DIFFERENTIAL IMPACT OF GAD67 SUPPRESSION IN DISTINCT GABA-ERGIC INTERNEURON POPULATIONS: IMPLICATIONS FOR MENTAL ILLNESS

By

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DEDICATION

For all of the great teachers I have had the honor of learning from, both inside and outside of classrooms and laboratories. For the mice who were the brains of this operation. For the patients who will hopefully benefit from research based on these and other experiments. And for my family, without whom this would not have been possible.

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LIST OF ABBREVIATIONS

a.u. ……..arbitrary units

ADHD…….....attention deficit hyperactivity disorder

AIS……..axon initial segment

ALR……..average log ratio

AMPA……..2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid

AMPH……amphetamine

ANOVA……..……...analysis of variance

BAC……..……..…bacterial artificial chromosome

BDNF……..……brain derived neurotrophic factor

BLA……..……basolateral amygdala

CA1……..……Cornu Ammonis area 1

CA3……..……Cornu Ammonis area 3

CB1R…….……cannabinoid receptor 1

CCK……cholecystokinin

CHCA……α cyano 4 hydroxycinnamic acid

CHO……Chinese hamster ovary cell line

CMV……..…cytomegalovirus

CNR1……..cannabinoid receptor 1 gene

CORPH……..…corpus callosum - hippocampus

CORPS……..…corpus callosum - striatum

CTXH……...cortex - hippocampus

CTXS……cortex - striatum

dB…….....decibel

DISC1……..…disrupted in schizophrenia 1

DNA……..…deoxyribonucleic acid

DNMT1……DNA methyl transferase 1

DSM……..…diagnostic and statistical manual

eGFP……...……enhanced green fluorescent protein

f……...…hippocampal fissure

FDA……..…food and drug administration

GABA……..gamma amino butyric acid

GABA-T……GABA-transaminase

GAD1……glutamic acid decarboxylase 1

GAD67……glutamic acid decarboxylase 1 / 67kDa isoform

GAT……...….GABA transporter

gcl…….granule cell layer

GIRKs……G-protein coupled inwardly rectifying potassium channels

GPCRs…….G-protein coupled receptors

h…….....hilus

HEK293……..……human embryonic kidney cell line 293

HIPP……..hippocampus

HYTH…….....hypothalamus

KCC2……potassium-chloride cotransporter 2

kDa……...kilodalton

LC-MS/MS……liquid chromatography – tandem mass spectrometry

LPS……...lipopolysaccharide

mA…….....milli amperes

MALDI-IMS……..matrix-assisted laser desorption ionization mass spectrometry

mCck……mouse cholecystokinin gene

mGad1……..mouse glutamic acid decarboxylase 1 gene

MIA……maternal immune activation

miRNA……micro ribonucleic acid

ml……..…….molecular layer

MNL……Vanderbilt murine neurobehavioral laboratory

mNPY……..……mouse neuropeptide Y gene

mRNA……messenger ribonucleic acid

nAchR……nicotinic acetylcholine receptor

NK1……..……neurokinin 1 receptor

NKCC1……..…sodium-potassium-chloride cotransporter 1

NMDA……N-methyl-D-aspartate

NMDAR……..N-methyl-D-aspartate receptor

NPY……neuropeptide Y

NR2B……...N-methyl D-aspartate receptor subtype 2B

ns……..…….not significant

NVHL……..neonatal ventral hippocampal lesion

PB…….....phosphate buffer

PCP4……Purkinje cell protein 4

PCR……polymerase chain reaction

PEP19……brain-specific polypeptide 19

PFC……...prefrontal cortex

PLP……pyridoxal 5’-phosphate

PPI……prepulse inhibition

PTSD……post-traumatic stress disorder

PV……....parvalbumin

RISC……..RNA induced silencing complex

ROI……..…region of interest

SEP……..……...septum

slm……...stratum lacunosum moleculare

SNRI……...serotonin and norepinephrine reuptake inhibitor

SNPs……..single nucleotide polymorphism

sp……stratum pyramidale

sr…….....stratum radiatum

SSA……succinic semialdehyde

SSRI……serotonin specific reuptake inhibitor

SST……..…somatostatin

STR…….....striatum

SVZ……..…subventricular zone

THAL……....thalamus

TRE……..tet-response element

TrkB…….receptor tyrosine kinase B

tVTA/RMTg……..tail of the ventral tegmental area / rostromedial tegmental area

VIP……...vasointestinal peptide

VTA……ventral tegmental area

CHAPTER 1

INTRODUCTION

"My investigations showed that the functional superiority of the human brain is intimately bound up with the prodigious abundance and unusual wealth of forms of the so-called neurons with the short axons."

- Santiago Ramon y Cajal

Cajal might be right. However, investigations presented here by our lab and others in the nearly 100 years since make it a complicated statement to judge. Did he intend to restrict his effusive comment to humans? If so, could he actually "show" that his cellular investigations translated to functional superiority? At that time, functional comparisons between species were limited to basic sensory and motor function, eating, fighting, and reproducing (Llinas, 2003) so it would be reasonable of him to assign higher functions to the complex human anatomy he saw through his microscope. Nevertheless, legions of researchers from his student Lorente de Nó, who described a multitude of interneuron types in mouse cortex (Lorente de No, 1922 (1992 trans.)), to The Petilla Interneuron Nomenclature Group, who convened in Cajal's hometown in 2008 attempting to classify the "prodigious abundance" of interneuron data from many species (Ascoli et al., 2008), have greatly elaborated the "wealth of forms" of interneurons in rodents. Similar advancements in behavioral science from demonstrations of self-awareness in pigeons (Epstein et al., 1981) to attentional and

affective set-shifting relying on primate-analogous brain regions in mice (Bissonette et al., 2008) to complex mood (Cryan and Holmes, 2005) and social behaviors (Silverman et al., 2010) in mice and rats have revealed that lower animals are capable of much more complex functions than Cajal might have considered. So if we revise his statement based on current knowledge, could the "functional superiority of *any* brain" be linked to the diversity of its interneurons? If so, what opportunities do the wealth of interneuron forms and behavioral complexities of mice present for modeling complex brain dysfunction found in human neuropsychiatric illness?

GABA-ergic inhibition in the brain

Neural action potentials are one of the most basic units of brain function. These electrical pulses are generated by moving ions across a membrane down their electrochemical gradient, typically through selectively permeable ion channels. Most of these action potentials are the results of cell signaling events. There are three general types of neural transmission in the brain that determine whether a given neuron will fire an action potential: excitatory, inhibitory, and modulatory. Excitatory transmission induces post-synaptic neurons to fire by opening ion channels that allow sodium and calcium (in some cases) to enter the cell. Movement of enough of these ions into the cell depolarizes the membrane and fires an action potential. Excitation in the brain chiefly involves the neurotransmitter glutamate through its ligand-gated ion channels. Conversely, inhibitory transmission prevents neurons from firing action potentials by hyperpolarizing the cell far below its threshold for firing action potentials, typically via the influx of chloride through channels opened by the neurotransmitter gamma-aminobutyric

acid, or GABA. Finally, modulatory transmission changes the probability that the cell will reach its firing threshold in the context of cell signaling events by altering electrochemical gradients in other ways such as modulating G-protein coupled inwardly rectifying potassium channels (GIRKs) or calcium channels. In this manner, neuromodulators such as dopamine, serotonin, and norepinephrine can influence the firing probability and/or rate of individual neurons. Research described here focuses on the inhibitory system and as such, we will assume a GABA-centric point-of-view.

GABA is produced exclusively from glutamate by glutamic acid decarboxylase (GAD). There are two isoforms of this enzyme that produce GABA in the brain. GAD67, the 67 kDa isoform, predominately localizes to cell bodies and dendrites while GAD65, the 65 kDa isoform predominately localizes to the axon terminals (Martin and Rimvall, 1993). These enzymes are encoded by two separate genes, *Gad1* and *Gad2* respectively, which are regulated independently (Erlander et al., 1991; Bu et al., 1992). Both enzymes require binding of a cofactor, pyridoxal 5'-phosphate (PLP), to be activated; however one of the major differences between the two isoforms is that GAD67 is nearly saturated with PLP, while only about 50% of the GAD65 in the brain is activated as a holoenzyme (Kaufman et al., 1991). This discrepancy ostensibly represents having one constitutively active form, GAD67, and a second dynamically-regulated isoform, GAD65 (Erlander et al., 1991; Feldblum et al., 1993; Esclapez et al., 1994). Dynamic binding of PLP to GAD (presumably GAD65 in this case) to activate the holoenzyme complex occurs in the presence of glutamate, with decreases of ATP concentrations, and/or with increases of inorganic phosphate concentrations which are

each consistent with augmented GABA production in response to increased neural activity (Martin and Rimvall, 1993). This phenomenon may explain observed increases in local GABA production resulting from excess glutamate spillover in the hippocampus (Stafford et al., 2010). The relative distribution and activation of these two isoforms promotes the interpretation that GAD67 supplies the bulk of cellular GABA for tonic inhibitory processes, cellular interactions other than those at axon terminals (such as dendrodendritic synapses), and cellular metabolism through the GABA shunt of the Kreb's cycle while GAD65 maintains excitatory/inhibitory balance at the axon terminal synapse (Erlander et al., 1991; Kaufman et al., 1991; Feldblum et al., 1993; Martin and Rimvall, 1993; Esclapez et al., 1994; Soghomonian and Martin, 1998). This view is supported by studies indicating that GAD67 loss causes a near complete reduction of brain GABA content and is not compatible with life (Asada et al., 1997) while GAD65 loss does not alter brain GABA content nor does it induce any other phenotypes except for an increased seizure susceptibility (Asada et al., 1996). Importantly, these seizures did not occur at baseline, but were induced by two separate classes of convulsant drugs that increase neuronal activity using different mechanisms (Asada et al., 1996). Inducing seizures in this manner shows that the dysfunction in GAD65-/- mice is caused by an inability to respond to the increase in neural activity rather than a general loss of inhibition. In a more recent study, conditional GAD67 loss in individual interneurons effectively eliminated the GABA content of those cells despite normal GAD65 expression (Chattopadhyaya et al., 2007).

Even more interesting is that these two isoforms appear to be differentially expressed in distinct interneuronal cell populations (Fish et al., 2011) that have different physiological properties coinciding nicely with the presumed roles of the GAD isoform they preferentially express. During a cell signaling event, GABA is released initially from vesicles like many neurotransmitters, but is followed by non-vesicular release likely through reverse action of the GABA transporter (GAT) (Soghomonian and Martin, 1998). Adding functional importance to the GAD65 and GAD67 distinction, it has been proposed that GAD65 is responsible for producing the vesicular pool of GABA and mediating its release during phasic firing while GAD67 provides cytosolic GABA that is released non-vesicularly during tonic firing (Esclapez et al., 1994; Soghomonian and Martin, 1998). Interestingly, GAD65 mRNA appears to be expressed highest in areas that exhibit phasic activity such as visual and neuroendocrine systems (Feldblum et al., 1993) while cells capable of both tonic and phasic firing modes, such as those in the thalamic reticular nucleus, express both isoforms (Contreras et al., 1992; Feldblum et al., 1993). At least one study combined electrophysiology and single cell PCR in rats to show that two cells with identical morphology and molecular expression, except for the presence of both GAD isoforms, differed in their firing patterns; the cell with both GADs fired consistent bursts in response to stimulation while the cell expressing only GAD65 fired few, irregular spikes (Ferezou et al., 2002). Taken together, these studies clearly show that the two GAD isoforms have different functions contributing to the diversity of GABA-ergic neurons and that GAD67 activity is required for normal GABA content in the brain.

GABA-ergic interneurons, postsynaptic cells, and glial cells (astrocytes) each have important roles at GABA-ergic synapses. All three of these cell types, and any excitatory synapses in the immediate area, participate in the glutamate/GABA-glutamine cycle to produce glutamate and GABA (Bak et al., 2006). The generation of GABA from glutamate by GAD isoforms was discussed in the previous section. When GABA is released into the synapse, it activates $GABA_A$ receptor complexes on the postsynaptic membrane. These are heteromeric ligand-gated chloride channels that typically hyperpolarize and inhibit the postsynaptic cell when activated (Macdonald and Olsen, 1994), but can also be excitatory during early development due to altered chloride gradients that will be discussed in the next section (Ben-Ari, 2002). Alternatively, GABA can activate $GABA_B$ G-protein coupled receptors (GPCRs) which act as inhibitory modulators on the postsynaptic membrane by opening GIRKs, closing calcium channels, and inhibiting adenylyl cyclase or as negative feedback sensors on the presynaptic membrane by similar mechanisms (Gassmann and Bettler, 2012). GABA that does not bind to these receptor types is taken up by GABA transporters (GAT) on either presynaptic cells where it can be recycled or on the astrocytes where it is metabolized by GABA-transaminase (GABA-T) to form succinic semialdehyde (SSA) (Bak et al., 2006). SSA is then converted to succinate and joins the Kreb's cycle where it is eventually converted to glutamate. Glutamate from this source or from astroglial uptake from nearby excitatory synapses is then converted by glutamine synthase into glutamine which is then exported back to the GABA-ergic interneuron (Bak et al., 2006). Glutamine is converted back into glutamate by glutaminase which can then be converted back into GABA by GAD as the cycle completes itself (Bak et al., 2006).

When all components of the cycle are working together, glutamate and GABA concentrations can regulate each other to maintain the excitatory/inhibitory balance necessary to prevent seizure activity and maximize the signal-to-noise ratio of neural communications.

Development of the Cortical GABA-ergic system

Perhaps not surprisingly, the interplay between excitation and inhibition requires careful coordination of these two systems from early in brain development. GABA-ergic interneurons and excitatory cortical pyramidal cells are generated in distant areas of the developing brain, migrate into their final positions in the cortex, and establish synaptic contacts during the course of development (Anderson et al., 1999). In rodents, where most of the data on cortical development has been collected for obvious ethical and practical reasons, nearly all GABA-ergic cells originate in the ganglionic eminences of the ventral subpallium, while glutamatergic cells originate in the subventricular zone (SVZ) of the dorsal telencephalon. Primates have identical mechanisms, but also have an additional source of GABA-ergic cells originating in the SVZ (Letinic et al., 2002; Petanjek et al., 2009b; Petanjek et al., 2009a).

Early in development, Cajal-Retzius cells migrate tangentially from multiple sources (Bielle et al., 2005) into the developing cortical preplate. Once established, they direct the radial migration of glutamatergic pyramidal neurons along radial glia by secreting reelin (Tissir and Goffinet, 2003) and coordinate the organization and positioning of radial glia and migrating pyramidal cells with cell adhesion molecules

cadherin and nectin (Gil-Sanz et al., 2013). Reelin signals migrating cortical cells to layer in an “inside-out” fashion with “younger” pyramidal neurons stacking on top of “older” cells until all of the layers of the cortex have developed (Tissir and Goffinet, 2003); however this is not the case for laminar determination and final positioning of GABA-ergic cells (Pla et al., 2006). GABA-ergic interneurons destined for the cortex migrate from the ganglionic eminences, avoid the striatum via semaphorin 3A- and 3F-mediated chemorepulsion (Marin et al., 2001), and settle into their final positions after the radial migration of pyramidal cells is complete (Pla et al., 2006). The overwhelming diversity of interneuron cell types (discussed in the next section) is predestined as different cell types originate in restricted portions of the medial, lateral, and caudal ganglionic eminences based on the combinatorial expression of different transcription factors (Wonders and Anderson, 2006; Flames et al., 2007). The specificity of their integration into cortical laminae is also predestined as different populations tend to cluster together in specific layers (Ciceri et al., 2013), likely via cell type specific expression of cell adhesion molecules including neuregulin and ErbB4 (Fazzari et al., 2010).

GABA itself also plays a role in the development of the cortex. GABA’s inhibitory effects on postsynaptic neurons depend on the equilibrium potential for chloride being lower than the resting membrane potential, resulting in hyperpolarization when $GABA_A$ receptor chloride channels are opened. Up until birth, this equilibrium is reversed because the sodium-potassium-chloride cotransporters (NKCC1) are expressed while the postassium-chloride exporter (KCC2) is not, the net effect of which raises

intracellular chloride concentrations (Ben-Ari, 2002). KCC2 expression typically increases around P0 and the chloride gradient switches; however, this may be dynamically regulated and there is evidence that local chloride gradients may be different at certain interneuron synapses and in disease states (Arion and Lewis, 2011; Hyde et al., 2011). GABA's excitatory role is important for the development of the cortex. GABA is released through non-vesicular release prior to the development of synapses (Manent et al., 2005; Cellot and Cherubini, 2013). Interestingly, this release mechanism correlates with the expression of the two GAD isoforms as GAD65, thought to provide vesicular GABA, is not expressed until after the gradient switch and development of synapses (Kiser et al., 1998). Tonic GABA stimulates and guides the migration of new neurons in a receptor type-dependent manner (Owens and Kriegstein, 2002). $GABA_A$ receptors are expressed on neural progenitors in the proliferative zone and GABA signaling inhibits DNA synthesis, promoting cell cycle exit before migration (LoTurco et al., 1995). $GABA_B$ and $GABA_C$ (an ion channel that is functionally identical to $GABA_A$ with different pharmacology) stimulation maintains migration through the cortical plate (Behar et al., 2001) while $GABA_A$ receptor stimulation finally provides a signal to stop migrating (Behar et al., 2000). This entire process is highly orchestrated by changing expression of GABA receptors as cells migrate from the proliferative zone to their final place in the cortical plate (Maric et al., 2001). Once in place, continued $GABA_A$ receptor stimulation signals cells to extend neurites (Barbin et al., 1993; Marty et al., 1996) and form excitatory pre-synaptic contacts to integrate with the developing cortical circuitry (Wang and Kriegstein, 2008). This GABA-mediated, activity-dependent process has important results for the organization of cortical circuits as it provides the

framework that sets cortical column spacing (Hensch, 2005). Somewhat unexpectedly, these same processes continue to regulate synaptic integration of cells in regions such as the dentate gyrus that continue to produce new neurons into adulthood (Ge et al., 2006).

Any mechanism of normal development provides an opportunity for abnormal development. Schizophrenia is marked by a reduction of reelin (Impagnatiello et al., 1998; Guidotti et al., 2000; Fatemi et al., 2005) and impaired neuregulin/ErbB4 (Harrison and Weinberger, 2005; Neddens et al., 2011) signaling that may contribute to the cortical migration and synaptic deficiencies seen in the disorder. Reelin deficiencies are also prominent in animal models of maternal immune activation (MIA; to be discussed) which mimics a risk factor for neurodevelopmental disorders (Meyer et al., 2008; Harvey and Boksa, 2012). Reelin-producing Cajal-Retzius cells express the cannabinoid receptor CB1 (Zurolo et al., 2010) which has become prominent in the neuropsychiatric literature (Andreasson et al., 1987; Henquet et al., 2008; Ferretjans et al., 2012). Dopamine D1 receptor stimulation promotes interneuron migration, while D2 stimulation slows it (Crandall et al., 2007). Dopamine receptor modulating drugs such as cocaine can affect this process (Crandall et al., 2004; Thompson et al., 2009; McCarthy and Bhide, 2012). Adenosine receptor antagonists such as caffeine similarly delay migration (Kabir et al., 2013; Silva et al., 2013). GABA system-targeting drugs such as anticonvulsants (Manent et al., 2007), anxiolytics (Haas et al., 2013), or alcohol (Cuzon et al., 2008; Thompson et al., 2009; Aronne et al., 2011) also alter cortical migration and development. Some of these effects appear to be cell type specific.

Neuregulin/ErbB4 signaling selectively affects development of GABA-ergic interneurons that express parvalbumin, cholecystokinin, calretinin, but not those that express calbindin in mice, rats, monkeys, and humans (Neddens et al., 2011). Parvalbumin+ interneurons are also selectively disrupted by exogenous GABA potentiation (Levav-Rabkin et al., 2010; Haas et al., 2013) or cocaine exposure (McCarthy and Bhide, 2012) during development while adenosine receptor agents and caffeine may target somatostatin+ cells (Silva et al., 2013). Finally, conditional GAD67 suppression in perisomatic-targeting basket cells during adolescence decreases axonal branching (Chattopadhyaya et al., 2007). Together, these studies highlight the importance of GABA-ergic system function for the proper migration and integration of inhibitory and excitatory cell types in the cortex and hippocampus as well as the sensitivity of this system to genetic and pharmacological insults.

There are some important caveats that must be acknowledged regarding the comparison of developing GABA-ergic circuitry in humans and mice and the methods used to study interneuron migration in mice. First, the proportion of interneurons to pyramidal cells in the human cortex is much greater than in the mouse (Jones, 2009). This discrepancy also raises the possibility that interneuron deficits in humans could have more robust effects on the brain and behavior than what is seen in rodent models. Second, up to 65% of human cortical interneurons arise from an alternate source of progenitors in the neocortical ventricular and subventricular zones that may have developed during primate evolution (Letinic et al., 2002; Petanjek et al., 2009b; Petanjek et al., 2009a). Therefore, some of the findings in rodents may be restricted to a limited

population of human neurons. There may also be deficits in humans arising from specific disruptions in this secondary interneuronal source that are not able to be studied in lower animals. However, since the gross majority of cells from this source express calretinin (Petanjek et al., 2009a) which appear to be spared in neuropsychiatric illnesses such as schizophrenia (Hashimoto et al., 2003b), rodent models assessing the differentiation, migration, and maturation of GABA-ergic interneurons from the ganglionic eminences remain very informative about the neurobiological processes underlying the developmental aspects of mental illnesses. Third, there are important caveats regarding methodologies used by many labs to generate data regarding interneuron development in mice that will be discussed in this section. GAD67-GFP mice were created by Tamamaki and colleagues to visualize GABA-ergic cells in the brain by knocking the eGFP gene into the transcriptional start site of the Gad1 gene (Tamamaki et al., 2003). However, Tamamaki, et al. downplayed the fact that GAD67-GFP mice have significantly reduced levels of GABA during development. They argued that these differences are unimportant since the GABA reduction was not statistically significant at 7 weeks of age ($p=0.09$) and suggested that a developmental compensatory mechanism, namely GAD65 expression, was able to overcome the differences (Tamamaki et al., 2003). These claims are difficult to evaluate since GABA content was measured in “samples from mouse brain” in their study (Tamamaki et al., 2003) and it is possible that regional differences across development influenced their results; nevertheless, studies discussed in the previous section argue against the ability of GAD65 to compensate for GAD67 reduction. Regardless of the accuracy of their explanation, the fact remains that GABA reduction

during early development of GAD67-GFP mice is highly likely to affect interneuron migration and cortical development. Since many groups have taken advantage of GABA-ergic neuron fluorescence in GAD67-GFP mice to track interneuron migration, data from such studies should be interpreted with the caveat that reduced GABA concentrations may have influenced the results.

Interneuron diversity

GABA-ergic cell types are so diverse that creating a nomenclature for their defining characteristics continues to be a tedious task (DeFelipe et al., 2013). However, classification of these diverse inhibitory cell types is necessary to describe the disparate functions performed by different classes and important because different types of these cells appear to be dysfunctional in neuropsychiatric disorders (Lewis et al., 2005; Marin, 2012). Categories can be broadly defined based on morphological, molecular, and physiological properties (Markram et al., 2004; Ascoli et al., 2008; DeFelipe et al., 2013) with the caveats that no single classification scheme is sufficient and integrating the growing amount of information is difficult in practice (DeFelipe et al., 2013).

Perhaps the most distinguishing feature of any cell type is its morphology. Laminar distribution, columnar distribution, soma size, dendritic arborization, axonal branching, orientation, and synaptic connectivity are some of the aspects of a cell's appearance that identify it as a member of a particular class (Markram et al., 2004; Ascoli et al., 2008; DeFelipe et al., 2013). Basket, bipolar, bitufted, chandelier, double bouquet, Martinotti and neurogliaform are terms that describe the major interneuron cell

types (Markram et al., 2004). Basket cells, which compose about half of all interneurons (Markram et al., 2004), can be further characterized as “large basket cells” that have larger soma and diffuse axonal arborization through multiple cortical lamina, “small basket cells” with smaller cell soma and arborization typically restricted to a local area, or “nest basket cells,” which are a similar size as “small basket cells” but have less axonal branching and fewer synaptic contacts (Markram et al., 2004). Bipolar, bitufted, and double bouquet cells are very similar in their size, distribution, synaptic contacts and molecular content, but vary in the branching and spread of their dendritic arbors (Markram et al., 2004; Ascoli et al., 2008; DeFelipe et al., 2013). They are typically small with oval-shaped cell bodies, and synapse onto dendrites of pyramidal cells in multiple layers. Their dendritic arbors span multiple layers (interlaminar) and vary based on the number and density of their dendrites with bipolar cells having one or very few tightly-packed dendrites and a vertically oriented, straight axon (Markram et al., 2004). Double-bouquet cells and bitufted cells are very similar with multiple, more diffuse branches of their axonal plexuses and dendritic trees, however the bitufted cells exhibit a wider branching of their axonal bundle that may cross over into other cortical columns (Markram et al., 2004; DeFelipe et al., 2013). Chandelier cells have broad axonal branching and distribution resembling the hanging structure for which they are named. These cells are easier to classify based on morphology alone but may have multiple functional modes that compound their diversity (to be discussed). Martinotti cells project from lower cortical lamina up to layer 1 to inhibit distal dendrites of pyramidal cells (Markram et al., 2004; Ascoli et al., 2008; Karagiannis et al., 2009; DeFelipe et al., 2013). Neurogliaform cells innervate dense, local regions and provide

tonic inhibition in an extra-synaptic manner (to be discussed), but also form synaptic networks with each other (Price et al., 2005) likely to coordinate previously-mentioned tonic processes. Since the cortex has segregated inputs and outputs into various layers and organized cortical information processing into modular column units, interlaminar (between layers of cortex) and intercolumnar (between cortical columns) connections may be especially important to coordinate and unify brain function (Lubke and Feldmeyer, 2007; DeFelipe et al., 2013). While many of these cells have been studied most extensively in the cortex, they are also found in other brain regions such as the hippocampus where they take similar forms and make analogous synaptic or extra-synaptic connections (Klausberger and Somogyi, 2008). These morphological categories can be further divided with additional information including selective molecular marker expression.

Research presented here relies on the organization of various interneuron cell types based on their molecular content since non-overlapping populations express various calcium-binding proteins or neuropeptides (Figure 1); the focus of this work will be on interneurons that express parvalbumin (PV), cholecystokinin, or neuropeptide Y (Lewis et al., 2005) which will be described here in detail. Identifying interneurons based on multiple parameters and determining their control of neural and behavioral processes yield important information directing the development of novel therapeutics for these disorders. Multiple methods can be used to determine the molecular content of interneuron cell types including single cell PCR, which measures the gene expression signature of selected cells, multiple-label immunohistochemistry, which measures

overlapping protein localization, or pharmacology, which measures the response of a cell to drugs that act on particular receptors to determine if they are present on or in the cell.

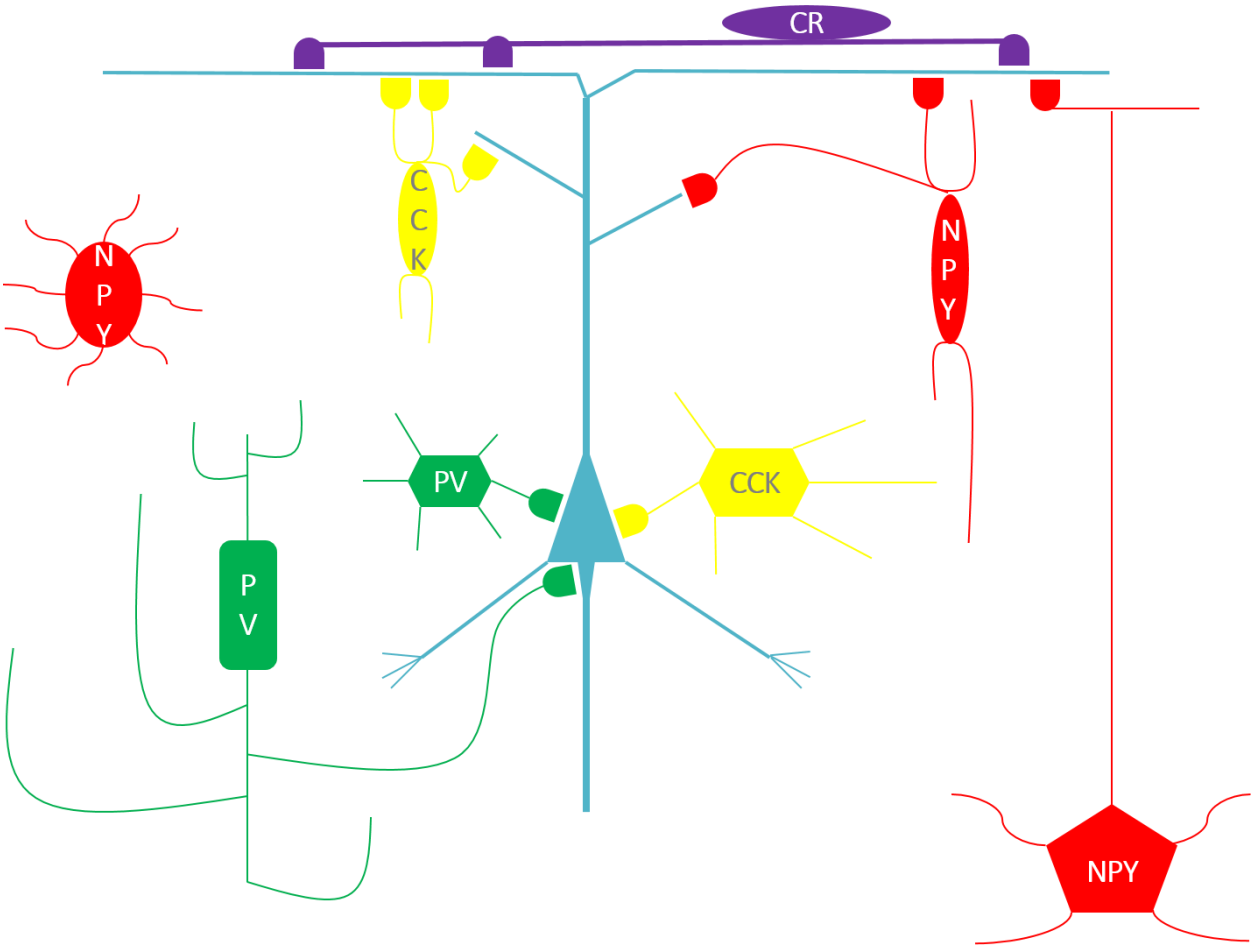


Figure 1: GABA-ergic cell types differentially express particular calcium-binding proteins or neuropeptides. Interneurons expressing parvalbumin (PV, green) regulate pyramidal cell soma and axon initial segments (AIS). Those expressing cholecystokinin (CCK, yellow) regulate pyramidal cell soma and distal dendrites. Cortical cells that express neuropeptide Y (NPY, red) regulate pyramidal cell distal dendrites and maintain tonic inhibition of a local area through extrasynaptic volume transmission. Cells that express calretinin (CR, purple) inhibit distal dendrites in layer 1. These cells do not appear to have altered GABA-ergic system components in neuropsychiatric disorders.

The calcium-binding protein parvalbumin is expressed by large basket cells and chandelier cells in multiple brain regions. PV+ basket cells form perisomatic synaptic contacts onto pyramidal cells (Freund and Katona, 2007) and also inhibit other dendrite-targeting interneuron populations (Lovett-Barron et al., 2012). PV+ chandelier cells synapse on axon initial segments to regulate the output of cortical pyramidal cells across multiple areas (Markram et al., 2004; Woodruff et al., 2010) and control gamma-oscillations within and between regions (to be discussed), which are believed to maintain working memory (Lewis et al., 2005). Their "fast-spiking" activity is determined by their heavy glutamatergic innervation and by their expression of P/Q-type voltage-gated calcium channels which cluster at synaptic active zones to support rapid vesicular release kinetics (Hefft and Jonas, 2005). Of course, parvalbumin itself may be involved in this process as a calcium buffer. Interestingly, the function of these cells has been described as "enigmatic" due to the possibility that they may also provide excitatory input to pyramidal cells in the cortex despite their GABA-ergic identities due to potential differences in local electrochemical gradients in normal and diseased brain (Woodruff et al., 2010; Arion and Lewis, 2011; Hyde et al., 2011). This possibility arises because the distribution of the chloride transporters that maintains these gradients may be different at chandelier cell synapses (Woodruff et al., 2010). Approximately 65% of PV+ interneurons express dopamine receptor D4 while nearly 20% express D2 (de Almeida and Mengod, 2010) which may be important for the regulation of PV+ GABA-ergic circuits in the amygdala (Woodruff and Sah, 2007; Bienvenu et al., 2012) by dopamine and for behaviors associated with dopamine signaling in the amygdala (Cao and

Rodgers, 1997; Royer et al., 1999; Marowsky et al., 2005; Pape, 2005; Likhtik et al., 2008; de la Mora et al., 2010).

In contrast, CCK+ basket cells regulate the activity of pyramidal cells directly via perisomatic synaptic transmission and indirectly via modulation of PV+ basket cells primarily in limbic and frontal circuits (Geola et al., 1981; Hornung et al., 1992; Meziane et al., 1997; Freund, 2003; Foldy et al., 2007; Karson et al., 2009). This distribution is remarkably similar in mice (Meziane et al., 1997), nonhuman primates (Oeth and Lewis, 1990), and humans (Geola et al., 1981; Hornung et al., 1992). Despite being morphologically similar to PV+ basket cells, this cell type is molecularly unique in several ways. In contrast to "fast-spiking" PV+ cells, CCK+ basket cells' slower "accommodating" activity is determined by their relatively diminished glutamatergic innervation and by their expression of N-type voltage-gated calcium channels which are dispersed throughout presynaptic terminals further away from active zones which leads to less precise regulation of vesicular release kinetics (Hefft and Jonas, 2005). CCK+ cells in the hippocampus selectively express alpha7 nicotinic receptors (nAchR) (Porter et al., 1999) and are thought to be necessary for the cognition-boosting effects of acetylcholine (Nagode et al., 2011). They are also the only interneuron type that expresses the serotonin type 3 receptor (Morales and Bloom, 1997) which positions them to integrate neuromodulatory information with fine-tuned network activity in multiple brain regions (Freund, 2003; Varga et al., 2009). Furthermore, CCK is expressed by five electrophysiologically distinct types of interneurons in the lateral amygdala (Sosulina et al., 2010) which are among those that modulate anxiety-like

behavior (Truitt et al., 2009). Finally, and perhaps most famously, CCK+ interneurons express the cannabinoid receptor CB1 (Katona et al., 1999; Marsicano and Lutz, 1999; Tsou et al., 1999; Katona et al., 2000; McDonald and Mascagni, 2001; Hill et al., 2007; Morales et al., 2008; Eggan et al., 2010) which has been shown to regulate both anxiety (Patel et al., 2005) and memory (Castellano et al., 1997; Castellano et al., 2003; Crombag et al., 2010; Tan et al., 2010). Combined, the molecular signature of CCK+ interneurons supports their role in integrating processes that are important for global brain function and behavior.

NPY+ neurogliaform interneurons release GABA via volume transmission (Olah et al., 2009; Manko et al., 2012) in diffuse cortical regions, the striatum, hippocampus, amygdala, and hypothalamus and play critical roles in maintaining tonic inhibition (Koos and Tepper, 1999; Markram et al., 2004; Tepper and Bolam, 2004; Karagiannis et al., 2009; Partridge et al., 2009; Truitt et al., 2009) and make synaptic contacts onto each other, likely to regulate tonic inhibition throughout a region (Price et al., 2005). The receptors expressed on these cells suggest that they are sensitive to endocrine changes. They express both mu (Krook-Magnuson et al., 2011) and delta opioid receptors, which are substantially reduced on NPY+ cells in the hippocampus of female rats compared to males and even further reduced during high-estrogen phases of estrous (Williams et al., 2011). Furthermore, NPY+ interneuron signaling involves the neurosteroid-sensitive GABA receptor delta subunit which implicates NPY+ neurogliaform cell function in response to stress and may be a component in the biology of gender differences in neuropsychiatric disorders (Lambert et al., 2003; Hosie et al.,

2006; Olah et al., 2009). Along with CCK+ basket cells, they are among those that regulate anxiety in the amygdala (Truitt et al., 2009). Finally, NPY+ interneurons in the striatum, compared to PV+ interneurons in the striatum, are directly responsible for the cholinergic modulation of striatal circuitry (Luo et al., 2013) which is critically important for regulating reward pathways (English et al., 2012). Their positioning in striatal circuitry (Ibanez-Sandoval et al., 2011) combined with our data implicating them in the control of amphetamine sensitivity may explain the mechanism of α4β2 nicotinic receptor drugs (Lippiello et al., 2008) and M4 muscarinic drugs (Brady et al., 2008) that modulate these behaviors with relevance for neuropsychiatric treatment.

While morphological and molecular markers can identify GABA-ergic cell types and differences between their receptor and channel expression can provide clues about the ways they might participate in systems modulating neural processes and behavior, they do not tell us what the cells do. In other words, interneuron function is not defined by firing rates or responses to drugs. What an interneuron cell type does is defined by its integration into brain circuitry and its role in shaping the output of that circuitry.

Interneuron Function

Classification of interneuron subpopulations is critical for all other studies of inhibition because diverse subtypes of these cells regulate relatively homogenous pyramidal cell populations in different brain regions and in different ways (Kawaguchi et al., 1997; Jinno and Kosaka, 2003; Tepper and Bolam, 2004; Lewis et al., 2005; Houser, 2007; Sosulina et al., 2010; Kubota et al., 2011). In the hippocampus, for

example, twenty one types of interneurons regulate the function of only three types of pyramidal cells (Klausberger and Somogyi, 2008). Therefore, it is likely that the diversity of interneuron form and function plays a direct role in generating diverse brain functions and behavior. Generally speaking, interneuron subpopulations modulate brain function at three levels: synchronizing neural populations, gating the activity of specific local circuits, and controlling the activity of neuromodulatory centers.

First, distinct interneuron classes have different physiological properties and maintain different aspects of network synchrony. Oscillations in the slow delta (0.5–3 Hz) and theta (3–8 Hz) ranges and fast gamma (30–90 Hz) and ultrafast (as high as 500 Hz) ranges maintain the signal-to-noise ratios and precise timing of neural communications that are critical for supporting cognition and other behavioral processes (Freund, 2003; Buzsaki and Draguhn, 2004; Bartos et al., 2007; Lewis et al., 2008). Synchronized network oscillations within and between brain regions are generated and maintained by different classes of interneurons (Whittington and Traub, 2003; Bartos et al., 2007; Freund and Katona, 2007; Gonzalez-Burgos and Lewis, 2008, 2012). For example, frequency-dependent burst activity of single pyramidal cells is able to activate and synchronize networks of nearby interneurons which then suppress surrounding pyramidal cells (Marshall et al., 2002). This process selects and propagates the original signal while suppressing noise from surrounding cells. An interesting interneuron population in this context is comprised of the perisomatic-targeting basket cells. These cells are heavily interconnected and coordinated through electrical coupling (Hestrin and Galarreta, 2005) and their oscillatory activity in the gamma and theta ranges is

necessary for maintaining network activity and behavior (Lewis et al., 2005; Fuchs et al., 2007; Gonzalez-Burgos and Lewis, 2008). There are two subtypes of basket cells that regulate each other (Karson et al., 2009) in addition to their pyramidal cell perisomatic contacts (Freund, 2003; Freund and Katona, 2007). The distinct physiological properties of PV+ and CCK+ basket cells are thought to maintain different aspects of input selection and integration as fast-spiking PV+ cells maintain the synchrony of the network and determine selectivity while accommodating CCK+ cells detect temporal binding and integrate the activity of the network (Freund, 2003; Hefft and Jonas, 2005; Freund and Katona, 2007). These functions are underscored by distinct synaptic contacts and receptor expression. PV+ basket cells are consistently activated by robust glutamatergic drive while CCK+ basket cells are activated by glutamatergic synapses that are susceptible to short-term depression (mediated by cannabinoid receptor 1 (CB1)) and are also modulated by serotonin and acetylcholine (Freund and Katona, 2007). In this manner, different interneuron classes, such as PV+ and CCK+ basket cells, distinguish signal from noise and integrate multiple signals in specific local circuits (Hefft and Jonas, 2005; Freund and Katona, 2007).

Interneuron populations also function to synchronize activity between brain regions. For example, the amygdala and hippocampus are heavily interconnected (Pitkanen et al., 2000). Homogenous subnetworks of NPY+ neurogliaform (Price et al., 2005) and PV+ basket (Klausberger et al., 2003) cells in the hippocampus separately participate in theta and gamma oscillations as well (Capogna, 2011). Recent studies found that NPY+ neurogliaform and calbindin+ dendrite-targeting interneurons

(Bienvenu et al., 2012; Manko et al., 2012), but not PV+ basket calls (Bienvenu et al., 2012) in the basolateral amygdala were phase-locked with hippocampal theta oscillations, leading to a transient reduction of inhibitory signaling in the amygdala (Manko et al., 2012) which would likely lead to specific alterations in behavior (discussed in the next section). These hippocampal theta oscillations also entrain the activity of pyramidal cells in the medial frontal cortex in an interaction that is functionally critical for working memory performance (Gordon, 2011). In this manner, particular classes of interneurons, such as NPY+ or calbindin+ cells in the amygdala or hippocampus or PV+ cells in the hippocampus or cortex function across networks and across brain regions to support a broad spectrum of behaviors from working memory to anxiety to social behavior (Herry et al., 2008).

Second, these classes are positioned to gate the flow of information within and between specific regional and subregional circuits. For example, the amygdala processes emotionally salient stimuli and relays this information to other regions to generate appropriate behavioral responses (Cardinal et al., 2002; Herry et al., 2008; Seymour and Dolan, 2008; Morrison and Salzman, 2010). It is composed of developmentally and functionally distinct subregions (basolateral, centromedial, central, etc.) that are connected by well-defined circuits (Sah and Westbrook, 2008). In addition to the cells that regulate network synchrony mentioned above, interneurons in the amygdala also act as critical switches within these circuits (Royer et al., 1999; Pape, 2005; Truitt et al., 2007; Likhtik et al., 2008; Ehrlich et al., 2009; Truitt et al., 2009) and are under the control of prefrontal cortical input and dopamine modulation (Rosenkranz

and Grace, 2001; Marowsky et al., 2005; Pape, 2005; de la Mora et al., 2010). Conversely, glutamatergic projections from the amygdala to the prefrontal cortex synapse on layer II/III interneurons providing reciprocal feed-forward and feed-back gating of information between regions (Saddoris et al., 2005; Cunningham et al., 2008; Benes, 2009) which is critical for everything from fear to addiction (Maren and Quirk, 2004; Peters et al., 2009; Koob and Volkow, 2010).

Localized interneuron function is also found in the hippocampus where twenty one types of interneurons regulate the function of only three types of pyramidal cells (Klausberger and Somogyi, 2008). These different interneuron types are distributed in different cellular layers and subregions of the hippocampus (Jinno and Kosaka, 2003; Klausberger and Somogyi, 2008) and dentate gyrus (Hefft and Jonas, 2005; Houser, 2007). It has been shown that the CA1, CA3, and dentate gyrus are responsible for different aspects of spatial representation, pattern separation, pattern completion, novelty detection, and short- or intermediate-term memory functions of the hippocampus (Kesner et al., 2004) and that various hippocampal functions map along a dorsoventral or anteroposterior axis in animals and humans (Bannerman et al., 2004; Fanselow and Dong, 2010; Poppenk et al., 2013). Several aspects of local hippocampal function are dependent on specific interneuron classes. The function of the dentate gyrus is dependent on the precise timing and co-regulation of PV+ and CCK+ cells discussed previously (Hefft and Jonas, 2005). These CCK+ cells are concentrated in the ventral hippocampus (Jinno and Kosaka, 2003) which is thought to be more involved in regulating emotion than memory (Bannerman et al., 2004) and fast

serotonergic modulation (Varga et al., 2009) of CCK+ interneurons in this subregion is likely a key component of that distinction (Morales and Bloom, 1997; Ferezou et al., 2002). Likewise, classes of striatal interneurons, particularly those expressing PV or NPY, regulate local circuits in a cell type-specific manner and directly influence striatal projection neurons and reward circuitry (Kubota and Kawaguchi, 1994; Kawaguchi et al., 1997; Koos and Tepper, 1999; Wilson, 2007; Tepper et al., 2010; Gerfen and Surmeier, 2011; English et al., 2012). In this manner, GABA-ergic interneurons function to regulate local circuitry and projections that are critical for normal behavior and are dysfunctional in mood disorders, PTSD, substance abuse, autism and schizophrenia (Truitt et al., 2007; Sah and Westbrook, 2008; Benes, 2009; Peters et al., 2009; Koob and Volkow, 2010; Mohler, 2012).

Third, different classes of interneurons regulate the release of neuromodulators such as dopamine and serotonin which have diffuse effects in multiple brain regions. The primary sources of these neuromodulators in the brain (the ventral tegmental area and substantia nigra and the raphe nuclei respectively) are under direct, monosynaptic control of GABA-ergic neurons. The tail of the ventral tegmental area/rostromedial tegmental nucleus (tVTA/RMTg) is composed of GABA-ergic cells that directly inhibit dopamine-producing cells in the VTA and substantia nigra to shut down dopaminergic signaling in response to aversive events (Jhou et al., 2009). In addition to the obvious functional implications of well-regulated dopamine release, balanced activity of neuromodulatory circuits is also critical for fundamental processes that determine stimulus salience (Schultz, 2011) which are underappreciated hallmarks of mental

illnesses (Morris et al., 2012). Similarly, dorsal raphe neurons that provide serotonergic innervation to most of the brain are under direct, local control of GABA-ergic cells (Inyushkin et al., 2010). These particular interneurons have been shown to be necessary for the acquisition of socially-relevant behavior (Challis et al., 2013). Consistent with this, we have also shown that silencing an interneuron subpopulation, causes specific disruption of serotonergic circuitry and changes in social behavior (Brown et al., 2013).

In addition to the direct, monosynaptic connections, GABA-ergic interneurons can affect modulatory systems via polysynaptic, systems level pathways. For example, NPY+ interneurons in the striatum control striatal projection neurons (Ibanez-Sandoval et al., 2011; English et al., 2012) and we have shown that silencing these neurons (and NPY+ interneurons in other regions) causes dopamine-dependent behavioral abnormalities and increased sensitivity to amphetamine (Schmidt et al., 2013). Disinhibition of these dopamine circuits reduces anxiety-like behaviors in rodents (Zweifel et al., 2011) likely by modulating the activity of the GABA-ergic interneurons in the amygdala (Marowsky et al., 2005; Pape, 2005; de la Mora et al., 2010) which include both the NPY+ and CCK+ cells described above (Truitt et al., 2009). It has also been suggested that disinhibition of the hippocampus by disruption of parvalbumin+ inhibitory circuitry can overdrive dopaminergic circuits in multiple systems (Lisman and Grace, 2005). In the case of serotonin, a growing body of evidence shows that the local GABA-ergic interneurons in the dorsal raphe (Inyushkin et al., 2010) actively participate in gating the glutamatergic drive of those cells (Soiza-Reilly et al., 2013) in response to,

or under the control of medial frontal cortical input (Varga et al., 2001). In this manner, GABA-ergic circuitry functions to directly and indirectly control the release of neuromodulators throughout the brain which has important consequences for a wide range of behaviors.

Given the challenges of comparing functional cortical microanatomy in rodents and humans, more work needs to be done to understand how the function of particular GABA-ergic circuits might influence disease-relevant behaviors. Nevertheless, there are long-accepted links between widespread biogenic amine system dysfunction and neuropsychiatric conditions such as dopaminergic changes in schizophrenia and substance abuse and serotonin disturbances in mood disorders, social behavior, and potentially psychosis. Advancing knowledge about the mechanisms whereby GABA-ergic interneuron function interacts with and controls these systems may identify diverse causes of these disorders and potential new treatments.

GABA and disease

Nearly all neuropsychiatric disorders include dysfunctional GABA system components: schizophrenia (Hashimoto et al., 2008b), bipolar disorder (Guidotti et al., 2000), anxiety (Rudolph et al., 1999; Low et al., 2000; Mohler, 2012), depression (Thompson Ray et al., 2011; Mohler, 2012), panic disorder (Malizia et al., 1998), post-traumatic stress disorder (Geuze et al., 2008), attention deficit hyperactivity disorder (Edden et al., 2012), autism (Fatemi et al., 2002), Rett syndrome (Blue et al., 1999), epilepsy (Lloyd et al., 1986), and others (Marin, 2012).

Researchers studying schizophrenia in particular have accumulated the largest body of evidence showing that GABA system dysfunction may be related to behavioral dysfunction and clinical diagnosis (Lewis et al., 2005). Discovering that GABA controls dopamine release in striatal and mesolimbic circuits prompted investigators in the 1970s to theorize that GABA dysfunction could cause schizophrenia (Roberts, 1972; Van Kammen, 1977). GABA-associated deficits steadily emerged in the clinical literature with the publication of studies that found reduced GABA content (Perry et al., 1979; Spokes et al., 1980), altered GABA receptor subunit protein levels and mRNA levels (Hanada et al., 1987; Benes et al., 1992; Impagnatiello et al., 1998; Volk and Lewis, 2002; Hashimoto et al., 2008b; Hashimoto et al., 2008a; Charych et al., 2009; Maldonado-Aviles et al., 2009), decreased GABA transporter protein levels (Simpson et al., 1989; Volk and Lewis, 2002; Hashimoto et al., 2008b), and altered interneuron densities (Benes et al., 1991; Daviss and Lewis, 1995; Wang et al., 2011) in the post-mortem brains of subjects with schizophrenia. In 1995, Akbarian and colleagues first reported a decrease in GAD67 mRNA in prefrontal cortex of post-mortem schizophrenic brain tissue that could not be accounted for by cell loss (Akbarian et al., 1995). The GAD67 expression deficit has become one of the most consistently replicated gene expression findings in schizophrenia across many different brain regions, patient cohorts, methods, and investigators which is remarkable given the complex genetics and diverse presentation of symptoms seen in patients (Akbarian et al., 1995; Impagnatiello et al., 1998; Guidotti et al., 2000; Mirnics et al., 2000; Volk et al., 2000; Knable et al., 2002; Volk and Lewis, 2002; Hashimoto et al., 2003a; Kalkman and

Loetscher, 2003; Costa et al., 2004; Fatemi et al., 2005; Lewis et al., 2005; Akbarian and Huang, 2006; Huang and Akbarian, 2007; Hashimoto et al., 2008b; Hashimoto et al., 2008a; Curley et al., 2011; Thompson Ray et al., 2011).

These studies of post-mortem brain tissue from subjects with schizophrenia also indicate that dysfunction of the GABA seems to be cell type-specific. Approximately 30% of GABA-ergic interneurons in the cortex of postmortem brains from individuals with schizophrenia do not express GAD67 mRNA (Akbarian et al., 1995; Volk et al., 2000); however those that do express detectable GAD67 appear to have normal levels (Volk et al., 2000). Among these dysfunctional cell types are interneurons that express the calcium binding protein parvalbumin or the neuropeptides CCK or NPY (Hashimoto et al., 2003a; Hashimoto et al., 2008b). The most overwhelming evidence is that PV+ interneurons, particularly cortical chandelier cells, are the predominantly affected cell type in schizophrenia. Hashimoto and colleagues reported that GAD67 was not detected in 55% of PV+ interneurons in the cortex despite observing normal PV+ cell density (Hashimoto et al., 2003b). The synaptic contacts made by these cells onto the axon initial segments (AIS) of pyramidal neurons are also abnormal in schizophrenia. Decreased presynaptic expression of GABA transporter 1 (GAT1) and increased postsynaptic expression of GABA receptor subunits suggests that GABA-ergic neurotransmission is diminished at these synapses (Volk and Lewis, 2002; Lewis et al., 2005). Furthermore, a 40% reduction in the total density of PV+ chandelier cell – AIS synaptic contacts has been observed in schizophrenia (Woo et al., 1998). The formation of these PV+ axon terminals and the formation of glutamatergic contacts that

drive the PV+ interneurons are dependent on Neuregulin—ErbB4 signaling (Fazzari et al., 2010; Wen et al., 2010) which is also dysfunctional in schizophrenia (Stefansson et al., 2004; Harrison and Weinberger, 2005; Mei and Xiong, 2008). The dysfunction of these cells likely contributes to impaired gamma oscillations (Fuchs et al., 2007; Gonzalez-Burgos and Lewis, 2012), learning (Chen et al., 2010), and working memory (Lewis et al., 2005; Fuchs et al., 2007; Haenschel et al., 2009).

Other GABA-ergic interneuron cell types are also clearly dysfunctional in schizophrenia. Many studies have found that CCK is downregulated in schizophrenic patients (Ferrier et al., 1983; Roberts et al., 1983; Beinfeld and Garver, 1991; Kerwin et al., 1992; Virgo et al., 1995; Bachus et al., 1997; Lewis et al., 2005; Hashimoto et al., 2008b; Curley and Lewis, 2012) and in animal models that may have relevance for schizophrenia including chronic dopamine receptor stimulation (Suzuki and Moroji, 1989) or NMDA receptor hypofunction (Arif et al., 2006). The strong correlation between reductions of CCK and GAD67 mRNA suggests that CCK+ interneurons are dysfunctional in schizophrenia (Hashimoto et al., 2008b). These are also the only interneuron cell type that expresses the cannabinoid receptor CB1 (Marsicano and Lutz, 1999; Tsou et al., 1999; McDonald and Mascagni, 2001; Eggan et al., 2010). CB1 receptor activation has been shown to silence CCK+ interneurons (Losonczy et al., 2004) and disrupt the functions of the hippocampus (Katona et al., 1999; Hajos et al., 2000) and amygdala (Katona et al., 2001; Tan et al., 2010) particularly in response to chronic stress (Patel et al., 2009). Since exposure to cannabis and chronic stress are both epidemiologically identified risk factors for developing schizophrenia (Horvath and

Mirnics, 2009), CCK+/CB1+ interneuron dysfunction may contribute to disease-relevant processes. However, it has also been suggested based on the post-mortem evidence that CCK+ basket cells may rely more heavily on GAD65 relative to GAD67 than other interneuron populations (Fish et al., 2011) and that CCK peptide and CB1 receptor downregulation observed in schizophrenic brain tissue (Lewis et al., 2005) may actually result in a net increase of inhibition produced by these cells (Curley and Lewis, 2012). This interpretation must be examined in the context of the circuitry where CCK+ interneurons inhibit both PV+ interneurons and pyramidal cells (Freund and Katona, 2007; Lee et al., 2011) by processes that are gated by the CCK peptide itself (Foldy et al., 2007). Further experiments are required to fully evaluate the role of CCK+ interneuron dysfunction presented by Curley and Lewis. Some of the results presented here will provide further information on this issue and the possibilities that GAD67 downregulation in these cells contributes to behavioral impairments.

A third GABA-ergic cell type that is dysfunctional in schizophrenia is the NPY+ population. Neuropeptide Y itself is downregulated in schizophrenia (Frederiksen et al., 1991; Gabriel et al., 1996; Kuromitsu et al., 2001; Hashimoto et al., 2008b; Mellios et al., 2009; Morris et al., 2009) although it is unclear whether antipsychotic medication (Huang et al., 2006; Mellios et al., 2009; Nikisch et al., 2012) or age and duration of illness (Peters et al., 1990) mediate this effect. Nevertheless, NPY+ interneuron density is altered in the cortex (Ikeda et al., 2004) and NPY interneuron dendrites are malformed in the hippocampus (Iritani et al., 2000) of schizophrenic brains indicating that these cells are specifically disrupted during development. Similar to the CCK+

cells, it has also been suggested that the strong correlation between reductions of NPY and GAD67 mRNA suggests that NPY+ interneurons are dysfunctional in schizophrenia (Hashimoto et al., 2008b). Furthermore, NPY+ interneurons mediate tonic inhibition of numerous brain regions as discussed previously (Olah et al., 2009). The $GABA_A$ receptor delta subunit is exclusively expressed in extrasynaptic receptors that mediate this tonic inhibition and is also downregulated in schizophrenia (Hashimoto et al., 2008b; Charych et al., 2009; Maldonado-Aviles et al., 2009). It is possible that this deficient molecular machinery supporting tonic inhibition and the corresponding dysfunction of NPY+ neurogliaform cells contributes to baseline hyperactivity in multiple brain regions seen in patients with schizophrenia (Molina et al., 2003; Fryer et al., 2013; Homan et al., 2013; Sorg et al., 2013). Interestingly, neurosteroids that act on these receptors (Hosie et al., 2006) have clinical efficacy in improving cognitive deficits in patients with schizophrenia and there is evidence that improving tonic GABA transmission is a component of this improvement; however glutamatergic systems are involved as well (Marx et al., 2009). There is also a growing body of evidence from animal models that NPY+ interneurons in the striatum and amygdala modulate reward pathways and mediate anxiety-like and risk aversion-related behaviors (Truitt et al., 2009; English et al., 2012) which have relevance for a number of disease states. Further clinical research will be required to fully evaluate the participation of these cells in other disorders and the potential for targeting these cells with new therapeutics including neurosteroids.

How might GAD67 expression deficits develop? Several studies of the gene that encodes GAD67, *GAD1*, have yielded a number of single nucleotide polymorphisms (SNPs) that are found more frequently in schizophrenic patients than controls (Addington et al., 2005; Straub et al., 2007; Du et al., 2008). The majority of SNPs in each study was found in gene regulatory sequences suggesting a role in regulating gene expression and not protein function. An analysis of patients with a *GAD1* genetic variant suggested that DNA sequence variation can effectively regulate mRNA expression levels in the postmortem tissue of subjects with schizophrenia (Straub et al., 2007). Epigenetic mechanisms may also contribute to decreased GAD67 expression in schizophrenia. Genes can be suppressed when promoters or other regulatory sequences are methylated causing changes in chromatin structure that prevent transcription (Veldic et al., 2004). Methylation is carried out by enzymes such as DNA methyltransferase 1 (DNMT1) which is overexpressed in GABA-ergic interneurons of schizophrenic patients and correlated with decreased GAD67 mRNA in the same cells suggesting that epigenetic regulation of the gene may be imbalanced; however, a causal relationship between increased DNMT1 and *GAD1* promoter methylation cannot be conclusively established in post-mortem studies (Veldic et al., 2004; Veldic et al., 2005; Huang and Akbarian, 2007; Ruzicka et al., 2007; Veldic et al., 2007). Finally, it has been suggested recently that GAD67 downregulation and other GABA-associated dysfunction measured in post-mortem tissue collectively reflect general dysfunction of GABA system development via changes in cell-cycle regulation (Benes, 2011), impairments in interneuron maturation (Hyde et al., 2011), and migration defects (Benes et al., 1991; Ikeda et al., 2004).

Complementing the genetic population-based studies and expression/epigenetic data from post-mortem research, the use of animal models is also able to shed light on the mechanisms by which GAD67 downregulation alters normal brain function. As mentioned previously, GAD67 knockout mice are not viable (Asada et al., 1997). However, data from rodent models using more subtle or restricted manipulations are useful. GAD67 expression can be reduced by chronic dopamine D2-receptor stimulation (Lindefors, 1993; Laprade and Soghomonian, 1995) or acute NMDA receptor antagonism (Qin et al., 1994) in multiple brain regions. These data mirror the ability of chronic dopamine stimulation (Sato et al., 1992) and acute NMDAR antagonism (Javitt and Zukin, 1991; Krystal et al., 1994) to precipitate psychosis in humans. Thus, the NMDA hypofunction hypothesis, the dopamine hypothesis, and the GABA dysfunction hypothesis of schizophrenia could be integrated with GAD67 deficiency being a player in each (Kalkman and Loetscher, 2003). Furthermore, some antipsychotic drugs demethylate the *GAD1* promoter which may enhance their therapeutic profile in some cases (Guidotti et al., 2009; Guidotti et al., 2011). According to one study, treating mice with nicotine suppressed DNMT1 expression, demethylated the *GAD1* promoter, and increased GAD67 protein levels which may explain in part the high incidence of cigarette smoking among individuals with schizophrenia (Satta et al., 2008). This study complements the human post-mortem epigenetic data and supports epigenetic modification as a potentially reversible mechanism of GAD67 deficiency. However, coincidence of GAD67 rescue with symptomatic improvement in the short- and long-term remains unclear (Thomsen et al., 2009; Guidotti et al., 2011).

Importantly, neither haloperidol nor olanzapine reduced GABA-associated gene expression in nonhuman primates, ruling out medication effects as a primary cause of GAD67 reduction in the human post-mortem brain of subjects with schizophrenia (Hashimoto et al., 2008b) and suggesting that GAD67 may be a primary mechanism of pathophysiology and behavior associated with mental illness.

In addition to the downstream effects of genetic manipulations, animal studies have also illuminated mechanisms that connect epidemiologically-identified environmental insults with cellular and behavioral dysfunction. Environmental disruptions during development including prenatal and perinatal infections, perinatal hypoxia, drug abuse during prenatal development or adolescence, and stress have all been shown to increase risk for schizophrenia diagnosis (Lewis and Levitt, 2002; Horvath and Mirnics, 2009). Among these, maternal immune activation and neonatal hippocampal damage have been the best characterized using animals. Maternal immune activation (MIA) is typically studied in animal models by administering bacterial infection mimetic lipopolysaccharide (LPS) or viral mimetic poly:IC during pregnancy (Canetta and Brown, 2012). GABA content (Bitanihirwe et al., 2010), GAD67 expression (Deslauriers et al., 2013; Richetto et al., 2013), and GABA receptor subunit expression (Nyffeler et al., 2006; Richetto et al., 2013) are all dysregulated in the offspring of MIA-treated rats and mice. Interestingly, these effects appear to be cell type specific and preferentially affect PV+ interneurons (Ibi et al., 2010; Ducharme et al., 2012; Piontkewitz et al., 2012). Even more interesting is that these effects are augmented by stress (Deslauriers et al., 2013; Giovanoli et al., 2013) or by DISC1

genotypes associated with neuropsychiatric illness (Ibi et al., 2010; Lipina et al., 2013) providing evidence for a two-hit model of risk factors with GABA system dysfunction as a common endpoint. Animal studies have also identified interleukin-6 as the harbinger of molecular and behavioral dysfunction resulting from MIA (Smith et al., 2007b; Garbett et al., 2012). DISC1 genotype plus MIA also has a supra-additive effect on interleukin-6 release, providing further support for the two-hit model of developmental dysfunction and providing a potential opportunity for intervention by targeting the interleukin-6 pathway.

The neonatal ventral hippocampal lesion (NVHL) rat models hippocampal damage during a period analogous to the third trimester in human pregnancy (Lipska and Weinberger, 2002; Tseng et al., 2009; O'Donnell, 2012). GABA system dysfunction is a core component of the NVHL model with multiple research groups reporting decreased GAD67 (Lipska et al., 2003) and increased GABAA receptor gene expression (Mitchell et al., 2005; Endo et al., 2007) as well as decreased interneuron cell number in some regions (Francois et al., 2009). One of the main findings in the NVHL model is altered dopaminergic regulation of prefrontal circuits; stimulating the VTA in these animals increased PFC pyramidal cell activity which was directly attributed to dysfunctional VTA inputs (O'Donnell et al., 2002) and loss of D2 receptor modulation (Tseng et al., 2008) on PFC interneurons. Each of these animal models has provided insight into the mechanisms governing the effects of disease-relevant environmental insults during development and highlights the involvement of GABA system dysfunction

as a common downstream component of multiple genetic and environmental disturbances.

It is unlikely that any single mechanism is solely responsible for the consistent GABA-system deficits observed in so many patient cohorts. The fact that several different mechanisms (genetic, environmental, and gene*environment interaction) can lead to decreases in GAD67 gene expression shows that diverse insults and influences can converge, giving rise to common GABA-ergic dysfunction. This concept is fundamental to understanding the neurobiology of psychiatric illness where heritability is clear (Lewis and Levitt, 2002; Harrison and Weinberger, 2005; Mirnics et al., 2006; Horvath and Mirnics, 2009), but genetics is less so (Purcell et al., 2009; Shi et al., 2009; Stefansson et al., 2009). Since polygenic risk factors interact with multiple environmental risk factors leading up to the delayed onset of symptoms, identifying "hubs" where these diverse factors exert common influence reveals the mechanisms that are likely responsible for symptoms of the disease and generates opportunities for directing therapeutic intervention to those systems (Mirnics et al., 2006; Horvath and Mirnics, 2009). These converging lines of evidence from human genetic, patient post-mortem brain, and rodent studies implicate GABA system dysfunction as a core "hub" feature of many neuropsychiatric illnesses and identify potential opportunities for novel therapeutic development. The diversity of interneurons is a critical factor in both the dysfunctional spectrum and the therapeutic potential and more data are needed addressing the functional consequences of restricted dysfunction in GABA-ergic interneuron subclasses.

Hypotheses

Directly or indirectly, many studies have addressed the activity of individual interneurons during normal and dysfunctional behavior (Costall et al., 1989; O'Donnell et al., 2002; Lewis et al., 2005; Marowsky et al., 2005; Chattopadhyaya et al., 2007; Fuchs et al., 2007; Likhtik et al., 2008; Tseng et al., 2008; Truitt et al., 2009; Polepalli et al., 2010; Bienvenu et al., 2012; Heldt et al., 2012; Alberi et al., 2013; Kvitsiani et al., 2013; Tukker et al., 2013) and recent findings suggest GAD67 expression and behavioral dysfunction are tightly correlated and that subtle decreases in GABA signaling give rise to behavioral changes (Chao et al., 2010; Heldt et al., 2012). GAD67 deficiency has been shown to effectively silence individual interneurons (Chattopadhyaya et al., 2007) and has been described as a primary mechanism of behavioral dysfunction in other animal models (Chao et al., 2010; Richetto et al., 2013). While one recent study examined two interneuron populations mediating different behavioral responses (Kvitsiani et al., 2013), systematic and comprehensive analyses of the molecular and behavioral consequences of restricted GAD67 suppression in distinct interneuron populations have not been conducted to date.

While no one will recreate complex human behavioral disorders in a rodent, simple research questions can be asked using transgenic mice generated in our lab and others to shed light on the functional implications of specific GABA-ergic dysfunction in neuropsychiatric disorders. Given the robust and consistent finding that GAD67 expression is correlated with neuropsychiatric diagnoses (described above), we are

particularly interested in several related questions. Does manipulating the expression of this gene affects behavior? If so, is one restricted cell type enough to induce the changes and do the effects depend on the interneuron cell type involved? Finally, are the answers to these questions meaningful for dysfunction associated with particular neuropsychiatric illness or is GAD67 suppression generalizable? Answers to these basic questions could direct future therapeutic interventions toward particular cell types and limit side effects. Experiments in the following chapters address these hypotheses. The targeting construct and mouse lines used to suppress GAD67 in restricted cell populations are described in Chapter 2. The cell type specific effects of GAD67 suppression on the lipidome and proteome are outlined in Chapter 3. Finally, the behavioral consequences of GAD67 suppression are detailed in Chapter 4. The results of these experiments, their relationships with the hypotheses described above, and their implications for understanding the brain, behavior, and behavioral dysfunction are discussed.

CHAPTER 2

NOVEL ANIMAL MODELS FOR STUDYING COMPLEX BRAIN DISORDERS: BAC-DRIVEN miRNA-MEDIATED *IN VIVO* SILENCING OF GENE EXPRESSION

Martin J. Schmidt, Szatmár Horváth, Krassimira A. Garbett, Philip Ebert, Levente Gellert, Monika Everheart, Khine Lwin, Pat Levitt, and Károly Mirnics

INTRODUCTION

To test the hypothesis that GAD67 downregulation affects brain systems and behavior in a cell type dependent manner, our lab created BAC-driven, miRNA-mediated technology that silences GAD67 expression in targeted cell populations in mice (Garbett et al., 2010). Bacterial artificial chromosomes (BACs) are large pieces of engineered DNA that are useful for inserting transgenes into mice (Heintz, 2001). miRNAs are naturally occurring small RNA molecules, often housed in introns of genes, that regulate the expression of genes by binding specifically to untranslated regions of mRNAs and either tagging the message for degradation by the RNA-induced silencing complex (RISC) or repressing translation by interfering with ribosomal function (Valencia-Sanchez et al., 2006). By engineering a synthetic miRNA to selectively target GAD1 mRNA, we are able to study the effects of GAD1/GAD67 deficiency in specific interneuronal populations *in vivo*.

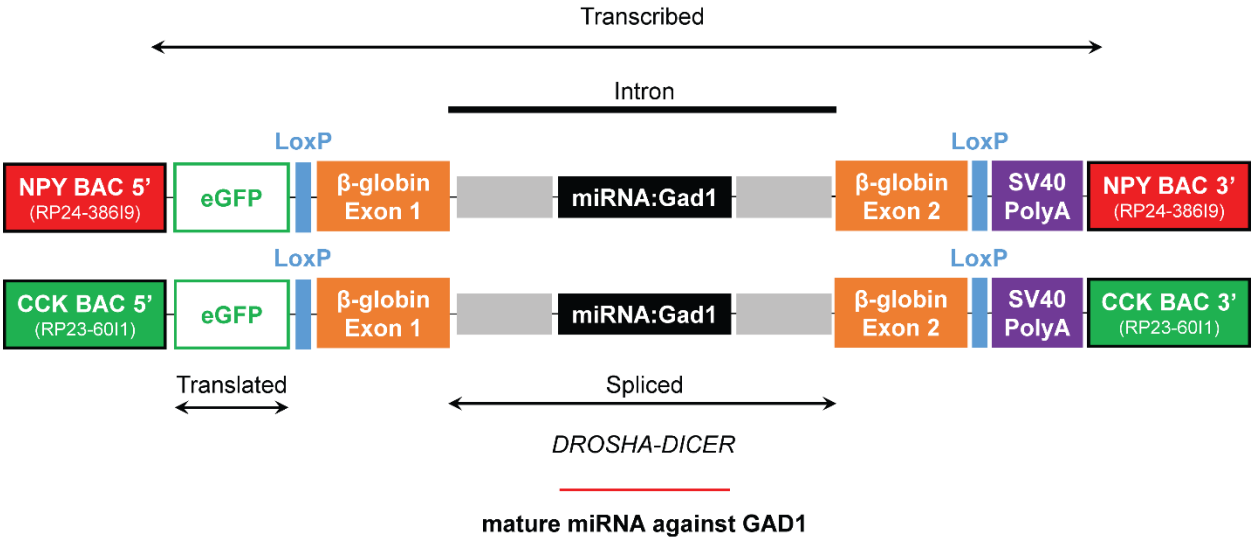


Figure 2: BAC-driven, miRNA-mediated *in vivo* silencing of GAD67 expression in mice. A synthetic miRNA targeted against GAD1 mRNA (black) was contained within an intron (gray) between the first two exons of the beta globin gene (orange) which were floxed (blue) to permit miRNA excision while maintaining the construct insertion site and fluorescent marker expression. An eGFP reporter (white with green border) was included to visualize construct expression and an SV40 polyadenylation signal (purple) ensured proper mRNA processing. This entire construct was inserted into bacterial artificial chromosomes (BAC) that contained gene regulatory sequences for the NPY (red) or CCK (green) genes to ensure that expression was restricted to either of those cell types.

METHODS

Mice

NPYGAD1 transgenic mice were generated and validated for construct expression and efficacy in a previous study (Garbett et al., 2010). CCKGAD1 transgenic mice were generated and validated in a similar manner described here. Briefly, RP23-60I1BAC containing the Cholecystokinin (*mCck*) locus (Chr9: 121,435,221 – 121,551,243, NCBI GRCm38.p1) was purchased from the Children's Hospital of Oakland Research Institute (http://bacpac.chori.org/). The BAC was isolated from the original DH10B E. coli strain and transformed into EL250 E. coli cells. The presence of the *mCck* locus in RP23-60I1 was verified by restriction enzyme digest mapping. GFP-miRNA:Gad1-FRT-neo-FRT was inserted at the start codon of *mCck*, ensuring that *mCck* promoter would control expression of an engineered construct described previously (Garbett et al., 2010). In essence, *mNpy* homology arms of the previous construct were swapped with *mCck* homology arms in pSTBlue-1 plasmid vector (Novagen). The *mCck* targeting construct carried Cnr1 5' (150 bp) and 3'(151 bp) homology arms surrounding the eGFP, β-globin minigene and an FRT-flanked neomycin-resistance cassette. The β-globin minigene contained miRNA:Gad1 in a position allowing the in vivo release of functional miRNA, which effectively reduced the GAD1 protein to undetectable levels in cell cultures (Garbett et al., 2010). After proper insertion of the targeting construct at the *mCck* start codon on the RP23-60I1 BAC, the selective marker neo was removed via FRT-directed recombination. BAC modifications were confirmed with restriction mapping and sequence analysis of the region of interest. The modified RP23-60I1 BAC was isolated with alkaline lysis and purified with

Sepharose CL-4B chromatography, described previously (Gong and Yang, 2005). Transgenic mice on congenic C57Bl/6 backgrounds were generated by injection of circular modified BAC into fertilized C57Bl/6 mouse oocytes by the Transgenic Mouse / ESC Shared Resource at Vanderbilt University (http://www.vicc.org/research/shared/tg-mouse.php) and identified by PCR using construct-specific primers.

Immunohistochemistry

Construct expression and efficacy were evaluated in NPYGAD1 transgenic mice in a previous study (Garbett et al., 2010). Mice were deeply anesthetized with isoflurane (IsoFlo, Abbott Animal Health) and transcardially perfused with ice-cold 1X PBS followed by 4% phosphate-buffered paraformaldehyde (PFA). Brains were removed and post-fixed in 4% PFA overnight before saturation in up to 30% phosphate-buffered sucrose. 50µM sections were cut on a cryostat (Leica Biosystems, Buffalo Grove, IL). Sections were washed extensively in PBS and blocked in 10% normal donkey serum in 0.1mM PB (pH 7.4) for 1 h. All primary antibody incubations were 72 h at 4°C and secondary incubations were 3 h at room temperature. Secondary antibodies were diluted 1:250 (Jackson Immunoresearch, West Grove, PA). For eGFP labeling, sections were incubated with either chicken anti-GFP (Abcam, Cambridge, MA; 1:2000) or rabbit anti-GFP (Invitrogen; 1:2000) primary antibodies and donkey anti-chicken DyLight488 or donkey anti-rabbit DyLight488 secondary. GAD1-stained sections were pre-incubated with 70 mg / ml of monovalent Fab' fragment of donkey anti-mouse immunoglobulin G (Jackson Immunoresearch) to block endogenous mouse immunoglobulins, then incubated with mouse anti-GAD1 (Millipore;1:2000) and donkey

anti-mouse Cy3 secondary. CCK-stained sections were incubated with either rabbit anti-proCCK (a generous gift from Dr. Andrea Varro) or rabbit anti-CCK8S (Immunostar, 1:1000) and donkey anti-rabbit Cy3 secondary. Images were acquired with a fluorescence microscope (Leica Microsystems Inc. Bannockburn, IL) and whole images were pseudocolored and adjusted for contrast in Photoshop (Adobe Systems, San Jose, CA).

RESULTS

Transgenic mice were generated containing a BAC construct with the promoter-enhancer elements of either the *NPY* or *CCK* genes, an eGFP reporter, and a synthetic miRNA targeted to Gad1 mRNA (**Figure 2**). These elements restricted eGFP expression and GAD1 suppression to either NPY+ or CCK+ interneurons and made the targeted cells fluorescent. Both transgenic lines were generated as previously described (Garbett et al., 2010) and construct expression and GAD1 suppression efficacy were verified with immunohistochemistry in Tg(Npy-eGFP/miRNA:GAD1)1KM (Garbett et al., 2010) and Tg(Cck-eGFP/miRNA:GAD1)2KM (**Figure 3;** Schmidt et al., 2013) transgenic mice, hereafter referred to as NPYGAD1 and CCKGAD1 respectively. These studies revealed that the transgene construct was expressed specifically in the targeted NPY+ and CCK+ cell populations and that these subpopulations had no detectable levels of GAD1 expression.

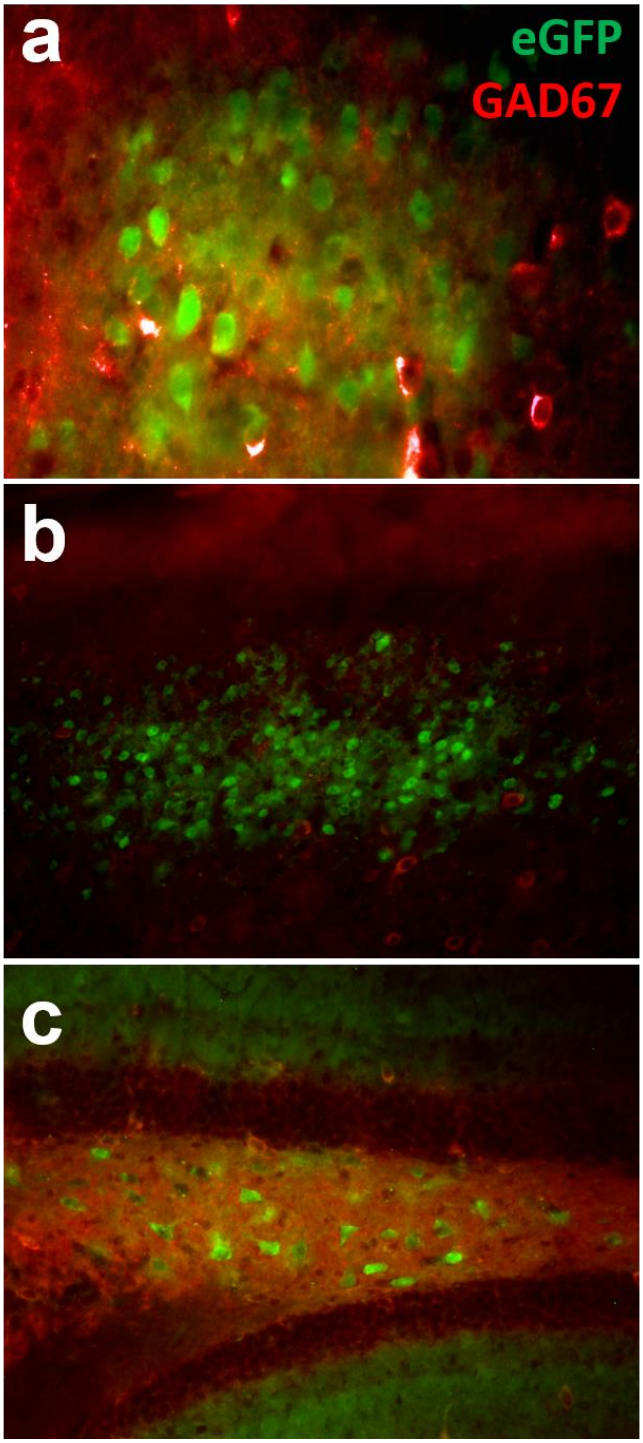


Figure 3: GAD67 is suppressed in CCK+ interneurons. GAD67 (red) was not detected in eGFP+ (green) cells in the amygdala (a), cortex (b), or hippocampus (c) of CCKGAD1 transgenic mice.

CHAPTER 3

MODULATION OF MOLECULAR NETWORKS BY SELECTIVE INTERNEURONAL INACTIVATION

Martin J. Schmidt, Philip Ebert, Jeremy L. Norris, Erin H. Seeley, Monika Everheart, Krassimira A. Garbett, Richard M. Caprioli, and Károly Mirnics

INTRODUCTION

Successfully suppressing GAD67 expression in two distinct interneuron populations with very different molecular and physiological properties (Garbett et al., 2010; Schmidt et al., 2013) allowed us to inventory the molecular changes that result from GAD67 deficiency and determine if these changes are dependent on the type of interneuron that is disrupted. Furthermore, since these interneuron populations are concentrated in different brain regions (Chronwall et al., 1985; Meziane et al., 1997), we chose to use a relatively new *in situ* proteomics technique (Cornett et al., 2007) that enables the comparison of region-specific changes of proteins, peptides, and lipids in our transgenic animals.

METHODS

Mice

NPYGAD1 and CCKGAD1 transgenic mice were generated and validated for construct expression and efficacy as described in previous studies (Garbett et al., 2010; Schmidt et al., 2013).

Matrix-Assisted Laser Desorption Ionization Imaging Mass Spectrometry (MALDI-IMS)

Tissue Preparation

Brains were harvested from male transgenic mice (n=6 per group) and wild type littermate controls (n=3 per group), snap frozen immediately on liquid nitrogen, and preserved at -80°C. Twelve micron thick coronal sections taken at the level of the striatum and hippocampus were cut in a cryostat (Leica Biosystems, Buffalo Grove, IL). The sections were thaw mounted onto gold-coated steel MALDI targets and stored in a vacuum desiccator until analysis.

Matrix Application

To prepare sections for protein analysis (m/z 2000-20,000), tissue was washed using 70%, 90%, and 95% ethanol solutions for 30 seconds and dried before matrix application. Dry, sinapinic acid powder was applied to seed the tissue which promoted uniform crystallization of the matrix on the tissue surface. Sinapinic acid solution (20 mg/mL in 50:49.9:0.1 acetonitrile, water, trifluoroacetic acid) was applied using an acoustic spotter (Aerni et al., 2006) in a 250 micron-spaced array pattern. A total of 45

drops were deposited in each position. Adjacent sections were prepared for lipid and peptide analysis (m/z 500-2000). α-cyano-4-hydroxy-cinnamic acid (CHCA) was used to seed as described above. CHCA solution (10 mg/mL in 50:49.9:0.1 acetonitrile, water, trifluoroacetic acid) was applied to the tissue using an acoustic spotter. Matrix was applied to each section in a 340 micron-spaced array pattern. A total of 60 drops were deposited in each position.

Mass Spectrometry Analysis

Low molecular weight species (m/z 500-2000) were analyzed using an ultrafleXtreme™ MALDI TOF/TOF (Bruker Daltonics) operating in reflector positive ion mode tuned for optimum resolution using the standard neurotensin (m/z 1672). Each position of the array was analyzed by summing 1000 spectra at each location. The protein data (m/z 2000-20,000) were collected using a linear autoflex™ speed MALDI TOF (Bruker Daltonics) tuned for optimum resolution of the standard, apomyoglobin (m/z 16,952). Protein identification was performed using LC-MS/MS as previously described (Schey et al., 2013).

Data processing

Mass spectrometry data were visualized using flexImaging software (Bruker Daltonics, version 3.0). Regions of interest (ROIs) were annotated and the data for each ROI were exported. Spectral data were processed using ClinProTools (Bruker Daltonics, version 2.2). Spectra were baseline corrected, recalibrated, normalized to total ion current, a peak-picking algorithm was applied, and p-values were calculated

using a pairwise two-tailed t-test and corrected using the Benjamini-Hochberg false-discovery rate (Benjamini and Hochberg, 1995). The pairwise t-test compared sections from transgenic and wild type animals on the same target plate to minimize the effects of inter-plate variability on the analysis. The magnitude of differences between transgenic and control mice was calculated using log2-based average log ratios (ALR; see statistical analysis section).

Statistical Analysis

For mass spectrometry analyses, p-values were calculated using two-tailed pairwise t-tests corrected for multiple comparisons using the Benjamini-Hochberg false discovery rate (Benjamini and Hochberg, 1995). The magnitude of significant differences was calculated using log2-based average log ratios (ALR) where ALR = mean(log2[NPYGAD1plate 1, section a], log2[NPYGAD1plate 1 section b]) - log2[NPYBACWTplate1] for each plate.

RESULTS

GAD1 suppression in NPY+ or CCK+ interneurons has differential effects on the lipidome and proteome

To determine if there are molecular changes downstream of GAD1 suppression and if they are dependent on interneuron cell type, we performed matrix assisted laser desorption ionization imaging mass spectrometry (MALDI-IMS) (Cornett et al., 2007) on brain tissue sections. We took advantage of the spatial resolution offered by this type of analysis and divided the sections into 10 regions of interest (ROIs; **Figure 4**): cortex (divided into CTXH for the neocortex in the hippocampal section, CTXS for the neocortex in the striatal section, and MFC for the cingulate area of the striatal section), corpus callosum (divided into CORPH and CORPS for the respective sections), hippocampus (HIPP), hypothalamus (HYTH), septum (SEP), striatum (STR), and thalamus (THAL). Using this method, we were able to reliably assess over 400 distinct proteins, peptides and lipids (0 – approx. 22,000 Da), in each brain region. GAD1 suppression in NPY+ interneurons lead to significant changes of 129 lipids, peptides, or proteins across the investigated regions (51 decreased, 65 increased, and 13 had region-specific changes; **Supplementary Table 1**) compared to wild type controls. GAD1 suppression in CCK+ interneurons induced region-specific expression changes of 52 lipids, peptides, or proteins (25 decreased, 23 increased, and 4 had region-specific changes; **Supplementary Table 2**) compared to wild type controls. Perhaps not surprisingly, there were only 15 that were common to both transgenic lines; of these, only 3 changed in the same direction, 6 changed in opposite directions, and 6 had region-specific differences. Highlighting the utility of MALDI-IMS spatial resolution and

regional specificity of the results, there were only two peaks (m/z 1583.09 and m/z 1907.27) that were significantly changed in at least three regions in NPYGAD1 mice and none in CCKGAD1 mice.

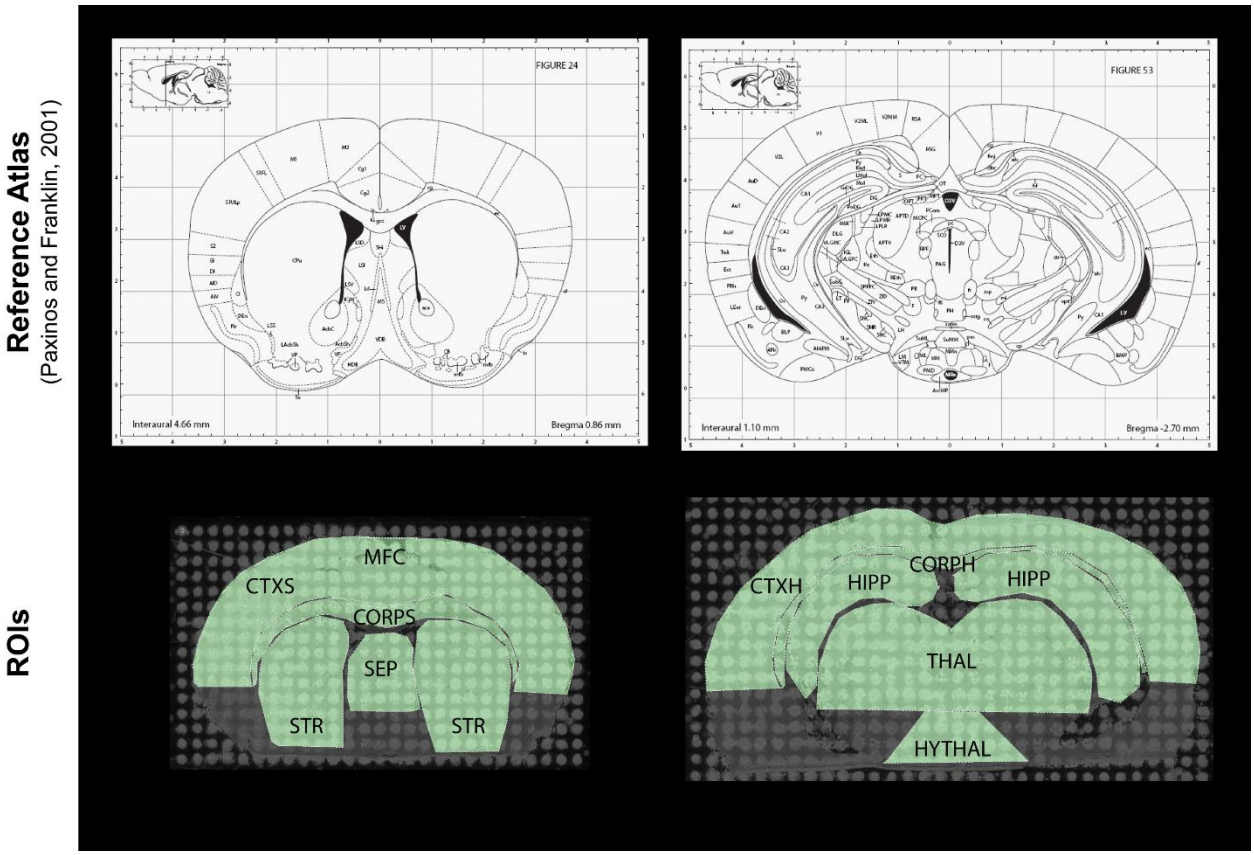


Figure 4. MALDI-IMS profiles lipids, peptides, and proteins with spatial resolution. Sections at the level of the striatum (left side) and hippocampus (right side) were taken from adult male mice from each BAC transgenic line and wild type littermates. Each section was imaged using matrix assisted laser desorption ionization mass spectrometry at 250-micron resolution. Sections were subdivided into regions of interest (ROIs) for the cortex, medial frontal cortex, corpus callosum, hippocampus, hypothalamus, septum, striatum, and thalamus (bottom row). Mass spectra for each ROI were then processed and analyzed for statistical significance. Atlas images from (Paxinos and Franklin, 2001).

We found that m/z 6725.9 was significantly upregulated in the hippocampus ($t_{(2)}$ = 4.471, p = 0.047) and cortex ($t_{(2)}$ = 6.796, p = 0.021) of NPYGAD1 mice (**Figure 5; Supplementary Table 1**). To determine its identity, protein was extracted directly from tissue sections and analyzed via LC-MS/MS similar to that described in Schey, et al (Schey et al., 2013) (data not shown). Using these methods, we were able to conclusively identify m/z 6725.9 as PEP19, also known as PCP4 (Harashima et al., 2011). Interestingly, PEP19/PCP4 overexpression has been shown to disrupt neurodevelopment (Mouton-Liger et al., 2011) and increase the release of neurotransmitters including dopamine and acetylcholine (Harashima et al., 2011). The peptide was also increased following chronic stress (Daniels et al., 2012) or amphetamine administration (Romanova et al., 2012) in rodents, while clinical gene expression studies found it to be increased in one study of patients with psychosis (Teyssier et al., 2011), but reduced in another (Guillozet-Bongaarts et al., 2013). Additional research is needed to fully identify and evaluate other significant changes in the dataset.

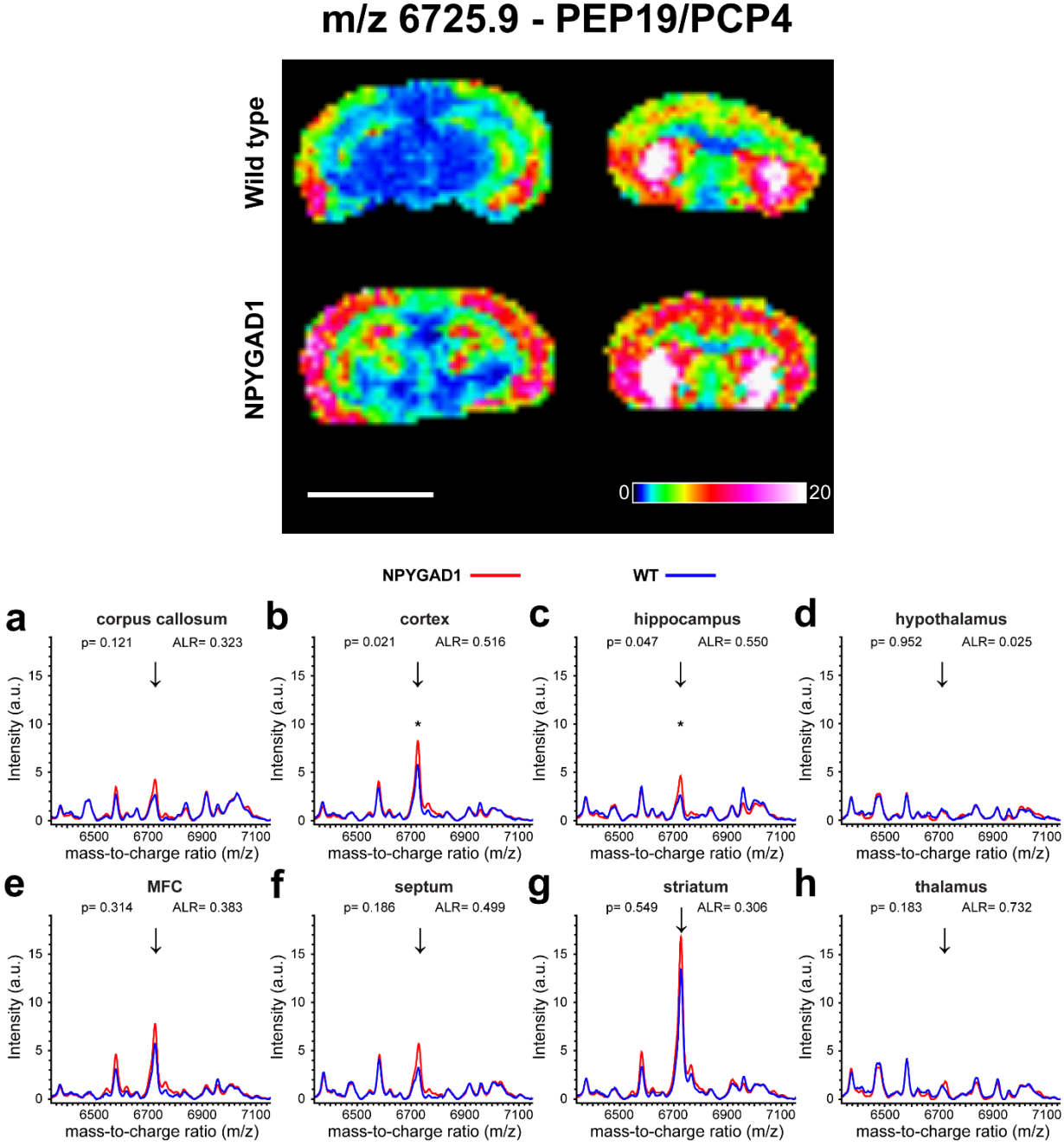

m/z 6725.9 - PEP19/PCP4
Wild type
NPYGAD1
0
20
NPYGAD1
WT
a
corpus callosum
p= 0.121
ALR= 0.323
b
cortex
p= 0.021
ALR= 0.516
c
hippocampus
p= 0.047
ALR= 0.550
d
hypothalamus
p= 0.952
ALR= 0.025
e
MFC
p= 0.314
ALR= 0.383
f
septum
p= 0.186
ALR= 0.499
g
striatum
p= 0.549
ALR= 0.306
h
thalamus
p= 0.183
ALR= 0.732
Intensity (a.u.)
mass-to-charge ratio (m/z)

Figure 5. GAD1 suppression in NPY+ interneurons leads to an increase in the expression of a 6725.9 Da peptide, identified as PEP19/PCP4. Representative MALDI-IMS expression intensity image of m/z 6725.9 from wild type (top row) and NPYGAD1 (bottom row) brain sections on a single MALDI target plate. m/z 6725.9 (arrows) was detected in the corpus callosum (**a**), cortex (**b**), hippocampus (**c**), medial frontal cortex (**e**), septum (**f**), and striatum (**g**). It was not detected in hypothalamus (**d**) or thalamus (**h**). m/z 6725.9 was significantly increased in the cortex (**b**) and hippocampus (**c**). The peptide was identified as PEP19, also known as PCP4, in a separate experiment. ALR, average log ratio; *, $p < 0.05$; a.u., arbitrary units, scale bar, 5mm.

CHAPTER 4

MODULATION OF BEHAVIORAL NETWORKS BY SELECTIVE INTERNEURONAL INACTIVATION

Martin J. Schmidt, Philip Ebert, Jacquelyn Brown, Monika Everheart, Krassimira A. Garbett, Taylor W. Grice, and Károly Mirnics

> *"However little it may be possible to identify human with animal brain-functions and illnesses, yet, from the effects produced by particular noxae in the brains of animals, conclusions can be drawn as to the issue of like processes in man."*
> - *E.Kraepelin* (Kraepelin, 1919 (1922 trans.))

INTRODUCTION

GABA system abnormalities have been identified in a number of neuropsychiatric disorders including schizophrenia (Hashimoto et al., 2008b), bipolar disorder (Guidotti et al., 2000), autism (Fatemi et al., 2002), Rett syndrome (Blue et al., 1999), and epilepsy (Lloyd et al., 1986). Among these, downregulation of glutamic acid decarboxylase 1 (GAD1), the enzyme responsible for producing the majority of the GABA in the brain (Martin and Rimvall, 1993), is a robust and consistent finding in the postmortem brains of subjects with schizophrenia (Lewis et al., 2005). However, what role(s) individual interneuronal cell types might play in normal or dysfunctional behavior are not well established. GABA-ergic interneurons are classified based on their morphology, physiology, receptor expression, brain distribution, and molecular markers (Ascoli et al., 2008). This diversity allows different classes of interneurons to regulate the input, signal

integration, output, and population synchrony of principle cells and other interneurons in distinct and dynamic ways (Markram et al., 2004). Given the variety of interneuron types and the spectrum of behavioral abnormalities among disorders with identified GABA-ergic pathophysiology, it is possible that dysfunction of one or more interneuron classes contributes to the variety of symptoms. Since CCK+ and NPY+ interneurons appear to be among those that are affected in subjects with schizophrenia (Hashimoto et al., 2008b) and since we found divergent molecular profiles in mice following GAD67 suppression in these populations (discussed in the previous chapter), we hypothesized that their dysfunction will lead to behavioral disturbances that are cell type-specific.

METHODS

Mice

NPYGAD1 and CCKGAD1 transgenic mice were generated and validated for construct expression and efficacy as described in previous studies (Garbett et al., 2010; Schmidt et al., 2013). Behavioral testing was performed in the Vanderbilt Murine Neurobehavioral Laboratory (MNL; http://vandymouse.org/) during the light cycle in accordance with the Vanderbilt Animal Care and Use Committee guidelines. Adult male mice (n = 10-12) were handled for 5 days prior to testing. Before each session, mice were acclimated for 1 hour under red light in an adjacent room. Tests were at least 24 hours apart. Experimenters were blinded to genotypes. All equipment was cleaned with Vimoba solution (Quip Labs, Wilmington, DE) between animals to reduce odor contamination and sanitize the equipment.

Irwin Screen, Grip Strength, Rotorod

A modified Irwin Screen assessed general health, neuromuscular function, and motor coordination (Irwin, 1968). To test grip strength, averaged across three trials, mice were held with their forepaws gripping metal mesh attached to a load cell and gently pulled away until they released the mesh. The rotorod (Ugo Basile, Comerio VA, Italy) accelerated from 2-40 rpm gradually over a 5 min period. Latency to fall was scored for each of three trials. In some cases animals can hold onto the rod and rotate around it when performing this task, especially during the low-speed portion. Since this result does not measure motor coordination and cerebellar function as desired, trials were stopped if the mouse rotated around the rod more than once.

Open Field Activity

Mice were placed in a white plastic box (50 x 50 x 40 cm) and allowed to explore freely for 10 min on two consecutive days. Video was recorded and locomotor activity and the amount of time in the center of the arena compared to the amount of time spent in the periphery was analyzed by ANY-maze software (Stoelting Co., Wood Dale, IL). The task measures locomotor activity and both within-session habituation, defined as a decrease in locomotion over time, and between-session habituation, defined as decreased locomotion at the beginning of the second session compared to the first. Failure to exhibit either type of habituation can indicate hippocampal dysfunction and/or general hyperactivity. Rodents have an innate aversion to open spaces that is adaptive for avoidance of their primary predators. Taking advantage of this behavior, many tasks compare time spent in a closed or protected area that is seemingly safer to an open

area that may be more dangerous (Rodgers et al., 1997; Belzung and Griebel, 2001; Rodgers, 2010). In this manner, comparing time spent in the center zone of the arena with time spent in the periphery of the arena can be considered a measure of “anxiety-like” (Cryan and Holmes, 2005) or “risk-avoidance” (Kim et al., 2013) behavior in mice.

Elevated Zero Maze

The white plastic zero maze was placed 60 cm above the floor in the center the testing room. The 5 cm wide runway was divided into four quadrants: two open and two closed with 15 cm high walls. Mice were placed in the center of one open area and allowed to explore freely for 6 min. Video was recorded and time spent in each zone and locomotor activity were analyzed by ANY-maze (Stoelting Co., Wood Dale, IL). Unprotected head dips, defined as the animal dipping its head over the side of the open or “unprotected” zone of the maze, and stretched attend postures, defined as the animal exhibiting an elongated body posture typically at the transition area between zones or in the open zone, (Shepherd et al., 1994) were scored by an experimenter blinded to genotype. Similar to the center/peripheral comparison in the open field task, comparing the amount of time spent in the closed zone with time spent in the open zone can be considered a measure of “anxiety-like” or risk-avoidance behavior in rodents. In addition, head dips and stretched attend postures are considered measures of risk assessment behavior as the animal is thought to be the determining whether or not immediate threats exist in the environment (Rodgers and Johnson, 1995; Rodgers et al., 1997; Cryan and Holmes, 2005).

Forced Swim

The Porsolt forced swim task measures an animal's propensity to continue to attempt to escape a stressful situation, in this case a container of water, over a relatively prolonged period of time. This task is thought to measure depression-like behavior in rodents, defined as decreased latency to float and/or increased total immobility time (considered acquiescence to the negative situation) based on its predictive validity for efficacy of antidepressant medications (Cryan and Holmes, 2005). Mice were placed into a 15 x 21 cm Plexiglas cylinder filled with room temperature water for 5 min. Each session was video recorded and scored for latency to float and total immobility time.

Light-Dark Boxes

The light-dark box is another paradigm designed to measure anxiety-like and risk-aversion behaviors in rodents through their innate avoidance of open spaces that may contain threats to their survival (Belzung and Griebel, 2001; Bourin and Hascoet, 2003; Cryan and Holmes, 2005). Mice were placed into the light side of a two-chambered box. The clear plastic light side (15 x 30 x 20 cm) was connected to a dark plastic chamber through a 5 x 7 cm opening. Boxes were enclosed inside ventilated sound-attenuating chambers and lit with overhead lights. Infrared photocells across each side recorded the location of the mouse and MED Activity computer software scored time in each box, locomotor activity, and number of transitions between boxes (MED Associates, Georgia, VT).

Prepulse Inhibition

Prepulse inhibition measures sensorimotor gating, considered by many to be translatable between rodents and people (Braff et al., 2001; Geyer et al., 2002), and has been used for this purpose in several patient populations including schizophrenia (Swerdlow et al., 1994; Braff et al., 1999; Braff et al., 2001). Mice were placed into cylinders affixed to force-transducers inside ventilated sound-attenuating chambers. The force transducers measured the motor startle response to a loud acoustic stimulus. After a 5 min acclimation, 45 trials were presented randomly with 20 ms of varying prepulse levels (0, 70, 76, 82, or 88 dB) followed by a 40 ms, 120 dB white noise burst. Nine null trials served as baseline measurements. Percent prepulse inhibition (startle during prepulse trials / startle during 0 dB trials x 100) and acoustic startle response (0 dB prepulse vs. null trials) were recorded and analyzed by StartleReflex software (MED associates, Georgia, VT).

Y-Maze Alternation

One method for evaluating spatial working memory is a three-armed-maze alternation task (Gerlai, 1998). Since rodents and other animals are exploratory by nature, they tend to prefer entering new arms of the maze instead of arms they had previously visited. If the animal cannot remember that it had just visited an arm or if it has no preference for novelty, it may show a reduced rate of spontaneous alternation. Mice were placed into an enclosed clear plastic y-maze (35 x 5 cm arms) and allowed to explore freely for 5 min. ANY-maze (Stoelting Co., Wood Dale, IL) scored arm entries when the mouse moved completely into an arm. Alternations were defined as entering

each of the arms once in any three consecutive entries. Percent alternation was determined by calculating the number of successful alternations out of the total possible alternations.

Social Interaction

As mentioned above, rodents are exploratory animals and tend to investigate new things. The three-chambered social interaction task, used as described by Yang et al. with minor modifications (Yang et al., 2011), quantifies that tendency in a social setting by introducing two novel social stimuli over the course of the task. In this study, the task involved three, 10 min phases. First, mice acclimated to the three chambered, clear plastic box (57 x 40 x 45 cm). Second, two wire pencil cups were placed in the two side chambers. In one cup a novel social stimulus mouse was placed while the second cup remained empty. Third, a second novel social stimulus mouse was placed in the empty cup. Social stimulus mice were naïve adult male wild type C57Bl/6 mice. Cup location and social stimulus mouse order were counterbalanced to overcome any potential bias for one side of the chamber or the other or for one novel stimulus mouse or the other in case any such bias existed. ANY-maze (Stoelting Co., Wood Dale, IL) tracked the position of the test mouse and scored interaction time when the head was <1 cm from the cups. Preference was calculated as 100 x (novel mouse 1 interaction time – novel object interaction time) / (novel mouse 1 interaction time + novel object interaction time) and 100 x (novel mouse 2 interaction time – familiar mouse interaction time) / (novel mouse 2 interaction time + familiar mouse interaction time). Typically, mice prefer the novel social stimulus mouse to the novel object (sociability) and prefer

the second novel social stimulus mouse to the familiar mouse (preference for social novelty) (Silverman et al., 2010).

Olfactory Habituation

A series of nonsocial (orange and almond extract, diluted 1:100 with water, McCormick and Co., Sparks, MD) and social odors (conspecific bedding) were presented via cotton swabs to each mouse (Silverman et al., 2010). Each presentation lasted 2 min with 1 min between trials. An experimenter, blinded to experimental conditions, measured the total time each mouse investigated the swab.

Fear Conditioning

Contextual and cued fear conditioning was tested using the protocol developed for mice by Smith et al.(Smith et al., 2007a) with minor modifications. Mice explored the chamber (20 x 15 x 10 cm) freely for 12 min. The next day, they received 6 tone-footshock pairings (70 dB, 2 kHz, 20 s tone and 2 s, 0.5 mA shock separated by 18 s). On day three, mice were placed into the “training context” for 15 min with no tones or shocks before being returned to a clean cage while the testing chamber was cleaned and outfitted with striped walls and covered floor. Mice were placed back into the chamber and allowed to explore this novel “testing context” for 3 min. Cued testing trials began immediately following the novel context exploration. Ten tones identical to those in the training phase were administered 80 s apart without shocks. Freezing, the absence of movement other than breathing, was scored objectively by VideoFreeze (MED Associates, Georgia, VT).

Amphetamine-Induced Locomotion

Mice were placed into clear plastic boxes (30 x 30 x 20 cm) inside ventilated sound-attenuating chambers lit with overhead white light, allowed to explore freely for 15 min, removed and injected with 3 mg/kg D-amphetamine hemisulfate (Sigma-Aldrich, St. Louis, MO) in 0.9% saline solution, and immediately returned to the chamber for 60 min. Infrared photocells measured locomotor activity and stereotypical behaviors (MED Activity software, MED Associates, Georgia, VT).

Statistical Analysis

Two-tailed groupwise t-tests and two-way repeated measures ANOVAs were used to compare transgenic mice with wild type littermate controls as appropriate for each test.

RESULTS

GAD1 suppression in NPY+ or CCK+ interneurons has differential effects on behavior

Adult male mice (n = 10-12 per group) were evaluated using a battery of tasks chosen to assess a broad range of behavioral traits (see Methods for full details). NPYGAD1 and CCKGAD1 lines were tested independently using identical testing parameters and results were compared against wild type littermate controls. Mice were visually indistinguishable from littermate controls and did not display any general health or neuromuscular problems (**Figure 6a,b**). CCKGAD1 mice exhibited decreased locomotor activity during the initial portion of the open field test (two-way repeated measures analysis of variance (ANOVA) time x genotype interaction, $F_{(9,198)} = 2.0938$, $p = 0.032$; **Figure 7a**). All groups displayed normal habituation to the open field arena (two-way repeated measures ANOVA main effect of time: NPYGAD1 $F(9,180) = 12.2300$ $p = 0.000$; CCKGAD1 $F(9,198) = 10.5489$, $p = 0.000$). CCKGAD1 mice also trended towards hypoactivity on the elevated zero maze (two-tailed independent samples t-test: $t(22) = -1.7822$ $p = 0.089$; **Figure 7b)**.

We assessed anxiety-like behavior using the open field, elevated zero maze, and light-dark box paradigms (Cryan and Holmes, 2005). Neither transgenic line showed significant differences when comparing the amount of time spent in the center of the open field to the amount of time spent in the periphery of the arena (data not shown). A ceiling effect of center-arena aversion due to the intensity and/or location of the overhead lighting prevented analysis of anxiety-like behavior in this paradigm since all

mice did not gradually increase their exploration of the center of the arena over the course of the session (data not shown). However, NPYGAD1 mice displayed reduced anxiety-like behavior in both the elevated zero maze and light-dark box paradigms. Although they did not spend significantly more time than WTs in the open zone of the zero maze, NPYGAD1 transgenics' lack of preference for a "safer," "less anxious" environment indicates an anxiolytic-like and/or less risk-averse phenotype. Wild type mice, including ours, typically spend significantly more time in the closed zone than the open zone of the zero maze, however NPYGAD1 mice failed to do so (paired t-test: $t(11) = 1.207$, $p = 0.253$; **Figure 7c**). Similarly, they had a significantly reduced aversion to the light box compared to littermate controls in the light-dark box task (two-tailed independent samples t-test: $t(20) = -2.247$, $p = 0.036$; **Figure 7f**).

The Porsolt forced swim task (Cryan and Holmes, 2005) measured depression-like behaviors by the duration each animal attempted to escape after being placed in a cylinder of water (latency to float) and the total immobility time during the session. Shorter latency to float and/or increased total immobility times are considered depression-like behaviors in rodents (Cryan and Holmes, 2005). There were no differences between groups in either latency to float (independent samples t-test: NPYGAD1 $t(20) = -0.538$, $p = 0.596$; CCKGAD1 $t(22) = 0.279$, $p = 0.783$; **Figure 7d**) or total immobility (independent samples t-test: NPYGAD1 $t(20) = -0.291$, $p = 0.774$; CCKGAD1 $t(22) = -0.078$, $p = 0.939$; **Figure 7e**).

Prepulse inhibition (PPI) tested the animals' sensorimotor-gating capabilities (Geyer et al., 2002). Briefly, varying levels of weaker auditory prepulse stimuli preceded stronger auditory bursts. The startle response elicited by the burst is typically inhibited by stronger prepulses. Data from one NPYGAD1 mouse was removed due to equipment malfunction. All groups showed appropriate levels of inhibition to each of four prepulse levels and there were no significant differences in percent PPI (two-way repeated measures ANOVA: NPYGAD1 $F(19) = 0.036$, $p = 0.852$; CCKGAD1 $F(22) = 0.000$, $p = 0.998$; **Figure 7g**) or in baseline acoustic startle in the absence of the prepulse (independent samples t-test: NPYGAD1 $t(19) = -0.788$, $p = 0.440$; CCKGAD1 $t(22) = 1.166$, $p = 0.256$; **Figure 7h**).

We tested hippocampal function using the y-maze spontaneous alternation task (Gerlai, 1998). All groups alternated at the expected level for mice, approximately 65-70%, and there were no differences between groups (independent samples t-test: NPYGAD1 $t(20) = -0.551$, $p = 0.587$; CCKGAD1 $t(22) = -1.370$, $p = 0.184$; **Figure 7i**).

Social behavior was evaluated using the three-chamber social task (Silverman et al., 2010). After an acclimation phase (10 min), the first interaction phase (10 min) tested sociability by comparing the test mouse's preference for investigating a social stimulus mouse (first novel mouse) placed in a pencil cup in one side chamber instead of an empty pencil cup in the opposite chamber (novel object). The second interaction phase (10 min) measured a preference for social novelty by introducing a second stimulus mouse (second novel mouse) and comparing the interaction time between the

test mouse and the second novel mouse or the first novel mouse (now the “familiar” mouse). All groups showed a preference for the first novel mouse over the novel object (**Figure 7j**). However, during the second interaction phase NPYGAD1 mice showed a significantly stronger preference for social novelty (two-tailed independent samples t-test; $t_{(20)} = 2.178$, $p = 0.042$; **Figure 7k**).

In addition to the social interaction task, we tested olfactory sensory capability and sensitivity to social cues (Silverman et al., 2010). This is an important control for the social interaction task since rodent social investigation is based mostly on olfaction. It is also a measure of a different aspect of exploratory behavior. Mice were presented with a series of social and nonsocial odors in a clean cage. CCKGAD1 mice spent significantly more time sniffing the almond (two-way repeated measures ANOVA, $F_{(1,22)} = 7.231$, $p<0.05$) and orange (two-way repeated measures ANOVA, $F_{(1,22)} = 5.831$, $p<0.05$) nonsocial odors than littermate controls (**Figure 7l**) and showed a trend towards increased investigation of the social odors (two-way repeated measures ANOVA, $F_{(1,22)} = 3.035$, $p = 0.095$).

Contextual and cued fear conditioning assessed hippocampus- and amygdala-dependent learning and memory in a single task developed for mice (Smith et al., 2007a) with minor modifications. Contextual fear conditioning measures the ability of an animal to associate an aversive event, in this case a mild footshock, with the environment or “context” in which it occurred. Similarly, cued fear conditioning measures the ability of an animal to associate an aversive event with a discrete

stimulus, in this case an auditory tone. “Learning” is the ability to form these associations and “memory” is the ability to exhibit them some time after. “Extinction” is a separate adaptive learning process that the previous association is no longer relevant. Contextual fear conditioning relies heavily on hippocampal circuitry (Fanselow, 2000) while cued fear conditioning is dependent on the amygdala (Fanselow and Gale, 2003). Interestingly, the amygdala appears to be involved in the consolidation of context fear learning, but not when animals are pre-exposed to the apparatus (Huff et al., 2005) as we did in this study. Extinction of fear memory involves neural plasticity in the cortex, amygdala, and hippocampus that supersedes the previously learned associations (Maren and Quirk, 2004; Likhtik et al., 2008; Peters et al., 2009). All groups rapidly learned to associate the tone with the shock, as shown by an increase in freezing behavior with each tone presentation, and reached high levels of freezing (**Figure 7m**). Elevated freezing during the initial 3 min in the training context compared to freezing in the testing context operationally defines contextual fear memory (Smith et al., 2007a). All groups displayed appropriate contextual fear memory (**Figure 7n**). Cued fear memory is defined by an increase in freezing behavior during the tone relative to the novel context baseline (Smith et al., 2007a). All groups displayed appropriate cued fear memory (**Figure 7o**). In addition, a significant decrease in freezing with repeated cue exposures in the absence of the shock indicated appropriate fear extinction (**Figure 7o**).

The dopamine system is dysregulated in a number of dysfunctional behaviors and neuropsychiatric disorders. One way to assess dopaminergic circuit function is to test the animals’ response to psychostimulants. In this experiment, we measured

locomotor responses following an amphetamine injection (3 mg/kg). Amphetamine sensitivity was defined here as the change in locomotor activity following the amphetamine injection. NPYGAD1 mice displayed an approximately 600% greater peak response to amphetamine than littermate controls (two-way repeated measures ANOVA, time x genotype interaction $F_{(11)} = 6.3646$, $p = 0.000$) while, in a separate experiment, CCKGAD1 mice showed an approximately 50% reduced peak response (two-way repeated measures ANOVA, time x genotype interaction $F_{(11)} = 2.015$, $p = 0.028$; **Figure 8a**). NPYGAD1 displayed significantly greater AMPH-induced locomotor activity compared to WT controls over the entire session ($t_{(20)} = 4.477$, $p = 0.000$; **Figure 8b**). Stereotypical behaviors, generally defined as repetitive movements in a spatially confined area, result from pronounced striatal activation which can be induced with a high dose of psychostimulants drugs or conceivably though a striatal system predisposed to higher levels of activity (Canales and Graybiel, 2000). When evaluating transgenic mice, a decrease in locomotor activation following psychostimulant drug exposure, as seen in the CCKGAD1 transgenic mice, can indicate either a reduction or increase in sensitivity to the drug. In the latter case, the same dose of drug may be in the locomotor activating range of the dose-response curve in a normal animal, but into the stereotypy-inducing higher range of the curve in a hypersensitive animal. In this study, there were no differences in motor stereotypies as scored by MED Activity software indicating that the CCKGAD1 transgenic mice are in fact hyposensitive to AMPH (data not shown).

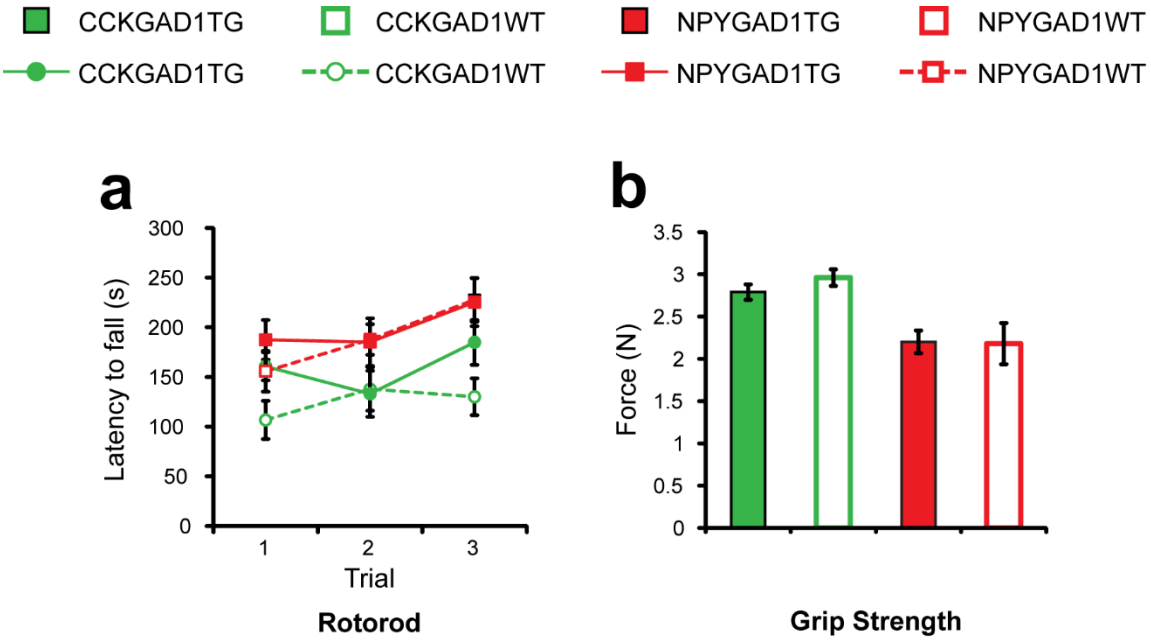


Figure 6. GAD1 does not affect basic neuromuscular performance. There were no differences between groups on the accelerating rotorod (**a**) or grip strength (**b**).

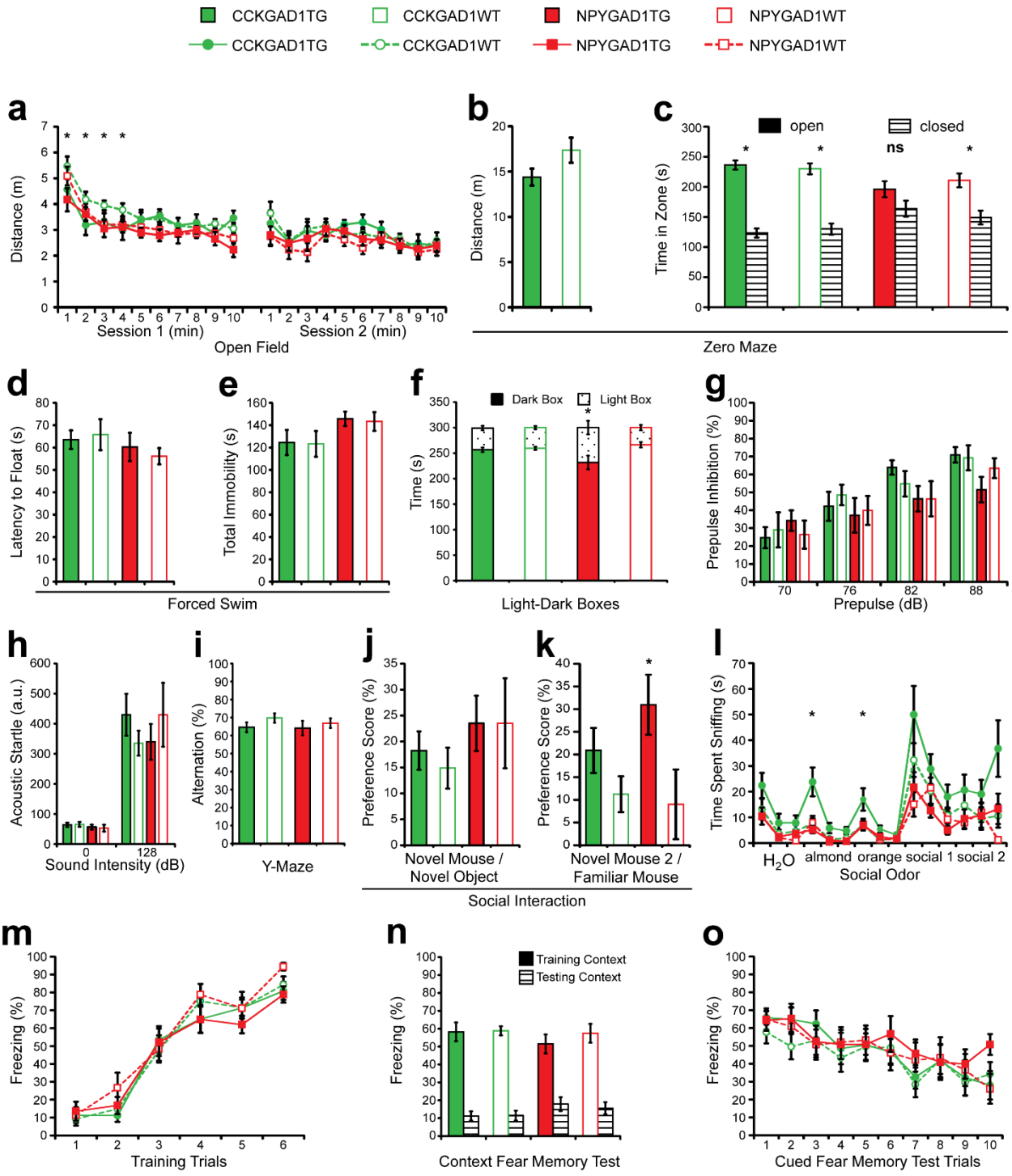

CCKGAD1TG
CCKGAD1WT
NPYGAD1TG
NPYGAD1WT
a
Distance (m)
Session 1 (min)
Session 2 (min)
Open Field
b
Distance (m)
c
open
closed
ns
Time in Zone (s)
Zero Maze
d
Latency to Float (s)
e
Total Immobility (s)
Forced Swim
f
Dark Box
Light Box
Time (s)
Light-Dark Boxes
g
Prepulse Inhibition (%)
Prepulse (dB)
h
Acoustic Startle (a.u.)
Sound Intensity (dB)
i
Alternation (%)
Y-Maze
j
Preference Score (%)
Novel Mouse / Novel Object
k
Preference Score (%)
Novel Mouse 2 / Familiar Mouse
Social Interaction
l
Time Spent Sniffing (s)
H2O
almond
orange
social 1
social 2
Social Odor
m
Freezing (%)
Training Trials
n
Training Context
Testing Context
Freezing (%)
Context Fear Memory Test
o
Freezing (%)
Cued Fear Memory Test Trials

Figure 7. GAD1 suppression has a cell type-specific impact on behavior. Adult male mice were evaluated in an extensive behavioral testing battery. Mice with GAD1 downregulation suppressing BAC constructs in CCK+ (CCKGAD1) or NPY+ (NPYGAD1) interneurons displayed different patterns of behavior compared to wild type littermate controls. CCKGAD1 mice were hypoactive in the open field **(a)** and on the zero maze **(b)**. NPYGAD1 mice did not spend more time in the closed zone of the zero maze **(c)**. There were no difference in latency to float **(d)** or total immobility **(e)** in the forced swim test. NPYGAD1 mice had a significantly reduced preference for the dark box in the light-dark box anxiety test **(f)**. There were no differences in prepulse inhibition **(g)** or acoustic starte response **(h)** (a.u., arbitrary units). All groups displayed normal alternation in the y-maze **(i)**. In the three-chambered social task, both mouse lines displayed normal sociability **(j)**, however NPYGAD1 mice had a significantly increased preference for social novelty **(k)**. CCKGAD1 mice spent significantly more time investigating a non-social olfactory stimulus but all groups investigated social olfactory stimuli similarly **(l)**. Finally, there were no differences in learning **(m)** or memory of contextual **(n)** or auditory cued **(o)** fear conditioning or cued fear extinction **(o)**. *, $p<0.05$; ns, non-significant; a.u., arbitrary units.

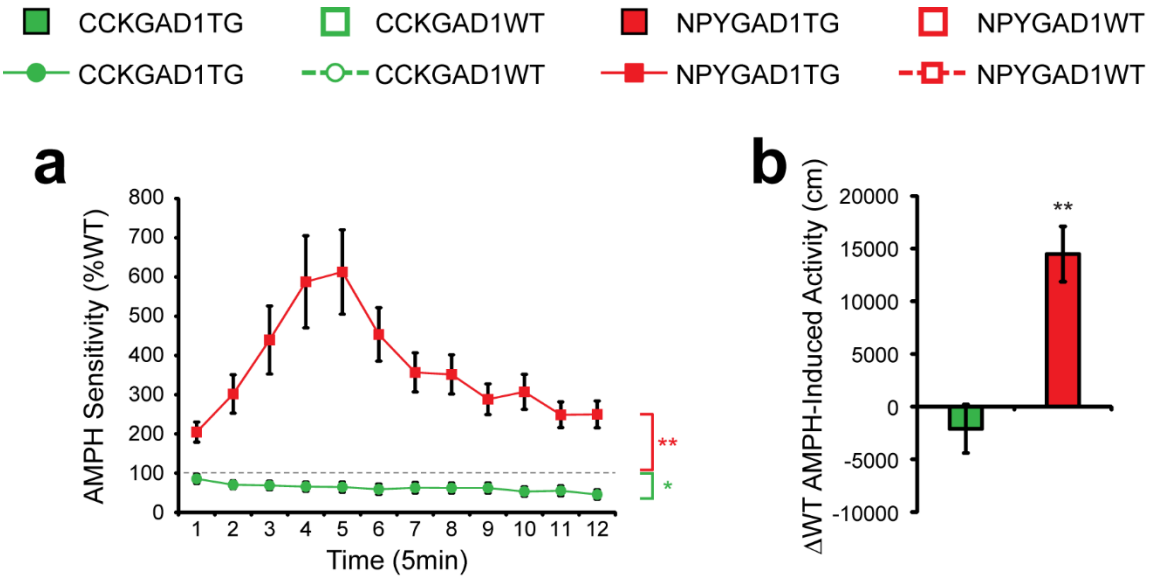


Figure 8. GAD1 suppression has cell type-specific augmentation or attenuation of amphetamine-induced locomotion. All mice displayed increased locomotor activity in response to a 3mg/kg injection of amphetamine. AMPH sensitivity was defined as the magnitude of the locomotor response after AMPH injection relative to baseline locomotor activity. NPYGAD1TG mice were approximately 600% more sensitive to AMPH compared to wild type littermate controls while CCKGAD1TG mice were approximately 50% less sensitive (**a**). Total locomotor activity across the entire post-injection session was decreased in CCKGAD1TG mice and increased in NPYGAD1TG mice compared to WT littermate controls (**b**) **, $p<0.01$. *, $p<0.05$.

CHAPTER 5

DISCUSSION

"Everything we hear is an opinion, not a fact. Everything we see is a perspective, not the truth."

— Marcus Aurelius

Cholecystokinin (CCK) and neuropeptide Y (NPY) are expressed in non-overlapping interneuron cell types with distinct morphology, connectivity, firing patterns, and distributions in both rodents and humans (Chronwall et al., 1985; Hornung et al., 1992; Meziane et al., 1997; Markram et al., 2004). Since these molecular markers do not overlap and since these distinct cell types have both been implicated in neuropsychiatric disorders (Hashimoto et al., 2008b; Truitt et al., 2009; Curley and Lewis, 2012), it is possible that their dysfunction will lead to distinct and independent effects and that dysfunction of one or both classes underlies different aspects of behavior spectrum disorders (Adam, 2013). GAD67 was suppressed below detectable levels in NPYGAD1 (Garbett et al., 2010) and CCKGAD1 transgenic mice (Schmidt et al., 2013). The development of these mice allows for the empirical study of the effects of GAD67 downregulation in specific cell types on molecular and behavioral processes of the brain. Using the BAC method simplifies the interpretation of the data because mice are generated on C57BL/6 congenic backgrounds, eliminating issues regarding strain background and backcrossing (Garbett et al., 2010).

Previous studies have detailed interneuron cell type diversity to the extent of their synaptic contacts onto principle cells and other interneurons (Markram et al., 2004; Ascoli et al., 2008). CCK+ and NPY+ interneurons represent only 8-17% of interneurons (Uematsu et al., 2008; Xu et al., 2010) and an even smaller percentage of all brain cells. Yet, we found that altering their GABA-ergic phenotype is sufficient to induce widespread, brain region-specific changes in a considerable number of lipids, peptides, and proteins. Since these two non-overlapping interneuronal subpopulations have vastly different morphology, physiology, receptor expression, brain distribution and molecular content, it is perhaps not surprising that their inactivation results in fundamentally different molecular changes.

However, it was somewhat unexpected that in both transgenic animal lines a single molecular manipulation (GAD1 suppression) led to region-specific molecular changes. Of these, we found PEP19/PCP4 to be increased in the hippocampus and cortex in NPYGAD1 transgenic line as a result of GAD1 suppression (1.55-fold, $p = 0.047$ and 1.516-fold, $p = 0.021$, respectively), but not in the CCKGAD1 animals. Discovering altered expression of a peptide previously linked to neurodevelopment (Mouton-Liger et al., 2011; Daniels et al., 2012), dopamine system function (Harashima et al., 2011; Romanova et al., 2012), and neuropsychiatric disorders (Teyssier et al., 2011; Daniels et al., 2012; Guillozet-Bongaarts et al., 2013) as a result of empirically modifying another (Lewis et al., 2005) supports the concept that diverse genetic factors can converge onto common molecular and behavioral dysfunction (Harrison and Weinberger, 2005; Mirnics et al., 2006; Horvath and Mirnics, 2009).

MALDI-IMS imaging mass spectrometry was developed with primarily for cancer research (Schwamborn and Caprioli, 2010). Comparing cancerous cells with normal adjacent tissue has benefits of isolating the molecular profile of the cancer cells from normal cells from the same region in the same individual. It also adds an underappreciated aspect of experimental design which is that all of the comparisons are done within sections on the same MALDI target plate during the same experimental scanning run. To our knowledge, this set of experiments is the first time that MALDI-IMS has been used to compare different groups of tissue with each other. This advancement of the technology represents a useful new application for studies in basic and clinical neuroscience that make between-groups comparisons. It presented a number of challenges, but we were able to rely on the Mirnics lab's expertise in gene expression analysis to use a pairwise average log ratio and false discovery rate statistical analyses typically used in microarray experiments to compare wild type and transgenic tissue from different animals on the same target plates during the same scanning run. This approach reduced some of the difficulties with standardization and variability between plates and scanning runs to yield usable data. However, further development will be useful to streamline this process. A standard solution or a reference section from the same wild type animal placed on every plate on every scanning run would benefit the normalization of data across plates. Further development of analysis and imaging software used for this type of analysis would generate images from this normalized data and introduce the application of fMRI and PET analysis techniques to the region-specific analysis of proteomics data. Between

plate variability and image-generation from data that are not normalized currently precludes the use of these useful analysis strategies.

We also found that GAD67 suppression in distinct interneuron cell types induced specific and in some cases opposing behavioral changes that may indicate region-specific dysfunction. Several interesting conclusions can be drawn from these data. First, suppressing GAD1 in as few as 8-12% of GABA-ergic interneurons is sufficient to induce molecular and behavioral changes that are dependent on the interneuron cell type affected. Second, GAD1 suppression in NPY+ and CCK+ interneurons leads to opposing effects on dopamine-dependent behavior highlighting the complex interplay between GABA-ergic and dopaminergic systems. Third, the pattern of behavioral results raises the possibility that dysfunction of distinct interneuron classes is related to different behavioral domains.

Recent findings suggest GAD1 expression and behavioral dysfunction are tightly correlated and that subtle decreases in GABA signaling give rise to behavioral changes (Chao et al., 2010; Heldt et al., 2012). Our data suggest that this modulation is dependent on the interneuron cell type and may be region specific. Some of our findings could have relatively straight-forward interpretations. For example, increased olfactory investigation in CCKGAD1 mice may be explained by disrupting the inhibition of primary olfactory cortex which has a high density of CCK+ interneurons (Meziane et al., 1997; Ekstrand et al., 2001). Yet our most intriguing finding, the opposing effects of amphetamine on locomotor behavior, is likely to be far more complex. We propose that

the opposite response of NPYGAD1 and CCKGAD1 mice to amphetamine is due to alterations in dopaminergic tone as a result of hippocampal dysfunction. NPY+ and CCK+ interneurons are both distributed in the hippocampus (Chronwall et al., 1985; Meziane et al., 1997), but they serve very different functions. NPY+ neurogliaform cells maintain tonic inhibition of entire regions through volume transmission (Olah et al., 2009; Karayannis et al., 2010). Disinhibiting hippocampal circuits by suppressing GAD1 in these cells can drive the activity of dopamine neurons in the ventral tegmental area (VTA) (Lisman et al., 2008) resulting in increased sensitivity to amphetamine and reduced anxiety-like behaviors (Zweifel et al., 2011). In contrast, CCK+ basket cells regulate parvalbumin+ (PV+) basket cells through synaptic contacts (Karson et al., 2009). By disinhibiting PV+ cells, GAD1 suppression in CCK+ interneurons could result in a net increase of inhibitory tone in the hippocampus that would diminish hippocampal-VTA loop signaling and lead to locomotor hypoactivity and reduced sensitivity to amphetamine. Alternatively, the divergent behavioral results could also be explained by disrupting amygdalar (Truitt et al., 2009) or striatal circuits (English et al., 2012), however GAD1 suppression in the amygdala alone does not change anxiety-like behavior (Heldt et al., 2012) and tonically disinhibiting the striatum would likely augment the hippocampal-VTA loop activity mentioned above. Clearly, these hypotheses will have to be tested in further experiments. However, regardless of the exact mechanism at work, it is fascinating to consider that specific GABA-ergic circuits can modulate and potentially unbalance the dopamine system in opposite directions and what that means for behavioral spectrum disorders.

Data presented here from our lab and others clearly demonstrate that diverse and distinct interneuron populations regulate a broad range of brain functions in region-specific ways and that these functions are critical for generating a large spectrum of behaviors. Therefore, and as we have shown, dysfunction of particular interneuron populations or combinations of multiple populations can result in specific dysfunction within the behavioral spectrum. It is well understood that psychiatric disorders are disorders and/or syndromes of this behavioral spectrum (Adam, 2013). We present the possibility that dysfunction of one or more classes of interneurons underlies behavioral dysfunction in some patients and that the diversity of this dysfunction may represent, at least in part, the diversity of symptoms along the behavioral spectrum.

These data have also broader, conceptual implications. The idea of the anatomical substrate defining specific brain function has been around for almost a century: in 1929, Herrick proposed that fundamental anatomical building blocks govern complex behavioral responses (Herrick, 1929). More recently, a hypothesis emerged that large networks can be controlled by diverse subnetworks (Liu et al., 2011) and further evidence suggests different network dynamics of interneuron classes underlie behavioral function (Kvitsiani et al., 2013). We believe that our findings provide strong behavioral and molecular support for this view while expanding the scope beyond a single brain region and elaborating upon the behavioral impact of cell type-specific interneuron dysfunction. In this context, we can consider the interneurons as anatomically encoded, critical modular building blocks that are directly responsible for various behavioral domains. Thus, silencing different inhibitory subnetworks, driven by various inhibitory interneuronal

subclasses, will lead to different behavioral phenotypes. Furthermore, modulating the inhibitory subnetworks will not only alter the behavior, but also result in brain and lipid composition disruption in the homogenous excitatory network. We also hypothesize that in pathological conditions, such as schizophrenia, the modular building blocks of the inhibitory subnetworks might be related to various symptom domains, either by direct effect on inhibitory subnetworks, or indirectly, through secondary effects on the relatively homogenous excitatory network.

In addition to the overarching conceptual implications arising from the diverging results following a common genetic manipulation, these data provide new information about the functional differences between these two interneuron cell types. NPY+ and CCK+ interneurons are fundamentally different and these studies provide interesting data regarding the development and function of each type and their participation in the development and function of the brain. NPY+ interneurons mediate tonic GABA signaling through extrasynaptic volumetric neurotransmission (Olah et al., 2009) while CCK+ interneurons regulate pyramidal cells as well as other interneurons via accommodating (long and integrated) synaptic input (Freund and Katona, 2007). These cell types demonstrate the concept that GAD67 maintains tonic GABA release while GAD65 mediates phasic release (Esclapez et al., 1994; Soghomonian and Martin, 1998). CCK+ interneurons fire phasically and appear to rely much more on GAD65 than other cell types (Fish et al., 2011). In fact, a subset of these cells do not express GAD67 at all in wild type tissue (Ferezou et al., 2002) suggesting that a portion of CCK+ interneurons would not be affected by our GAD67-targeting miRNA. Our results in

CCKGAD1 mice show that GAD67 downregulation in these cells does have an impact on the molecular and behavioral profile of brain function. However, the effects were modest in comparison to NPYGAD1 mice which is consistent with the interpretation that CCK+ interneurons may rely more on GAD65 than GAD67 (Fish et al., 2011) and that tonic inhibition mediated by NPY+ interneurons relies on GAD67.

Our results in CCKGAD1 mice also inform the presumed interaction of CCK+ and PV+ basket cells in the context of mental illness. It is generally assumed that GAD67 downregulation observed in mental illness has a disinhibitory effect. However GAD67 downregulation in CCK+ interneurons could disinhibit PV+ basket cell-mediated perisomatic inhibition and result in a net increase of inhibition (Freund and Katona, 2007). Curley and Lewis proposed that gene expression changes in these cells in the cortex of individuals with schizophrenia augment CCK+ and decrease of PV+ synaptic strength with respect to perisomatic modulation of pyramidal cells (Curley and Lewis, 2012) and this interaction is critical for working memory function (Fuchs et al., 2007; Timofeeva and Levin, 2011; Curley and Lewis, 2012). As stated previously, this is difficult to interpret since feedback inhibition of CCK+ basket cells by pyramidal cells is likely impaired by concurrent decreases of CB1 mRNA and CCK mRNA (Lewis et al., 2005; Lee et al., 2011). Furthermore, CB1 mRNA reduction in CCK+ cells could result in increased GABA release onto PV+ basket cells (Katona et al., 1999) while CCK mRNA reduction could result in decreased PV+ cell stimulation (Foldy et al., 2007; Lee et al., 2011). If the interaction between these two cell types mediates working memory, we might expect to see a disruption or enhancement of working memory function. We

found no changes in learning, memory, or working memory following GAD67 suppression in CCK+ interneurons. These data can be interpreted in two ways. First, this synapse might not require GAD67, which would be supported by the relatively high expression of GAD65 in CCK+ basket cells (Fish et al., 2011). Second, CCK+ basket cell regulation of PV+ interneurons in general is not necessary for learning, memory, and working memory processes *in vivo*, which would be contradictory to *in vitro* data that suggests this interaction integrates subcortical inputs to maintain working memory (Freund, 2003; Freund and Katona, 2007; Varga et al., 2009). Further experiments measuring gamma and theta oscillations in CCKGAD1 mice *in vivo* or increasing the working memory load of the behavioral analysis may clarify the interpretation of these results.

Experiments with NPYGAD1 transgenic mice also provide insight into potential mechanisms of neuropsychiatric disease and treatment. NPY+ interneurons express neurosteroid-sensitive $GABA_A$ delta receptor subunits (Olah et al., 2009). Stimulation of these receptors inhibits networks of NPY+ neurogliaform interneurons that maintain tonic inhibition through volume transmission (Olah et al., 2009) and disinhibits a region. Glutamatergic neurons also express neurosteroid-sensitive receptors at different levels across development (Shen et al., 2007; Smith, 2013). Allopregnanolone is a neurosteroid that is increased during stress. Our experimental manipulation, which mimics the same effect of neurosteroid receptor stimulation on NPY+ neurons consistently throughout development and adulthood in male mice by silencing neurogliaform cells, is interesting in the context of gender differences in development

and neuropsychiatric disorders. We show that GAD67 downregulation in these cells *in vivo* leads to an anxiolytic-like phenotype in multiple behavioral assays and a dramatically increased sensitivity to amphetamine. This result is similar to the effects of allopregnanolone administration in male rodents during early postnatal development (Darbra and Pallares, 2012) and adulthood (Rodgers and Johnson, 1998), however changing levels of neurosteroid-sensitive GABA receptors on projection neurons during female adolescence produces anxiogenic effects (Shen et al., 2007). Similar anxiogenic behavior results from fluctuations of neurosteroid circulation during the menstrual cycle in females (Smith, 2001; Gulinello and Smith, 2003; Smith et al., 2006). Taken together, these results and ours suggest that neurosteroid receptors on NPY+ interneurons mediate anxiolytic effects of neurosteroids in males. It is possible that anxiogenic effects of neurosteroids in females relate to changing expression in glutamatergic neurons (Shen et al., 2007). Comprehensive analysis of NPYGAD1 female animals has not been done to date, but could shed light on this issue. Administration of neurosteroids to NPYGAD1 male mice could also shed light on the relative contributions of these receptors on interneurons and pyramidal cells on anxiety-like behavior. Another possible explanation for these anxiogenic-like effects is that NPY+ interneurons in the striatum directly regulate striatal projection neurons and VTA reward circuitry (Koos and Tepper, 1999; Tepper and Lee, 2007; Tepper et al., 2010; Ibanez-Sandoval et al., 2011; English et al., 2012) and silencing these cells by GAD67 suppression or neurosteroid application could increase dopaminergic tone and reduce anxiety-like behavior (Zweifel et al., 2011). Clarification of these possibilities relies on the interpretation of the behavioral data.

Anxiety-like behavior in rodents relies on the innate avoidance of bright, open spaces (Rodgers et al., 1997; Cryan and Holmes, 2005; Rodgers, 2010). Other measures in these types of tasks claim to measure "risk-assessment" by the rodents' cautious exploration of the interface between a "safe" area and an open "risky" area (Rodgers and Johnson, 1995; Griebel et al., 1997) however these terms rely on anthropomorphism. Regardless, "risk-assessment" and "risk-avoidance" or "risk-taking" are not the same thing. Risk-taking refers to the probability of executing behavior that could have a dangerous or negative outcome (Schultz, 2011). It does not refer to the amount of time an organism takes to make that decision. Increased exploration of an open environment that poses an innate predatory risk to a rodent can demonstrate increased risk-taking behavior and/or decreased risk-avoidance behavior in the absence of any change in risk-assessment or anxiety. In fact, there is some evidence that anxiety and "anxiety-related risk-avoidance behavior" involve separate circuitry (Kim et al., 2013). These distinctions may sounds like semantics, but have real world consequences. Blocking nicotinic receptors (nAchR) on NPY+ interneurons, effectively silencing them as we have, induced a similar pattern of behavior to what we have shown (Lippiello et al., 2008). Targacept and Astra Zeneca, the companies developing the nAchR antagonist used by Lippiello and colleagues, interpreted the data as an anti-anxiety-like and possible anti-depressant-like effect and ran clinical trials for patients with depression which failed (data unpublished). Removing anthropomorphism from the analysis would have lead to a dopamine-centric interpretation of the rodent data since dopamine clearly regulates these behaviors (Wall et al., 2003; Marowsky et al., 2005;

Pape, 2005; Diaz et al., 2011; Zweifel et al., 2011) and may have resulted in exploring this compound as a potential treatment for a number of disorders with dopamine system abnormalities. For neuropsychiatric disorders, symptom improvement is behavior-based by definition. The careful interpretation of behavioral phenotypes in our mice and other rodent models is critically important to understand mechanisms of neuropsychiatric disease and developing treatments. In fact, the number of first-in-class drugs approved by the FDA between 1998 and 2008 increasingly favors phenotypic screening (Swinney and Anthony, 2011) which will make behavioral analyses and interpretation even more important in the years to come.

In summary, we have shown that GAD67 downregulation in as few as 8-17% of GABA-ergic interneurons has specific molecular and behavioral effects that are dependent on the type of interneuron affected. Molecular changes downstream of GAD67 suppression include proteins that have also been identified in neuropsychiatric disorders, indicating that they may be part of a convergent molecular network leading to brain and behavioral dysfunction. Behavioral analyses showed that the effects of GAD67 downregulation are most prominent in cells that mediate tonic inhibition of brain regions and less prominent in cells that have relatively abundant GAD65. Bi-directional modulation of amphetamine sensitivity by two distinct interneuron classes suggests that dopaminergic dysfunction in a number of brain disorders may modulate positioning on the neuropsychiatric spectrum. However, over-interpretation of data from rodent studies can be misleading and further clinical research in multiple patient populations will be required to fully understand these mechanisms. In many cases, these future studies will

require the development of objective methods to measure and quantify analogous behaviors and brain functions in rodents and humans which may prove difficult. However, experiments presented here, in conjunction with information about cell type-specific receptor expression, may provide opportunities to target different domains of behavioral dysfunction to reduce side effects and improve clinical symptoms.

SUPPLEMENTARY TABLES

Supplementary Table 1.

	m/z	602.2	606.4	621.4	658.1	663.3	705.4	706.4
THAL	p-value	0.291	0.333	0.891	0.004*	0.556	0.221	0.249
	ALR	0.189	-0.099	-0.009	0.275	0.060	-0.220	-0.157
STR	p-value	0.280	0.375	0.345	0.455	0.496	0.258	0.220
	ALR	-0.549	0.254	0.151	-0.352	-0.044	0.435	0.393
SEPTUM	p-value	–	0.598	0.392	–	–	0.481	0.521
	ALR	–	0.218	0.161	–	–	0.239	0.193
MFC	p-value	0.326	**0.011**	**0.029**	0.400	–	**0.014**	**0.046**
	ALR	-0.613	0.254	0.104	-0.370	–	0.271	0.221
HYTH	p-value	–	0.371	0.300	–	0.990	0.403	0.406
	ALR	–	-0.501	-0.157	–	0.002	-0.380	-0.341
HIPP	p-value	0.813	0.412	0.482	0.089	0.410	0.472	0.540
	ALR	-0.032	-0.114	-0.065	0.128	-0.070	-0.144	-0.103
CTXS	p-value	0.362	0.169	0.268	0.510	0.501	0.091	0.083
	ALR	-0.628	0.203	0.137	-0.339	-0.099	0.360	0.321
CTXH	p-value	**0.018**	0.925	0.603	**0.040**	**0.012**	0.441	0.487
	ALR	0.337	-0.015	-0.042	0.255	0.124	-0.207	-0.160
CORPS	p-value	0.398	0.190	0.090	0.657	0.564	0.129	0.100
	ALR	-0.683	0.460	0.312	-0.142	-0.124	0.693	0.556
CORPH	p-value	0.036*	0.708	0.572	0.871	**0.003**	0.622	0.698
	ALR	0.146	-0.113	-0.067	-0.029	0.250	-0.194	-0.126

767.7	784.7	822.8	824.7	834.8	838.9	850.9	866.9	884.7	896.7	909.2
0.232	0.234	0.669	0.495	0.936	0.671	0.743	0.954	**0.026**	_	_
-0.230	-0.303	-0.070	-0.105	-0.007	-0.071	-0.051	-0.008	0.180	_	_
0.040	0.126	**0.040**	0.139	0.563	**0.013**	**0.032**	0.726	_	_	_
0.585	0.490	0.311	0.335	0.104	0.103	0.123	-0.044	_	_	_
0.160	0.282	**0.044**	**0.035**	0.671	0.632	0.317	0.377	_	_	_
0.417	0.407	0.333	0.338	0.117	0.075	0.181	0.166	_	_	_
0.231	**0.028**	0.440	0.275	0.699	0.688	0.880	0.923	_	_	0.185
0.341	0.210	0.279	0.299	0.083	0.268	0.086	0.084	_	_	-0.509
0.070	0.077	0.209	0.255	0.205	0.330	0.359	0.338	_	_	_
-0.494	-0.161	0.289	0.155	0.224	0.509	0.435	0.480	_	_	_
0.270	0.257	0.411	0.323	0.179	0.334	0.364	0.423	_	_	**0.018**
-0.206	-0.211	-0.220	-0.261	-0.104	-0.265	-0.191	-0.215	_	_	0.221
0.146	0.095	0.284	0.354	**0.043**	0.525	0.765	0.976	_	0.419	_
0.383	0.313	0.279	0.246	0.110	0.204	0.089	-0.016	_	-0.213	_
0.079	0.232	0.254	0.262	0.325	0.378	0.498	0.616	_	**0.018**	_
-0.349	-0.300	-0.325	-0.287	-0.120	-0.274	-0.175	-0.159	_	0.125	_
0.172	0.159	0.357	0.871	0.133	0.194	0.354	0.357	0.147	_	_
0.676	0.668	-0.322	0.030	-0.468	-0.582	-0.465	-0.542	-0.325	_	_
0.202	0.424	0.050	0.246	0.030*	0.064	0.053	**0.048**	0.067	_	_
-0.370	-0.204	0.563	0.184	0.487	0.846	0.680	0.753	0.445	_	_

963.8	966.9	968.8	972.6	1069.9	1085.0	1114.0	1114.8	1122.9	1157.8	1158.8
0.358	0.058	0.284	0.803	_	0.492	0.253	0.190	0.684	0.459	0.289
0.152	0.167	0.095	0.039	_	0.143	0.214	0.220	0.056	0.174	0.234
0.326	**0.032**	**0.010**	_	_	0.114	0.208	0.168	0.067	0.174	_
-0.368	-0.172	-0.152	_	_	-0.475	-0.640	-0.583	-0.470	-0.429	_
0.606	0.566	0.141	_	_	0.175	0.670	_	0.541	0.092	_
-0.172	-0.021	-0.085	_	_	-0.573	-0.126	_	-0.257	-0.413	_
0.459	_	_	_	_	0.296	_	_	_	0.683	_
-0.304	_	_	_	_	-0.455	_	_	_	-0.024	_
0.047	0.060	0.143	_	0.134	0.490	0.145	_	0.072	0.238	_
0.122	0.214	0.142	_	0.477	0.149	0.479	_	0.083	0.352	_
0.167	0.485	0.993	_	_	0.563	_	_	0.562	0.064	_
0.099	0.047	0.001	_	_	-0.069	_	_	0.070	0.315	_
0.366	0.367	0.394	_	0.150	0.232	0.253	0.251	0.200	0.270	_
-0.388	-0.262	-0.165	_	-0.394	-0.335	-0.489	-0.427	-0.278	-0.187	_
0.175	0.054	0.314	_	**0.020**	**0.021**	**0.000**	**0.005**	**0.007**	**0.009**	_
0.197	0.149	0.093	_	0.428	0.262	0.399	0.413	0.297	0.442	_
0.104	0.161	0.072	**0.004**	_	0.087	0.482	0.274	0.344	0.182	**0.046**
-0.504	-0.495	-0.497	-0.389	_	-0.544	-0.270	-0.321	-0.270	-0.228	-0.486
0.953	0.032*	0.042*	0.106	_	0.506	0.305	0.754	0.687	0.293	0.164
-0.012	0.564	0.517	0.329	_	0.097	0.075	0.044	0.101	0.204	0.213

1173.0	1185.9	1188.9	1200.0	1201.9	1223.9	1242.0	1246.1	1259.0	1274.1	1275.9
0.511	0.200	0.402	0.167	0.120	0.293	0.261	**0.044**	0.251	_	0.205
0.145	0.194	0.151	0.198	0.253	0.102	0.202	0.295	0.193	_	0.255
0.068	0.179	0.148	**0.021**	0.244	_	0.137	0.207	0.149	0.192	_
-0.196	-0.365	-0.325	-0.200	-0.514	_	-0.448	-0.665	-0.662	-0.656	_
0.071	0.149	0.222	0.168	0.288	_	**0.035**	0.123	0.131	0.250	_
-0.320	-0.452	-0.289	-0.262	-0.291	_	-0.379	-0.474	-0.609	-0.464	_
0.206	0.241	_	0.354	0.231	_	0.207	0.357	0.310	0.276	_
-0.243	-0.314	_	-0.223	-0.139	_	-0.357	-0.123	-0.483	-0.480	_
0.237	0.040*	0.023*	0.328	0.185	_	0.070	0.188	0.118	0.172	_
0.175	0.362	0.197	0.075	0.468	_	0.217	0.669	0.247	0.220	_
0.575	0.303	0.587	0.335	**0.003**	0.367	0.286	0.112	0.441	0.297	_
0.100	0.136	0.113	0.163	0.224	0.114	0.136	0.322	0.157	0.208	_
0.095	0.165	0.138	**0.026**	0.195	_	0.188	0.227	0.233	0.258	0.223
-0.192	-0.329	-0.308	-0.200	-0.376	_	-0.336	-0.388	-0.461	-0.499	-0.516
0.049	**0.026**	**0.043**	0.071	**0.015**	_	0.085	0.069	**0.033**	**0.008**	**0.002**
0.273	0.340	0.378	0.255	0.422	_	0.296	0.428	0.320	0.404	0.394
0.271	0.346	_	_	0.064	**0.017**	_	**0.005**	0.086	0.444	_
-0.184	-0.238	_	_	-0.087	-0.598	_	-0.475	-0.514	-0.240	_
0.096	0.088	_	_	0.113	0.575	_	0.025*	0.963	0.253	_
0.193	0.258	_	_	0.370	0.052	_	0.393	-0.013	0.177	_

1278.2	1290.1	1294.2	1318.1	1322.1	1349.7	1352.9	1353.8	1357.2	1362.1	1394.0
0.336	**0.037**	0.098	**0.027**	0.325	0.437	0.553	0.551	0.578	0.225	0.530
0.122	0.271	0.180	0.275	0.189	0.114	0.107	0.111	0.109	0.239	0.102
0.064	0.299	**0.015**	0.232	**0.028**	0.063	_	_	0.140	0.143	0.075
-0.309	-0.691	-0.276	-0.472	-0.305	-0.255	_	_	-0.334	-0.416	-0.354
0.163	0.486	0.758	0.154	_	0.167	_	_	0.220	0.134	0.487
-0.119	-0.212	-0.020	-0.443	_	-0.319	_	_	-0.318	-0.362	-0.253
0.337	0.648	0.284	0.435	_	0.111	_	_	0.089	0.074	_
-0.262	-0.063	-0.232	-0.291	_	-0.202	_	_	-0.383	-0.291	_
0.039*	0.416	0.164	0.335	0.190	0.296	0.264	_	0.087	0.154	0.060
0.272	0.496	0.210	0.251	0.117	0.196	0.170	_	0.215	0.396	0.278
0.471	**0.023**	0.123	0.223	0.516	0.268	_	_	0.131	0.144	0.421
0.131	0.340	0.161	0.190	0.134	0.122	_	_	0.185	0.205	0.044
0.194	0.238	0.131	0.196	0.088	0.219	_	_	0.139	0.100	0.168
-0.269	-0.408	-0.154	-0.378	-0.248	-0.131	_	_	-0.260	-0.291	-0.281
0.034	0.086	**0.007**	**0.012**	0.060	**0.018**	_	_	0.058	**0.002**	**0.048**
0.346	0.593	0.329	0.441	0.322	0.235	_	_	0.250	0.397	0.338
0.716	0.202	**0.034**	0.075	0.269	**0.013**	**0.007**	**0.043**	**0.049**	0.350	0.167
-0.047	-0.219	-0.236	-0.634	-0.397	-0.404	-0.390	-0.280	-0.282	-0.173	-0.462
0.764	0.165	0.201	0.204	0.332	**0.001**	0.036*	0.023*	0.391	0.040*	0.129
-0.043	0.531	0.285	0.151	0.222	0.200	0.184	0.202	0.155	0.288	0.143

1406.2	1431.1	1432.9	1450.2	1466.3	1468.1	1491.4	1492.2	1517.2	1538.3	1549.1
0.334	0.549	0.992	0.145	0.109	0.434	_	_	0.831	0.200	0.922
0.180	0.151	0.002	0.207	0.212	0.070	_	_	-0.024	0.219	0.015
0.162	0.142	_	0.178	0.327	0.108	**0.000**	0.611	0.265	0.217	0.268
-0.381	-0.476	_	-0.609	-0.657	-0.302	-0.257	0.102	-0.139	-0.545	-0.220
0.282	0.111	_	0.177	0.287	**0.010**	_	**0.049**	0.192	0.309	0.141
-0.253	-0.437	_	-0.557	-0.191	-0.363	_	-0.164	-0.231	-0.267	-0.347
0.287	_	_	0.321	0.625	0.791	0.401	0.114	0.562	0.193	**0.002**
-0.127	_	_	-0.243	0.279	0.131	-0.163	-0.283	0.095	-0.227	-0.254
0.108	0.053	0.464	0.276	0.619	_	_	0.564	_	0.219	0.152
0.425	0.367	0.154	0.380	0.329	_	_	0.045	_	0.490	0.185
0.106	0.376	0.954	**0.028**	0.147	**0.005**	0.502	_	0.866	0.157	0.909
0.215	0.095	-0.007	0.162	0.450	0.178	0.116	_	0.019	0.138	-0.011
0.111	0.272	_	0.203	**0.040**	0.974	**0.000**	0.309	0.838	0.025*	0.450
-0.236	-0.294	_	-0.331	-0.212	0.010	-0.176	-0.074	-0.014	-0.219	-0.094
0.012	0.052	_	**0.023**	0.096	0.296	0.157	0.339	**0.017**	0.087	0.148
0.528	0.289	_	0.457	0.572	0.193	0.235	0.192	0.171	0.393	0.161
0.153	**0.003**	**0.016**	0.523	0.882	_	_	_	_	**0.005**	0.205
-0.391	-0.499	-0.610	-0.193	0.033	_	_	_	_	-0.494	-0.114
0.259	0.810	0.527	0.249	0.410	_	_	_	_	0.212	0.948
0.230	0.040	0.109	0.178	0.617	_	_	_	_	0.415	-0.012

1578.1	1583.1	1593.0	1604.1	1613.3	1626.5	1627.2	1671.1	1695.1	1715.1	1721.8
0.787	0.166	_	0.514	0.478	_	0.230	0.336	0.694	0.516	0.650
0.046	0.191	_	0.133	0.150	_	0.197	0.130	0.074	0.088	0.080
0.043	0.104	_	_	_	0.163	0.194	0.163	0.071	0.149	_
-0.372	-0.368	_	_	_	-0.569	-0.466	-0.347	-0.305	-0.389	_
0.536	0.335	0.291	_	_	**0.015**	0.097	0.122	0.416	0.306	_
-0.252	-0.198	-0.412	_	_	-0.411	-0.472	-0.341	-0.268	-0.310	_
_	0.189	_	_	_	0.844	0.522	0.435	0.385	0.195	_
_	-0.134	_	_	_	-0.049	-0.146	-0.081	-0.206	-0.189	_
0.888	0.444	_	_	_	_	0.392	0.354	0.604	0.410	_
0.028	0.158	_	_	_	_	0.260	0.329	0.117	0.274	_
0.989	**0.043**	_	0.506	_	_	0.103	0.143	0.649	0.343	_
-0.003	0.224	_	0.094	_	_	0.112	0.141	0.074	0.112	_
0.068	**0.045**	0.184	0.093	_	0.129	0.314	0.357	**0.002**	**0.036**	**0.043**
-0.291	-0.226	-0.250	-0.259	_	-0.232	-0.246	-0.120	-0.167	-0.218	-0.112
0.025	**0.003**	**0.007**	0.073	_	**0.044**	0.156	**0.012**	0.063	0.071	0.061
0.271	0.353	0.185	0.251	_	0.570	0.262	0.410	0.299	0.373	0.233
0.342	0.476	_	**0.032**	**0.008**	_	**0.005**	0.337	0.241	0.065	0.416
-0.295	-0.032	_	-0.359	-0.307	_	-0.372	-0.052	-0.268	-0.303	-0.238
0.498	0.056	_	0.154	0.447	_	0.220	0.308	0.778	0.084	0.776
0.109	0.226	_	0.093	0.112	_	0.355	0.143	0.059	0.299	0.059

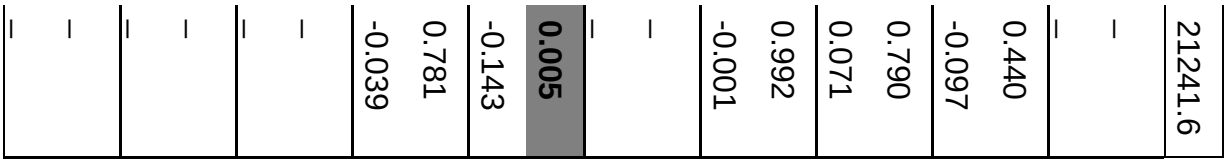
1751.3	1759.2	1794.2	1854.3	1857.6	1907.3	1934.8	1951.3	1979.3	1995.4	2039.4
0.640	_	0.996	_	0.810	0.347	0.447	0.473	_	0.438	0.420
-0.122	_	0.001	_	-0.090	0.150	0.132	0.130	_	0.162	0.140
0.113	0.159	**0.042**	**0.015**	_	0.267	**0.026**	0.229	_	0.188	0.193
-0.311	-0.416	-0.330	-0.392	_	-0.522	-0.316	-0.559	_	-0.542	-0.541
0.116	0.145	0.359	0.200	0.232	0.470	0.066	0.107	_	0.215	0.176
-0.479	-0.455	-0.288	-0.399	-0.392	-0.133	-0.311	-0.462	_	-0.437	-0.354
0.433	0.394	_	_	0.477	0.362	0.264	0.547	_	0.264	0.424
-0.279	-0.133	_	_	-0.201	0.282	-0.322	-0.030	_	-0.083	-0.144
0.319	0.194	0.447	_	_	0.351	0.246	0.269	_	0.358	0.349
0.187	0.318	0.108	_	_	0.324	0.137	0.287	_	0.301	0.186
0.398	0.755	_	_	_	**0.016**	0.316	0.070	_	0.094	0.058
-0.247	0.049	_	_	_	0.371	0.141	0.175	_	0.213	0.197
0.193	0.104	_	0.053	_	**0.039**	0.127	0.108	0.200	0.061	0.074
-0.214	-0.185	_	-0.163	_	-0.144	-0.256	-0.269	-0.346	-0.307	-0.301
0.255	**0.008**	_	0.085	_	**0.040**	0.073	**0.002**	**0.034**	**0.021**	**0.009**
0.147	0.366	_	0.172	_	0.500	0.277	0.429	0.332	0.408	0.408
0.007	0.154	_	_	**0.031**	0.921	0.136	**0.025**	_	0.168	0.063
-0.503	-0.240	_	_	-0.563	-0.005	-0.264	-0.385	_	-0.245	-0.545
0.782	0.125	_	_	0.285	0.151	0.327	0.085	_	0.085	0.022*
0.060	0.376	_	_	0.275	0.450	0.132	0.363	_	0.418	0.270

2062.2	2083.5	2127.5	2387.6	2406.5	2442.1	3097.6	3214.9	3728.0	3769.6	4118.4
0.566	_	_	0.535	0.855	0.795	0.439	0.904	**0.016**	0.759	_
0.110	_	_	0.156	0.027	0.042	0.219	0.034	-0.326	-0.033	_
0.133	0.151	0.106	_	_	0.075	_	0.815	0.085	0.802	**0.001**
-0.403	-0.463	-0.490	_	_	-0.354	_	-0.038	-0.293	0.018	-0.142
0.185	0.144	0.326	_	_	0.077	_	0.602	0.065	0.241	_
-0.527	-0.466	-0.363	_	_	-0.398	_	0.106	-0.236	-0.118	_
0.394	0.080	0.224	0.462	_	0.394	0.413	0.947	0.044*	0.797	0.563
-0.463	-0.422	-0.441	-0.355	_	-0.243	-0.493	-0.011	-0.157	0.017	-0.059
_	0.327	0.800	0.244	0.624	_	0.531	_	0.297	0.773	_
_	0.224	0.060	0.142	0.104	_	0.097	_	-0.285	-0.096	_
0.887	0.423	_	_	0.867	0.599	_	0.367	0.141	0.347	0.981
0.020	0.150	_	_	-0.023	0.109	_	0.151	-0.173	0.130	0.008
0.305	0.134	0.129	0.129	0.266	**0.029**	_	0.962	0.081	0.202	0.457
-0.341	-0.329	-0.385	-0.418	-0.259	-0.279	_	0.003	-0.101	0.185	-0.068
0.080	**0.028**	**0.040**	0.147	**0.034**	0.099	_	**0.044**	0.259	0.657	0.328
0.258	0.419	0.335	0.326	0.282	0.320	_	0.167	-0.205	0.045	0.168
0.042	**0.012**	**0.025**	**0.028**	0.051	0.155	**0.025**	_	_	**0.045**	_
-0.695	-0.593	-0.390	-0.559	-0.516	-0.316	-0.608	_	_	0.126	_
0.216	0.448	0.114	0.391	0.451	0.586	0.306	_	_	0.615	_
0.193	0.086	0.160	0.169	0.139	0.211	0.156	_	_	-0.049	_

4138.1	4275.7	4284.8	4338.6	4419.3	4510.1	4570.5	4667.4	5021.6	5061.1	5105.3
_	_	**0.010**	**0.006**	0.281	0.875	**0.008**	0.428	**0.038**	**0.016**	0.895
_	_	0.112	-0.215	-0.197	0.035	-0.419	0.158	-0.100	-0.354	-0.012
0.022	0.833	0.623	0.207	0.317	0.229	0.218	**0.029**	0.151	0.933	0.068
-0.198	0.029	0.101	-0.103	-0.568	-0.220	-0.503	0.291	-0.327	-0.023	0.148
0.423	_	0.084	0.327	0.672	0.138	0.627	0.087	0.261	0.557	**0.004**
-0.098	_	0.356	0.060	0.095	-0.251	-0.135	0.288	-0.459	-0.176	0.158
0.323	**0.026**	0.123	0.564	0.495	0.025*	0.558	0.125	0.245	0.813	0.249
-0.119	0.135	0.220	-0.081	-0.051	-0.389	-0.173	0.439	-0.389	-0.039	0.120
_	0.482	_	0.146	_	0.132	_	0.389	0.375	_	_
_	-0.519	_	-0.267	_	0.104	_	-0.222	0.130	_	_
0.003	_	**0.010**	**0.007**	0.203	0.076	0.173	0.923	0.524	_	_
-0.302	_	0.108	-0.201	-0.125	0.197	-0.442	0.020	-0.050	_	_
0.208	0.207	0.243	0.528	**0.005**	0.052	0.612	0.087	0.285	0.514	0.144
-0.156	0.122	0.215	-0.057	-0.153	-0.286	-0.133	0.402	-0.249	-0.070	0.121
0.073	**0.006**	0.365	0.263	0.092	0.710	0.132	0.814	0.187	0.400	_
-0.178	0.184	0.134	-0.112	-0.087	0.064	-0.495	0.056	-0.154	-0.088	_
_	0.352	0.092	0.921	_	**0.010**	_	**0.041**	0.230	_	_
_	0.079	0.251	0.020	_	-0.223	_	0.444	-0.206	_	_
_	0.773	0.059	0.168	_	**0.047**	_	0.280	0.043*	_	_
_	-0.021	0.159	-0.209	_	-0.116	_	0.292	-0.180	_	_

5289.9	5378.1	5829.0	6174.0	6332.6	6725.9	6956.5	7244.8	7758.9	7864.7	8260.4
_	**0.038**	0.337	0.240	**0.014**	0.183	_	_	0.536	0.601	0.237
_	-0.127	-0.302	0.175	-0.268	0.732	_	_	0.092	0.100	-0.239
_	0.925	_	0.754	_	0.549	**0.023**	0.213	0.216	_	0.902
_	0.013	_	0.049	_	0.306	-0.282	0.115	0.270	_	0.025
0.035	0.704	**0.023**	0.790	_	0.186	0.289	**0.016**	0.964	_	0.950
-0.271	0.064	-0.171	0.026	_	0.499	-0.253	0.210	0.005	_	0.019
0.131	0.208	0.436	_	_	0.314	0.357	_	0.259	_	0.153
-0.185	0.254	0.138	_	_	0.383	-0.200	_	0.350	_	0.171
_	0.712	0.123	0.662	_	0.952	_	_	0.429	**0.048**	0.929
_	0.137	-0.493	0.109	_	0.025	_	_	-0.107	0.507	-0.019
_	0.581	0.900	**0.031**	_	**0.047**	**0.018**	_	0.840	_	**0.022**
_	-0.083	-0.020	0.164	_	0.550	-0.795	_	0.032	_	-0.287
_	0.261	0.656	_	_	0.370	_	0.639	0.403	_	0.833
_	0.132	-0.028	_	_	0.325	_	0.077	0.197	_	0.027
_	0.844	0.498	_	_	**0.021**	0.069	_	**0.044**	_	0.532
_	-0.032	0.104	_	_	0.516	-0.650	_	0.266	_	-0.078
_	0.513	_	0.655	_	0.489	0.376	_	0.243	_	0.624
_	-0.152	_	0.078	_	0.226	-0.029	_	0.387	_	0.042
_	0.345	_	0.216	_	0.121	0.376	_	0.039*	_	0.034*
_	-0.188	_	0.317	_	0.323	-0.281	_	0.212	_	-0.176

8498.9	8799.0	9087.9	9922.1	10271.3	10495.8	12421.7	12683.5	14746.8	14995.4	21150.2
0.375	0.463	**0.011**	0.392	0.194	_	0.851	**0.016**	0.555	**0.006**	_
0.139	0.205	0.167	0.067	0.177	_	-0.045	-0.192	-0.074	0.731	_
0.296	**0.013**	0.688	0.577	0.344	0.685	0.818	0.401	0.504	0.397	0.564
0.127	0.235	-0.084	0.065	0.139	0.067	-0.042	-0.091	-0.064	0.353	-0.070
0.015	**0.031**	0.854	**0.025**	0.212	0.716	0.959	0.898	0.988	0.491	0.924
0.283	0.409	-0.060	0.289	0.342	0.102	-0.013	-0.032	0.004	0.229	0.025
0.360	0.354	0.813	0.412	**0.007**	**0.038**	**0.047**	0.807	0.842	0.280	0.964
0.120	0.233	-0.051	0.181	0.326	0.279	0.234	-0.026	0.020	0.365	0.005
0.457	0.044*	0.228	0.079	0.185	_	0.883	0.348	_	0.108	_
-0.201	0.618	0.138	-0.421	-0.301	_	-0.070	-0.171	_	1.178	_
0.585	0.201	0.641	0.577	0.818	_	0.399	**0.007**	**0.001**	0.201	**0.006**
0.093	0.365	0.058	-0.100	0.042	_	-0.108	-0.202	-0.130	0.461	-0.172
0.392	0.087	0.683	0.818	0.805	0.556	0.644	0.704	0.943	0.318	0.830
0.154	0.356	-0.079	0.030	0.057	0.100	-0.061	-0.060	-0.009	0.473	-0.030
0.718	0.235	0.329	0.936	0.794	0.784	0.177	0.611	0.534	0.987	0.403
-0.072	0.129	0.101	-0.020	0.062	0.064	0.102	-0.067	-0.034	0.006	-0.098
0.001	0.542	0.312	0.349	0.291	_	0.748	_	_	0.378	_
0.215	-0.205	-0.088	0.153	0.261	_	0.092	_	_	0.362	_
0.985	0.713	0.301	0.518	0.278	_	0.215	_	_	0.839	_
-0.002	-0.089	0.052	0.166	0.168	_	0.148	_	_	0.070	_



Supplementary Table 1. MALDI-IMS analysis identifies region-specific changes in NPYGAD1 transgenic mice. *In situ* proteomic analysis identified 129 lipids, peptides, and proteins (0 – approximately 22,000 Da) with significantly altered expression in NPYGAD1 mice compared to wildtype controls. 51 were decreased, 65 were increased, and 13 had region-specific changes. Only two results (m/z 1583.09 and m/z 1907.27) were significant across more than three regions. *, significant results assessed by Benjamini-Hochberg correction for false discovery rate; _ , not significant.

Supplementary Table 2.

	m/z	606.4	705.4	706.4	724.4	757.7	773.7	790.7	804.7
THAL	p-value	0.152	0.171	0.178	0.606	0.155	0.752	_	0.768
	ALR	-0.155	-0.221	-0.197	0.074	-0.072	0.053	_	0.033
STR	p-value	0.808	0.546	0.482	0.465	_	0.655	_	0.457
	ALR	-0.070	-0.150	-0.158	-0.049	_	-0.053	_	-0.104
SEPTUM	p-value	0.966	0.323	0.286	0.560	0.298	0.493	_	**0.035**
	ALR	-0.008	-0.121	-0.135	-0.083	-0.124	-0.114	_	-0.153
MFC	p-value	0.946	0.894	0.821	**0.010**	_	_	_	0.812
	ALR	-0.020	-0.038	-0.056	0.172	_	_	_	0.026
HYTH	p-value	**0.033**	0.023*	0.018*	0.551	**0.002**	0.645	_	0.905
	ALR	0.418	0.336	0.323	0.168	0.143	0.105	_	-0.037
HIPP	p-value	0.593	0.355	0.348	**0.004**	0.192	**0.012**	_	0.898
	ALR	-0.115	-0.195	-0.191	-0.091	-0.173	-0.120	_	0.010
CTXS	p-value	0.899	0.785	0.741	0.270	0.855	0.402	_	0.904
	ALR	-0.034	-0.067	-0.074	0.180	-0.021	0.159	_	-0.010
CTXH	p-value	0.900	0.824	0.825	0.428	0.856	0.598	_	0.380
	ALR	-0.024	-0.064	-0.056	0.078	-0.026	0.046	_	-0.089
CorpS	p-value	**0.011**	**0.017**	**0.045**	0.484	0.134	0.493	**0.014**	0.122
	ALR	-0.541	-0.527	-0.439	-0.307	-0.563	-0.317	-0.272	-0.184
CorpH	p-value	0.248	0.329	0.371	0.416	0.485	0.170	0.371	0.536
	ALR	-0.272	-0.383	-0.289	-0.074	-0.104	-0.096	0.132	0.069

812.8	856.7	868.7	1099.9	1135.0	1157.8	1210.9	1246.1	1321.9	1362.1	1408.1
0.029*	0.545	0.406	**0.008**	0.977	0.791	0.565	0.760	0.761	0.861	0.916
-0.058	0.024	0.062	0.506	0.010	0.107	0.040	0.109	0.042	-0.068	-0.022
0.381	_	0.610	0.754	0.413	0.981	_	**0.010**	0.752	0.343	_
-0.086	_	-0.097	0.180	-0.167	-0.002	_	0.229	0.066	0.122	_
0.215	0.365	0.725	0.696	0.408	0.444	_	0.938	0.799	0.818	_
-0.083	-0.132	-0.047	0.083	-0.237	-0.258	_	0.035	-0.057	0.041	_
0.994	0.533	0.564	0.693	0.040*	0.184	_	0.615	_	0.239	**0.049**
-0.001	-0.101	0.090	-0.116	-0.327	-0.380	_	-0.125	_	-0.237	-0.188
0.951	0.465	0.482	0.609	0.646	0.589	0.225	0.978	0.841	0.480	_
0.009	-0.178	-0.253	-0.181	0.096	0.218	-0.105	0.013	-0.023	-0.357	_
0.091	**0.050**	0.082	0.110	0.377	0.800	0.038*	0.960	**0.003**	0.654	_
-0.053	0.171	0.297	0.384	0.183	0.096	0.174	0.022	0.074	-0.100	_
0.759	_	0.649	0.980	**0.012**	**0.015**	_	0.314	0.428	0.411	_
0.019	_	-0.142	-0.014	-0.294	-0.180	_	0.096	-0.168	-0.080	_
0.667	_	**0.049**	0.717	0.728	0.020*	_	0.361	0.499	0.691	_
-0.052	_	-0.058	0.112	-0.055	0.165	_	0.176	-0.119	0.035	_
0.029	0.768	0.884	0.435	0.987	0.622	0.453	0.057	0.762	**0.019**	0.130
-0.344	-0.069	0.025	1.074	0.004	0.137	0.439	0.670	0.137	0.244	0.706
0.197	0.471	0.527	0.302	0.857	0.934	**0.047**	0.777	0.314	0.878	0.944
0.044	0.077	0.092	0.152	-0.039	0.036	-0.090	0.112	0.084	-0.058	-0.025

1415.1	1450.2	1495.2	1629.0	1700.9	1835.0	1840.2	1847.5	1854.3	1873.0	1891.4
0.047	0.960	0.768	**0.002**	0.121	0.229	0.073	0.735	_	0.515	_
0.102	0.021	-0.070	-0.163	-0.162	0.631	-0.324	0.084	_	0.232	_
_	**0.013**	**0.002**	_	0.950	0.416	0.368	**0.034**	0.614	0.572	0.105
_	0.421	0.264	_	0.011	0.548	0.276	0.558	0.085	0.339	0.328
_	0.726	0.378	_	0.865	0.289	0.558	**0.024**	**0.009**	0.312	0.339
_	0.139	0.123	_	0.017	0.522	0.422	0.558	0.294	0.570	0.247
_	0.555	0.693	_	0.458	0.751	0.544	0.968	_	0.902	0.639
_	-0.142	0.061	_	-0.168	0.122	0.279	-0.008	_	0.044	-0.082
_	0.691	0.450	_	**0.040**	0.694	**0.032**	0.423	_	0.359	0.419
_	-0.279	-0.337	_	-0.354	-0.298	-0.367	-0.430	_	-0.491	-0.454
_	0.974	0.855	0.852	0.927	**0.003**	0.535	0.460	_	0.159	_
_	-0.016	-0.040	0.028	-0.014	0.436	-0.159	0.147	_	0.375	_
_	0.719	0.913	_	0.642	0.658	0.430	0.723	0.549	0.842	0.484
_	-0.072	-0.018	_	-0.140	0.303	0.356	0.075	-0.075	0.096	-0.074
_	0.431	0.547	_	0.207	0.063	0.209	0.809	0.231	**0.027**	0.990
_	0.111	0.070	_	-0.438	-0.160	-0.491	-0.025	-0.254	-0.243	-0.002
_	0.106	0.704	_	0.366	0.351	0.968	0.192	_	0.393	**0.030**
_	0.610	0.215	_	0.395	1.294	0.034	1.036	_	1.018	0.654
_	0.898	0.653	_	0.785	0.659	0.435	0.827	_	0.996	0.725
_	-0.064	-0.240	_	-0.061	0.221	-0.205	-0.072	_	-0.002	-0.063

1934.8	1990.1	2083.5	2127.5	2224.5	3051.3	3246.0	3347.1	4138.1	6124.3	6189.9
0.797	0.583	_	_	0.328	0.674	_	0.393	_	**0.008**	**0.045**
-0.049	0.062	_	_	-0.066	-0.069	_	0.520	_	0.241	0.264
0.270	_	**0.010**	**0.004**	0.531	_	**0.027**	0.388	0.381	0.593	0.199
0.140	_	0.561	0.436	0.103	_	-0.289	-0.223	-0.157	0.082	-0.194
0.120	0.366	0.314	0.479	**0.032**	_	_	0.064	**0.004**	0.678	0.023*
0.139	0.228	0.455	0.314	0.335	_	_	-0.220	-0.191	-0.037	-0.210
0.697	_	0.750	0.754	_	_	0.545	0.275	**0.013**	_	0.784
-0.057	_	0.096	-0.061	_	_	-0.090	0.131	0.085	_	-0.064
0.471	**0.044**	0.902	0.796	0.149	_	_	0.094	_	0.381	0.635
-0.316	-0.125	-0.078	-0.132	-0.232	_	_	0.764	_	-0.227	0.198
0.969	0.536	0.290	_	0.644	0.876	_	0.341	0.879	_	0.199
-0.005	0.047	0.349	_	-0.055	0.034	_	0.581	0.026	_	0.159
0.748	_	0.195	0.123	0.555	_	0.307	0.394	0.639	0.605	0.474
-0.037	_	0.220	0.156	-0.131	_	-0.137	0.216	0.047	-0.137	-0.122
0.384	_	0.924	0.975	0.288	_	0.409	**0.039**	0.704	0.280	0.217
-0.136	_	0.023	0.006	-0.238	_	0.299	0.672	0.097	0.181	0.336
0.000	0.462	**0.042**	**0.028**	0.308	**0.034**	_	0.383	_	0.888	**0.041**
0.469	0.460	0.821	0.862	0.345	0.355	_	0.068	_	0.029	-0.186
0.278	0.461	0.808	0.618	0.234	0.299	_	0.239	_	0.093	0.183
-0.166	-0.100	0.051	0.098	-0.188	-0.175	_	0.592	_	0.318	0.228

6916.6	10271.3	12421.7	13905.5	14541.4	14746.8	15631.1	16291.2	17279.8	22185.7	22385.1
0.824	0.396	0.951	0.502	0.159	**0.010**	0.251	**0.037**	0.629	**0.038**	_
-0.078	0.241	-0.024	-0.424	-0.440	-0.318	-0.821	-0.285	-0.138	-0.436	_
0.165	0.477	0.156	0.179	0.556	0.400	0.264	0.819	**0.025**	0.207	0.096
0.312	0.081	-0.457	0.196	-0.197	0.085	-0.114	0.050	-0.122	0.332	0.273
0.029	0.736	0.362	0.488	0.793	0.505	0.631	0.932	0.571	0.023*	**0.030**
0.456	-0.045	-0.333	0.275	-0.078	0.125	-0.186	-0.024	0.123	0.170	0.281
_	0.969	0.641	0.431	0.592	0.349	0.489	0.417	0.904	0.479	0.148
_	0.003	-0.146	-0.288	-0.145	0.196	0.201	0.178	-0.028	0.161	0.178
0.513	**0.037**	0.885	**0.037**	0.689	_	0.990	0.711	0.824	0.394	_
-0.099	0.447	-0.120	-0.520	-0.149	_	0.008	0.217	0.103	0.283	_
0.407	0.458	0.979	0.598	0.107	0.045*	0.093	0.459	0.444	0.095	0.065
-0.117	0.064	0.014	-0.300	-0.632	-0.346	-0.675	-0.112	-0.148	-0.419	-0.327
_	0.922	0.434	0.318	**0.048**	0.662	0.434	0.624	0.961	0.079	0.305
_	0.009	-0.240	0.059	-0.109	0.119	0.188	0.155	0.005	0.190	0.235
0.359	0.100	0.591	0.468	0.007*	0.735	0.195	0.784	0.993	0.012*	0.344
-0.218	-0.234	0.221	-0.421	-0.341	-0.042	-0.347	0.058	-0.002	-0.528	-0.247
0.484	0.952	**0.024**	0.945	0.662	_	0.688	0.748	0.678	0.163	_
0.098	-0.011	-0.379	0.019	-0.101	_	0.095	0.078	-0.034	0.098	_
0.160	0.678	0.958	0.295	0.094	_	**0.005**	0.425	0.400	0.071	_
-0.566	0.023	0.030	-0.836	-0.538	_	-0.644	-0.121	-0.162	-0.469	_

Supplementary Table 2. MALDI-IMS analysis identifies region-specific changes in CCKGAD1 transgenic mice. *In situ* proteomic analysis identified 52 lipids, peptides, and proteins (0 – approximately 22,000 Da) with significantly altered expression in NPYGAD1 mice compared to wiltype controls. 25 were decreased, 23 were increased, and 4 had region-specific changes. No results were significant across more than three regions. *, significant results assessed by Benjamini-Hochberg correction for false discovery rate; _ , not significant.

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