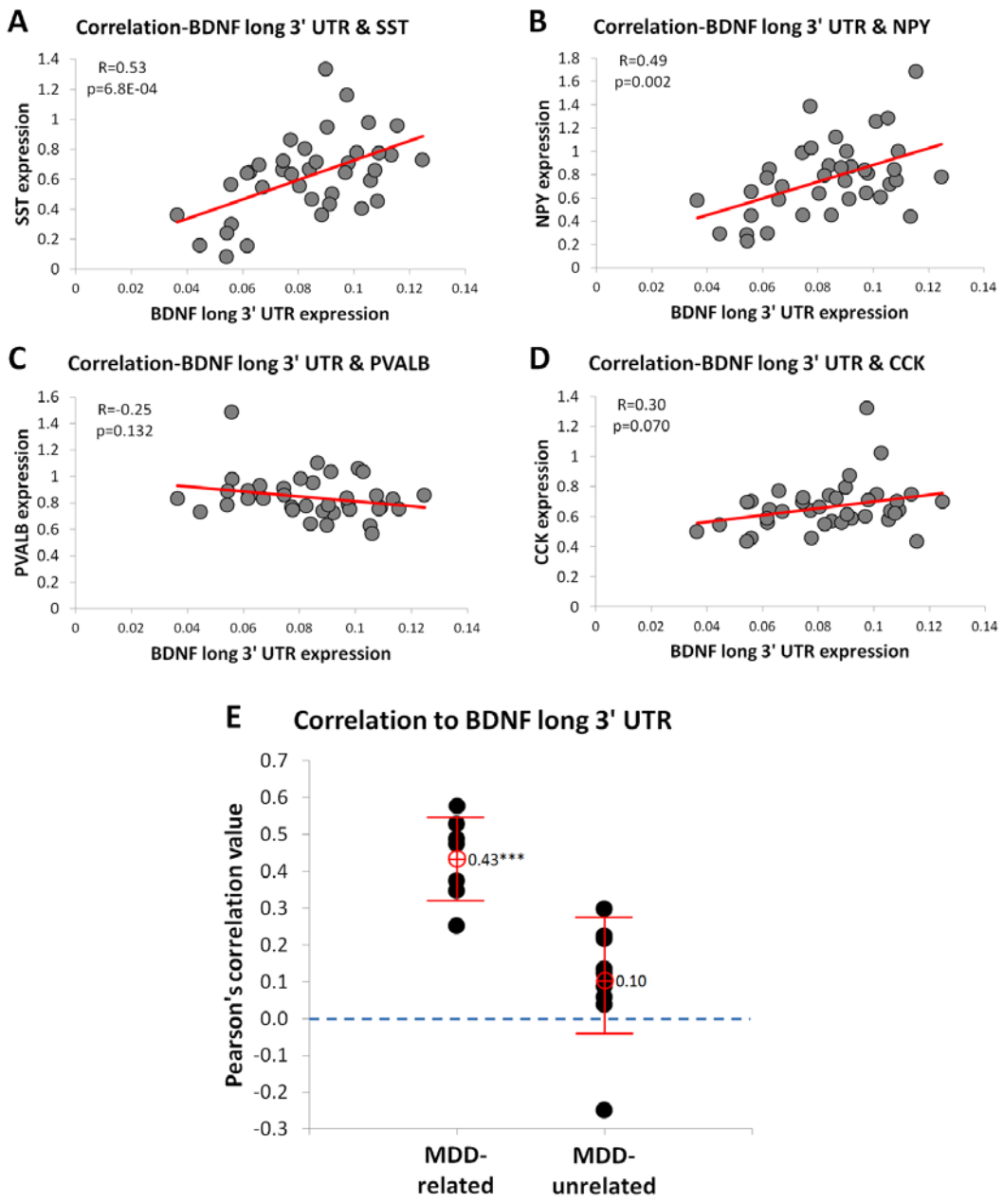


Figure 12. Association between BDNF long 3' UTR and MDD-related synaptic gene changes

(A) Pearson's correlation between BDNF long 3' UTR and SST, (B) NPY, (C) PVALB, (D) CCK. BDNF long 3' UTR is positively correlated with dendritic targeting interneuron markers (SST, NPY), but not with markers of perisomatic targeting interneurons (PVALB, CCK). (E) Overall correlation between BDNF long 3' UTR and MDD-related or unrelated synaptic markers. Significant positive correlation between BDNF long 3' UTR level and MDD-associated gene expression changes suggest contribution of dendritic BDNF to transcriptome alterations (one sample t-test to 0, *p<0.05 **p<0.01 ***p<0.001).

4.3.2 Chronic stress-related downregulation of dendritic BDNF transcript in the mPFC of rodents

Although chronic stress is a significant risk factor for major depression, MDD is associated with a joint contribution of environmental and genetic factors. Furthermore, BDNF can be regulated by various etiological factors such as exercise, circadian rhythm, and diet, all factors which cannot be controlled in human postmortem studies. Hence, we tested if chronic stress was sufficient to induce MDD-like gene expression changes using an animal model of depression, UCMS (Unpredictable Chronic Mild Stress). Male C57BL/6J mice were exposed to chronic mild stressors or control housing conditions for 6 weeks (n=12/group) and common behavioral tests measuring anxiety-/depressive-like behavior were performed. UCMS-induced depressive- and anxiety-like phenotype indicated by significantly higher emotionality z-score in stressed mice than control (z-score = 0.57, p=2.6E-03; Figure 13.A). Chronic stress significantly increased the latency to bite in NSF (control: 120±27s, UCMS: 212±37s, p=0.03) and in the cookie test (control: 287±72s, UCMS 471±54s, p=0.03). Individual behavioral test results are available in Figure 27 in Appendix D.

In medial prefrontal cortex (mPFC), long 3' UTR of BDNF (-24.9±7.5%, p=0.029) and CCK (+11.0±3.3%, p=0.045) were significantly changed by chronic stress (Figure 13.B and C). Despite significant reduction of dendritic BDNF transcripts, none of the dendritic-targeting interneuron markers showed stress-related change (Figure 13.C): SST (-4.3±2.3%, p=0.288), NPY (+9.2±7.9%, p=0.201), CORT (-14.6±4.5%, p=0.208) (expression levels of other synaptic genes are available in Figure 28 in Appendix D). Due to the discrepancy between human and animal results, we calculated Pearson's correlation values between SST and BDNF long 3' UTR to see if SST is closely associated to BDNF-long 3' UTR in the rodent brain. A significant

positive correlation suggested that close links between SST and BDNF long 3' UTR exists in the mouse tissue (R=0.43, p=0.018; Figure 13.D). Next, we examined whether the depressive-/anxiety-like trait is associated with gene expression level. Emotionality z-score was negatively correlated to both BDNF long 3' UTR (R=-0.43, p=0.018; Figure 13.E) and SST (R=-0.41, p=0.024; Figure 13.F). Nonetheless, PVALB and CCK did not show a significant correlation either to BDNF long 3' UTR or to emotionality z-score: correlation value between PVALB and BDNF long 3' UTR (R=-0.23, p=0.141) and emotionality (R=0.17, p=0.211), correlation value between CCK and BDNF long 3' UTR (R=0.31, p=0.070) and emotionality (R=-0.06, p=0.390).

Together, chronic stress induced downregulation in dendritic BDNF mRNA, but not in SST. These subtle gene expression changes in the stressed mouse brain might be caused by differences in timescale; human MDD subjects suffered for years to decades whereas mice were exposed to stress for only six weeks. A significant positive correlation between SST and BDNF long 3' UTR suggests that dendritic BDNF function is closely linked to SST-expressing interneurons in the mouse brain. In addition, depressive-/anxiety-related traits were inversely correlated with both BDNF long 3' UTR and SST. These data suggest that dendritic BDNF expression decreases at the early stage of stress-induced transcriptome changes, and SST alteration is a later event, maybe as a downstream target of decreased BDNF signaling on dendrites.

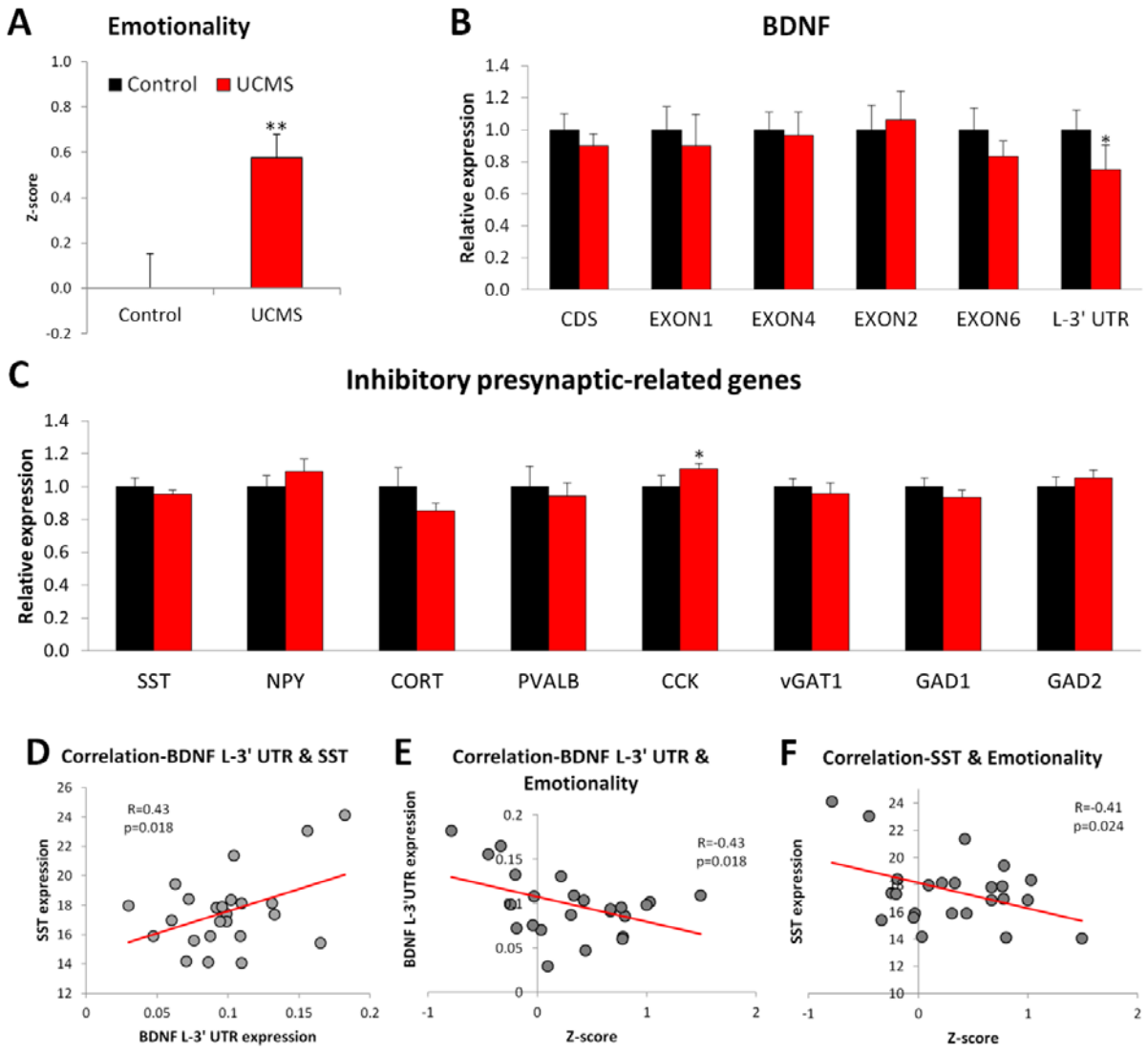


Figure 13. Chronic stress-related changes of BDNF transcript variants and synaptic markers in mouse mPFC

(A) Increased depressive-/anxiety-like phenotype in stressed mice compared to control, (B) Relative expression level (stress/control) of BDNF transcript variants, (C) inhibitory presynaptic-related genes. Similar to MDD, BDNF long 3' UTR was reduced by chronic stress. (D) Positive correlation between BDNF long 3' UTR and SST suggest a close link between two genes in mouse brain. (E) Significant inverse correlation between emotionality and BDNF-long 3' UTR and (F) SST. (n=12/group; *p<0.05, **p<0.01, ***p<0.001)

4.3.3 Knock-down of dendritic BDNF transcript is sufficient to induce MDD- and stress-associated phenotype

As significant downregulation of BDNF-long 3' UTR was observed in the prefrontal cortices of MDD subjects as well as in UCMS mice, we next investigated whether altered dendritic BDNF was causal to the MDD/stress-related phenotype. Structural abnormalities including synaptic loss, dendritic shrinkage and atrophy in brains of MDD patients and stressed animals have been well documented. To address whether low dendritic BDNF could explain these anatomical changes, we utilized shRNA targeting long 3' UTR of BDNF, which was already verified to decrease dendritic BDNF transcripts by other group (Orefice et al., 2013). Using GFP as a co-expressed marker gene (Figure 14.A, B), shRNA-expressing primary mouse cortical cells were collected with Fluorescence-activated cell sorting (FACS) to assess the knockdown yield of shRNA. qPCR showed that shRNA treatment decreased BDNF long 3' UTR level to below detection limit without affecting total BDNF (Figure 14.C). Five days of BDNF knockdown significantly impaired dendritic integrity of primary cortical neurons (control: n=14, BDNF long 3' UTR shRNA: n=10). Sholl analysis revealed that the distal dendrite was mainly affected by gene manipulation; number of dendrites crossing circles was not different in the proximal dendrite of both groups (0~30 μm from cell body), but shRNA expressing cells had smaller number of neurites between 40 to 250 μm (Figure 14.D). Furthermore, total dendritic length of shRNA-treated cells was decreased to 83.6±5.3% compared to scrambled treated neurons ($p=0.009$, Figure 14.E) while number of dendritic segments was unchanged (98.5±13.9%, $p=0.91$; Figure 14.F). These data indicate that BDNF long 3' UTR suppression decreases the length of individual branches rather than the branching pattern.

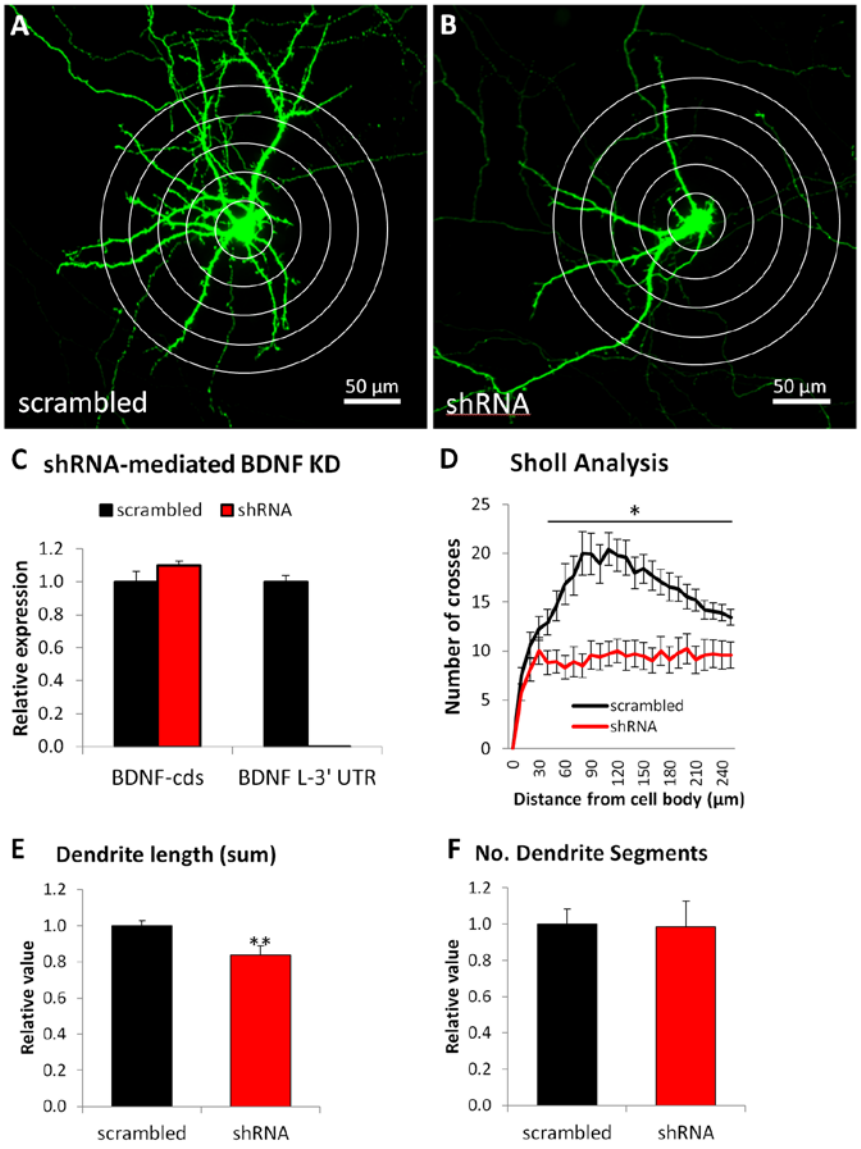


Figure 14. BDNF long 3' UTR knockdown-induced structural changes of primary cortical neurons

(A) Representative image of primary mouse cortical neuron expressing scrambled shRNA and (B) BDNF long 3' UTR-targeting shRNA. (C) Relative expression (shRNA/scrambled) of BDNF-cds and BDNF long 3' UTR in shRNA expressing cells compared to control. Expression of long 3' UTR was decreased below detection limit by shRNA treatment. (D) Sholl analysis for shRNA-treated and control neurons showed that shRNA treatment disrupted integrity of distal dendrite. (E) Relative value of total length of dendrites of shRNA-treated and control neurons. (F) Relative value of number of dendritic segments of shRNA-expressing neurons compared to control. Total dendritic length was significantly decreased without changes in branch number (control: n=14, shRNA: n=10; *p<0.05, **p<0.01, ***p<0.001).

To investigate whether decreased BDNF long 3' UTR is associated with behavioral changes, we injected AAV expressing either BDNF long 3' UTR shRNA or scrambled shRNA to the mPFC of wild type mice bilaterally (Figure 15.A). After 2~3 weeks of recovery, baseline emotionality was measured. No significant group difference was observed in any behavioral tests we performed (scrambled: n=9, shRNA: n=13; Figure 15.E-F). Therefore, we exposed mice to chronic stress for 6 weeks to see if low BDNF long 3' UTR altered stress susceptibility (Figure 15.B). While body weight was not changed with prolonged stress, we observed progressive worsening of coat state, indicating that both groups of mice responded to UCMS. Interestingly, shRNA treated mice displayed coat state changes earlier than control group (Figure 15.C, D). The behavioral tests performed in UCMS week 6 revealed significantly greater depressive-/anxiety-related behaviors in shRNA treated mice compared to control. In the open field test, shRNA treated mice did not differ from control group in time spent in center in pre-stress period (scrambled 54.7±2.9 s, shRNA 42.0±5.8 s, p=0.052), however, shRNA treated mice spent significantly shorter time in the center area after stress exposure (scrambled 41.3±5.6 s, shRNA 26.5±2.8 s, p=0.009. Figure 15.E). Overall emotionality also showed significant group differences after stress exposure (scrambled 0.95±0.22, shRNA 1.5±0.17, p=0.029; Figure 15.F). Results from other behavioral tests are available in Figure 29 in Appendix D.

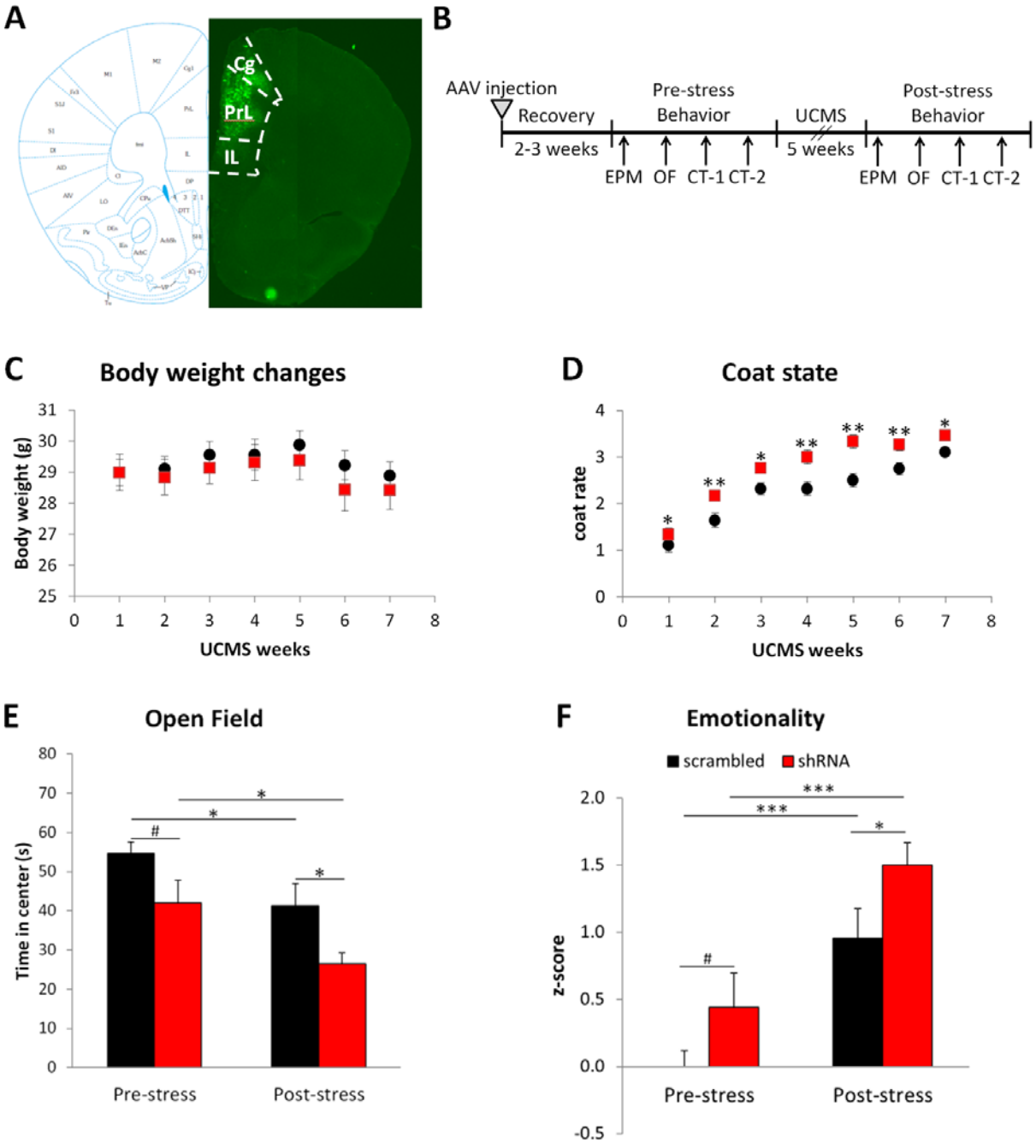


Figure 15. Combinational effect of BDNF long 3' UTR knockdown and chronic stress on behavioral changes

(A) Representative image of GFP expression following AAV injection in mPFC. (B) Schematic of the experimental design. (C) Body weight changes with chronic stress (D) Progressive coat state changes with stress exposure. (E) Stress X shRNA interaction on time spent in center in open field test. shRNA treated mice spent less time in center after stress. (F) Emotionality z-score. ShRNA showed additive effect on stress-induced emotionality increase (scrambled: n=9, shRNA: n=13; *p<0.05, **p<0.01, ***p<0.001).

To examine whether knockdown of dendritic BDNF is sufficient to induce MDD-associated synaptic gene changes, we injected BDNF long 3' UTR shRNA in one hemisphere and scrambled shRNA in the other (Figure 16.A, n=6/group). Mice were sacrificed at 2, 4, and 6 weeks after surgery, gene expression level was measured and compared in an intra-subject manner (Figure 16.B). qPCR analysis revealed that shRNA treatment changed synaptic gene expression in a manner similar to the MDD-associated transcriptome profile. Although no obvious gene expression changes were found after 2 weeks of shRNA expression (Figure 16.C). BDNF long 3' UTR as well as total BDNF level were significantly reduced in week 4 (Figure 16.D): BDNF-cds: -24.1±5.9% (p=0.030), BDNF long 3' UTR: -27.3±11.0% (p=0.013). And finally, SST level was changed along with BDNF mRNAs in post-surgery week 6: BDNF-cds: -52.9±4.9% (p=0.009), BDNF-long 3' UTR: -40.7±6.6% (p=0.030), SST: -31.2±7.0% (p=0.002) (Figure 16.E). In support of the mRNA changes, immunofluorescent signal intensity of SST was significantly decreased in shRNA injected region than control region (-5.7±1.7%, p=0.026; Figure 30 in Appendix D). A significant positive correlation between SST and BDNF long 3' UTR expression (R=0.66, p=0.009) in week 6 confirmed that reduced BDNF long 3' UTR is responsible for SST changes (Figure 16.F). Again, PVALB did not show such relationship to BDNF long 3' UTR (R=0.05, p=0.433; Figure 16.G). Although total BDNF was significantly reduced by shRNA, its expression level was not related to either SST or PVALB (correlation to SST R=0.18, p=0.284; correlation to PVALB R=0.15, p=0.324).

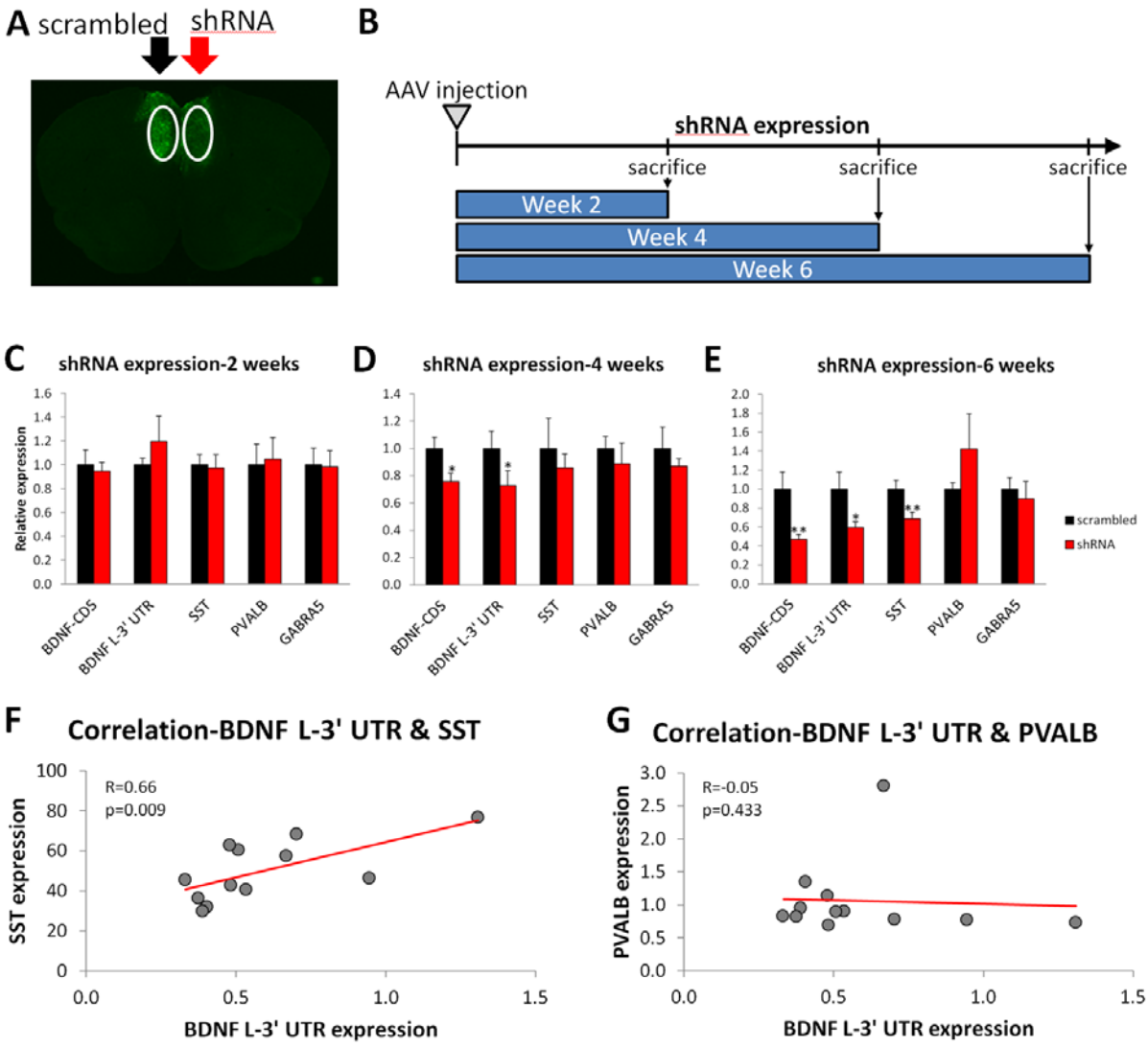


Figure 16. BDNF long 3' UTR knockdown-related gene expression changes

(A) Representative image of GFP expression following AAV injection in mPFC. (B) Schematic of the experimental design. (C-E) Expression changes in core genes at 2, 4, 6 weeks after surgery. Expression changes in total BDNF and BDNF-long 3' UTR were observed from week 4 and SST changed in week 6. (F-G) Pearson's correlation between BDNF long 3' UTR and SST/PVALB at post-surgery week 6. Significant correlation to BDNF long 3' UTR was observed in SST, not in PVALB. (n=6/time point; *p<0.05, **p<0.01, ***p<0.001).

We analyzed other BDNF/TrkB isoforms and synapse-related genes in mouse cortices from post-surgery week 6. None of the tested genes were altered by shRNA treatment except for BDNF exon4 (-37.0±9.5%, p=0.035; Figure 31 in Appendix D). Overall, BDNF long 3' UTR KD-induced gene expression changes were similar to transcriptome profile in MDD (R=0.45, p=0.009; Figure 32 in Appendix D).

In summary, knockdown of dendritic BDNF recapitulated MDD- and chronic stress-associated phenotypes: impaired integrity of distal dendrites, increased emotionality and reduced SST. Our observations that SST reduction occur several weeks after successful knockdown of dendritic BDNF, and that shRNA treated mice showed transcriptome profiles more similar to MDD than UCMS mice clearly suggest that MDD-induced dendritic BDNF downregulation is the cause of GABAergic gene changes.

4.3.4 7,8-dihydroxyflavone, a selective TrkB agonist prevented development of depressive-/anxiety-like behavior by chronic stress, but not through dendritic BDNF

To test whether stress-induced changes can be prevented by enhanced BDNF signaling, mice received daily intraperitoneal injection of TrkB agonist, 7,8-dihydroxyflavone (7,8-DHF), for 4 weeks starting in the third week of UCMS (Figure 17.A). Drug efficacy of 7,8-DHF was confirmed with increased phospho-TrkB in mouse forebrain (relative signal intensity to control: 2.9, Figure 17.B).

We found that development of the depression/anxiety-like phenotype was prevented by pharmacological potentiation of TrkB with 7,8-DHF. In novelty suppressed feeding, 7,8-DHF treatment significantly reduced latency to eat a food pellet (non-stress+vehicle: 120±27 s, UCMS+vehicle: 212±37 s, UCMS+7,8-DHF: 114±7 s, group difference between UCMS+vehicle

and 7,8-DHF: p=0.013; Figure 17.C). Other behavioral test results are available in Figure 33 in Appendix D. Emotionality z-score was also decreased by drug treatment (Non-stress+vehicle: 0.0±0.2, UCMS+vehicle: 0.6±0.1, UCMS+7,8-DHF: 0.2±0.1, group difference between UCMS+vehicle and 7,8-DHF: p=0.035; Figure 17.D.). These data suggest that maintaining integrity of BDNF-TrkB signaling is critical for mood regulation.

Next, we examined whether increased TrkB activity restored UCMS-related gene expression changes. While stress did not change TrkB-FL mRNA expression in mPFC, it reduced TrkB protein level in the forebrain. Both mRNA and protein showed significant upregulation after 4 weeks of 7,8-DHF treatment increased both mRNA and protein (Figure 17.E-G). Overall, TrkB protein and mRNA showed a significant positive correlation (Figure 34 in Appendix D). 7,8-DHF treatment recovered stress-related CCK downregulation and upregulated GRIN2A, and decreased three GABA receptors (GABRA4, 5 and GABRB3) compared to vehicle-treated stressed mice (Table 12 in Appendix D). Neither BDNF long 3' UTR nor SST were changed by drug treatment.

In conclusion, a chronic mild stress-induced depressive-/anxiety-like trait was prevented by pharmacological potentiation of TrkB with 7,8-DHF. Nevertheless, the therapeutic effect of 7,8-DHF seems to be related to enhanced TrkB activity rather than dendritic BDNF transcript and SST.

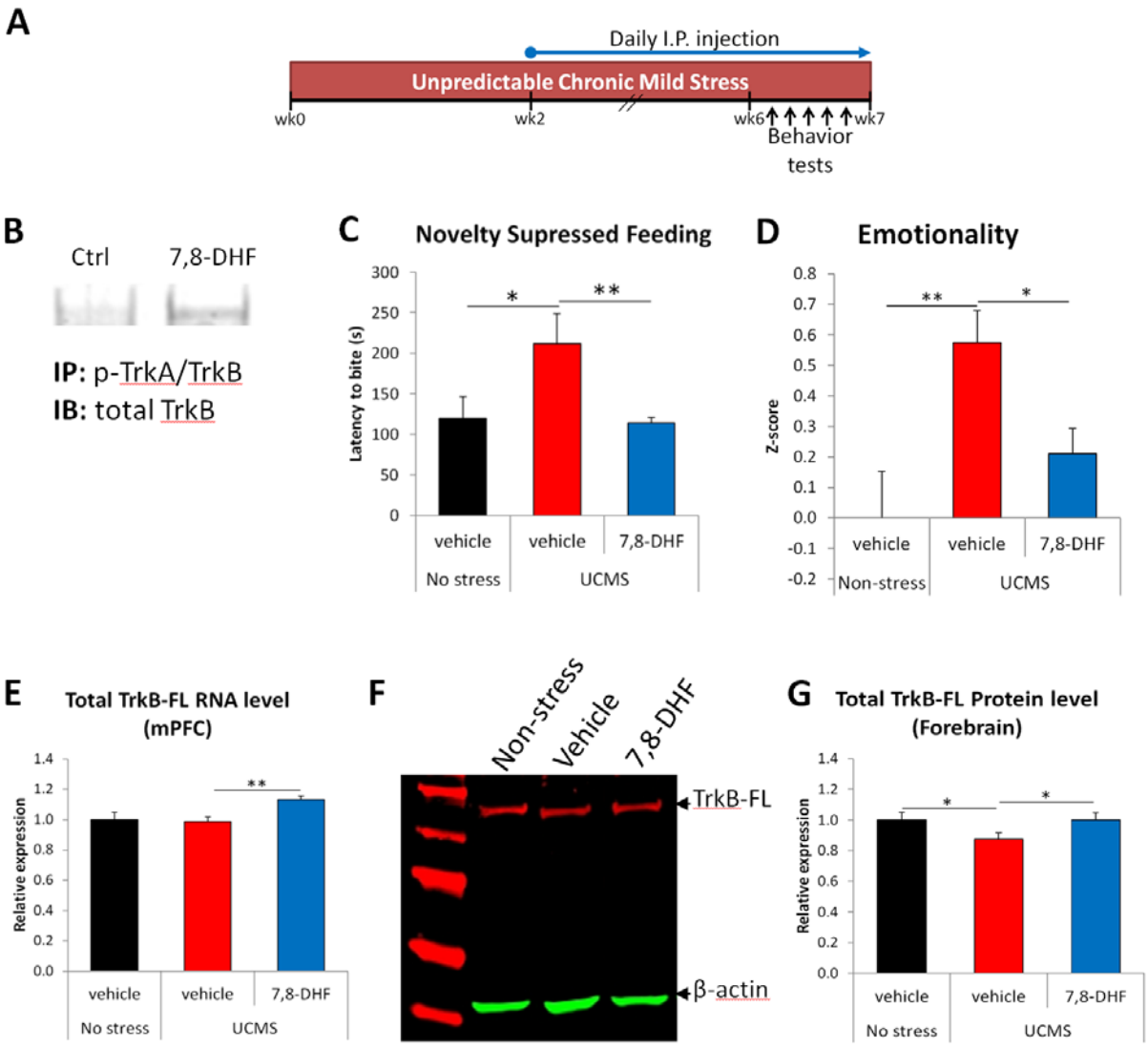


Figure 17. Pharmaceutical effect of 7,8-DHF, a selective TrkB agonist, on stress-induced behavioral, molecular changes

(A) Experimental design. (B) Western blot of immunoprecipitated phosphor-TrkB in forebrain of control or 7,8-DHF treated mice. 7,8-DHF significantly increased p-TrkB in the forebrain of treated mice. (C) Effect of chronic 7,8-DHF treatment on latency to eat food pellets in novelty suppressed feeding test, (D) overall integrated emotionality z-score. NSF and z-score suggest an antidepressant effect of 7,8-DHF. (E) TrkB-FL mRNA level in mPFC, (F-G) total TrkB-FL level measured by western blot. TrkB mRNA and protein were significantly increased by 7,8-DHF treatment. (n=12/group; *p<0.05, **p<0.01, ***p<0.001)

4.4 DISCUSSION

Major depression disorder is a devastating disorder characterized by low mood, anhedonia, cognitive deficits and physiological changes. Human and animal studies suggest that impaired neural plasticity and GABA dysfunction is associated with the disease; however, the mechanism underlying these two phenomena has not been clearly defined. In the current study, we observed selective downregulation of dendritic BDNF mRNA without changes in total BDNF level in the dlPFC of human MDD subjects and paralleled downregulation of dendritic-targeting interneuron markers (SST, NPY, CORT) without affecting perisomatic-targeting interneuron markers (PVALB, CCK) (Figure 11). High correlation between BDNF long 3' UTR and inhibitory synaptic genes suggests that an MDD-related decrease in dendritic BDNF transcripts contributes to impairment of dendritic-targeting interneurons (Figure 12). A selective deficit of BDNF long 3' UTR was also observed in the mPFC of stressed mice (Figure 13). Although SST expression did not show significant changes after 6 weeks of stress exposure, it was positively correlated to BDNF long 3' UTR and showed significant negative correlation to emotionality. Knockdown of BDNF long 3' UTR using shRNA replicated MDD-associated structural (Figure 14) and molecular (Figure 16) phenotypes. Obvious depression-/anxiety-like behavioral phenotypes were not observed in the shRNA treated mice at baseline (Figure 15); however, a time course study revealed that BDNF expression remained unchanged after 2 weeks of AAV expression (Figure 16). Supportive to the significant BDNF expression changes in post-surgery week 4, shRNA-expressing mice showed fur degradation earlier than control mice. When the shRNA-treated group was divided into two based on their surgery date, group differences in open field test and emotionality z-score became significant: Time spent in center (OF)- control (n=9): 54.7±2.9s, shRNA-1 (first half, n=7): 40.0±5.4s (p=0.012), shRNA-2 (second half, n=6): 44.42±11.5s);

Emotionality z-score- control: 0±0.3, shRNA-1: 0.7±0.6 (p=0.029), shRNA-2: -0.5±0.3 (p=0.267). Seven weeks of UCMS increased emotionality in both BDNF long 3' UTR shRNA- and scrambled shRNA-treated group; however, the final emotionality was slightly higher in shRNA treated mice than control, suggesting that dendritic BDNF KD increased stress susceptibility. The fact that group differences in coat state reached maximum at the 5th week of UCMS and decreased with prolonged stress suggests that we might have lost the time window to see the maximum group difference in behavioral phenotype. Pharmacological activation of TrkB with 7,8-DHF prevented development of depression-/anxiety-like behaviors following chronic stress, but the anti-depressant effect seems to be mediated by potentiated TrkB function rather than normalization of dendritic BDNF and GABA system (Figure 17).

4.4.1 Contribution of low dendritic BDNF on reduced neuroplasticity and function in the dlPFC of depressed subjects

Knockdown of BDNF long 3' UTR was sufficient to induce stress- and MDD-associated morphological changes in cortical neurons. In agreement with a human postmortem study reporting reduced length of dendritic processes but no change in the number of processes in basilar dendrite of deep layer 3 pyramidal neurons in dlPFC of subjects with psychiatric illnesses including MDD (Glantz and Lewis, 2000) and animal studies showing stress-induced morphological changes mainly occur on the distal portion of apical dendrites (Bloss et al., 2010; Bloss et al., 2011; Cook and Wellman, 2004; Radley et al., 2008; Radley et al., 2004), small hairpin RNA targeting BDNF long 3' UTR induced retraction of distal dendrites of primary cortical neurons.

In addition to the impaired neuronal plasticity, dendritic BDNF downregulation is known to induce functional and behavioral abnormalities such as impaired long term potentiation (An et al., 2008), learning and memory (Stein et al., 2006). Studies on the genetic polymorphism of BDNF also corroborate the idea that the dendritic action of BDNF is essential to structural stability and normal brain function. The most common single nucleotide polymorphism in humans at codon 66, resulting in the Val to Met substitution, has been implicated in cognitive impairment (Bath and Lee, 2006), defective fear extinction (Soliman et al., 2010), and increased susceptibility to psychiatric diseases (Gatt et al., 2009). Human BDNF Val66Met knock-in mice exhibit impairment in the dendritic transport of BDNF mRNA (Chiaruttini et al., 2009; Mallei et al., 2015), decreased activity-dependent BDNF secretion (Egan et al., 2003), decreased dendritic complexity and anxiety-like behavior that is not normalized by fluoxetine (Chen et al., 2006), impaired synaptic plasticity in the central amygdala (Galvin et al., 2015), mPFC (Pattwell et al., 2012), hippocampus (Ninan et al., 2010) and lower ketamine-induced synaptogenesis in the PFC (Liu et al., 2012). Our finding that emotionality is significantly correlated to BDNF long 3' UTR expression provides evidence that dendritic BDNF function is important for the mood regulatory system.

Then how can the loss of dendritic BDNF and associated structural changes contribute to functional abnormalities? One of the mechanisms could be the loss of the distal dendrite itself. The degree of structural changes is negatively related to cognitive flexibility and working memory performance (Hains et al., 2009). Stress-related structural alterations are reversed in the absence of stress (Bloss et al., 2011) and this structural recovery is accompanied by functional restoration (Goldwater et al., 2009). Furthermore, serotonin receptors are highly expressed in the distal portion of the dendrite (Liu and Aghajanian, 2008). Repeated mild restraint stress and

chronic corticosterone treatment decrease 5-HT-induced excitatory postsynaptic currents and this effect is negatively correlated with the length of apical dendrites and spine density on the distal branches (Liu and Aghajanian, 2008). With dendritic shrinkage, neurons might lose serotonin synapses and connections to other brain areas. Our finding that shRNA-treated mice spent less time in the center of the open field than controls suggests that dendritic BDNF KD may compromise mPFC function to suppress amygdala, the fear center. Second, defects in activity-dependent BDNF supply might play a role. Recent study found that over 2,000transcripts were localized in dendrites and/or axons, maybe for faster protein supply on demand by local synaptic translation (Cajigas et al., 2012). Not only does long 3' UTR (+) BDNF mRNA exist in the dendrite; it stays dormant in resting state, yet binds to a polyribosome responding to neuronal activity (Lau et al., 2010). Given that MDD is considered a disease of adaptation, impaired activity-dependent neuroplasticity may underlie the inability to cope with stress. A third possible mechanism could be through reduced GABA transmission. Knockdown of dendritic BDNF transcript and/or blockade of dendritic transport downregulates GABA receptors and GABA synthesizing enzymes (Stein et al., 2006; Waterhouse et al., 2012). These data imply that the functional outcome may derive from low GABA transmission at least in part. Related, further discussion continues in the next section.

4.4.2 Close link between dendritic BDNF transcripts and dendritic-targeting interneurons and its implication for pathology of major depressive disorder

SST and PVALB are expressed in distinct classes of cortical interneurons. Multiple lines of evidence imply that BDNF/TrkB signaling plays a crucial role in interneuron development and

maintenance of GABA function. Our studies consistently found that low BDNF function is responsible for reduced GABAergic genes in the depressed brain (Guilloux et al., 2012; Sibille et al., 2011; Tripp et al., 2011; Tripp et al., 2012). As TrkB is predominantly expressed in PVALB (+) interneurons rather than other interneuron populations (Cellerino et al., 1996; Gorba and Wahle, 1999), higher BDNF dependency of SST than PVALB raised questions about the mechanism. Our current study provides a clue that dendritic BDNF function is associated with neuronal plasticity, behavior and SST expression, which may indicate the health and function of dendritic-targeting interneurons.

To date, it is not clearly defined how dysfunction of SST (+) interneurons contribute to pathophysiology of mood disorders. Distal dendrites mainly receive inputs from other cortical areas and regulation of dendritic excitability is crucial for functional computations (reviewed in (Larkum, 2013)). Considering that dendritic excitation mainly occurs in actively-behaving animals, one can assume that proper control of activation/inactivation of dendrite is crucial for regulation of active behavior. For instance, SST (+) interneurons in barrel cortex are inactivated during active whisking which leads to increased excitability for the sensorimotor stimuli (Gentet et al., 2012). In the BLA during contextual fear learning, SST (+) interneurons are inactivated by multisensory environmental context (conditioned stimulus, CS), whereas aversive event (unconditioned stimulus, US) inactivates both PV (+) and SST (+) interneurons (Tovote et al., 2015; Wolff et al., 2014). In the hippocampus, US activates CA1 dendritic targeting interneurons such that pyramidal cells do not receive aversive sensory signal from entorhinal cortex during conditioning (Lovett-Barron et al., 2014). Loss of dendritic inhibition caused by dysfunctional SST (+) interneurons may result in failure to discriminate different stimuli. Furthermore, hyper-excitability of pyramidal cells may potentially contribute to sustained arousal and vigilance.

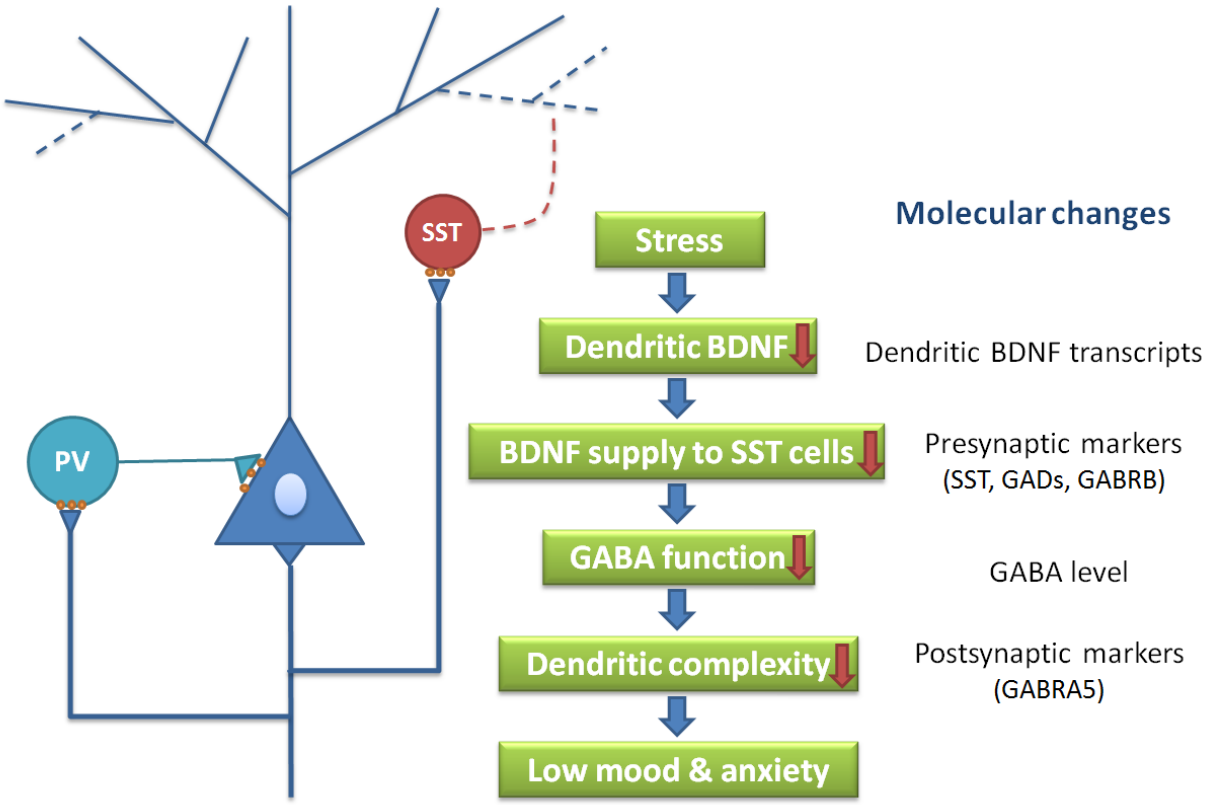


Figure 18. Proposed model

Results from current study suggest sequential events associated with MDD-related brain changes. First, prolonged stress induces selective deficit of dendritic BDNF indicated by low BDNF long 3' UTR expression in the PFC. Reduced BDNF supply to dendritic compartments and GABA interneurons targeting distal dendrites will lead to impaired function of dendritic-targeting interneurons (e.g. downregulation of SST and GABA synthesizing enzyme), and disrupted dendritic integrity, and finally, results in mood dysregulation.

Combining current knowledge and our experimental results, we propose a biological model linking low BDNF, impaired neural plasticity and GABA dysfunction, three phenomena commonly observed in the PFC of MDD subjects and stressed animals (Figure 18). MDD and stress may disturb dendritic BDNF, which leads to low neurotrophic supply to dendrite and dendritic targeting interneurons. Reduced dendritic BDNF signaling may contribute to low

GADs expression (i.e. low GABA production) and decreased dendritic complexity, finally culminating in affect dysregulation. To the best of our knowledge, this is the first study investigating the mechanism underlying the high BDNF dependency of dendritic-targeting interneurons observed across diverse brain diseases. Results from this study could have a major impact on our understanding of BDNF function in normal and disease-state subjects.

In conclusion, our results provide evidence that MDD-related downregulation of dendritic BDNF contributes to selective impairment of dendritic-targeting interneurons in depressed subjects, and that maintaining integrity of the BDNF-TrkB system is critical for mood regulation.

5.0 GENERAL DISCUSSION

MDD is a serious mental illness associated with high comorbidity, poor quality of life, and considerable economic burden; however, the detailed biological mechanisms are far from being understood. Evidence suggesting low BDNF function and GABA deficits are widely reported in MDD brains at multiple levels ranging from molecule to function, although these two lines of evidence have been mostly investigated separately. The research in this dissertation investigated a mechanism underlying a close link between BDNF signaling and GABA dysfunction using translational approaches encompassing cell, animal and human postmortem studies. We demonstrate that GABA interneuron markers display different BDNF dependencies which may be involved with local action of BDNF, at least in part, and that different interneuron populations are implicated in the disease in a region-specific manner. We also show that, distinct from related mechanisms occurring during normal aging of the brain, the ability to maintain E/I homeostasis may be impaired in the brain of MDD subjects. In the following sections, I summarize our findings and discuss their implications for our understanding of disease mechanisms. I also discuss limitations of the current studies and possible future directions.

5.1 BDNF- AND GABA-RELATED GENE CHANGES IN THE DLPFC AND SG-ACC OF MDD PATIENTS

Growing evidence suggests that dysfunction of corticolimbic circuitry is implicated in MDD. Reduced gray matter volume (Drevets, 2000), impaired functional connectivity to other brain regions (Mayberg, 1997; Siegle et al., 2007), activity changes in disease state (Alcaro et al., 2010; Fitzgerald et al., 2007) and in treatment response (Mayberg, 2002; Mayberg et al., 1999) have been observed in the sgACC and dlPFC of depressed brains. Impaired E/I balance, potentially caused by GABA dysfunction in these brain areas has been thought to be associated with the clinical symptoms of affect dysregulation (Sibille et al., 2011; Tripp et al., 2011). Guilloux et al showed a close association between MDD-related GABAergic gene changes and low BDNF function in the female amygdala (Guilloux et al., 2012). Therefore, we investigated whether BDNF signaling is responsible for expression changes of GABAergic genes in these two brain regions in male and female MDD subjects (Chapter 2 and 4).

Our data suggest low BDNF signaling and reduced GABA function in both brain areas. Yet, the gene profile displays similarities as well as differences: SST, NPY, CORT, GAD1 and GAD2 were commonly decreased. However, PVALB was selectively decreased in the sgACC. The BDNF signaling module also showed regional differences. Whereas only dendritic BDNF (long 3' UTR–containing) mRNA was altered in the dlPFC, the sgACC was related to changes in TrkB rather than BDNF (Table 2 and Figure 11, summarized in Figure 19). These data are in agreement with animal studies that PVALB expression depends on the expression of TrkB (Hashimoto et al., 2005a) whereas SST and NPY are highly BDNF-dependent (Glorioso et al., 2006; Guilloux et al., 2012).

SST, NPY, CORT are expressed in the overlapping populations of interneurons that innervate the dendritic compartment of pyramidal cells and regulate incoming information from other cortices. In contrast, PVALB is expressed in the fast-spiking, perisomatic-targeting interneurons which regulate the output of targeted neurons. Expression changes in interneuron markers are thought to reflect the functional alterations of the corresponding interneuron group. Hence, one can assume that perturbed GABA inhibition caused by different interneuron subtypes may have different consequences for the sgACC and dlPFC. The hypothesized contribution of GABA dysfunction in these two brain areas is discussed in the conclusion (5.4) and further reviewed elsewhere in the context of internal and external awareness (Northoff and Sibille, 2014).

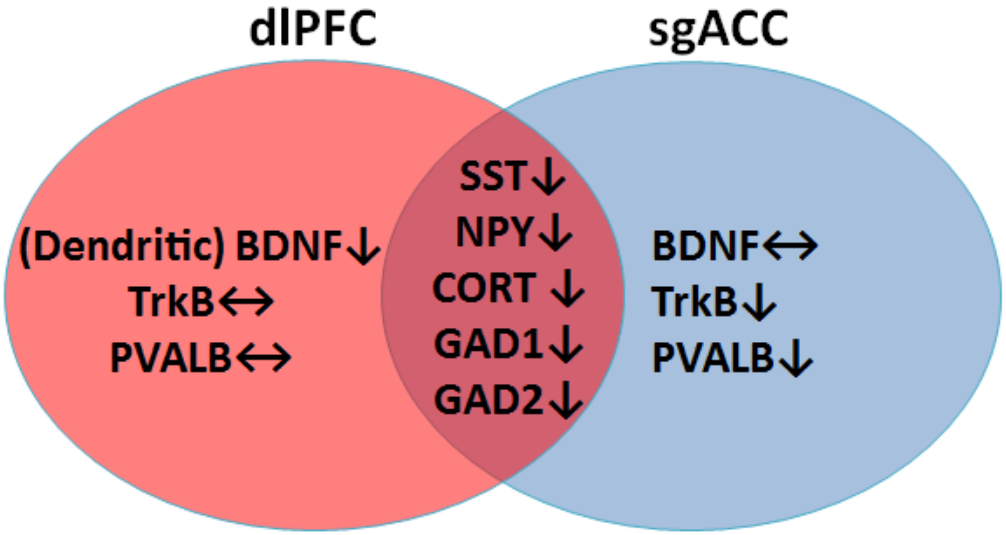


Figure 19. Region-specific expression changes in GABAergic genes

5.2 LESSONS FROM NORMAL BRAIN AGING

5.2.1 Impaired homeostatic plasticity: differences between normal aging and pathological changes in the brains of MDD subjects

While the incidence rate of clinical depression varies depending on the diagnostic criteria, depressive symptoms are commonly found in the older subjects. Previous studies in our lab showed that great portion of age-regulated genes in the human brain is reported to be associated with various neurobiological disorders (Glorioso et al., 2011), suggesting that the brain becomes more prone to illness with age. However, not every old subject develops psychiatric or neurodegenerative disorders.

In paper 2, we studied the role of BDNF in healthy brain aging to gain insights into its role on pathological brain changes. We found that aging was associated with changes in both inhibitory and excitatory synapse-related genes (Table 3, Figure 8). The concurrent expression changes in glutamatergic- and GABAergic-genes imply that the E/I balance is well-maintained during normal aging. In contrast, a meta-analysis integrating eight independent microarray studies performed with different cohorts, different brain regions and platforms shows that MDD is associated with preferential changes in GABA-related genes rather than excitatory synaptic gene changes (Ding et al., 2015).

Neurons maintain appropriate levels of activity by adjustment of synaptic strength and intrinsic cellular excitability. Recent studies suggest the involvement of dynamic crosstalk between excitatory and inhibitory neurons in these processes. Following chronically reduced network activity, pyramidal cells restore their excitability to control levels by increasing amplitude of miniature excitatory postsynaptic currents (mEPSCs) (Rutherford et al., 1998), or

decreasing miniature inhibitory postsynaptic currents (mIPSCs) or spontaneous IPSC amplitude (Kilman et al., 2002; Rutherford et al., 1997; Swanwick et al., 2006). On the other hand, prolonged activation of network activity increases the numbers of excitatory synapses on interneurons as well as GABAergic synapses on pyramidal cells (Lin et al., 2008; Spiegel et al., 2014). BDNF appears to play an important role in homeostatic plasticity, because exogenous BDNF application or an antibody against TrkB prevents or recapitulates these compensatory changes, respectively.

Our study with pharmaceutical blockade of TrkB activity showed that low BDNF signaling impacts the GABA system first (Figure 9). The same event might happen in brain aging as well as in MDD; however, the compensatory changes may not occur in the pathological condition for some reason. Together, the absence of excitatory compensation might be one of the core features of neuropathology of MDD (See detailed description in Chapter 2).

5.2.2 Dendrite: the link between SST and BDNF

Human and animal studies consistently indicate that SST downregulation accompanies BDNF and/or TrkB changes. PVALB is also affected by BDNF function, but is not directly BDNF-dependent. Glorioso et al suggested that interneuronal gene expression is regulated in a complex manner (Glorioso et al., 2006) but this model has not been tested yet.

In paper 2, we tried to find a molecular link between BDNF and SST using microarray data from the prefrontal cortex of control subjects through gene coexpression network analysis, a useful tool to identify a functional module, i.e., a set of genes that are involved in a common function. BDNF coexpression network analysis revealed that SST and GABRA5 were closely associated with BDNF function. Intriguingly, GABRA5 displays the highest correlation among ~

300,000 probes obtained with the arrays. This data leads us to hypothesize that the local action of BDNF may play a role in differential regulation of distinct interneuron populations. This proposed mechanism was tested in Chapter 4 and seems to be true, at least in the PFC, based on the experimental results.

5.3 LESSONS FROM ANIMAL STUDIES

5.3.1 Various BDNF dependency of GABA-related genes and implications for the stress model

Throughout studies, we utilized mouse models to address the questions of whether and how BDNF regulates GABAergic genes (results are summarized in Figure 20). Various BDNF dependency of GABA related genes, and possible sequential events were revealed by different animal models.

It seems obvious that SST, NPY, CORT have a higher BDNF dependency than PVALB. All these three interneuron markers changed by constitutive BDNF knockdown ($BDNF^{Het}$ and $BDNF^{KIV}$; Table 1) as well as by temporal TrkB blockade in adult brain ($TrkB^{F616A}$; Figure 9). Virus-mediated BDNF long 3' UTR interference provides evidence that low BDNF-induced SST downregulation happens earlier than that of the other two genes, NPY and CORT. This data is further supported by results of a coexpression network analysis in which SST was the only dendritic-targeting interneuron marker included in the top 200 genes closely linked to BDNF function.

GAD2 is enriched in synaptic boutons whereas GAD1 is expressed across all cellular compartments of interneurons. Given that exon IV containing BDNF transcripts are restricted to soma (Pattabiraman et al., 2005), it is possible that selective change of GAD2 in $BDNF^{Het}$ compared to $BDNF^{KIV}$ (Table 1) might be caused by perturbed dendritic BDNF function.

Three weeks of TrkB blockade in a developed brain was sufficient to change all three interneuron markers as well as GAD1 (Figure 9). Considering that TrkB binds to not only BDNF but also NT-3 and 4, it is not surprising that TrkB activity blockade shows greater impact than BDNF knockdown. No change was observed in PVALB expression in animal experiments, suggesting that PVALB is the most resistant one among the GABAergic genes tested in my dissertation study.

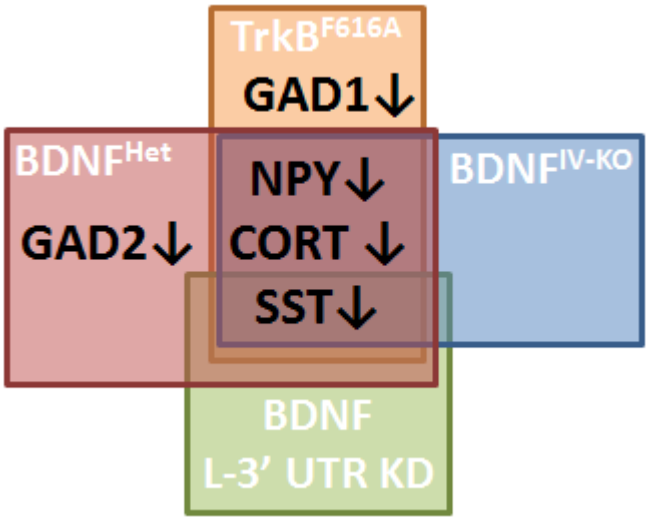


Figure 20. Different GABAergic genes changed in each mouse model

5.3.2 Stress-induced downregulation of BDNF, the cause of impaired GABA circuitry

Postmortem studies are useful for identifying biological factors relevant to the disease; however, they only allow us to see the outcome of pathological changes. Therefore, it is essential

to use animal models to delineate neuropathological changes related to the disease. In chapter 4, I used the UCMS mouse model of depression to investigate stress-related changes in BDNF and GABAergic genes. As discussed earlier, presumably because of shorter duration of stress exposure than humans experience in the context of the disease, we did not find many differences in the UCMS mouse brain. Nonetheless, a clear finding from the UCMS mouse model is that a BDNF reduction is one of the earliest events following chronic stress exposure in the prefrontal cortex (Figure 13). These gene changes may be responsible for anatomical (Figure 14), behavioral (Figure 15), and molecular changes (Figure 16) observed in brains of human MDD subjects and stressed mice (more detailed discussion is available in Chapter 4).

5.4 LIMITATIONS AND FUTURE DIRECTIONS

Most of the limitations of Chapter 2 and 3 have already been covered in each respective chapter. Here, I will discuss limitations and future directions of Chapter 4 in addition to common limitations across studies.

5.4.1 Confirmation of cell type specific alterations

In all of the three studies, we utilized total RNA from brain homogenates to analyze gene expression changes. Interneuron markers like SST and correlation profiles provide some evidence about population-specific impairments; however, it is hard to define the cellular origin for other common GABAergic transcripts changes. Gene expression analysis with laser

microdissection-collected SST (+) and PV (+) cells will be used in future work to provide further in-depth information.

5.4.2 Identification of the cause of selective downregulation of BDNF long 3' UTR in MDD

We observed a selective decrease in BDNF long 3' UTR in MDD and mouse brains after chronic exposure, but the underlying mechanism was not yet investigated in this study. As the 3' UTR is a common target of trans-acting factors, changes in such molecules are of interest. For instance, a recent study identified several micro RNAs (miRNAs) regulate BDNF long and short 3' UTRs (Varendi et al., 2014). HuD, a neuronal RNA binding protein, is reported to interact with BDNF long 3' UTR (Allen et al., 2013) and promote dendritic translation of BDNF long 3' UTR (+) mRNA (Vanevski and Xu, 2015). Therefore, the level of BDNF long 3' UTR targeting miRNA and HuD should be measured in future studies.

5.4.3 Confirmation of dendritic trafficking of human long 3' UTR (+) BDNF mRNA

While dendritic trafficking of long 3' UTR (+) BDNF transcripts was observed in primary mouse neurons, it has not been tested in human brains. Since primary human neuronal culture is not technically available, alternative experiments such as expressing a fusion protein, GFP-human BDNF short or long 3' UTR, in rodent primary cortical neurons could be performed to address this question.

5.4.4 Confirmation of effect of BDNF-long 3' UTR knockdown on dendritic complexity

The effects of BDNF long 3' UTR knockdown on dendritic stability was only investigated in vitro, not in vivo. Although studies suggest that autocrine action of BDNF is critical for dendritic stability (English et al., 2012; Wang et al., 2015), BDNF from other cells also can play a role. We were able to see strong marker gene (GFP) expression in dendrites of virus-infected neurons (Figure 30). However, the density of GFP-expressing neurons was too high to analyze the dendritic structure of an individual cell. Therefore, a small amount of viruses should be injected into the mouse cortex to obtain sparsely distributed infected neurons displaying Golgi-like GFP staining.

5.4.5 Confirmation of gene expression changes in protein level

Although we examined protein expressions of key genes (BDNF in Chapter 2, phospho-TrkB in Chapter 3 and 4, SST immuno-signal intensity in Chapter 4), most gene expression was only measured with qPCR. There were two reasons that I focused on the mRNA analysis. First, protein expression analysis in human postmortem brain is quite challenging because of the long postmortem interval which causes loss of post-translational modifications and protein degradation. I tried immunoblot against total TrkB for Chapter 2, but could not find an optimal condition. Second, due to the small size of mouse brains, I could not extract a sufficient quantity of protein from tissue punches to perform a protein assay.

Nonetheless, the functional implications of mRNA changes can only be confirmed with concurrent protein level changes. Furthermore, post-translational modifications such as phosphorylation, acetylation, and proteolytic cleavage also can change protein function. More

sensitive protein-detection methods than immunoblot, such as ELISA and selected reaction monitoring, will help to overcome this limitation.

5.4.6 Identification of regulators of homeostatic plasticity

We suggested that disturbed homeostatic plasticity would be one of the main differences between normal brain aging and pathological brain changes. The underpinning mechanism is not clear, but disturbed transcription factors could be a good candidate. A recent study found that a decreased number of spine synapses in the dlPFC of MDD subjects was associated with an elevated level of GATA1 which represses transcription of synapse-related genes (Kang et al., 2012). Indeed, several transcription factors have been implicated in homeostatic plasticity. For example, CREB mediates activity-regulated transcription in response to calcium influx. MEF2 activates transcription of proteins that disrupt synaptic protein complexes and reduce synapse number (reviewed in (Flavell and Greenberg, 2008)). Npas4 is upregulated in both excitatory and inhibitory neurons in response to sustained network activity; however, the transcription factor activates distinct transcription programs in a cell type-specific manner (Spiegel et al., 2014). Determination of common promoter elements in the excitatory synapse-related genes selectively reduced in aged brains could be used to find candidate transcription factors dysregulated in MDD.

5.4.7 Verification of the influence of low BDNF signaling on E/I balance

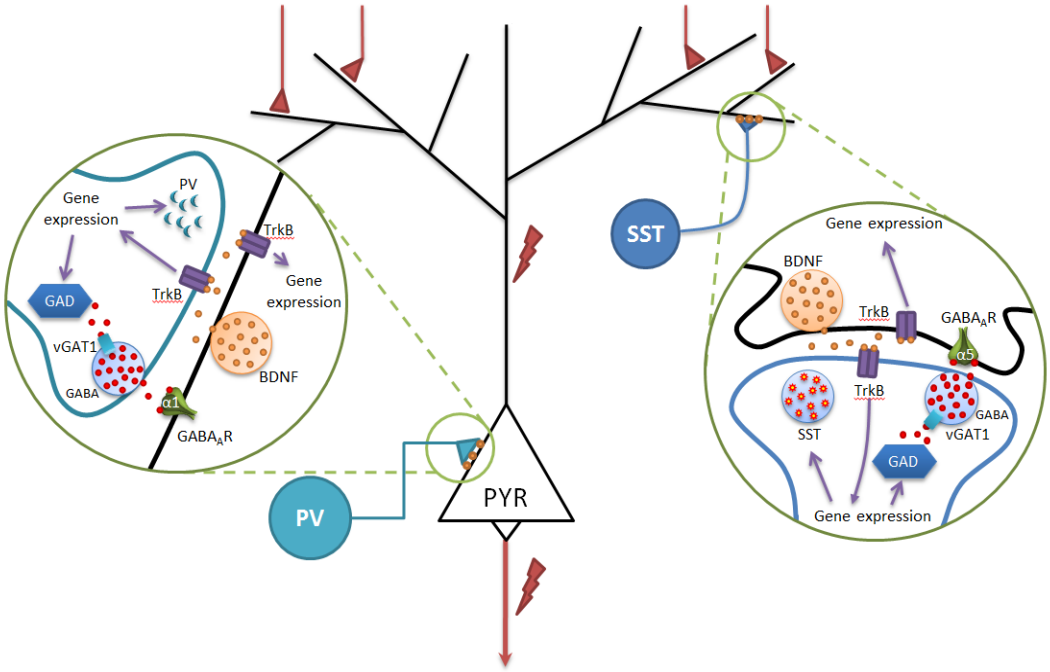
I argued that the E/I balance is impaired by low BDNF expression (BDNF knockdown or knockout mouse) and short-term TrkB blockade (TrkB transgenic mouse) based on selective gene expression changes in GABA interneuron markers. However, like excitatory neurons,

interneurons receive excitatory as well as inhibitory input. Simple expression changes in synaptic function-related genes do not identify the precise circuits which are dysregulated. Therefore, synaptic connectivity should be investigated with morphometric analysis using electron microscopy, and synaptic function needs to be addressed by analyzing synaptic protein clustering patterns. Measurement of electrophysiological properties may provide further information about functional changes of each cell population. If my hypothesis is correct, the IPSCs elicited by SST (+) interneurons would be selectively reduced after genetic/pharmaceutical manipulation while IPSPs activated by PVALB cells remain unchanged.

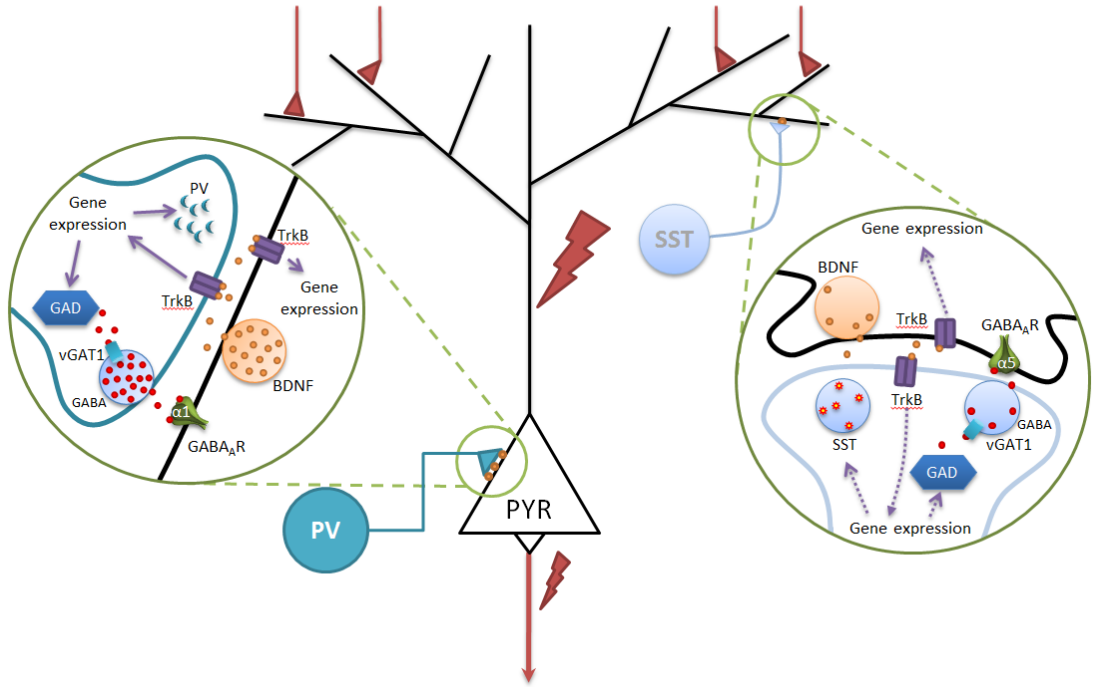
5.5 CONCLUSION AND PROPOSED MODEL

MDD is characterized by a syndrome encompassing diverse physiological and emotional symptoms, indicating involvement of multiple brain regions. The goals of this dissertation were first to characterize the molecular specificities, and then to investigate mechanisms linking two different hypotheses implicated in the pathophysiology of MDD; (1) low neurotrophic factor and (2) a GABA deficit in the sgACC and dlPFC.

Normal subjects



MDD subjects-dlPFC



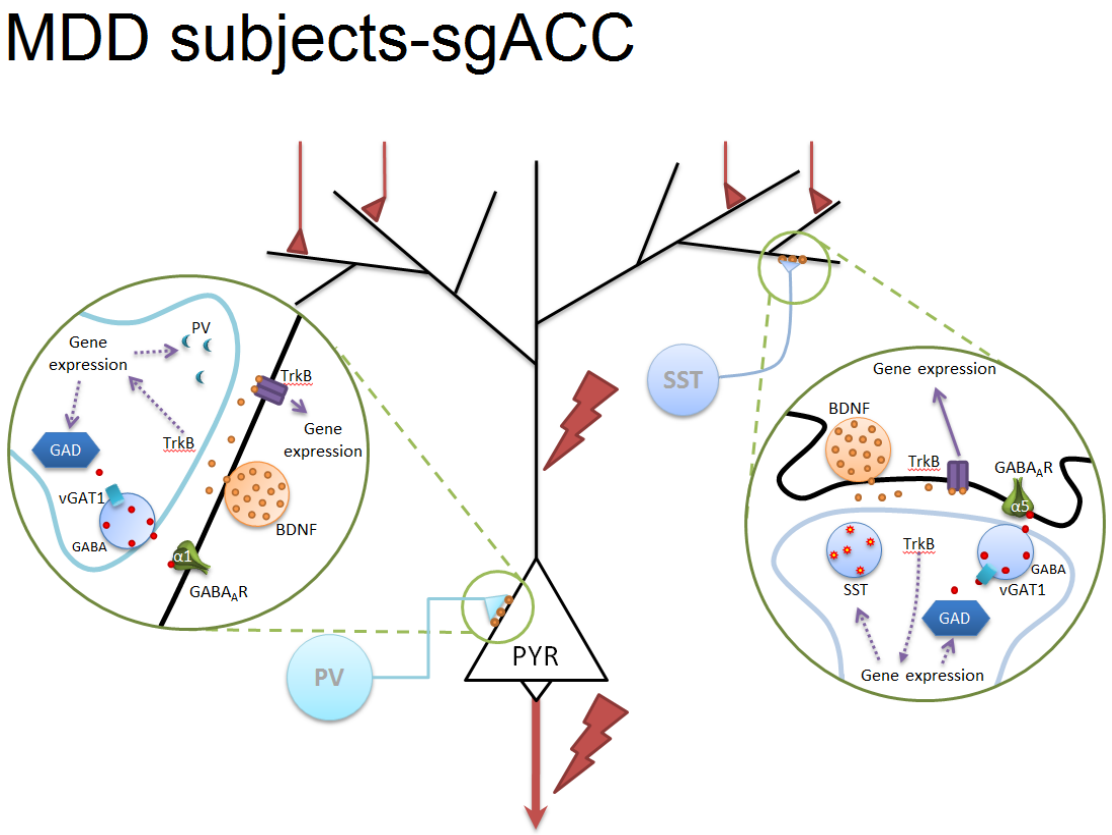


Figure 21. Proposed model of brain region-specific cellular microcircuit changes

SST cells innervate the dendritic compartment of pyramidal cells and regulate levels of excitatory input from other cortical areas. PVALB neurons target the cell body of pyramidal cells and regulate signal output. Region-specific BDNF signaling changes in MDD will lead to population-specific GABA neuron disturbance, which finally results in distinctive input/output changes in the sgACC and dlPFC (modified from (Northoff and Sibille, 2014)).

Integrating a model suggested in a research review (Northoff and Sibille, 2014) and findings from my dissertation study, I propose a model of impaired function of SST- and PVALB-expressing cells as a downstream consequence of reduced local BDNF signaling. In the dlPFC of depressed subjects, there is a selective disturbance in dendritic BDNF which results in low neurotrophin supply to SST interneurons. Impaired function of SST (+) interneurons might compromise their ability to provide tonic inhibition to pyramidal cells, which results in increased

information input. However, BDNF supply to PVALB (+) cells remains intact; therefore, signal output from pyramidal cells maintains a proper level. On the other hand, in the sgACC of MDD subjects, the function of both interneuron groups is disrupted because of general downregulation of TrkB, and sgACC become hyperactive in general (Figure 21).

This impaired cellular circuit may contribute to functional impairment at the regional level, and alterations in both brain areas may give rise to improper informational computation which contributes to affect dysregulation.

APPENDIX A

AGE-BY-DISEASE BIOLOGICAL INTERACTIONS: IMPLICATIONS FOR LATE-LIFE DEPRESSION

Brandon C. McKinney[1], Hyunjung Oh[1,2] and Etienne Sibille[1,2*]

[1]Department of Psychiatry, University of Pittsburgh, PA 15312, USA

[2]Center For Neuroscience, University of Pittsburgh, PA 15312, USA

[*]To whom correspondence should be addressed. E-mail:sibilleel@upmc.edu

Note on my contributions to this paper: I helped with writing the manuscript.

INTRODUCTION

Among elderly individuals, depressive symptoms are common and burdensome. Approximately 1% of individuals over the age of 65 meet criteria for major depressive disorder (MDD), as defined by the diagnostic and statistical manual of mental disorders, fourth edition, text revision (DSMIV-TR; American Psychiatric Association, 2000) a prevalence lower than that in younger adults (Kessler et al., 2003). Another 15–25%, however, experience depressive symptoms that, while not meeting criteria for MDD, do cause significant distress and interfere with daily functioning (Koenig and Blazer, 1992). In this article, the term late-life depression (LLD) will be used to refer to individuals over the age of 65 who for the first time in their lives meet criteria for MDD or display clinically significant depressive symptoms. Individuals with LLD experience greater functional disability (Dombrovski et al., 2007) and cognitive decline (Lenze et al., 2005) than those without. Further, they are at increased risk of morbidity and mortality from medical illness (Ganguli et al., 2002). LLD also appears to contribute to increased rates of suicide among older individuals (Van Orden and Conwell, 2011).

The biological substrates of LLD are being characterized and several hypotheses for the etiology and pathophysiology of LLD have been proposed, including the vascular hypothesis (Alexopoulos et al., 1997), inflammation hypothesis (Alexopoulos and Morimoto, 2011), and dementia prodrome hypothesis ((Byers and Yaffe, 2011); reviewed in (McKinney and Sibille, 2013)). Here, we propose an alternative and complementary hypothesis, which we termed the age-by-disease biological interaction hypothesis of LLD. Central to this hypothesis is the concept of molecular aging of the human brain. An earlier version of this hypothesis has been described elsewhere (McKinney and Sibille, 2013).

MOLECULAR AGING OF THE HUMAN BRAIN

Despite its critical importance to a population that is growing older, “normal” brain aging is understudied. This may be due to the often expressed, but false belief held by many that aging is inescapable, broad-ranging and non-specific. Studies that have investigated biological aging have revealed specific changes and thus avenues for intervention. At the cellular level in the human brain, morphological and stereological studies reveal a decrease in neuron volumes, a small loss or no change in cell numbers (Morrison and Hof, 1997; Pakkenberg and Gundersen, 1997), and a progressive thinning of cortical thickness, affecting both gray and white matter (Resnick et al., 2003; Sowell et al., 2003). Similar structural changes with age have been demonstrated in the brains of animal models (Jucker and Ingram, 1997; Peters, 2002). At the molecular level in animal models, less than 10% of brain-expressed genes exhibit age-related changes in gene expression (Blalock et al., 2003; Jiang et al., 2001; Lee et al., 1999; Lee et al., 2000; Sibille et al., 2007). Similar numbers have been reported in studies of human tissue (Avramopoulos et al., 2011; Lu et al., 2004). In one such study of human tissue from the prefrontal cortices of subjects aged 13–79, our group used gene microarray technology to investigate age-related changes in gene expression and reported that approximately 7.5% of genes changed significantly with aging (Erraji-Benchekroun et al., 2005). Other studies have confirmed these results, identifying a maximum of ~10% of all detected genes, depending on sample size and analytical power of the respective studies (Glorioso et al., 2011; Yankner et al., 2008). Of note, not only is the identity of the genes and gene classes that are affected with aging consistent among studies, but so are the directions of change.

Interestingly, the identity of the genes whose expression changes with age suggests that specific cellular populations and biological processes are selectively affected by the aging

process. For instance, expression of genes playing a role in glial-mediated inflammation, oxidative stress responses, mitochondrial function, synaptic function and plasticity, and calcium regulation and neuropeptide signaling have consistently been shown across multiple studies to be affected by aging, while numerous other neuronal and glial genes remain apparently unchanged (Glorioso and Sibille, 2011; Yankner et al., 2008). Overall, age-upregulated genes are mostly of glial origin and related to inflammation and cellular defenses, while downregulated genes display mostly neuron-enriched transcripts relating to cellular communication and signaling (Erraji-Benchekroun et al., 2005).

Using the expression levels of the age-dependent genes and their expected trajectories with age, we have generated predicted ages of individual subjects from which the brain tissue was sampled, and demonstrated that this predicted age is highly correlated with the chronological age of that individual (Erraji-Benchekroun et al., 2005; Glorioso et al., 2011; Glorioso and Sibille, 2011). We have termed this predicted age the “molecular age”. These findings suggest that gene expression changes with age can be used as biomarkers for brain aging.

MOLECULAR AGING AND BRAIN-RELATED DISEASE PATHWAYS

This correlation between molecular and chronological ages is robust in individuals without neurodegenerative and neuropsychiatric disorders (Erraji-Benchekroun et al., 2005), but investigations of individual genes suggest that the molecular age or individual gene trajectory can deviate from chronological age in individuals with these disorders. To illustrate this phenomenon, one can look at somatostatin (SST), a signaling neuropeptide that is expressed in a subpopulation of gamma-aminobutyric acid (GABA)-positive inhibitory interneurons (Viollet et al., 2008). We have demonstrated that expression of SST decreases progressively with age in

individuals without neurodegenerative and neuropsychiatric disorders such that expression levels at 70 years of age are approximately 40–50% of those at 20 years of age (Erraji-Benchekroun et al., 2005; Tripp et al., 2011; Figure 1A). SST is also downregulated in individuals with MDD. Interestingly, the magnitude and direction of change in SST expression with age is similar to that of control individuals, but the absolute values of SST expression are lower at most ages in individuals with MDD compared to those without (Sibille et al., 2011; Tripp et al., 2011; Figure 1A, top panel). Similar findings have been observed in subjects with schizophrenia (Morris et al., 2008; Figure 1A, bottom panel).

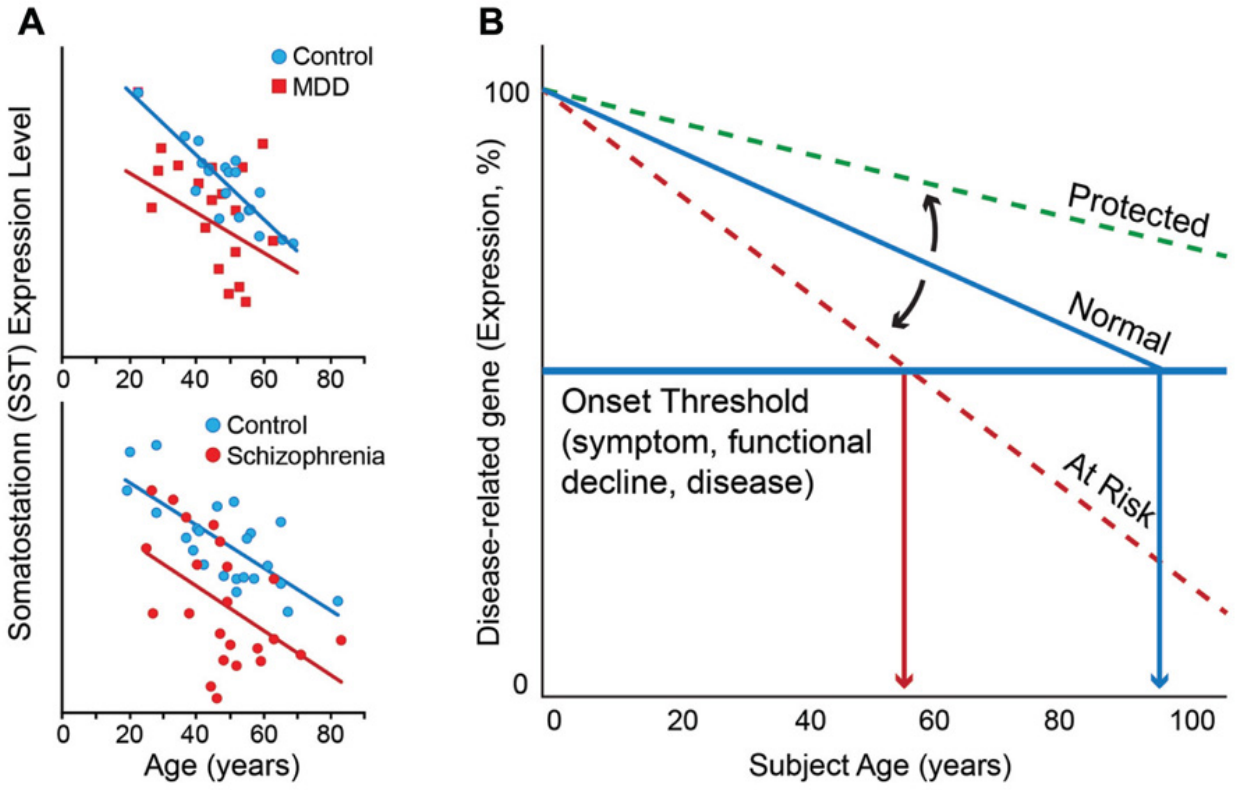


Figure 22

(A) Somatostatin expression decreases with age in control subjects. Individuals with MDD (top panel) and schizophrenia (bottom panel) display lower levels of expression than control subjects at most ages, together prompting the hypothesis that decreased expression of somatostatin in depression and schizophrenia may represent early brain age-related molecular phenotypes in these individuals, which would render these subjects more

vulnerable to developing psychiatric diseases. (B) Based on similar age and disease observations for numerous additional genes, we have proposed a model of "age-by-disease biological interaction" (Glorioso et al., 2011). In this model, change in expression of disease-related genes (a decrease is shown) across a threshold (horizontal blue line) marks the onset of disease symptoms. Changes in the trajectory of age-related changes in expression of disease-related genes (Y-axis) determine at what age (X-axis), or even if, an individual develops disease symptoms (vertical red arrows). Modulators (black arrows) may thus place an individual on "at risk" or "protected" trajectory. Per this model, individuals with LLD may have modulators, genetic or environmental, that place them on an "at risk" trajectory for developing mood symptoms. Figures are adapted from Morris et al. (2008), Tripp et al. (2011), Glorioso and Sibille (2011), and McKinney and Sibille (2012).

The relationship between gene expression of a disorder-associated gene and aging is not limited to SST. In fact, genome-wide investigations have reported that up to a third of age-regulated genes in the human brain have been at some point associated in the literature with neurodegenerative (Alzheimer's, Parkinson's, and Huntington's diseases, amyotrophic lateral sclerosis) and neuropsychiatric disorders (bipolar depression, major depression, and schizophrenia; see Figure 3 in (Glorioso et al., 2011) for details). Not only do the genes relevant to these brain disorders show age-related changes, but the direction of the changes that occur with age is almost always in the direction thought to cause or promote diseases. See Table 1 in Glorioso et al. (2011) for a list of approximately 40 top candidate disease genes that exhibit agreement between age- and disease-related changes. Studies of gene expression in Alzheimer's disease (AD) and normal aging find that most of the changes in gene expression that occur in normal aging also occur in AD, only in greater magnitude (Avramopoulos et al., 2011; Miller et al., 2008). The difference in the magnitude of change in gene expression between individuals with brain disease and those without during aging is attributable to the disease. Our hypothesis posits that it is these changes that drive the disease process; however, it cannot be ruled out that

these additional changes in magnitude are the result of the disease process rather than the cause. That said, there are certainly genes for which it is known that the age-related direction of change in gene expression directly causes, rather than is a consequence of, the disease, for example, the Parkinson's disease genes PINK1 and DJ1/PARK7. The expression of these genes progressively declines with age and it is known that individuals with loss of expression/function mutations in these genes develop Parkinson's disease (Schapira, 2008). In contrast, relatively fewer of the larger pool of genes that do not display age-dependent changes (<5%), are associated with neurodegenerative and neuropsychiatric diseases.

Recently, we have further investigated the relationship between age, gene changes, and neuropsychiatric disorders, specifically in the context of MDD (Douillard-Guilloux et al., 2013). Results demonstrated that most MDD-related genes were frequently age-regulated in both MDD and in control subjects, and that the effects of MDD and age were positively correlated. Moreover, most genes that are age-dependent in control subjects displayed greater age effects in MDD subjects, and the overall increased prevalence of age effects on genes in MDD subjects corresponded to similar trends in controls, rather than representing de novo age effects. This systematic correlation between age-dependent and depression-related changes, with greater effects in depressed subjects, further suggests that normal brain aging is a risk factor for biological changes observed in MDD subjects.

One interpretation of these observations is that age-dependent changes (i.e., molecular aging) are on an earlier trajectory in individuals who develop MDD and potentially other neuropsychiatric disorders. However, it is important to note that these studies are cross-sectional and do not follow the longitudinal progression of gene changes within individuals, so it is not known whether age-dependent changes are on an earlier trajectory, or whether changes occurred

at earlier time points and were fixed at lower levels, for instance in the case of SST. So while we hypothesize that age may be pushing the expression of genes in disorder-causing directions, an alternate scenario is that of earlier and fixed changes, which then act as latent vulnerability factors that are revealed with advancing age, resulting in increased vulnerability to develop neurodegenerative and psychiatric disorders, including LLD.

AGE-RELATED CHANGES IN GENE EXPRESSION APPEAR TO BE, IN PART, GENETICALLY MODULATED

While molecular and chronological ages are highly correlated, we have also reported individual deviations from predicted ages (Erraji-Benchekroun et al., 2005; Glorioso et al., 2011). The fact that molecular age can deviate from its chronological age suggests that modulating factors exist and may contribute to one's vulnerability to brain aging and to developing late-life brain disorders, such as LLD. In the age-by-disease biological interaction hypothesis we have proposed that those individuals with older predicted molecular ages compared to their chronological age may not only display greater biological brain aging, but may also be at greater risk of age-gated brain diseases, because gene expression of disease-related genes would have proceeded further in disease-promoting directions. Conversely, subjects with younger age-dependent gene trajectories and lower predicted molecular ages would be at lower risk, and may in fact display resiliency against LLD and other late-life disorders (Figure 1B). Environmental and genetic factors represent obvious candidates to modulate the trajectory of biological aging.

In a proof-of-principle study, our laboratory sought to demonstrate a genetic role in modulating the aging process. The above-described "molecular age" assay was used to characterize the brain tissue of individuals carrying different polymorphisms of the sirtuin genes

(Glorioso et al., 2011), a family of genes previously demonstrated to modulate age and longevity in nematodes, insects, and rodents. We focused on SIRT5, due to prior report of altered expression for that gene in a rodent model of anticipated brain aging (Sibille et al., 2007). This study found that subjects carrying a low-expressing polymorphism of the SIRT5 gene had molecular ages that were older than actual chronological age, as measured in the three different areas of the cerebral cortex (i.e., BA9, BA24, and BA47) of human postmortem samples (Glorioso et al., 2011). Interestingly, this effect was not demonstrated in the amygdala, a brain area in which the low-expressing polymorphism of the SIRT5 gene does not appear to affect expression levels of SIRT5 as it does in the cerebral cortex. These findings at the molecular level are consistent with findings from studies of brain structure with age that show robust decreases in gray matter in the cerebral cortex but more variable decreases in the amygdala (Good et al., 2001). The effect of the low-expressing SIRT5 polymorphism on molecular age was accompanied by expression changes for a set of genes whose products are localized to the mitochondria, including PINK-1 and DJ-1, two Parkinson's disease-associated genes, in ways that would promote mitochondrial dysfunction-related diseases, including Parkinson's disease. This (correlative) proof-of-principle study suggests that factors that affect brain aging can potentially place an individual at higher risk for disease, through a mechanism by which it accelerates brain biological aging, and thus promotes changes in expression of disorder-relevant genes in disease-causing directions. With respect to Figure 1B, the low-expressing polymorphism of the SIRT5 gene can be thought of a modulator that puts one on the "at risk" trajectory. The converse of this model is that factors delaying age trajectories of gene changes may lead to younger brain molecular aging and potential resiliency toward developing functional declines and age-related disorders, including LLD (Figure 1B).

PUTATIVE MECHANISMS FOR AGE-RELATED CHANGES IN GENE EXPRESSION

The mechanisms by which age-related changes in gene expression occur are unknown. Candidate mediators include among others, loss of telomere integrity, increased oxidative stress, and epigenetic modifications.

Loss of Telomere Integrity

Telomeres are regions of repetitive nucleotide sequences at each end of a chromosome. One of the hypothesized functions of telomeres is to deter the degradation of genes near the ends of chromosomes by instead allowing repetitive telomeres to shorten, a necessary part of chromosome replication. Telomeres are highly susceptible to oxidative stress because of their high content of guanines. As both chromosome replication and oxidative stress increase with age, one would expect telomeres to shorten with increased age. Indeed, in peripheral tissues, it has been consistently demonstrated that telomeres become shorter as one ages and once telomeres reach a critical length, irreversible arrest of cell division or apoptosis is triggered (Chin et al., 1999; Flores and Blasco, 2009; Hayflick and Moorhead, 1961; Sahin and DePinho, 2012; Sharpless and DePinho, 2004). Although telomere shortening has not yet been observed in the human brain, studies suggest that peripheral telomere length is a biomarker for aging. Leukocyte telomere shortening is positively correlated with the chronicity of stress and depression (Epel et al., 2004; Wolkowitz et al., 2011a) and is associated with incidence of age-related diseases such as myocardial infarction, stroke, and shorter lifespan (reviewed in (Wolkowitz et al., 2011b). It is not known whether telomere shortening increases the risk of LLD, however, given the common pathway of aging and depression, the possibility cannot be excluded.

Recent animal studies suggest that the putative link between telomere integrity and depression-like behaviors extends to the brain, and that this link may be mediated by telomerase activity (Zhou et al., 2011). In that study, the expression of telomerase was decreased in the hippocampi of mice subjected to chronic mild stress, and hippocampal infusion of a telomerase inhibitor induced depressive-like behaviors that did not respond to antidepressant treatment (Zhou et al., 2011). Given that neurogenesis has been implicated in antidepressant responses in mice (Santarelli et al., 2003) and that the proliferation capacity of neural stem cells highly depend on telomerase activity (Ferron et al., 2009), the authors suggested telomerase activity may play a role in linking mechanisms of aging and depression (Zhou et al., 2011); although the translation of these observations to humans is contentious, due to very low rates of neurogenesis in adult human subjects (Bhardwaj et al., 2006).

The anti-apoptotic role of telomerase is thought to reflect its capacity of maintaining DNA integrity, however, recent studies have reported other putative functions (reviewed in (Saretzki, 2009). Overexpression of telomerase reverse transcriptase (TERT), protects mouse neurons from excitotoxicity by improving basal mitochondrial membrane potential and Ca^{2+} uptake into mitochondria (Kang et al., 2004) and decreases cellular reactive oxygen species (ROS; (Ahmed et al., 2008). Furthermore, TERT mediates the neurotrophic action of BDNF (Fu et al., 2002) and affects the global pattern of gene expression in the direction of cell survival, including upregulation of growth promoting genes (FGF5, EGFR, and etc.) and downregulation of cell-growth inhibitors such as IGFBPs (Choi et al., 2008; Smith et al., 2003). Changes in telomerase activity with age and MDD have yet to be explored in the human brain. However, given the fact that neurotrophic growth signaling, including reduced BDNF, is decreased in MDD (Guilloux et al., 2012; Sen et al., 2008; Tripp et al., 2011), we cannot exclude the

possibility that telomeres and telomerase activity may significantly contribute to the mediation of stress and brain aging.

Increased Oxidative Stress

Oxidative stress is the damage caused to cells as a result of an imbalance between the production of ROS and the ability of the cells to reduce the ROS or repair the resulting damage. The degree of oxidative stress to cellular components, including DNA, correlates positively with age (Epel, 2009; Joseph et al., 2005; Wolkowitz et al., 2011b). Because of their high demand for energy and postmitotic status, neurons appear to be particularly sensitive to oxidative stress and thus aging.

One way in which oxidative stress may contribute to age-related changes in gene expression is via its direct effect on DNA. Lu et al. (2004) showed that age-related decrease in gene expression is related to the accumulation of oxidative DNA damage. The underlying mechanism was suggested that promoter regions with high GC contents are specifically vulnerable to oxidative damage. Oxidated promoter regions may potentially adopt different conformation and lose affinity for transcription factors (Lu et al., 2004). Damage on mitochondrial DNA (mtDNA), which is considered extremely vulnerable to oxidation due to its proximity to the site of ROS production, respiratory chain, and the absence of protective histone (Lee and Wei, 2007), results in downregulation of genes related to respiratory chain and further, energy metabolism impairment (Lin et al., 2002). Several studies showed that psychiatric diseases including MDD, bipolar disorder, and schizophrenia are associated with mitochondrial dysfunction (Rezin et al., 2009) and that accumulation of mtDNA damage induces mood disorder-like phenotypes as well as premature aging in mice (Kasahara et al., 2006; Trifunovic et

al., 2004). These results support the idea that oxidative stress plays a role as a link between aging and depression.

Another direct way in which oxidative stress may contribute to age-related changes in gene expression is via its effect on transcription factors. For example, ROS are able to activate nuclear factor kappa B (NF-κB) by decreasing binding affinity of the inhibitory subunit, Iκ-B, to NF-κB, an observation made relevant by the fact that NF-κB activity has been demonstrated to increase with aging and depression (Koo et al., 2010; Toliver-Kinsky et al., 1997). Also, transcription factors containing the zinc-finger DNA binding motif appear to be especially susceptible to damage from oxidative stress due to their high cysteine residue content. As intracellular ROS accumulate, oxidation of the thiol residues in cysteine occurs and binding affinity for DNA is lost. One of the zinc-finger transcription factors, Sp1, an ubiquitous transcription factor for housekeeping genes and enzymes involved in glucose metabolism and DNA synthesis, has been demonstrated to have decreased DNA binding affinity with advancing age (Ammendola et al., 1992, 1994; Wu et al., 1996). Interestingly, genes with lower affinity binding sites to Sp1 are more influenced by oxidative stress than those containing high-affinity sites (Ammendola et al., 1992, 1994; Wu et al., 1996). Furthermore, ROS decreases telomerase activity. Antioxidant treatment normalizes catalytic activity of TERT and delays cellular senescence (Haendeler et al., 2004).

In addition to the conformational change of gene and transcription factors, ROS can act on various cellular signaling pathways to control gene expression. For example, ROS increase p53 signaling, which has been implicated in various neurodegenerative diseases and thought to mediate its effect by increasing expression of genes related to cell cycle arrest, DNA repair, and apoptosis in response to cellular stressors such as DNA damage and hypoxia (Lundberg et al.,

2000). Another example is illustrated by p38 MAP Kinase (MAPK). When p38 MAPK is activated by oxidative stress, it promotes lamin B1 accumulation and expression of several transcription factors related to cellular senescence and apoptosis such as p53, CREB, C/EBPβ, and ATF2 (Barascu et al., 2012; Wagner and Nebreda, 2009). Interestingly, selective p38 MAPK deletion in serotonergic neurons produces stress resilience in an animal model of depression by inhibiting serotonin transporter translocation to plasma membrane (Bruchas et al., 2011).

In summary, the main effect of oxidative stress on aging has been thought to be the accumulation of toxic, inactive molecules produced randomly by ROS. However, oxidative stress may also have an active role in aging and related diseases, through direct modification of DNA and transcription factor integrity and through indirect pathways regulating upstream modulators. These observations suggest that antioxidants may contribute to preventing biological changes and/or associated symptoms of depression, in addition to their potential anti-aging effects.

Epigenetic Modifications

Epigenetic modifications, including DNA methylation and histone modification, regulate gene expression without changing the primary DNA sequence. Though classically viewed as a permanent event, recent data indicates that such modifications are influenced by genetic and environmental factors in adult organisms, including changes in methylation patterns across the lifespan (Numata et al., 2012). At the genome level, DNA methylation decreases with age. In contrast, CpG islands of many specific promoter regions that are typically not methylated become methylated with aging, including in promoters of tumor suppressor genes, estrogen receptor (ER), and insulin-like growth factor 2 (IGF2; (Issa et al., 1994; Issa et al., 1996; Numata

et al., 2012; So et al., 2006). Changes in DNA methylation at the glucocorticoid receptor, potentially due to early-life stress, was also correlated with altered vulnerability to psychiatric disorders and death by suicide in adults (McGowan et al., 2009).

Similarly, histone modifications such as acetylation, phosphorylation, symoylation, and methylation change with age. It was recently demonstrated that the aging-related deficit of long-term synaptic plasticity in the rodent hippocampus is related to decreased BDNF expression secondary to decreased acetylation of histones residing at the BDNF promoter region (Zeng et al., 2011). This observation fits well with human studies demonstrating reduced BDNF function in aging and depression (Guilloux et al., 2012; Webster et al., 2002). Also, changes in histone modifications in rodent systems may be protective against the effects of stress (Covington et al., 2011; Uchida et al., 2011). In support of a specific role for histone acetylation in age-related changes in gene expression, sirtuins (NAD-dependent histone deacetylases) have been implicated in longevity in yeast and invertebrates (Longo and Kennedy, 2006). A recent rodent study directly links SIRT1 to the risk of anxiety-like behaviors through its activity on monoamine oxidase A and serotonin levels (Libert et al., 2011). In the same study, the authors reported association between a single nucleotide polymorphism in the human SIRT1 gene and the risk of various psychiatric disorders such as anxiety disorder, panic disorder, and major depression. Furthermore, overexpression of SIRT6 in mice has been reported to increase lifespan and protect from diet-induced physiological damage (Kanfi et al., 2012; Kanfi et al., 2010) and SIRT6 knockout mice exhibit accelerated aging. Interestingly, decreased expression of SIRT6 has been observed during MDD (Abe et al., 2011). How SIRT6 mediates its effect on aging or is involved in MDD is not clear, but its functions as an HDAC (Kawahara et al., 2009; Michishita et al., 2008; Tennen et al., 2011) and in DNA repair (Mao et al., 2011) suggest that it may

contribute to protecting against aging and psychiatric illness by maintaining telomere integrity or protecting against and repairing the effects of oxidative stress.

Together, the occurrence of epigenetic modifications during aging and in the context of neuropsychiatric disorders may thus provide mechanistic underpinnings for the proposed age-by-disease biological interaction hypothesis, through the dual role of longevity and other age-associated genes.

SUMMARY AND IMPLICATIONS

We propose a novel framework for investigating the development of late-life brain disorders, including LLD, which we term the age-by-disease biological interaction hypothesis. This paper expands upon an earlier version described elsewhere (McKinney and Sibille, 2013). This hypothesis posits that symptoms of LLD and other late-life brain disorders are the emerging properties of underlying biological changes, which in turn are supported by normal changes in the expression of multiple genes with age, including disease-related genes changing in disease-causing directions. Here, in addition to presenting the gene expression data on which the hypothesis is based, we discussed molecular mechanisms that may account for age-dependent gene expression changes, including loss of telomere integrity, increased oxidative stress, and epigenetic modifications. Importantly, this hypothesis complements existing hypotheses, including the vascular, inflammatory, and dementia prodrome hypotheses of LLD, but it differs in that it positions age-dependent gene expression changes as the mechanism potentially driving dysfunctions in multiple biological pathways, including vascular, inflammatory, and neurotrophic functions. A potential sequence of events is summarized in Figure 2. The purpose of this paper was to discuss the general framework. Examples of gene changes at the intersection

of depression and aging were provided (e.g., SST and BDNF), but the exact nature and complexity of changes in multiple genes and pathways and their relevance to disease pathways will be described in details elsewhere.

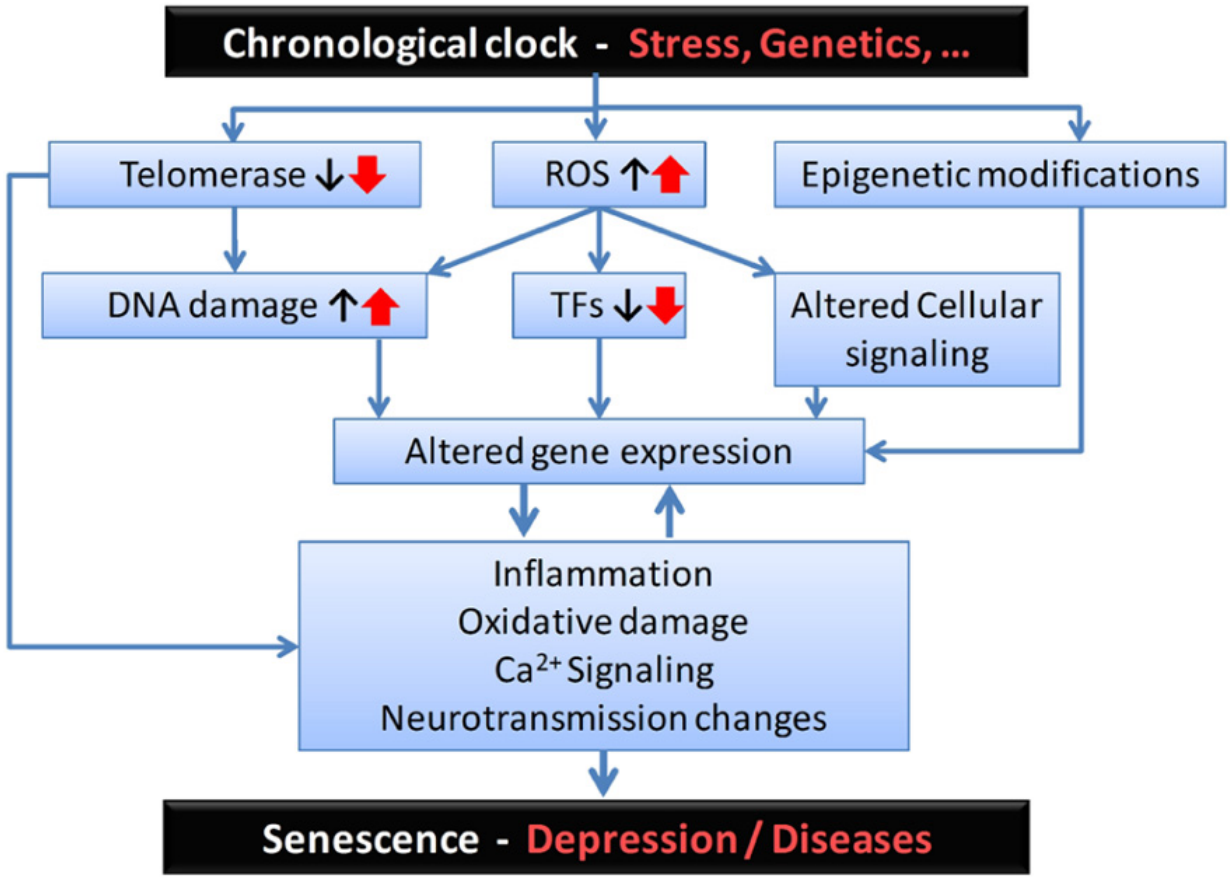


Figure 23. Proposed sequence of biological events and putative mediators for the age-by-disease biological interaction hypothesis.

From the top: Although its biological substrates are unknown, a chronological clock drives age-related changes in gene expression. These changes can be exacerbated by psychophysiological stress and/or genetic variants, and placed on accelerated age trajectories. In this model, age-related changes in telomeres, oxidative load and epigenetic landscape, among other putative mechanisms, may represent a first level of biological events, which in turn affect basic cellular processes involved in regulating gene expression (i.e., including DNA damage, altered structure and function of transcription factors (TFs), and associated local cellular signaling). The resulting changes in the global pattern of age-dependent gene expression mediate the next set of deleterious biological events, exemplified here by

increased inflammation, oxidative damage-related signaling, and changes in neurotransmission. These two levels of changes are likely to reciprocally interact. At the neural network and brain levels, the emerging properties of those specific cellular events are expressed as senescence in normal aging subjects and, in subjects at risk, as age-related symptom dimensions and diseases, including depression. Notably, the degree of individual vulnerability is thought to be under genetic and environmental control, so decreased vulnerability may mediate resiliency through the same pathways.

The implications of this hypothesis for the prevention and treatment of LLD and other late-life brain disorders are exciting. Understanding the mechanisms mediating age-related changes in gene expression is expected to provide insight into pathophysiological mechanisms and potential targets for intervention into these disorders. Identifying key upstream hub genes mediating patterns of altered age-dependent changes would provide novel targets for further investigations. Although sirtuins and BDNF may represent obvious candidates, the large set of age-dependent genes (~10% of all genes; (Erraji-Benchekroun et al., 2005)) and its overlap with genes previously implicated in brain disorders (Glorioso et al., 2011) should be viewed as an enriched pool of candidate genes. Targeting such upstream factors (transcription or function) should represent productive research avenues. Early candidate interventions may include known interventions such as antidepressant medications, psychotherapy, exercise, and others. Investigating how these known interventions affect age-dependent changes in the function of critical genes may help in optimizing their implementation with respect to timing and duration of intervention for age-dependent disorders. Further, identifying genetic and environmental factors that slow or accelerate age-related changes in gene function may lead to individualized strategies aimed at promoting resilience and successful aging. Other entry points and targets for intervention are likely to arise out of understanding the mechanisms by which gene expression

changes with age, including determining the role of telomere integrity, oxidative stress, and epigenetic modifications. Finally, for the broader fields of aging and gerontology, the implication of this hypothesis is that it brings together research on normal aging more closely with the investigation of neuropsychiatric and neurodegenerative diseases. Indeed, our data firmly support the assertion that they may in fact be related facets of similar biological processes, and also provide the basis for a putative mechanism of age-by-disease biological interactions.

CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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APPENDIX B

SUPPLEMENTAL INFORMATION PAPER 1

Table 5. Demographics of Post-Mortem Subjects

	Control Group								Major Depression Group										
	Case	Race[2]	Age	PMI	pH	RNA ratio	RIN		Case	Race[2]	Age	PMI	pH	RNA ratio	RIN	Suicide	Recurrent Episode	PTOD[1]	MOD[3]
Male	795	W	68	12.0	6.6	1.2	6.7		598	W	69	5.9	7.3	1.6	8.8	Y	N	D	S
	615	W	62	7.2	6.4	1.4	7.8		600	W	63	9.9	6.7	1.7	7.1	Y	N	N	S
	713	W	58	37.5	7.0	1.6	8.4		698	W	59	13.0	6.8	1.5	9.0	Y	N	D	N
	736	W	54	15.5	6.9	1.6	8.3		783	W	63	11.5	6.5	1.4	8.8	N	Y	N	S
	1031	W	53	23.1	6.8	1.5	8.2		809	W	50	20.0	6.9	1.5	8.5	N	N	D	A
	1086	W	51	24.2	6.8	1.4	8.1		863	W	51	28.3	7.2	1.5	8.4	N	N	N	S
	1122	W	55	15.4	6.7	1.4	7.9		926	W	56	19.0	7.0	1.4	7.3	N	N	D	S
	1047	W	43	12.0	6.9	1.8	9.0		943	W	56	15.4	6.6	1.5	8.2	Y	Y	N	S
	852	W	54	8.0	6.8	1.8	9.1		1001	W	53	7.3	6.6	1.4	7.6	N	N	N	N
	1067	W	49	6.0	6.6	1.4	8.2		1049	W	48	5.4	6.6	1.5	8.4	N	N	D	N
	604	W	39	19.3	7.1	2.1	8.6		1060	W	30	11.1	6.6	1.3	8.3	Y	N	N	N
	634	W	52	16.0	7.0	1.8	8.1		619	W	55	18.8	6.9	1.3	7.9	Y	U	B D	N
	551	W	62	7.2	6.4	1.4	7.8		613	W	59	15.6	7.0	1.9	9.1.	Y	Y	N	N
	685	W	57	14.0	6.6	1.5	8.0		505	W	57	12.8	7.1	1.8	8.9	Y	Y	N	S
	789	W	22	20.0	7.0	1.4	7.4		513	W	24	13.1	6.9	1.5	7.0	Y	U	N	S
	857	W	48	16.6	6.7	1.8	8.9		868	W	47	10.5	6.8	1.5	9.3	N	Y	N	S
	1462	W	47	17.2	6.2	2.0	8.5		1013	W	46	16.1	6.3	1.5	8.0	Y	Y	N	N
	1129	W	54	21.0	6.3	1.5	9.0		1161	W	57	15.9	6.6	2.0	7.6	N	Y	D	S
	1374	W	43	21.7	6.6	1.8	7.2		1226	W	44	19.3	6.5	1.7	7.5	N	Y	N	N
	1439	W	56	16.1	6.8	2.1	7.7		1253	W	58	12.5	6.8	1.9	8.1	N	Y	D	N
	1444	W	46	22.0	6.3	2.1	8.4		1261	W	46	22.8	6.6	1.9	8.8	N	Y	D	A
	1031	W	53	23.1	6.8	1.5	8.9		1312	W	51	24.6	6.5	1.6	8.1	N	Y	N	A
	1317	W	56	22.9	6.4	1.3	6.9		1320	W	55	24.4	6.5	1.3	7.2	N	Y	N	N
	1214	W	57	16.4	6.2	1.7	7.5		1389	W	61	16.0	6.6	1.9	8.4	N	Y	N	S
	1394	W	45	17.3	6.6	1.9	7.3		10010	W	42	14.3	6.4	1.8	7.6	Y	Y	D	S
	1372	W	37	20.5	6.6	1.6	9.0		10031	W	36	20.0	6.8	2.0	8.9	N	Y	D	A
Female	731	W	63	10.5	6.2	1.6	8.2		565	W	62	12.5	6.9	2.0	9.2	Y	Y	D	S
	1270	W	73	19.7	6.7	1.4	7.7		860	W	74	23.0	6.2	1.2	8.1	N	Y	B D	N
	1293	W	65	18.5	6.6	1.3	7.0		1272	W	64	12.1	6.6	1.4	7.8	N	Y	B D	N
	1466	B	64	20.0	6.7	2.0	8.8		803	W	65	18.0	7.0	1.9	9.0	N	Y	D	A
	1247	W	58	22.7	6.4	1.3	8.4		934	W	54	17.9	6.2	1.2	8.2	N	Y	D	N
	1282	W	39	24.5	6.8	1.3	7.5		967	W	40	22.2	6.6	1.6	7.4	N	Y	N	N
	575	B	55	11.3	6.8	1.8	9.6		986	W	53	11.9	6.7	1.8	8.7	N	Y	D	N
	1391	W	51	7.8	6.6	1.6	7.1		1041	W	52	10.3	6.5	1.5	8.4	N	Y	B D	A
	686	W	52	22.6	7.1	1.9	8.5		1143	W	49	23.4	6.4	1.8	8.1	N	Y	B D	A
	1034	W	23	8.5	6.1	2.0	7.8		1157	W	26	13.4	6.4	1.5	7.8	Y	Y	D	S
	567	W	46	15.0	6.8	2.3	8.9		1190	W	47	22.3	6.6	1.6	8.0	Y	Y	N	S
	546	W	37	23.5	6.7	2.0	8.6		1221	B	28	24.8	6.6	1.8	7.2	N	Y	N	N
	1092	B	40	16.6	6.8	1.7	8.0		1249	W	40	11.2	6.5	2.0	9.0	N	Y	B D	A
	1403	W	45	12.3	6.7	1.8	8.2		1254	W	39	12.8	6.4	1.9	9.0	Y	Y	D	S
	1318	W	58	18.8	6.7	2.0	7.4		1271	W	50	18.6	6.4	1.8	8.6	N	Y	D	A
	1196	W	36	14.5	6.4	1.8	8.2		1408	W	37	15.5	6.6	1.6	7.0	N	Y	B D	A
	568	W	60	9.5	6.9	1.9	8.7		564	W	56	16.8	7.0	1.9	9.2	Y	N	B D	S
	10013	W	16	9.3	6.7	1.8	9.0		666	W	16	10.0	7.3	2.0	9.4	N	N	D	A
	840	W	41	15.4	6.8	2.0	9.1		1202	W	39	11.2	6.4	1.8	8.0	N	Y	D	N
	1280	W	50	23.5	6.7	1.3	7.7		1289	W	46	25.0	6.3	1.4	7.3	N	N	U	N
	1099	W	24	9.1	6.5	1.9	8.6		1315	W	28	12.4	7.0	1.5	7.9	Y	N	N	S
	627	B	43	14.1	7.1	1.0	7.0		1332	W	46	17.5	6.7	1.6	8.9	N	Y	B D	N
	818	W	67	24.0	7.1	1.5	8.4		1356	W	60	20.6	6.1	1.8	8.5	N	Y	D	A
	1081	W	57	14.9	6.8	1.8	9.0		1360	W	59	18.1	6.4	1.4	7.6	Y	N	D	S
	1355	W	74	24.9	6.6	1.9	7.0		10028	W	72	23.1	6.7	1.4	7.0	Y	N	N	S
		Mean	50	16.9	6.7	1.7	8.1			Mean	50	16.2	6.7	1.6	8.2				
		SEM	2	0.9	0.0	0.0	0.1			SEM	2	0.8	0.0	0.0	0.1				

[1]Psychotropic Medications at Time of Death (D is antidepressant, B is benzodiazepine, N is no, U is unknown)

[2]Race: W = Caucasian American B = African American

[3]cause of death: S = Suicide A = Accidental N = natural

Table 6. Cofactor Analysis in Post Mortem Subjects

		Sex	Age	PMI[1]	pH	RNA ratio	RIN[2]	Suicide	ATOD[3]
BDNF-IX	All	.494	.054	.207	.620	.022	.265	.157	0.611
	Male		.279	.822	.827	.532	.844	.568	0.588
	female		.118	.098	.382	.012	.096	.161	0.664
TrkB-F	All	.167	.480	.141	.304	.066	.529	.906	0.804
	Male		.059	.203	.061	.084	.501	.743	0.944
	female		.099	.364	.911	.375	.808	.940	0.348
CORT	All	.084	.038	.592	.889	.150	.277	.449	0.244
	Male		.005	.209	.500	.078	.628	.733	0.313
	female		.390	.325	.464	.527	.165	.189	0.728
SST	All	.016	.010	.488	.069	.925	.997	.005	0.399
	Male		.049	.962	.851	.813	.394	.170	0.109
	female		.191	.312	.042	.622	.510	.012	0.560
VGF	All	.032	.672	.525	.708	.534	.794	.654	0.537
	Male		.697	.513	.080	.193	.910	.450	0.131
	female		.332	.836	.215	.625	.624	.830	0.677
SNAP25	All	.302	.860	.092	.618	.127	.924	.359	0.110
	Male		.084	.129	.248	.014	.990	.268	0.211
	female		.086	.453	.713	.847	.990	.307	0.025
NPY	All	.010	.199	.762	.006	.718	.402	.420	0.375
	Male		**.002**	.978	.101	.543	.397	.863	0.546
	female		.691	.618	.041	.624	.546	.081	0.993
TAC1	All	**1.27E-04**	.222	.985	.078	.170	.210	.225	0.506
	Male		**.001**	.914	.378	.531	.563	.703	0.629
	female		.913	.954	.245	.015	.079	.263	0.933
RGS4	All	.602	.677	.550	.326	**.002**	.595	.828	0.610
	Male		.186	.685	.028	.111	.910	.453	0.454
	female		.843	.657	.802	.014	.613	.860	0.413
GAD1	All	.121	.073	.187	.364	.019	.270	.998	0.551
	Male		.016	.122	.114	.013	.772	.389	0.812
	female		.702	.948	.605	.487	.221	.110	0.985
GAD2	All	.609	.732	.551	.413	.012	.112	.230	0.325
	Male		.491	.377	.375	.162	.181	.554	0.350
	female		.886	.792	.592	.046	.273	.196	0.843
PVALB	All	**.004**	.124	.008	.742	**.001**	.775	.275	0.883
	Male		**.001**	.043	.092	**.001**	.555	.170	0.745
	female		.588	.088	.138	.157	.989	.707	0.280
SLC6A1	All	.013	.010	.348	**.004**	.739	.940	.668	0.287
	Male		.314	.532	**1.7E-04**	.327	.729	.955	0.829
	female		**.001**	.424	.252	.380	.954	.455	0.339
CALB2	All	.589	.591	.818	.576	.592	.713	.799	0.776
	Male		.190	.479	.866	.286	.974	.685	0.148
	female		.888	.292	.396	.135	.663	.671	0.316
GABRA1	All	.006	.157	.226	.527	.065	.363	.910	0.488
	Male		.009	.503	.698	.006	.081	.889	0.583
	female		.808	.299	.394	.760	.879	.969	0.081
BDNF ELISA	All	**3.3E-04**	.695	.007	.064	.582	.046	.521	0.069
	Male		.952	.007	.028	.263	.163	.250	0.126
	female		.260	.534	.766	.190	.054	.351	0.755

[1]Post Mortem interval (hours)

[2]RNA Integrity Number

[3]antidepressants at time of death

Grey highlighted $p < 0.05$ uncorrected

Bold are included in ANCOVA modeling after Bonferonni-Holm Correction as Cofactors

Table 7. Suicide Effect on BDNF and BDNF-Dependent Gene Expression

Changes in suicide and non-suicide MDD subjects for genes with significant MDD effects are shown below. Highlighted in light grey are statistically significant $p < 0.05$. Dark grey indicates significance after correction for multiple testing.

	All		MDD & Suicide		MDD not Suicide	
	Alr	*p value*	Alr	*p value*	Alr	*p value*
TrkB	-0.52	0.003	-0.51	0.039	-0.52	0.015
CORT	-0.58	1.69E-05	-0.54	0.002	-0.68	2.63E-06
VGF	-0.63	0.001	-0.69	0.007	-0.58	0.010
SST	-0.59	0.001	-0.04	0.861	-0.83	0.005
TAC1	-0.42	0.016	-0.26	0.285	-0.55	0.008
NPY	-0.66	1.01E-04	-0.53	0.015	-0.72	4.25E-05
SNAP25	-0.69	4.53E-04	-0.82	0.008	-0.65	0.001
GAD2 (GAD65)	-0.48	2.00E-03	-0.37	0.116	-0.54	0.001
GAD1 (GAD67)	-0.41	1.00E-03	-0.43	0.019	-0.40	0.003
PVALB (PV)	-0.58	0.005	-0.86	0.004	-0.42	0.038

APPENDIX C

SUPPLEMENTAL INFORMATION PAPER 2

Table 8. Top 200 genes positively correlated to BDNF expression

GABRA5, SLC2A13, SCG2, ROBO2, HTR2A, ACSL4, KCNK1, DLG3, PTPRR, LPPR4, TLN2, RASGRP1, KCNAB1, KIAA1239, CACNA2D1, AKAP5, GABRA4, EPHA5, PPEF1, FAM102B, LANCL2, CA10, RAB27B, GRIA1, PCDH19, GGCT, OLFM3, GABRB3, PRKCB, NRXN3, DCLK1, BZW2, PCDH20, NETO1, NPTX2, ELMOD1, ELMO1, SYT5, GRIN2B, SCN3B, KIAA1244, NRN1, FABP3, SPCS3, NELL2, DPP6, LNX1, NCAM2, FAM19A1, METAP1, LUZP6, HS6ST3, PPP4R4, CRYM, CACNB1, HMGCR, TBC1D9, SVOP, CACNA1E, SPHKAP, ATXN10, RBP4, CAMK4, RBFOX2, CNTN4, MAL2, FAM49A, ANO3, FAM131A, CMAS, GRIN2A, FZD3, PIH1D1, ATP6V0D1, PGM2L1, CACNG3, VAMP2, PCMT1, ST8SIA5, SCN2A, SORCS3, NEUROD6, FBXL2, RGS4, LARGE, NUDT4P1, CRHBP, SYT4, MMD, CAP2, KCNV1, WBSCR17, PAK1, FRMPD4, LMO3, PHACTR1, PPP2CA, PTPRO, CDH8, HCCS, RAB3C, SLC17A7, KIAA0317, FSTL5, REEP1, DUSP3, TMEFF1, HMP19, ARPC5L, CDC40, PRKCD, SST, EFNB3, GLRA3, RASGRF1, GABBR2, YWHAB, SCAMP5, CDK17, ENC1, LRRTM2, MYH10, STMN2, NLK, MTMR9, GLCE, XKR4, INPP4A, RBM18, NEDD4L, NNAT, ITFG1, HSPA13, EXT1, CALB1, CLVS1, VLDLR, AZIN1, NLGN1, LIG4, ODZ3, EPHA4, ARHGEF9, CDKL5, PSMB7, TUSC3, CCNA1, RNMT, CLSTN1, COPS3, CSRNP2, FGF12, CDH13, CASK, CADM3, PLK2, PAK3, VKORC1L1, PPP5C, ITPKA, MTMR6, PAFAH1B1, GNPTAB, PRKCE, PDE1A, HIVEP2, HN1, DIRAS2, ZNF711, CCT5, KCND2, PTDSS1, CDK5R2, RAPH1, IDH3B, ZNF238, ACVR2A, RTN4RL1, ASNS, GDA, HMOX2, BRMS1L, PRKCZ, RPS6KA3, MAGI1, HIAT1, SLC39A10, ST8SIA3, RNF11, JAZF1, SCN8A, PCSK1, LCMT1, SERPINI1, ACTR3B, CHL1, PRICKLE1, SV2B, STYK1, SCG5

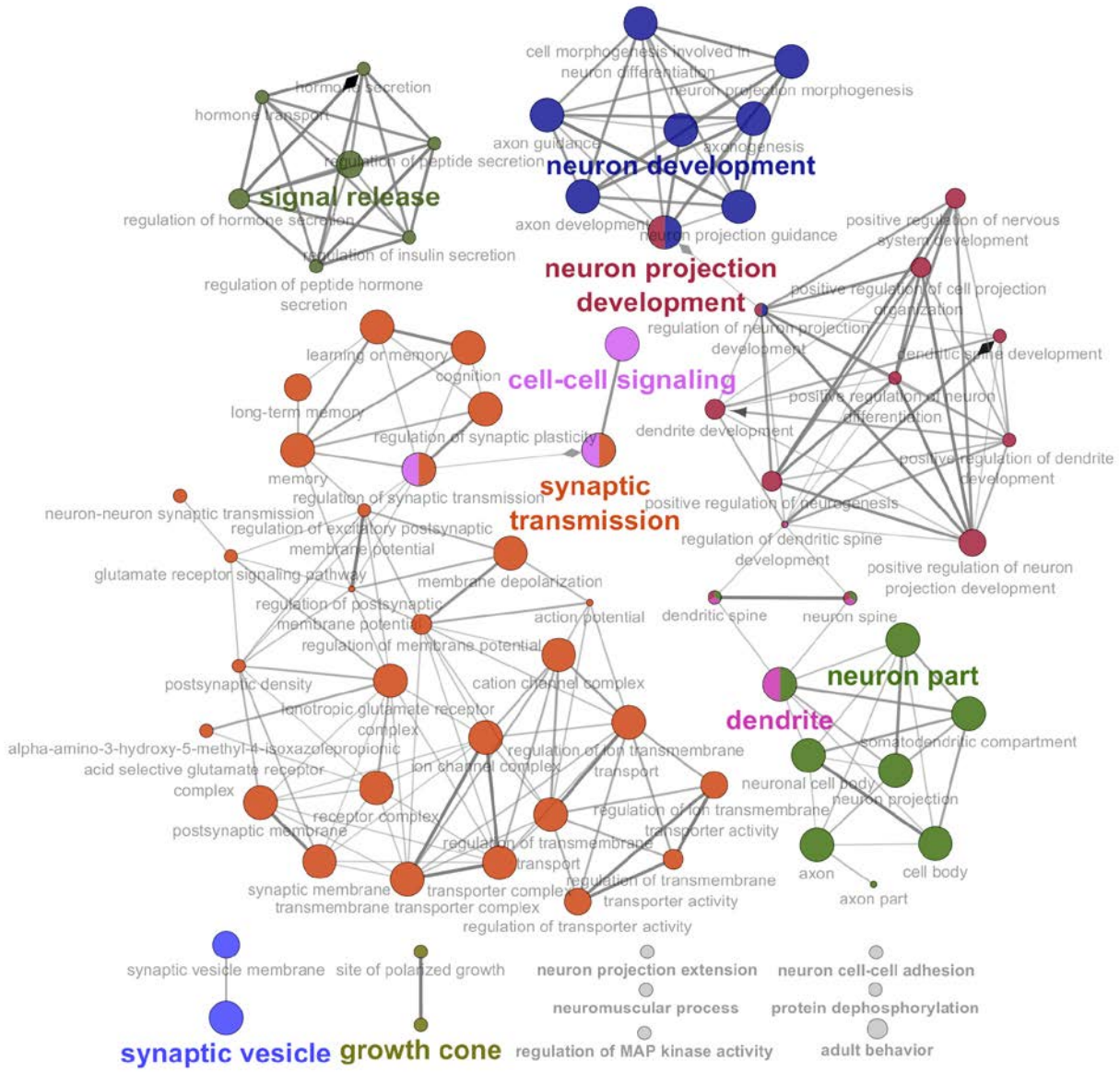


Figure 24. Full list of GO terms enriched in top 200 genes correlated with BDNF in human frontal cortex as identified by Cytoscape with ClueGO

Table 9. Top 3 annotation cluster containing full GO terms identified by DAVID

Annotation Cluster 1 **Enrichment Score: 6.93**

Category	Term	Count	%	PValue	Fold Enrichment
SP_PIR_KEYWORDS	synapse	19	9.55	8.11E-12	8.62
GOTERM_CC_FAT	synapse	24	12.06	1.02E-10	5.33
SP_PIR_KEYWORDS	cell junction	22	11.06	1.03E-09	5.33
GOTERM_BP_FAT	synaptic transmission	20	10.05	5.25E-09	5.37
GOTERM_CC_FAT	synapse part	18	9.05	1.28E-08	5.77
GOTERM_BP_FAT	transmission of nerve impulse	21	10.55	1.31E-08	4.80
GOTERM_CC_FAT	cell junction	25	12.56	3.11E-08	3.81
SP_PIR_KEYWORDS	postsynaptic cell membrane	10	5.03	2.05E-06	8.79
GOTERM_BP_FAT	cell-cell signaling	23	11.56	5.09E-06	3.07
GOTERM_BP_FAT	neurological system process	34	17.09	1.22E-05	2.25
GOTERM_CC_FAT	postsynaptic membrane	10	5.03	5.15E-05	5.84
KEGG_PATHWAY	Neuroactive ligand-receptor interaction	9	4.52	0.043934	2.23

Annotation Cluster 2 **Enrichment Score: 4.68**

Category	Term	Count	%	PValue	Fold Enrichment
SP_PIR_KEYWORDS	ionic channel	19	9.55	5.54E-09	5.78
GOTERM_MF_FAT	gated channel activity	19	9.55	3.70E-08	5.04
GOTERM_MF_FAT	ion channel activity	20	10.05	2.05E-07	4.26
GOTERM_MF_FAT	substrate specific channel activity	20	10.05	3.29E-07	4.13
GOTERM_MF_FAT	channel activity	20	10.05	5.57E-07	3.99
GOTERM_MF_FAT	passive transmembrane transporter activity	20	10.05	5.78E-07	3.98
SP_PIR_KEYWORDS	ion transport	22	11.06	5.89E-07	3.68
GOTERM_MF_FAT	voltage-gated cation channel activity	12	6.03	1.64E-06	6.71
GOTERM_CC_FAT	ion channel complex	14	7.04	1.89E-06	5.39
SP_PIR_KEYWORDS	voltage-gated channel	11	5.53	3.57E-06	7.09
GOTERM_MF_FAT	voltage-gated channel activity	12	6.03	2.46E-05	5.06
GOTERM_MF_FAT	voltage-gated ion channel activity	12	6.03	2.46E-05	5.06
GOTERM_BP_FAT	ion transport	25	12.56	2.68E-05	2.61
GOTERM_MF_FAT	cation channel activity	14	7.04	2.92E-05	4.18
GOTERM_MF_FAT	metal ion transmembrane transporter activity	15	7.54	4.41E-05	3.76
GOTERM_BP_FAT	metal ion transport	17	8.54	2.22E-04	2.93
GOTERM_CC_FAT	cation channel complex	9	4.52	2.62E-04	5.38
SP_PIR_KEYWORDS	transport	33	16.58	4.48E-04	1.91
GOTERM_BP_FAT	cation transport	18	9.05	5.23E-04	2.61
GOTERM_BP_FAT	monovalent inorganic cation transport	10	5.03	0.017867	2.52
GOTERM_MF_FAT	alkali metal ion binding	8	4.02	0.02087	2.88
GOTERM_BP_FAT	transmembrane transport	14	7.04	0.025129	1.97
INTERPRO	Ion transport	5	2.51	0.038445	3.92

Annotation Cluster 3 **Enrichment Score: 3.72**

Category	Term	Count	%	PValue	Fold Enrichment
GOTERM_CC_FAT	neuron projection	22	11.06	1.88E-09	5.08
GOTERM_CC_FAT	cell projection	26	13.07	1.98E-06	2.94
GOTERM_CC_FAT	axon	10	5.03	1.80E-04	4.96
GOTERM_CC_FAT	cell projection part	8	4.02	0.028691	2.70
GOTERM_CC_FAT	axon part	4	2.01	0.030469	5.84
GOTERM_CC_FAT	nerve terminal	3	1.51	0.078856	6.40

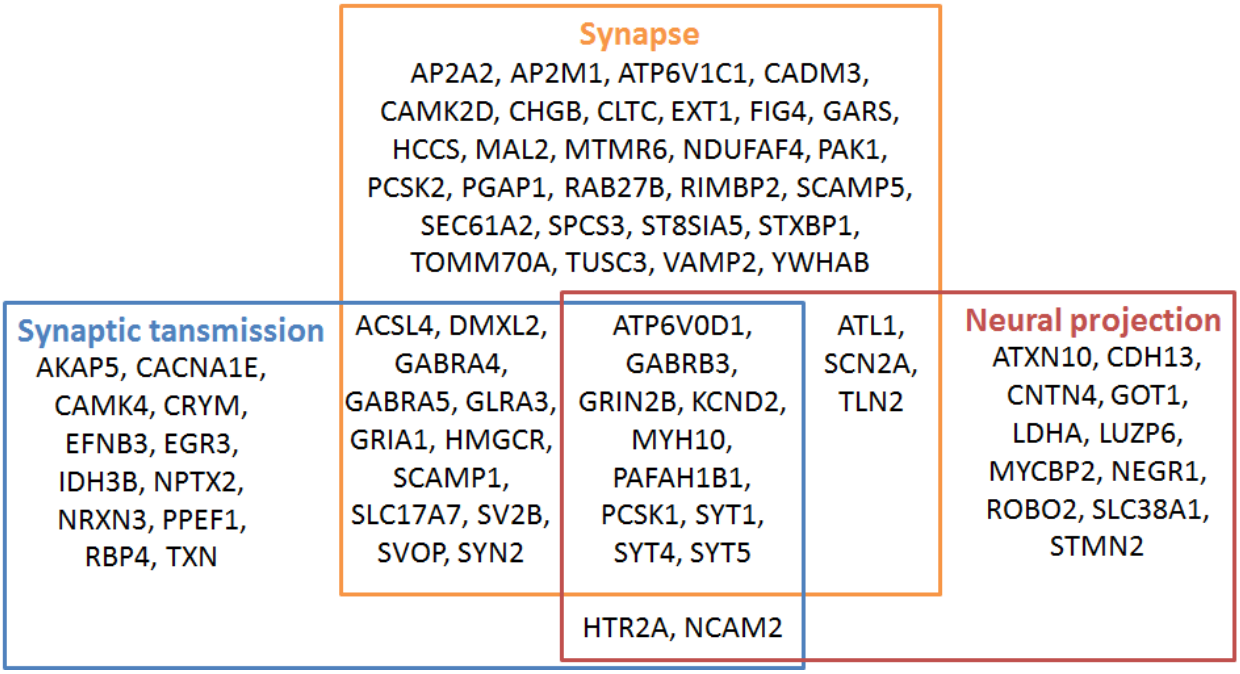


Figure 25. Synapse related genes enriched in BDNF coexpression network established with age-residual expression values

Close link between BDNF and synaptic genes were remained after age effect on gene expression was removed.

	ALR	
	array	qPCR
BDNF	-0.40	-0.17
vGLUT1	-0.29	-0.21
GRIA1	-0.30	-0.08
GRIN2A	-0.47	-0.41
GRIN2B	-0.49	-0.41
DLG4	-0.28	-0.30
SST	-0.77	-0.96
NPY	-0.30	-0.42
PVALB	-0.15	-0.09
CCK	-0.18	-0.31
vGAT1	-0.39	-0.64
GAD1	-0.19	-0.22
GAD2	-0.34	-0.05
GABRA4	-0.31	-0.24
GABRA5	-0.45	-0.39
GABRB3	-0.36	-0.53

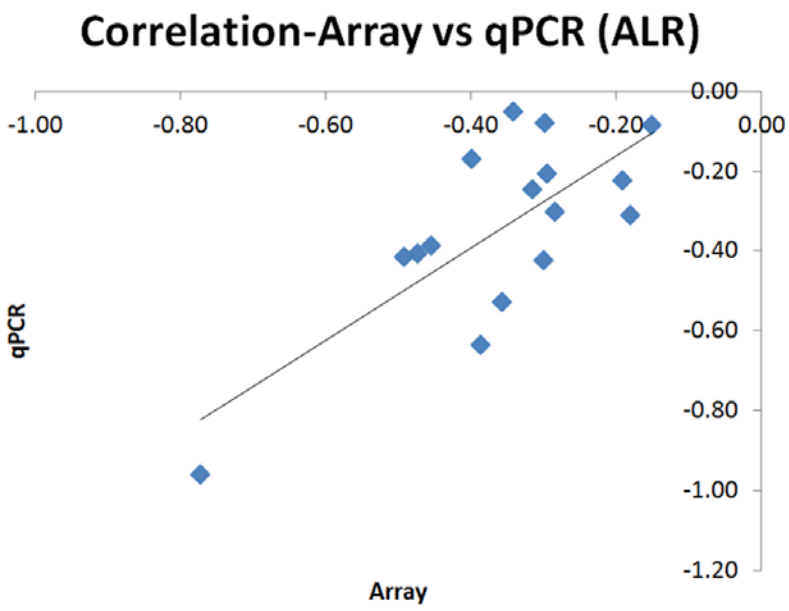


Figure 26. Correlation between altered gene expressions measured in array and qPCR

Pearson's correlation value between array and qPCR result was 0.74 (p=4.8E-04).

APPENDIX D

SUPPLEMENTAL INFORMATION PAPER 3

Table 10. Characteristics of human postmortem brain samples

	Control	MDD
n	19	19
Age (years)	48.1 ± 10.6	45.2 ± 10.1
Sex	M 10, F 9	M 10, F 9
Race	W 18, B 1	W 18, B 1
Postmortem interval (hours)	19.5 ± 5.1	20.1 ± 6.0
pH	6.6 ± 0.2	6.6 ± 0.2
RNA integrity number	8.0 ± 0.6	8.0 ± 0.5

Table 11. Cofactor analysis in postmortem subjects

Cofactor analysis		Age	PMI	pH	RNA ratio	RIN	Sex	Suicide	AD ATOD
BDNF LONG 3' UTR	All (n=38)	0.296	0.403	0.006	0.015	0.275	0.361		
	Control (n=19)	0.007	0.368	0.797	0.030	0.333	0.138		
	MDD (n=19)	0.950	0.505	0.001	0.392	0.347		0.302	0.327
SST	All (n=38)	0.023	0.473	0.127	0.030	0.794	0.215		
	Control (n=19)	0.010	0.596	0.706	0.675	0.920	0.860		
	MDD (n=19)	0.068	0.726	0.031	0.005	0.565		0.110	0.320
NPY	All (n=38)	0.541	0.586	0.073	0.293	0.747	0.077		
	Control (n=19)	0.642	0.720	0.808	0.912	0.924	0.190		
	MDD (n=19)	0.194	0.424	0.002	0.137	0.263		0.024	0.190
CORT	All (n=38)	0.158	0.368	0.175	0.011	0.588	0.139		
	Control (n=19)	0.005	0.536	0.700	0.044	0.820	0.305		
	MDD (n=19)	0.414	0.562	0.090	0.217	0.273		0.059	0.796
vGAT1	All (n=38)	0.383	0.980	0.989	0.191	0.066	0.103		
	Control (n=19)	0.533	0.788	0.465	0.902	0.072	0.551		
	MDD (n=19)	0.103	0.885	0.731	0.007	0.707		0.046	0.596
GAD1	All (n=38)	0.811	0.848	0.517	0.239	0.737	0.146		
	Control (n=19)	0.813	0.934	0.761	0.626	0.510	0.558		
	MDD (n=19)	0.803	0.967	0.171	0.400	0.205		0.305	0.832
GAD2	All (n=38)	0.463	0.766	0.783	0.284	0.886	0.218		
	Control (n=19)	0.822	0.844	0.541	0.583	0.902	0.302		
	MDD (n=19)	0.500	0.977	0.589	0.758	0.684		0.906	0.187
GABRA5	All (n=38)	0.395	0.954	0.490	0.040	0.714	0.119		
	Control (n=19)	0.371	0.700	0.647	0.297	0.421	0.041		
	MDD (n=19)	0.152	0.903	0.155	0.088	0.177		0.072	0.316

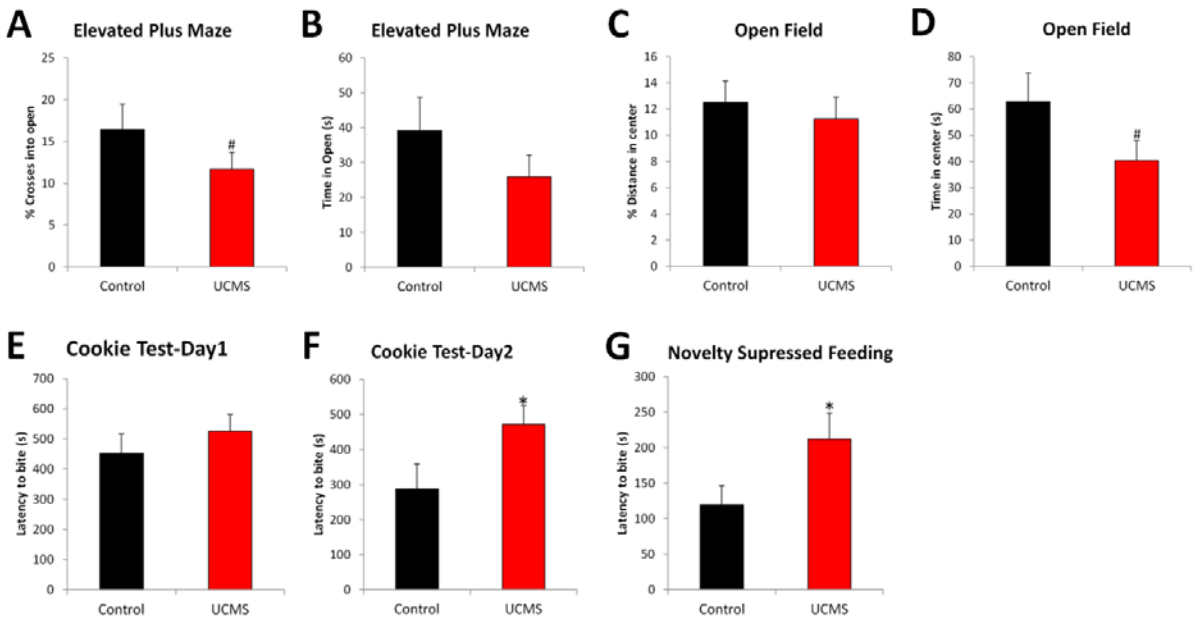


Figure 27. Chronic stress-induced behavioral changes in mice

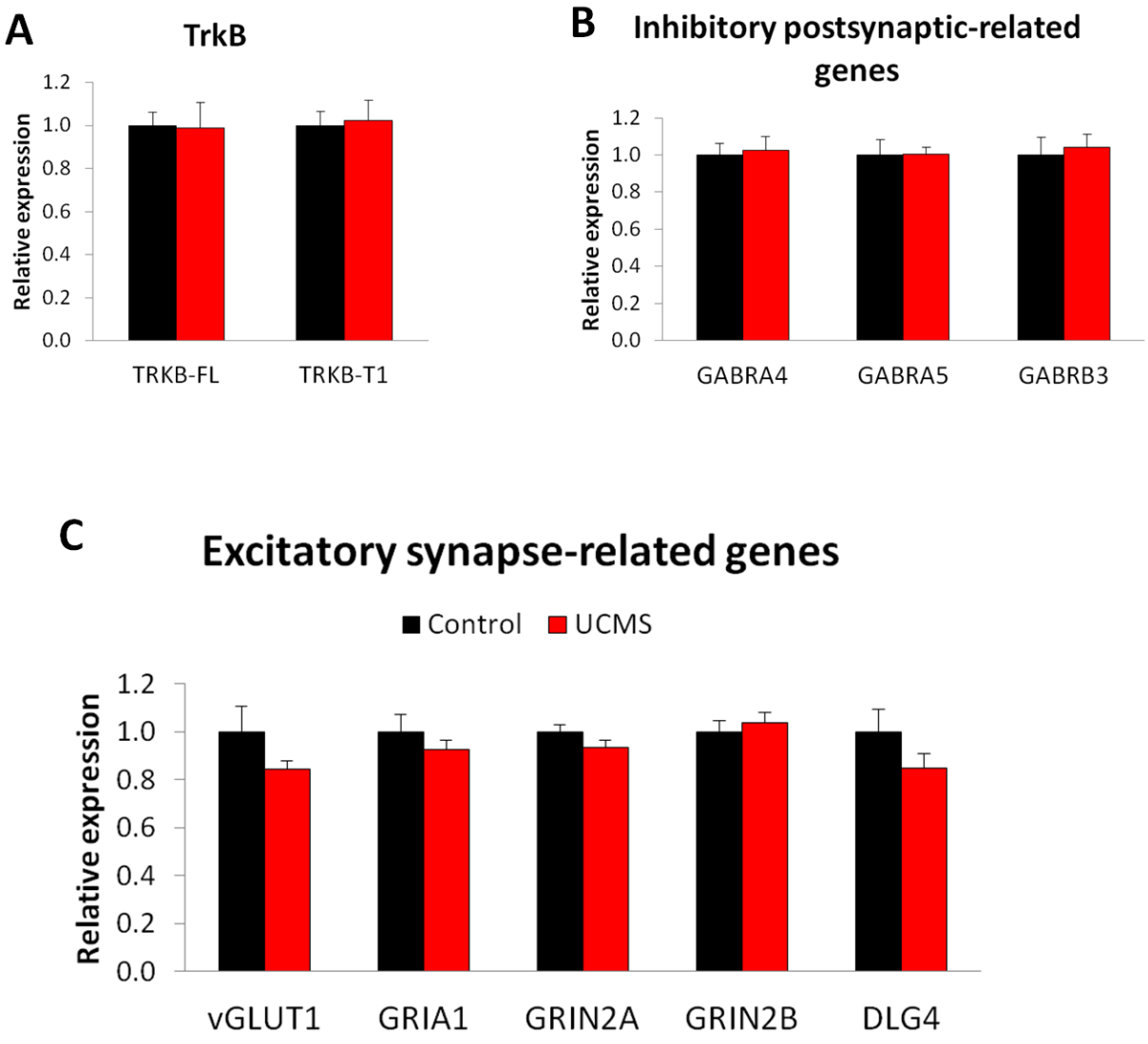


Figure 28. UCMS-induced gene expression changes in mouse mPFC

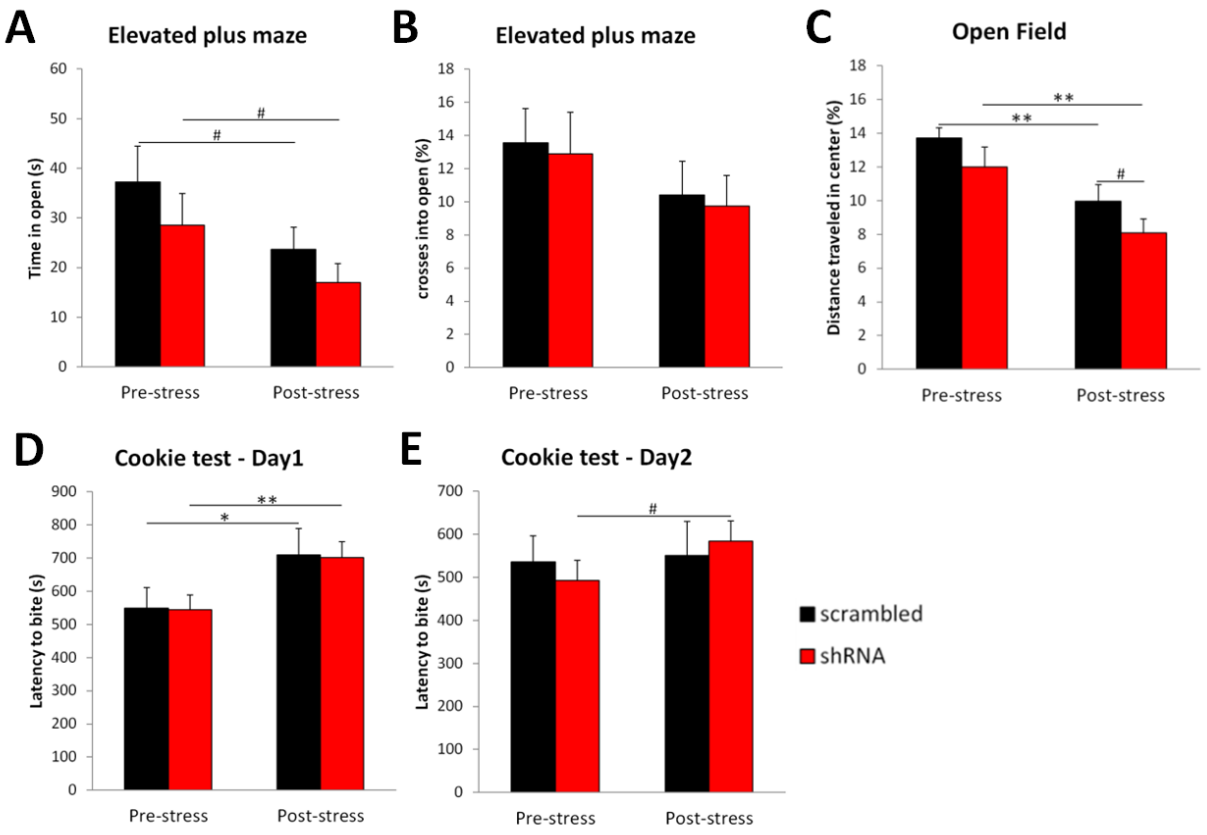
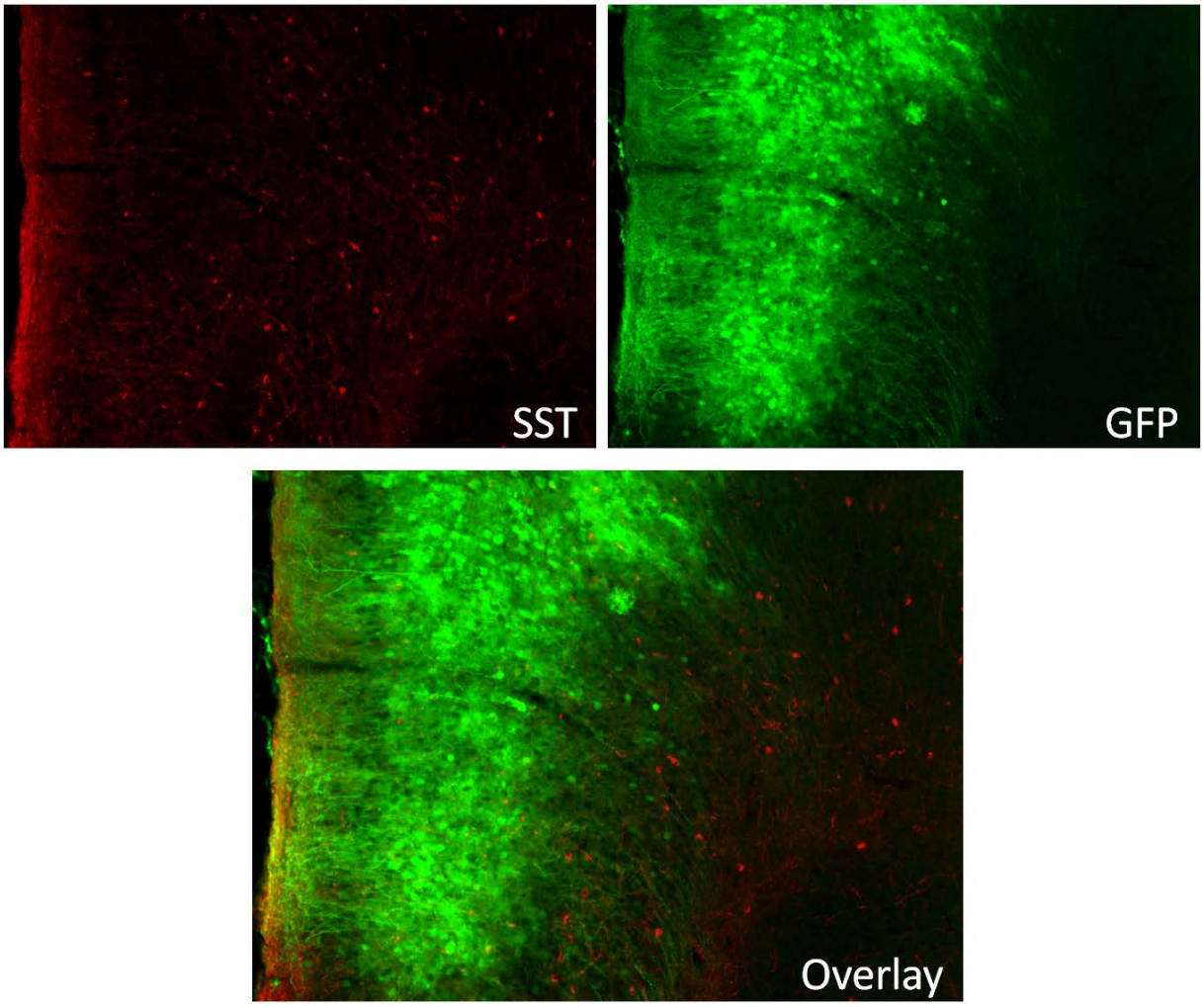


Figure 29. BDNF 3' UTR knockdown-induced behavioral changes in mice



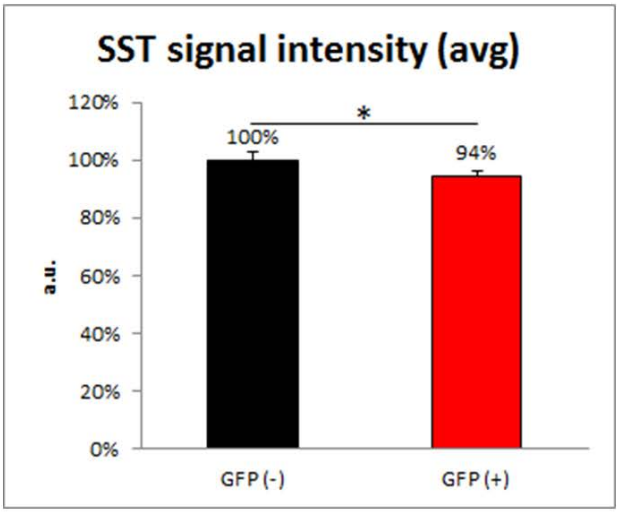


Figure 30. SST immunofluorescent signal intensity changes by shRNA treatment

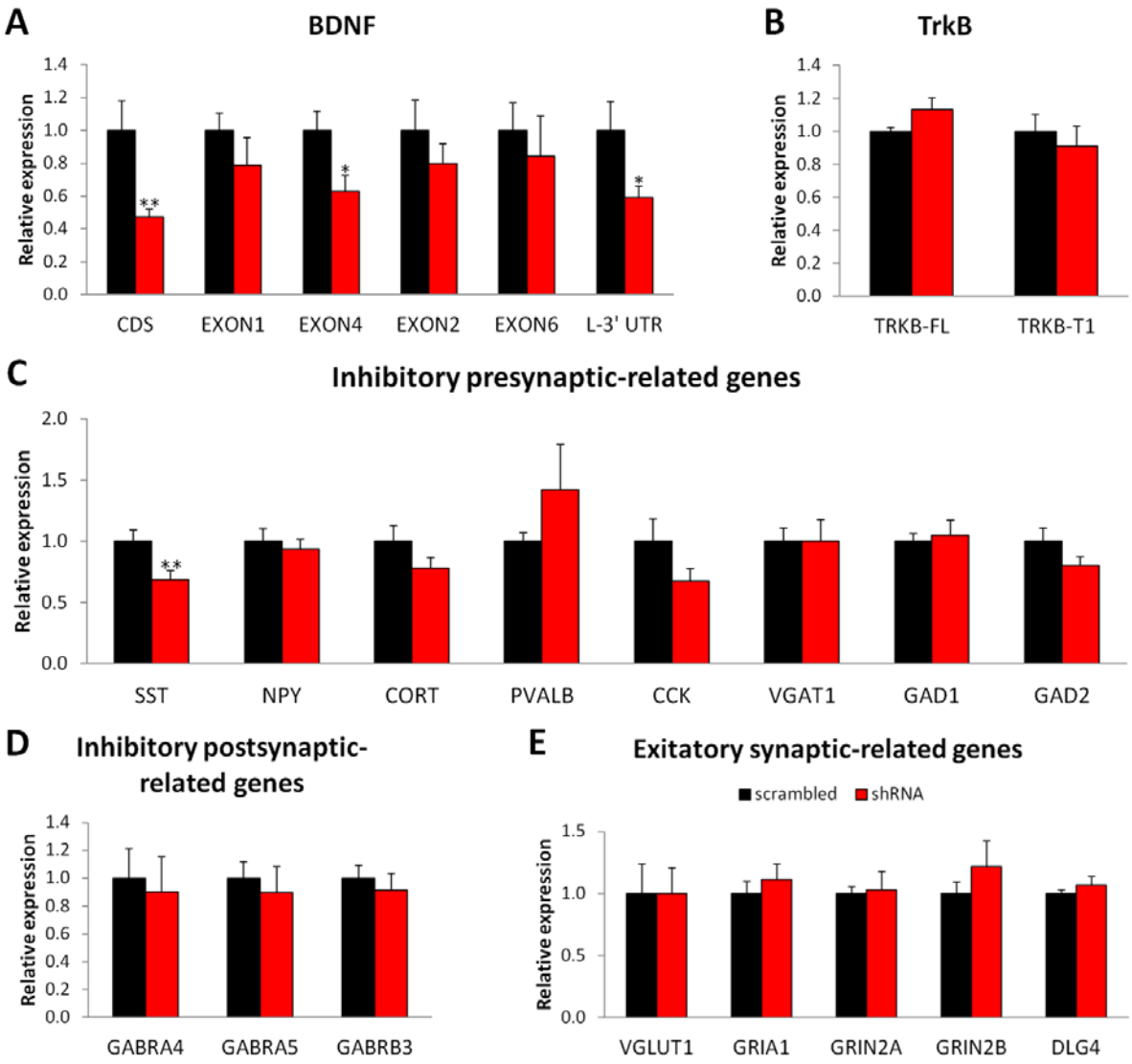


Figure 31. Gene expression changes by shRNA treatment in mouse cortices

	Cohort	MO1		BDNF-long 3' UTR KD	
	Brain region	dlPFC (BA46)		mPFC	
	n/group (sex)	19 (10M, 9F)		6 (M)	
	Gene symbol	ALR	p-value	ALR	p-value
BDNF	BDNF-CDS	-0.02	0.45	-1.01	2.1E-03
	BDNF-EXON1	-0.87	0.06	-0.49	0.15
	BDNF-EXON4	-0.16	0.23	-0.69	0.03
	BDNF-EXON2	-0.88	0.23	-0.29	0.12
	BDNF-EXON6	-0.25	0.40	-0.41	0.23
	BDNF-long 3' UTR	-0.37	3.7E-03	-0.69	0.01
TrkB	TrkB-FL	0.27	0.14	0.16	0.09
	TrkB-T1	0.14	0.18	-0.16	0.30
Inhibitory presynaptic-related genes	SST	-0.75	1.5E-03	-0.54	8.0E-04
	NPY	-0.60	3.4E-04	-0.07	0.40
	CORT	-0.56	5.0E-06	-0.34	0.16
	PVALB	0.12	0.07	0.34	0.18
	CCK	-0.08	0.21	-0.52	0.15
	GAD1	-0.30	0.01	0.03	0.44
	GAD2	-0.27	0.02	-0.28	0.08
	vGAT1	-0.45	0.01	-0.09	0.40
Inhibitory postsynaptic-related genes	GABRA4	0.18	0.12	-0.19	0.37
	GABRA5	-0.30	3.2E-04	-0.24	0.25
	GABRB3	0.29	0.09	-0.16	0.26
Excitatory synapse-related genes	DLG4	0.18	0.25	0.08	0.18
	GRIA1	0.31	0.10	0.14	0.31
	GRIN2A	0.38	0.08	-0.03	0.45
	GRIN2B	0.39	0.09	0.19	0.30
	vGLUT1	0.22	0.22	0.03	0.47

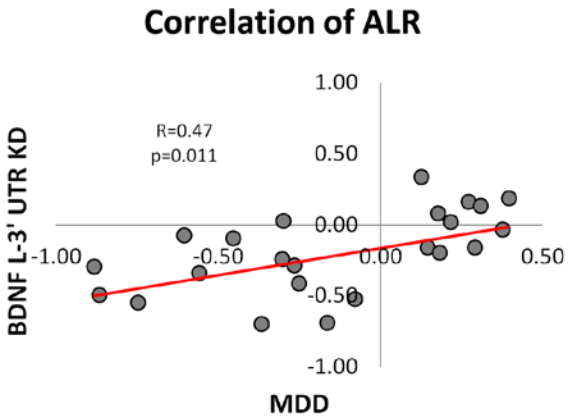


Figure 32. Similarity between MDD- and BDNF long 3' UTR knockdown-induced gene expression changes

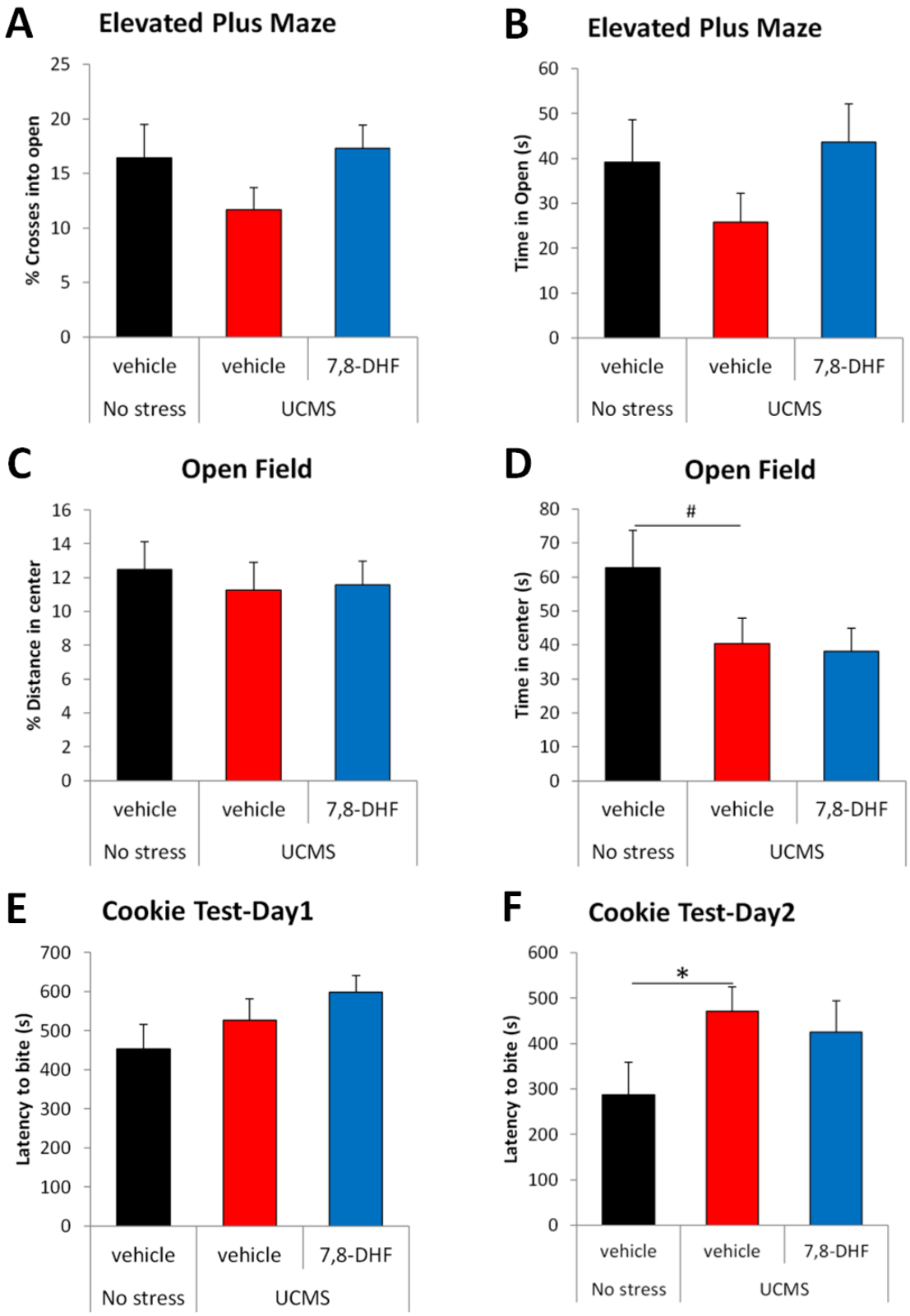


Figure 33. Effect of 7,8-DHF treatment on stress-induced behavior in mice

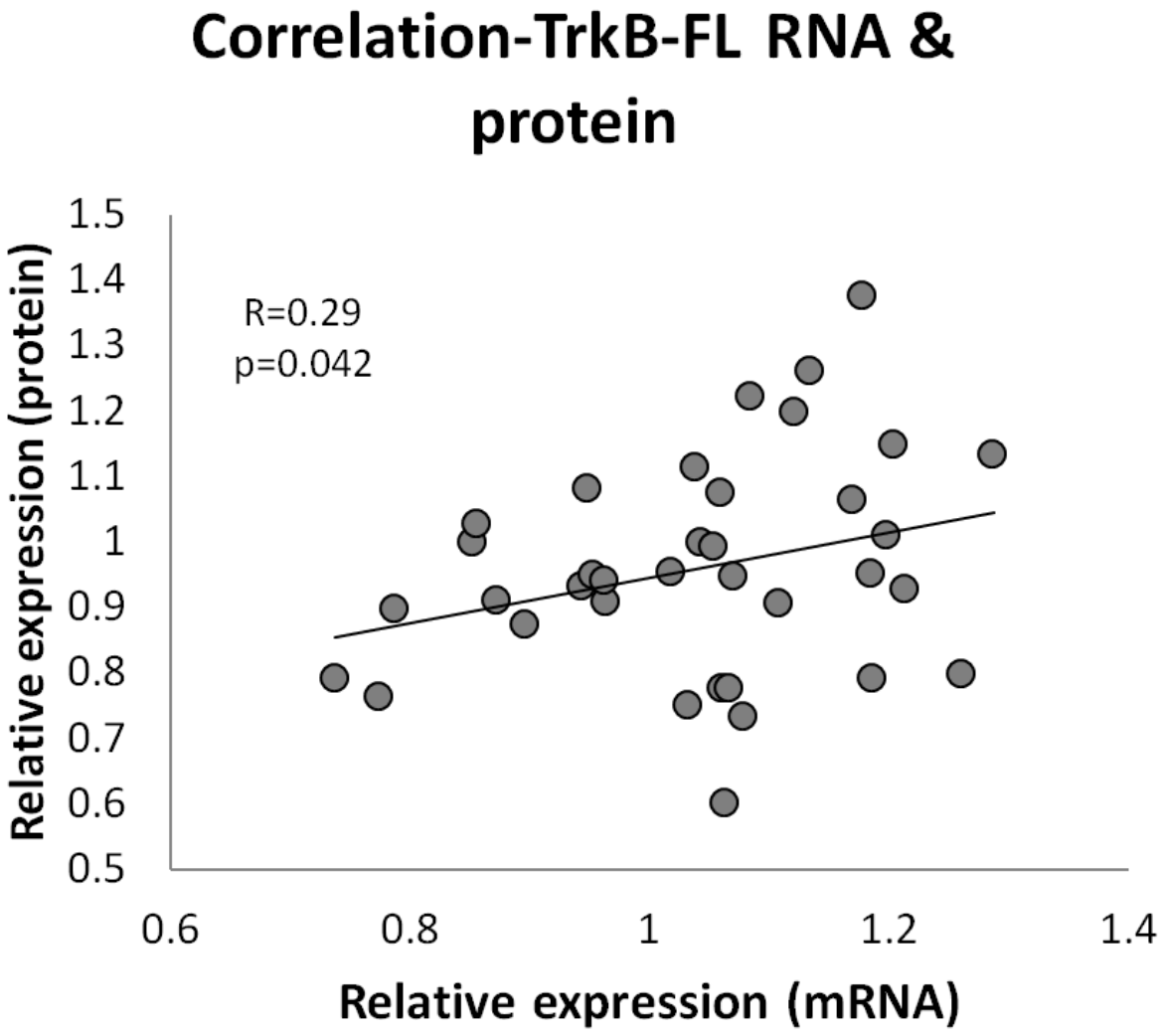


Figure 34. Correlation between TrkB-FL mRNA and protein expression in the mouse brain

Table 12. 7,8-DHF-induced gene expression changes

		Stress effect		7,8-DHF effect	
		% changes	p-value	% changes	p-value
BDNF	CDS	-9.9±3.6%	0.163	+0.7±2.6%	0.439
	EXON1	-9.9±8.8%	0.334	+19.6±10.2%	0.061
	EXON4	-3.4±3.5%	0.455	-6.2±5.8%	0.155
	EXON2	+6.5±8.0%	0.279	+3.9±12.1%	0.476
	EXON6	-16.6±9.2%	0.075	+8.2±10.2%	0.358
	L-3' UTR	-24.9±7.5%	0.029	+6.2±5.1%	0.344
TrkB	TRKB-FL	-1.2±2.9%	0.473	+13.4±2.4%	0.003
	TRKB-T1	+2.2±5.1%	0.438	-4.4±4.1%	0.390
Inhibitory presynaptic-related genes	SST	-4.3±2.3%	0.288	-1.2±2.5%	0.173
	NPY	+9.2±7.9%	0.201	+5.4±3.9%	0.199
	CORT	-14.6±4.5%	0.208	+2.7±5.0%	0.390
	PVALB	-5.6±7.9%	0.459	-11.7±8.6%	0.166
	CCK	+11.0±3.3%	0.045	-22.9±3.4%	1.2E-05
	vGAT1	-4.1±6.4%	0.261	+14.6±6.8%	0.059
	GAD1	-6.6±4.7%	0.168	-5.5±3.2%	0.183
	GAD2	+5.3±4.8%	0.214	+7.4±6.9%	0.180
Inhibitory postsynaptic-related genes	GABRA4	+2.3±3.7%	0.331	-14.4±2.6%	0.002
	GABRA5	+0.4±3.0%	0.410	-8.5±4.4%	0.033
	GABRB3	+4.3±2.5%	0.139	-5.1±1.7%	0.042
Excitatory synapse-related genes	vGLUT1	-15.6±3.5%	0.144	-8.2±4.3%	0.081
	GRIA1	-7.5±3.7%	0.226	-0.9±3.8%	0.413
	GRIN2A	-6.5±2.8%	0.057	+12.1±5.2%	0.046
	GRIN2B	+3.5±4.5%	0.291	-1.4±4.1%	0.444
	DLG4	-15.3±6.2%	0.091	-10.6±4.5%	0.135

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